ORIGINAL ARTICLE

Induced Pluripotent Stem Cell-derived Mesenchymal Stem Cell Seeding on Biofunctionalized Calcium Phosphate Cements

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Induced pluripotent stem cells (iPSCs) have great potential due to their proliferation and differentiation capability. The objectives of this study were to generate iPSC-derived mesenchymal stem cells (iPSC-MSCs), and investigate iPSC-MSC proliferation and osteogenic differentiation on calcium phosphate cement (CPC) containing biofunctional agents for the first time. Human iPSCs were derived from marrow CD34+ cells which were reprogrammed by a single episomal vector. iPSCs were cultured to form embryoid bodies (EBs), and MSCs migrated out of EBs. Five biofunctional agents were incorporated into CPC: RGD (Arg-Gly-Asp) peptides, fibronectin (Fn), fibronectin-like engineered polymer protein (FEPP), extracellular matrix Geltrex, and platelet concentrate. iPSC-MSCs were seeded on five biofunctionalized CPCs: CPC-RGD, CPC-Fn, CPC-FEPP, CPC-Geltrex, and CPC-Platelets. iPSC-MSCs on biofunctional CPCs had enhanced proliferation, actin fiber expression, osteogenic differentiation and mineralization, compared to control. Cell proliferation was greatly increased on biofunctional CPCs. iPSC-MSCs underwent osteogenic differentiation with increased alkaline phosphatase, Runx2 and collagen-I expressions. Mineral synthesis by iPSC-MSCs on CPC-Platelets was 3-fold that of CPC control. In conclusion, iPSCs showed high potential for bone engineering. iPSC-MSCs on biofunctionalized CPCs had cell proliferation and bone mineralization that were much better than traditional CPC. iPSC-MSC-CPC constructs are promising to promote bone regeneration in craniofacial/orthopedic repairs.

Keywords: induced pluripotent stem cells; RGD; fibronectin; platelet concentrate; biofunctionalized calcium phosphate cement; bone tissue engineering

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Introduction

Bone repair and regeneration are frequently needed in cases of trauma, tumor resections, infections and con-

genital malformations. More than six million bone fractures occur annually in the United States (1). Furthermore, the need for bone repair is increasing rapidly as baby boomers enter into retirement with an aging population (2-3). The development of novel tissue engineering methods is promising to provide viable therapies (4-7). The use of stem cells and scaffolds offer great potential for tissue regeneration (4-5, 8-9). While mesen-

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chymal stem cells (MSCs) are frequently investigated, their harvest requires an invasive procedure, and their potency decreases due to aging and diseases. Therefore, other types of stem cells are needed. Recently, human induced pluripotent stem cells (iPSCs) were successfully generated and represented a major breakthrough in stem cell research (10-13). iPSCs have received intense interest in biomedical applications and regenerative medicine (10-13). Researchers have obtained iPSCs via transfecting mouse cells with the reprogramming transcription factors Oct3/4, Sox2, c-Myc, and Klf4 (10), or human somatic cells with factors Oct4, Sox2, Nanog, and Lin28 (11). In many aspects, iPSCs appeared to be similar to natural pluripotent stem cells such as embryonic stem cells (ESCs). Their similar features include the expression of certain stem cell genes and proteins, doubling time, chromatin methylation patterns, embryoid body formation, teratoma formation, viable chimera formation, potency, and differentiability (10, 12). Similar to ESCs, iPSCs also possess nearly unlimited potential for proliferation, and can differentiate into not only all derivatives of the three primary germ layers (ectoderm, endoderm, and mesoderm), but also many mature cells in vitro (13). iPSCs can be relatively easily obtained, and they can be autogeneic which avoids immunological rejections. Therefore, iPSCs provide an invaluable resource for regenerative medicine (13). Efforts were made to employ iPSCs to regenerate cardiac myocytes (14), renal lineage cells (15), pancreatic insulin-producing cells (16), motor neurons (17), and other distinct tissues. However, there have been only a few studies on the use of iPSCs for bone regeneration applications (18-19).

Hydroxyapatite (HA) and other CaP bioceramics are important for bone repair due to their similarity to bone minerals and ability to bond to bone to form a functional interface (20-24). Calcium phosphate cements can be injected and set in situ to achieve intimate adaptation to complex-shaped defects (25-30). The first calcium phosphate cement consisted of tetracalcium phosphate and dicalcium phosphate anhydrous (referred to as CPC) (26, 31). Other calcium phosphate cements were also developed with various compositions (25, 27-30). Recently, biofunctional agents were incorporated into CPC to enhance cell attachment (32). Biofunctional agents such as fibronectin (Fn) and Arg-Gly-Asp (RGD) have been shown to improve cell adhesion (33-37). RGD is an integrin-recognition site that promotes cell attachment (35-37). Fn is a general cell adhesion molecule that can anchor cells to collagen and proteoglycan (33-34). In addition, genetically-engineered proteins, such as fibronectin-like engineered protein poly-

mer (FEPP), can also enhance cell adhesion (38-39). FEPP includes 13 copies of the cell attachment epitope of Fn between repeated structural peptides. It has a stable three-dimensional conformation resistant to thermal and chemical denaturation. Furthermore, extracellular matrices (ECMs) can also enhance stem cell function (40-41). In this regard, Geltrex is a basement membrane ECM, which is a soluble form of reduced growth factor basement extract and consists of laminin, collagen IV, entactin, and heparin sulfate proteoglycan. In addition, platelet concentrate, which is a fraction of the plasma in which platelets are concentrated, can also improve cell functions (42-43). Platelet concentrate is obtained by withdrawing blood from the vein of the patient. It contains many bioactive molecules and was used in bioceramics to improve cell proliferation (42-43). A recent study investigated bone morphogenic protein 2 (BMP2) gene modification of iPSC-MSCs seeded on a CPC scaffold (44). It was shown that the iPSC-MSCs on CPC successfully underwent osteogenic differentiation, and the overexpression of BMP2 in iPSC-MSCs enhanced differentiation and bone mineral production (44). That previous study focused on BMP2 gene modification and used one CPC-based scaffold without varying the scaffold composition. The present study investigated the effects of the aforementioned five different types of biofunctional agents in CPC on iPSC-MSC seeding and osteogenic differentiation.

Accordingly, the objectives of this study were to generate MSCs from human iPSCs, and investigate the osteogenic differentiation of iPSC-MSCs seeded on biofunctionalized CPCs containing RGD, Fn, FEPP, Geltrex, and platelet concentrate for the first time. The following hypotheses were tested: (1) The attachment and proliferation of iPSC-MSCs on CPC can be significantly enhanced via the incorporation of biofunctional agents in CPC; (2) The osteogenic differentiation and mineral synthesis of iPSC-MSCs will be improved via biofunctionalized CPC scaffolds, compared to control CPC.

Materials and Methods

Human iPSC culture and the derivation of MSCs

Human iPSCs were obtained from the Johns Hopkins University and were generated as described recently (45-46). Briefly, iPSC BC1 line was derived from adult bone marrow CD34+ cells. Human primary mononuclear cells (MNCs) 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 (MACS) system and cultured for 4 days (d) with hematopoietic

cytokines before being reprogrammed by a single episomal vector pEB-C5 (45-46). 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 per well on Nunclon Δ Surface six-well culture plates (Nunc, Rochester, NY). Mitotic inactivation was achieved through exposure MEF to $10 \mu\text{g}\cdot\text{mL}^{-1}$ Mitomycin C (Sigma, St. Louis, MO) for 2 h. The iPSC culture medium consisted of 80% (v/v) Dulbecco's modified Eagle medium (DMEM)/F12 (Invitrogen, Carlsbad, CA), 20% Knockout Serum Replacement (a serum-free formulation; Invitrogen) mixed with 50% MEF conditioning medium, 1% MEM non-essential amino acids solution (Invitrogen), $10 \text{ ng}\cdot\text{mL}^{-1}$ basic fibroblast growth factor (b-FGF, Invitrogen), $1 \text{ mmol}\cdot\text{L}^{-1}$ L-glutamine (Sigma) and $0.1 \text{ mmol}\cdot\text{L}^{-1}$ β -Mercaptoethanol (Sigma). 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 embryoid bodies (EBs) (46). Briefly, iPSC colonies were dissociated into clumps through treatment with $1 \text{ mg}\cdot\text{mL}^{-1}$ collagenase type IV in DMEM/F12 at 37°C for 6 min, followed by mechanical scraping. The dissociated iPSC clumps were collected and resuspended in differentiation medium (iPSC medium without b-FGF), and transferred to 25 cm^2 ultra-low attachment flasks (Corning, Corning, NY). The medium was changed every 2 d. After 10 d, the EBs were transferred into 0.1% gelatin-coated plates and cultured for 10 d. At 2 d 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% FBS (HyClone, Logan, UT), $2 \text{ mmol}\cdot\text{L}^{-1}$ L-glutamine (Gibco), $100 \text{ U}\cdot\text{mL}^{-1}$ penicillin, and $100 \text{ mg}\cdot\text{mL}^{-1}$ streptomycin (Gibco). The differentiated cells which outgrew from the EBs derived from these culture conditions were termed iPSC-derived MSCs (iPSC-MSCs).

Immunophenotyping of iPSC-MSCs via flow cytometry

To confirm the derivation of MSCs, the expression of surface antigen profile of iPSC-MSCs was characterized via flow cytometry (47-49). 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 approximately 1×10^6 cells in $50 \mu\text{L}$ of cold PBS containing 1% BSA. Cell samples were separately labeled on ice with optimal dilution of fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAbs, all from Invitrogen, except when indicated)

against CD29, CD31, CD34 (BD, San Jose, CA), CD44, CD73 (BD), TRA-1-81 (BD), HLA-ABC, phycoerythrin (PE)-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. Nonspecific 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, San Jose, CA). 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 (47-49).

Development of biofunctionalized CPCs

CPC powder consisted of a mixture of tetracalcium phosphate [TTCP, $\text{Ca}_4(\text{PO}_4)_2\text{O}$] and dicalcium phosphate anhydrous (DCPA, CaHPO_4). TTCP was synthesized using DCPA and calcium carbonate (J.T. Baker, Phillipsburg, NJ). TTCP was ground to obtain particles of 1 to $80 \mu\text{m}$, with a median of $17 \mu\text{m}$ (50). DCPA was ground to obtain a median particle size of $1 \mu\text{m}$. TTCP and DCPA powders were mixed at 1:1 molar ratio to form the CPC powder. Chitosan was used to render CPC fast-setting and improve mechanical properties (50). Chitosan lactate (Vanson, Redmond, WA) was mixed with water at a chitosan/(chitosan + water) mass fraction of 15% to form the cement liquid (51). A resorbable suture fiber (Vicryl, polyglactin 910, Ethicon, NJ) was cut to filaments of a length of 3 mm and mixed with CPC paste at a fiber volume fraction of 20% to reinforce CPC (52). A CPC powder to liquid mass ratio of 2:1 was used to form a flowable paste. The CPC paste was filled into a disk mold with a diameter of 12 mm and a thickness of 1.5 mm. The disks were incubated in the mold at 37°C for 1 d in a humidifier, and then demolded and used for subsequent experiments. This CPC is referred to as "CPC control".

In addition, five biofunctionalized CPCs were prepared by incorporating RGD, Fn, FEPP, Geltrex, and platelet concentrate. Each biofunctional agent was mixed with the chitosan liquid, which was then mixed with the CPC powder. The concentration of RGD (Sigma, St. Louis, MO) was $50 \mu\text{g}$ of RGD per 1 g of CPC paste, yielding 0.005% by mass, following a previous study (36). For Fn (human plasma Fn, Invitrogen, Carlsbad, CA) and FEPP (Sigma), the same 0.005% concentration was used in CPC. Geltrex (Invitrogen) was added to CPC at $100 \mu\text{L}$ Geltrex per 1 g of CPC paste (0.1% by mass). This percentage was selected because preliminary study showed that it did not compromise the CPC setting time and mechani-

cal property, while greatly improving cell function (32). Similarly, human platelet concentrate (1.2×10^6 platelets per μL , Biological Specialty, Colmar, PA) was added to CPC at 100 μL of platelet concentrate per 1 g of CPC paste (0.1% by mass) (32). The five biofunctionalized CPCs are termed CPC-RGD, CPC-Fn, CPC-FEPP, CPC-Geltrex, and CPC-Platelets, respectively.

iPSC-MSC adhesion and proliferation on biofunctionalized CPCs

Each CPC disk was placed in a well of a 24-well plate, and iPSC-MSCs at passage 4 were seeded at a seeding density of 50 000 cells in 2 mL of medium per well. An osteogenic medium was used for cell culture, which consisted of the MSC growth medium supplemented with 100 nmol $\cdot$ L⁻¹ dexamethasone, 10 mmol $\cdot$ L⁻¹ β -glycerophosphate, 0.05 mmol $\cdot$ L⁻¹ ascorbic acid, and 10 nmol $\cdot$ L⁻¹ 1 α ,25-Dihydroxyvitamin (Sigma) (47, 53). After 1, 4, and 8 d, the constructs were washed in Tyrode's Hepes buffer, live/dean stained and viewed by epifluorescence microscopy (TE2000S, Nikon, Melville, NY) (52). Three randomly-chosen fields of view were photographed for each disk. Five disks yielded 15 photos for each CPC at each time point. The live cell density was measured as the number of live cells on the specimen divided by the specimen surface area.

Actin cytoskeleton formation in iPSC-MSCs

Actin fibers in the iPSC-MSC cytoskeleton were examined to understand its interaction with CPC and to determine if the addition of biofunctional agents in CPC would enhance cell attachment and increase the amount of actin stress fibers. iPSC-MSCs were seeded on CPC disks as described above. After 1 d culture, the disks were washed with PBS, 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 (BSA) for 30 min (48). An actin cytoskeleton and focal adhesion staining kit (Chemicon, Temecula, CA) was used, which stained actin fibers into a red color. After incubating the construct with diluted (1:400) TRITC-conjugated phalloidin, cell nuclei were labeled with 4'-6-diamidino-2-phenylindole (DAPI) into a blue color. The actin fluorescence was increased when the actins stress fiber density was increased. Fluorescence microscopy (Nikon) was used to examine the specimens. The fluorescence of actin fibers in the cells was measured via a NIS-Elements BR software (Nikon) as in previous studies (48).

Osteogenic differentiation of iPSC-MSCs seeded on biofunctional CPCs

Quantitative real-time reverse transcription polymerase

chain reaction measurement (qRT-PCR, 7900HT, Applied Biosystems, Foster City, CA) was performed. A seeding density of 150 000 cells per well was used following previous studies (52, 54). The constructs were cultured in osteogenic media for 1, 4, and 8 d (54-55). The total cellular RNA on the scaffolds was extracted with TRIzol reagent (Invitrogen). RNA (50 ng $\cdot$ μL^{-1}) was reverse-transcribed into cDNA. TaqMan gene expression kits were used to measure the transcript levels of the proposed genes on human alkaline phosphatase (ALP, Hs00758162_m1), Runx2 (Hs00231692_m1), collagen type I (Coll I, Hs00164004), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Hs99999905). Relative expression for each target gene was evaluated using the $2^{-\Delta\Delta C_t}$ method (55). The C_t values of target genes were normalized by the C_t of the TaqMan human house-keeping gene GAPDH to obtain the ΔC_t values. These values were subtracted by the C_t value of iPSC-MSCs cultured on tissue culture polystyrene in control medium for 1 d (the calibrator) to obtain the $\Delta\Delta C_t$ values (52, 54-55).

Mineral synthesis by iPSC-MSCs seeded on biofunctional CPCs

Osteogenesis of iPSC-MSCs was determined by bone matrix formation via Alizarin Red S (ARS) staining (Millipore, Billerica, MA) (48, 54). ARS stained calcium-rich deposits by cells into a red color. At 4, 14 and 21 d of culture, the iPSC-MSC-CPC constructs were stained with ARS. After staining, the cell-scaffold constructs were washed with deionized water for several times with gentle shaking for 5 min for each wash, until no dye extraction in the used water was observed. An osteogenesis assay (Millipore, Billerica, MA) was used to extract the stained minerals and measure the ARS concentration at OD₄₀₅, following the manufacturer's instructions (48, 54). Control scaffolds with the same CPC compositions and treatment including immersion in the same culture medium for the same period of time, but without cell seeding, were also measured. The control's ARS concentration was subtracted from the ARS concentration of the scaffold with iPSC-MSCs, to yield the net mineral concentration synthesized by the cells. The time points of 14 d and 21 d were selected because previous studies found a large increase in calcium content from 12 d to 21 d (48, 54).

One-way and two-way ANOVA were performed to detect significant ($\alpha=0.05$) effects of the variables. Tukey's multiple comparison procedures were used to group and rank the measured values, and Dunn's multiple comparison tests were used on data with non-normal distribution or unequal variance, both at a family

confidence coefficient of 0.95.

Results

Figure 1 plots the flow cytometry results of the iPSC-MSCs.

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 greater than 75%. On the other hand, the expressions of hematopoietic markers, CD31 and CD34,

Figure 1 Immunophenotyping of human iPSC-MSCs. Flow cytometry showed that the iPSC-MSCs expressed a number of cell surface markers characteristic of MSCs, and were negative for pluripotent, typical hematopoietic and endothelial cell markers. iPSC-MSCs with labeling (cells stained with surface markers) were shown as red curves. Control iPSC-MSCs with no labeling were shown as blue curves.

were less than 0.6% in the iPSC-MSCs, while hESC pluripotency markers, TRA-1-81 and Oct3/4, were less than 0.2%. Furthermore, human leukocyte antigen (HLA) HLA-ABC, present on the surface of all nucleated cells and platelets, were also expressed. These results showed that the typical MSC surface markers were consistently and highly expressed in the iPSC-MSCs.

Figure 2 plots the live cell density of iPSC-MSCs on CPCs measured from the live/dead staining images (mean $\pm$ SD; $n=5$). The cell density was increased by nearly an order of magnitude from 1 to 8 d on CPC-Fn and CPC-Platelets. At each time point, the live cell density on biofunctional CPCs was higher than that on CPC control ($P<0.05$).

Figure 3 shows the results on fluorescence of actin fibers in iPSC-MSCs seeded on CPCs. The actin fibers in the cell cytoskeleton were stained into a red color. The cell nuclei appeared blue. Compared to CPC control in (A), the red fluorescence was increased in CPCs with biofunctional agents (B-F), indicating a greater number of actin stress fibers. The area of red fluorescence was measured for each image and divided by the image area to yield the area fraction of actin fiber fluorescence in (G) (mean $\pm$ SD; $n=5$). The biofunctional CPCs had increased area fractions of actin fiber fluorescence, compared to CPC control ($P<0.05$).

Osteogenic gene expressions of iPSC-MSCs seeded on CPCs are plotted in Figure 4 for: (A) ALP, (B) Runx2, and (C) collagen I (mean $\pm$ SD; $n=5$). The iPSC-MSCs cultured

on tissue culture polystyrene in control medium for 1 d served as the calibrator with a value of 1. The ALP expressions in (A) were higher than 1, indicating osteogenic differentiation. CPC-Platelets had the highest ALP at 8 d ($P<0.05$), which was 7-fold that of the calibrator, and 2-fold that of CPC control. In (B), Runx2 had a similar trend as ALP of peaking at 8 d. In (C) for collagen I, there was also a general trend of the gene expression peaking at 8 d. The peak value for CPC-Platelets was higher than the other groups ($P<0.05$).

The mineralization results of iPSC-MSCs on CPCs are shown in Figure 5. Staining images are shown for CPC control, CPC-Fn and CPC-Platelets at 4 d (A), and 21 d (B), as examples. ARS stained a red color for minerals at 4 d. At 21 d, a thick matrix mineralization was synthesized by the cells, and the red staining became much thicker over time. Examination of all the disks indicated that a layer of mineral matrix synthesized by the cells covered the entire disk, and the mineralization increased with the addition of biofunctional agents in CPC. The results of osteogenesis assay are plotted in (C) (mean $\pm$ SD; $n=5$). CPC disks without cells had baseline values of 0.5–0.7 mmol·L⁻¹. The values in (C) had their baseline values subtracted to represent the net mineral synthesized by the cells. At 21 d, mineral synthesis by iPSC-MSCs seeded on CPC-RGD, CPC-Fn and CPC-Platelets were significantly higher than other groups ($P<0.05$). The mineral synthesis by iPSC-MSCs on CPC-Platelets was nearly 3-fold that on CPC control.

Figure 2 Live cell density of human iPSC-MSCs cultured on the biofunctionalized CPCs. Each value is mean $\pm$ SD; $n=5$. Values with dissimilar letters are significantly different from each other ($P<0.05$).

Figure 3 Fluorescence of actin fibers in iPSC-MSCs on biofunctionalized CPCs. In (A-F), the actin stress fibers in the iPSC-MSCs were stained red. The cell nuclei (blue fluorescence) indicated the location and distribution of the iPSC-MSCs on the scaffolds. The red color was brighter and denser with the addition of biofunctional agents in CPC. (G) Actin fiber fluorescence area fraction (mean $\pm$ SD; $n=5$). Values with dissimilar letters are significantly different from each other ($P<0.05$).

Figure 4 RT-PCR results of osteogenic differentiation of iPSC-MSCs on biofunctionalized CPCs. (A) ALP, (B) Runx2, (C) collagen I gene expressions (mean $\pm$ SD; $n=5$). In each plot, values with dissimilar letters are significantly different from each other ($P<0.05$).

Figure 5 Mineral synthesis by iPSC-MSCs on biofunctionalized CPCs. ARS stained minerals into a red color. (A) ARS staining of iPSC-MSC-scaffold constructs after 4 d on CPC control, CPC-Fn, and CPC-Platelets, as examples. (B) ARS staining of iPSC-MSC-scaffold constructs after 21 d. (C) Mineral concentration synthesized by the iPSC-MSCs was measured by an osteogenesis assay (mean ± SD; $n=5$). Dissimilar letters indicate values that are significantly different from each other ($P<0.05$).

Discussion

This study investigated iPSC-MSC seeding on CPCs containing five different bioactive agents for bone tissue engineering for the first time. MSCs were migrated out of EBs from human iPSCs. The iPSC-MSCs on CPC scaffolds successfully differentiated into the osteogenic lineage, and the biofunctionalized CPCs significantly enhanced iPSC-MSC attachment and mineralization. iPSCs offer a promising approach to obtaining pluripotent stem cells by reprogramming somatic cells, without the controversy of embryonic materials (10). A major advantage of iPSCs is that patient-specific or disease-specific cells of any lineage can be generated for therapeutic use. Recent studies indicated that iPSCs can be generated without using viral vectors; this could move the iPSC technology to be closer to clinical applications (56-57). Regarding tissue engineering and regenerative medicine, iPSCs hold great potential because of their ability to provide unlimited amounts of stem cells with easier cell resources, as well as their lack of immunologic rejection and ethical controversy. Indeed, iPSCs were shown to be able to differentiate into the osteogenic lineage (58-59). Currently there have been only a few reports on the use of iPSCs for bone tissue engineering (18-19). Furthermore, bone engineering requires suitable scaffolds, and CPC biofunctionalized via the five agents showed great potential for enhancing cell function (32). Therefore, the iPSC-MSC-CPC constructs with five biofunctional agents of the present study are promising for bone tissue engineering.

iPSCs were induced into EBs which were further cultured to generate MSCs. These MSCs can then be proliferated and differentiated into specific lineages such as bone, cartilage or fat, which can potentially provide large numbers of progenitor cells to regenerate skeletal defects. In the present study, the 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 (60-61). The iPSC-MSCs lacked the expression of hematopoietic lineage markers (60), hESC pluripotency markers TRA-1-81 and Oct3/4 (62-63), and the marker of professional antigen-presenting cells, HLA-DR (64). Furthermore, the results showed that the iPSC-MSCs appeared to be suitable for bone tissue engineering, consistent with recent studies indicating that iPSCs had the potential to differentiate down the osteogenic lineage (19).

Previous studies guided osteogenic differentiation by culturing iPSC-MSCs in osteogenic media seeded on silk scaffolds (18-19). The use of CPC as a scaffold for iPSC-MSC seeding in the present study has several advantages.

First, CPC has good mechanical strength for moderate load-bearing bone repair applications. Previous studies showed that the strength and elastic modulus of CPC scaffolds matched those of natural cancellous bone (52). Second, CPC can be injected to fill complex-shaped bone defects with intimate adaptation to neighboring bone, and can be molded or contoured in situ to the desired shapes to achieve esthetics for dental, craniofacial and orthopedic repairs (22, 31, 52). Third, CPC can be gradually resorbed and replaced by new bone (30-31). Forth, bioactive agents can be readily mixed into the CPC paste to enhance cell function (8, 32). Bioactive agents can provide a favorable extracellular matrix environment to enhance cellular functions and promote tissue regeneration. For example, Fn and RGD were coated on the surfaces of calcium phosphate bioceramics in previous studies (35-37). Our previous study examined BMP2 gene modification of iPSC-MSCs seeded on a single type of CPC scaffold (44). The present study tested the effects of five different types of biofunctionalized CPC scaffolds on iPSC-MSC seeding and bone tissue engineering. The live cell density of iPSC-MSCs was greatly increased on CPC-RGD, CPC-Fn, CPC-FEPP, CPC-Geltrex, and CPC-Platelets, compared to CPC control. The actin stress fiber density was also increased via biofunctional agents in CPC, which is important because the actin stress fibers anchor to the cell membrane at focal adhesions which are connected to the extracellular matrix or the scaffold (48, 65-66). Furthermore, osteogenic differentiation and bone matrix mineralization were also increased via biofunctional agents in CPC. Among these biofunctional agents, RGD is the principle integrin-binding domain present in ECM proteins and is able to bind multiple integrin species, and hence is the most widely studied adhesive peptide in the development of biomimetic scaffolds (37). RGD has less risk of immune reaction or pathogen transfer comparing with the use of native ECM proteins. This study showed that RGD in CPC enhanced iPSC-MSC proliferation and mineralization, consistent with a previous study showing that RGD-grafted biphasic calcium phosphate ceramics induced more new bone than that without RGD in a rabbit model (67).

Fn is also a cell adhesion molecule and is an important ECM protein. Previous studies showed that Fn could regulate cellular functions and direct cell adhesion, proliferation, and differentiation via direct interactions with cell surface integrin receptors (33). Fn is synthesized by the adherent cells which assemble into a fibrillar network through integrin-dependent and fibronectin-integrin interactions. Indeed, incorporating Fn into CPC enhanced iPSC-MSC attachment and mineralization. In

addition, FEPP is genetically-engineered and has a three-dimensional structure (38-39). For example, FEPP-coated polyurethane graft was shown to enhance cell adhesion (39). Geltrex is another three-dimensional membrane and a human-derived ECM that can support stem cell culture (40-41). The incorporation of FEPP and Geltrex improved cell attachment and bone mineralization via iPSC-MSCs on CPC. However, the enhancement of cell attachment (Figure 2) and mineralization (Fig. 5C) via FEPP and Geltrex was not as robust as those via Fn and platelets.

Several studies have investigated the use of autologous platelets to facilitate healing (68-69). Platelets have many bioactive proteins responsible for attracting macrophages, MSCs and osteoblasts, which promote the removal of necrotic tissue and enhance tissue regeneration and healing. Platelet concentrate is advantageous because it contains a mixture of growth factors which play key roles in wound healing and tissue regeneration (42-43). Previous studies indicated that platelet-rich plasma could be an effective additive in periodontal and oral surgical procedures including bone grafts, implants and maxillofacial reconstructions by facilitating the healing rate (68). For example, 3-6 mL of platelet-rich plasma can be collected from the initial 30-60 mL of blood withdrawal from the vein, hence self-production of platelet-rich plasma can be achieved (68-69). The present study showed that CPC-Platelets had the highest iPSC-MSC attachment and proliferation, as well as the highest mineralization. Among the six CPCs tested, CPC-Platelets, CPC-RGD and CPC-Fn had the highest mineralization amount. They were followed by CPC-FEPP and CPC-Geltrex, while iPSC-MSCs on CPC control had the lowest mineralization. These results suggest that incorporation autologous platelets from the plasma of the patient into the CPC paste could be a promising approach to enhance iPSC-MSC attachment and bone regeneration. The present study showed that MSCs could be generated from iPSCs with excellent cell proliferation and differentiation on CPC, and the iPSC-MSC functions could be greatly enhanced with bio-functional agents in CPC. Further study should investigate iPSC-MSC-CPC constructs containing biofunctional agents for bone regeneration in animal models.

The present study derived MSCs from human iPSCs and investigated iPSC-MSC proliferation and osteogenic differentiation on CPC containing five different biofunctional agents for the first time. MSCs were generated by culturing iPSC colonies and EBs. The new biofunctionalized CPCs, including CPC-RGD, CPC-Fn, CPC-FEPP, CPC-Geltrex and CPC-Platelets, enhanced iPSC-MSC attachment, proliferation and bone mineral synthesis.

Among them, CPC-Platelets, CPC-RGD and CPC-Fn had the best cell attachment and the highest mineralization, followed by CPC-FEPP and CPC-Geltrex, which were all higher than CPC control. These results show that human iPSCs are promising for bone engineering applications, and the iPSC-MSC-CPC constructs have potential to enhance bone regeneration. The iPSC-MSC-seeded bio-functionalized CPCs with greatly-enhanced cell proliferation and mineralization are promising to enhance bone regeneration efficacy in craniofacial and orthopedic repairs.

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References

- 1 WHO Technical Report Series 919. The burden of musculoskeletal conditions at the start of the new millennium. Geneva, Switzerland: WHO. 2003; 102-103.
- 2 Johnson PC, Mikos AG, Fisher JP, Jansen JA. Strategic directions in tissue engineering. *Tissue Eng.* 2007;13:2827-2837.
- 3 Mao JJ, Vunjak-Novakovic G, Mikos AG, Atala A. *Translational Approaches in Tissue Engineering and Regenerative Medicine*. Boston, MA: Artech House. 2007.
- 4 Mao JJ, Giannobile WV, Helms JA, Hollister SJ, Krebsbach PH, Longaker MT, Shi S. Craniofacial tissue engineering by stem cells. *J Dent Res.* 2006;85:966-979.
- 5 Mikos AG, Herring SW, Ochareon P, Elisseeff J, Lu HH, Kandel R, Schoen FJ, Toner M, Mooney D, Atala A, Van Dyke ME, Kaplan D, Vunjak-Novakovic G. Engineering complex tissues. *Tissue Eng.* 2006;12:3307-3339.
- 6 Benoit DS, Nuttelman CR, Collins SD, Anseth KS. Synthesis and characterization of a fluvastatin-releasing hydrogel delivery system to modulate hMSC differentiation and function for bone regeneration. *Biomaterials.* 2006;27:6102-6110.
- 7 Simon CG Jr, Lin-Gibson S. Combinatorial and high-throughput screening of biomaterials. *Adv Mater.* 2011;23:369-387.
- 8 Jansen JA, Vehof JW, Ruhé PQ, Kroeze-Deutman H, Kuboki Y, Takita H, Hedberg EL, Mikos AG. Growth factor-loaded scaffolds for bone engineering. *J Control Release.* 2005;101:127-136.
- 9 Huebsch N, Arany PR, Mao AS, Shvartsman D, Ali OA, Bencherif

- SA, Rivera-Feliciano J, Mooney DJ. Harnessing traction-mediated manipulation of the cell/matrix interface to control stem-cell fate. *Nat Mater*. 2010;9:518-526.
- 10 Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006;126:663-676.
- 11 Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. Induced pluripotent stem cell lines derived from human somatic cells. *Science*. 2007;318:1917-1920.
- 12 Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochiduki Y, Takizawa N, Yamanaka S. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat Biotechnol*. 2008;26:101-106.
- 13 Amabile G, Meissner A. Induced pluripotent stem cells: current progress and potential for regenerative medicine. *Trends Mol Med*. 2009;15:59-68.
- 14 Mauritz C, Schwanke K, Reppel M, Neef S, Katsirntaki K, Maier LS, Nguemo F, Menke S, Hausteiner M, Hescheler J, Hasenfuss G, Martin U. Generation of functional murine cardiac myocytes from induced pluripotent stem cells. *Circulation*. 2008;118:507-517.
- 15 Morizane R, Monkawa T, Itoh H. Differentiation of murine embryonic stem and induced pluripotent stem cells to renal lineage in vitro. *Biochem Biophys Res Commun*. 2009;390:1334-1339.
- 16 Zhang D, Jiang W, Liu M, Sui X, Yin X, Chen S, Shi Y, Deng H. Highly efficient differentiation of human ES cells and iPS cells into mature pancreatic insulin-producing cells. *Cell Res*. 2009;19:429-438.
- 17 Dimos JT, Rodolfa KT, Niakan KK, Weisenthal LM, Mitsumoto H, Chung W, Croft GF, Saphier G, Leibel R, Golland R, Wichterle H, Henderson CE, Eggan K. Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Science*. 2008;321:1218-1221.
- 18 Duan X, Tu Q, Zhang J, Ye J, Sommer C, Mostoslavsky G, Kaplan D, Yang P, Chen J. Application of induced pluripotent stem (iPS) cells in periodontal tissue regeneration. *J Cell Physiol*. 2011;226:150-157.
- 19 Ye JH, Xu YJ, Gao J, Yan SG, Zhao J, Tu Q, Zhang J, Duan XJ, Sommer CA, Mostoslavsky G, Kaplan DL, Wu YN, Zhang CP, Wang L, Chen J. Critical-size calvarial bone defects healing in a mouse model with silk scaffolds and SATB2-modified iPSCs. *Biomaterials*. 2011;32:5065-5076.
- 20 Ginebra MP, Driessens FC, Planell JA. Effect of the particle size on the micro and nanostructural features of a calcium phosphate cement: a kinetic analysis. *Biomaterials*. 2004;25:3453-3462.
- 21 Ginebra MP, Traykova T, Planell JA. Calcium phosphate cements as bone drug delivery systems: a review. *J Control Release*. 2006;113:102-110.
- 22 Bohner M, Baroud G. Injectability of calcium phosphate pastes. *Biomaterials*. 2005;26:1553-1563.
- 23 Deville S, Saiz E, Nalla RK, Tomsia AP. Freezing as a path to build complex composites. *Science*. 2006;311:515-518.
- 24 Reilly GC, Radin S, Chen AT, Ducheyne P. Differential alkaline phosphatase responses of rat and human bone marrow derived mesenchymal stem cells to 45S5 bioactive glass. *Biomaterials*. 2007;28:4091-4097.
- 25 Ginebra MP, Rilliard A, Fernandez E, Elvira C, San RJ, Planell JA. Mechanical and rheological improvement of a calcium phosphate cement by the addition of a polymeric drug. *J Biomed Mater Res*. 2001;57:113-118.
- 26 Brown WE, Chow LC. A new calcium phosphate water setting cement//Brown PW. *Cements Research Progress*. Westerville, OH: Am Ceram Soc. 1986:352-379.
- 27 Barralet JE, Gaunt T, Wright AJ, Gibson IR, Knowles JC. Effect of porosity reduction by compaction on compressive strength and microstructure of calcium phosphate cement. *J Biomed Mater Res*. 2002;63:1-9.
- 28 Bohner M, Gbureck U, Barralet JE. Technological issues for the development of more efficient calcium phosphate bone cements: a critical assessment. *Biomaterials*. 2005;26:6423-6429.
- 29 Link DP, van den Dolder J, Wolke JG, Jansen JA. The cytocompatibility and early osteogenic characteristics of an injectable calcium phosphate cement. *Tissue Eng*. 2007;13:493-500.
- 30 Bohner M. Design of ceramic-based cements and putties for bone graft substitution. *Eur Cell Mater*. 2010;20:1-12.
- 31 Friedman CD, Costantino PD, Takagi S, Chow LC. BoneSource hydroxyapatite cement: a novel biomaterial for craniofacial skeletal tissue engineering and reconstruction. *J Biomed Mater Res*. 1998;43:428-432.
- 32 Thein-Han W, Liu J, Xu HHK. Calcium phosphate cement with biofunctional agents and stem cell seeding for dental and craniofacial bone repair. *Dent Mater*. 2012;28:1059-1070.
- 33 Mosher DF. *Fibronectin*. San Diego, CA: Academic Press. 1989.
- 34 van den Dolder J, Bancroft GN, Sikavitsas VI, Spauwen PH, Mikos AG, Jansen JA. Effect of fibronectin- and collagen I-coated titanium fiber mesh on proliferation and differentiation of osteogenic cells. *Tissue Eng*. 2003;9:505-515.
- 35 Sawyer AA, Hennessy KM, Bellis SL. Regulation of mesenchymal stem cell attachment and spreading on hydroxyapatite by RGD peptides and adsorbed serum proteins. *Biomaterials*. 2005;26:1467-1475.
- 36 Schneiders W, Reinstorf A, Pompe W, Grass R, Biewener A, Holch M, Zwipp H, Rammelt S. Effect of modification of hydroxyapatite/collagen composites with sodium citrate, phosphoserine, phosphoserine/RGD-peptide and calcium carbonate on bone remodelling. *Bone*. 2007;40:1048-1059.
- 37 Bellis SL. Advantages of RGD peptides for directing cell association with biomaterials. *Biomaterials*. 2011;32:4205-4210.
- 38 Esty A. Receptor-specific serum-free cell attachment using a high

- stable engineered protein polymer. *Am Biotechnol Lab.* 1991;9:44.
- 39 Tiwari A, Kidane A, Salacinski H, Punshon G, Hamilton G, Seifalian AM. Improving endothelial cell retention for single stage seeding of prosthetic grafts: use of polymer sequences of arginine-glycine-aspartate. *Eur J Vasc Endovasc Surg.* 2003;25:325-329.
- 40 Ilic D. Culture of human embryonic stem cells and the extracellular matrix microenvironment. *Regen Med.* 2006;1:95-101.
- 41 Kim J, Efe JA, Zhu S, Talantova M, Yuan X, Wang S, Lipton SA, Zhang K, Ding S. Direct reprogramming of mouse fibroblasts to neural progenitors. *Proc Natl Acad Sci U S A.* 2011;108:7838-7843.
- 42 Vogel JP, Szalay K, Geiger F, Kramer M, Richter W, Kasten P. Platelet-rich plasma improves expansion of human mesenchymal stem cells and retains differentiation capacity and in vivo bone formation in calcium phosphate ceramics. *Platelets.* 2006;17:462-469.
- 43 Kasten P, Vogel J, Beyen I, Weiss S, Niemeyer P, Leo A, Luginbuhl R. Effect of platelet-rich plasma on the in vitro proliferation and osteogenic differentiation of human mesenchymal stem cells on distinct calcium phosphate scaffolds: the specific surface area makes a difference. *J Biomater Appl.* 2008;23:169-188.
- 44 Liu J, Chen W, Zhao Z, Xu HH. Reprogramming of mesenchymal stem cells derived from iPSCs seeded on biofunctionalized calcium phosphate scaffold for bone engineering. *Biomaterials.* 2013;34:7862-7872.
- 45 Chou BK, Mali P, Huang X, Ye Z, Dowey SN, Resar LM, Zou C, Zhang YA, Tong J, Cheng L. Efficient human iPS cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. *Cell Res.* 2011 ;21:518-529.
- 46 Cheng L, Hansen NF, Zhao L, Du Y, Zou C, Donovan FX, Chou BK, Zhou G, Li S, Dowey SN, Ye Z, NISC Comparative Sequencing Program, Chandrasekharappa SC, Yang H, Mullikin JC, Liu PP. Low incidence of DNA sequence variation in human induced pluripotent stem cells generated by nonintegrating plasmid expression. *Cell Stem Cell.* 2012;10:337-344.
- 47 Tang M, Chen W, Weir MD, Thein-Han W, Xu HH. Human embryonic stem cell encapsulation in alginate microbeads in macroporous calcium phosphate cement for bone tissue engineering. *Acta Biomater.* 2012;8:3436-3445.
- 48 Thein-Han W, Xu HH. Collagen-calcium phosphate cement scaffolds seeded with umbilical cord stem cells for bone tissue engineering. *Tissue Eng Part A.* 2011;17:2943-2954.
- 49 Chen W, Zhou H, Weir MD, Tang M, Bao C, Xu HH. Human embryonic stem cell-derived mesenchymal stem cell seeding on calcium phosphate cement-chitosan-RGD scaffold for bone repair. *Tissue Eng Part A.* 2013;19:915-927.
- 50 Xu HH, Takagi S, Quinn JB, Chow LC. Fast-setting calcium phosphate scaffolds with tailored macropore formation rates for bone regeneration. *J Biomed Mater Res A.* 2004;68:725-734.
- 51 Xu HH, Simon CG Jr. Fast setting calcium phosphate-chitosan scaffold: mechanical properties and biocompatibility. *Biomaterials.* 2005;26:1337-1348.
- 52 Zhao L, Weir MD, Xu HH. An injectable calcium phosphate-alginate hydrogel-umbilical cord mesenchymal stem cell paste for bone tissue engineering. *Biomaterials.* 2010;31:6502-6510.
- 53 Hwang NS, Varghese S, Lee HJ, Zhang Z, Ye Z, Bae J, Cheng L, Elisseeff J. In vivo commitment and functional tissue regeneration using human embryonic stem cell-derived mesenchymal cells. *Proc Natl Acad Sci U S A.* 2008;105:20641-20646.
- 54 Zhou H, Weir MD, Xu HH. Effect of Cell Seeding Density on Proliferation and Osteodifferentiation of Umbilical Cord Stem Cells on Calcium Phosphate Cement-Fiber Scaffold. *Tissue Eng Part A.* 2011;17:2603-2613.
- 55 Kim K, Dean D, Mikos AG, Fisher JP. 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.* 2009;10:1810-1817.
- 56 Kaji K, Norrby K, Paca A, Mileikovsky M, Mohseni P, Woltjen K. Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature.* 2009;458:771-775.
- 57 Zhou H, Wu S, Joo JY, Zhu S, Han DW, Lin T, Trauger S, Bien G, Yao S, Zhu Y, Siuzdak G, Schöler HR, Duan L, Ding S. Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell.* 2009;4:381-384.
- 58 Tashiro K, Inamura M, Kawabata K, Sakurai F, Yamanishi K, Hayakawa T, Mizuguchi H. Efficient adipocyte and osteoblast differentiation from mouse induced pluripotent stem cells by adenoviral transduction. *Stem Cells.* 2009;27:1802-1811.
- 59 Kao CL, Tai LK, Chiou SH, Chen YJ, Lee KH, Chou SJ, Chang YL, Chang CM, Chen SJ, Ku HH, Li HY. Resveratrol promotes osteogenic differentiation and protects against dexamethasone damage in murine induced pluripotent stem cells. *Stem Cells Dev.* 2010;19:247-258.
- 60 Tocci A, Forte L. Mesenchymal stem cell: use and perspectives. *Hematol J.* 2003;4:92-96.
- 61 Barry FP, Murphy JM. Mesenchymal stem cells: clinical applications and biological characterization. *Int J Biochem Cell Biol.* 2004;36:568-584.
- 62 Gruenloh W, Kambal A, Sondergaard C, McGee J, Nacey C, Kalomoiris S, Pepper K, Olson S, Fierro F, Nolte JA. Characterization and in vivo testing of mesenchymal stem cells derived from human embryonic stem cells. *Tissue Eng Part A.* 2011;17:1517-1525.
- 63 Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet.* 2000;24:372-376.
- 64 Le BK, Tammik C, Rosendahl K, Zetterberg E, Ringden O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp Hematol.* 2003;31:

- 890-896.
- 65 Amano M, Chihara K, Kimura K, Fukata Y, Nakamura N, Matsuura Y, Kaibuchi K. Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. *Science*. 1997;275:1308-1311.
- 66 Grosheva I, Vittitow JL, Goichberg P, Gabelt BT, Kaufman PL, Borrás T, Geiger B, Bershadsky AD. Caldesmon effects on the actin cytoskeleton and cell adhesion in cultured HTM cells. *Exp Eye Res*. 2006;82:945-958.
- 67 Fricain JC, Pothuaud L, Durrieu MC, Pallu S, Bareille R, Renard M, Jeanfils J, Dard M, Amédée J. Effects of cyclic RGD peptide functionalization on the quantitative bone ingrowth process in cellularized biphasic calcium phosphate ceramics. *Key Eng Mater*. 2005;284-286:647-650.
- 68 Carlson NE, Roach RB Jr. Platelet-rich plasma: clinical applications in dentistry. *J Am Dent Assoc*. 2002;133:1383-1386.
- 69 Sampson S, Gerhardt M, Mandelbaum B. Platelet rich plasma injection grafts for musculoskeletal injuries: a review. *Curr Rev Musculoskelet Med*. 2008;1:165-174.