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Umbilical cord and bone marrow mesenchymal stem cell seeding on macroporous calcium phosphate for bone regeneration in rat cranial defects

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Abstract

Human umbilical cord mesenchymal stem cells (hUCMSCs) are inexhaustible and can be harvested at a low cost without an invasive procedure. However, there has been no report on comparing hUCMSCs with human bone marrow MSCs (hBMSCs) for bone regeneration *in vivo*. The aim of this study was to investigate hUCMSC and hBMSC seeding on macroporous calcium phosphate cement (CPC), and to compare their bone regeneration in critical-sized cranial defects in rats. Cell attachment, osteogenic differentiation and mineral synthesis on RGD-modified macroporous CPC were investigated *in vitro*. Scaffolds with cells were implanted in 8-mm defects of athymic rats. Bone regeneration was investigated via micro-CT and histological analysis at 4, 12, and 24 weeks. Three groups were tested: CPC with hUCMSCs, CPC with hBMSCs, and CPC control without cells. Percentage of live cells and cell density on CPC *in vitro* were similarly good for hUCMSCs and hBMSCs. Both cells had high osteogenic expressions of alkaline phosphatase, osteocalcin, collagen I, and Runx2. Bone mineral density and trabecular thickness in hUCMSC and hBMSC groups *in vivo* were greater than those of CPC control group. New bone amount for hUCMSC-CPC and hBMSC-CPC constructs was increased by 57% and 88%, respectively, while

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blood vessel density was increased by 15% and 20%, than CPC control group at 24 weeks. hUCMSC-CPC and hBMSC-CPC groups generally had statistically similar bone mineral density, new bone amount and vessel density. In conclusion, hUCMSCs seeded on CPC were shown to match the bone regeneration efficacy of hBMSCs *in vivo* for the first time. Both hUCMSC-CPC and hBMSC-CPC constructs generated much more new bone and blood vessels than CPC without cells. Macroporous RGD-grafted CPC with stem cell seeding is promising for craniofacial and orthopedic repairs.

Keywords

Calcium phosphate cement; Stem cells; RGD; Bone regeneration; Athymic rats; Critical-sized cranial defect

1. Introduction

Stem cell-based tissue engineering approaches have the potential to regenerate damaged and diseased tissues. Bone defects often arise from skeletal diseases, congenital malformations, trauma, and tumor resections which require bone reconstruction [1-4]. Studies have shown exciting results in stem cell delivery via scaffolds for bone regeneration [5,6]. Human bone marrow-derived mesenchymal stem cells (hBMSCs) are multipotent and able to differentiate into osteoblasts, chondrocytes, neurons, myoblasts, adipocytes, and fibroblasts [7]. hBMSCs can be harvested from bone marrow, expanded in culture, induced to differentiate and combined with a scaffold to repair bone defects.

However, autogenous hBMSCs require an invasive procedure to harvest and are limited in cell numbers [8]. Furthermore, hBMSCs have lower self-renewal and proliferative ability due to patient aging [9-11] and diseases such as osteoporosis and arthritis [12,13]. Therefore, other sources of stem cells are needed for tissue engineering. Recently, human umbilical cord MSCs (hUCMSCs) were derived and shown to differentiate into adipocytes, osteoblasts, chondrocytes, neurons, and endothelial cells [14-20]. Umbilical cords can provide an inexhaustible and low-cost source of stem cells, without the invasive procedure of hBMSCs [21]. Furthermore, hUCMSCs appeared to be primitive MSCs and exhibited a high plasticity and developmental flexibility [19]. In addition, in preliminary studies the hUCMSCs had minimal immunorejection *in vivo* and were not tumorigenic [19]. These advantages make hUCMSCs a highly attractive alternative to hBMSCs for bone regeneration. Although a few reports used hUCMSCs for bone tissue engineering research [18,22-25], there is still a lack of *in vivo* studies comparing the bone regenerative efficacy of hUCMSCs with hBMSCs.

A scaffold serves as a template for cell attachment, proliferation, differentiation and bone growth *in vivo*. Calcium phosphate scaffolds mimic bone minerals and can facilitate cell attachment and function [26,27]. They are bioactive and can bond to bone to form a functional interface [28,29]. Calcium phosphate cements have injectability, biocompatibility, osteoconductivity and bioresorbability [30-34]. The first calcium phosphate cement (referred to as CPC) was developed in 1986 and consisted of tetracalcium phosphate (TTCP) and dicalcium phosphate anhydrous (DCPA). CPC was approved in 1996 by the Food and Drug

Administration (FDA) for repairing craniofacial defects [30,35]. Recent studies created macroporous CPC scaffolds to increase the resorption rate and facilitate cell access to fluids [24]. Furthermore, incorporation of biofunctional agents into CPC could improve cell attachment, which is important for cellular functions such as proliferation, migration, and differentiation. The tripeptide arginyl-glycyl-aspartic acid (RGD), a key cell-adhesion motif, mediates cell attachment and promotes cell adhesion to biomaterials [24,36,37]. Indeed, recent studies showed the effect of RGD in CPC on attachment and osteogenic differentiation of stem cells *in vitro* [37,38]. However, a literature search revealed no report on *in vivo* comparison of hUCMSCs with hBMSCs seeded on CPC for bone regeneration in animals.

Therefore, the objectives of this study were to investigate the *in vivo* behavior of stem cell-seeded CPC scaffolds in an animal model, and compare the bone regeneration efficacy of hUCMSCs with hBMSCs for the first time. RGD was grafted in chitosan which was then incorporated into CPC. A gas-foaming method was used to create macropores in CPC. A critical sized cranial defect model in athymic rats was used to evaluate and compare the bone regeneration efficacy of hUCMSCs and hBMSCs. Three hypotheses were tested: (1) hUCMSCs and hBMSCs will have similarly good attachment and osteogenic differentiation *in vitro* on macroporous CPC-RGD scaffold; (2) hUCMSCs seeded on CPC will match the *in vivo* bone regeneration efficacy of hBMSCs which require an invasive procedure to harvest; (3) Both hUCMSCs and hBMSCs seeded with CPC scaffolds will generate significantly more new bone *in vivo* than CPC control without stem cells.

2. Materials and methods

2.1 Fabrication of RGD-grafted macroporous CPC

CPC powder consisted of an equimolar mixture of TTCP ($\text{Ca}_4[\text{PO}_4]_2\text{O}$) and DCPA (CaHPO_4). TTCP was synthesized from a solid-state reaction between equimolar amounts of DCPA and CaCO_3 (J. T. Baker, Phillipsburg, NJ), which were mixed and heated at 1500 °C for 6 h in a furnace (Model 51333, Lindberg, Watertown, WI). The heated mixture was quenched to room temperature, ground in a ball mill (Retsch PM4, Brinkman, NY) and sieved to obtain TTCP particles with sizes of approximately 1-80 μm , with a median of 17 μm . DCPA was ground for 24 h to obtain particle sizes of 0.4-3.0 μm , with a median of 1.0 μm . TTCP and DCPA powders were mixed in a blender at a molar ratio of 1:1 to form the CPC powder. The CPC liquid consisted of RGD-grafted chitosan mixed with distilled water at a chitosan/(chitosan + water) mass fraction of 7.5%. RGD grafting was performed by coupling G4RGDSP (Thermo Fisher) with chitosan malate (Vanson, Redmond, WA). This was achieved by forming amide bonds between carboxyl groups in peptide and residual amine groups in chitosan using 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC, Thermo Fisher) and sulfo-N-hydroxysuccinimide (Sulfo-NHS, Thermo Fisher) as coupling agents [37,39,40]. After dissolving G4RGDSP peptide (24.8 mg, 32.64×10^{-6} mol) in 0.1 mol/L of 2-(N-Morpholino) ethanesulfonic acid (MES) buffer (4 mL) (Thermo Fisher), EDC (7.52 mg, 39.2×10^{-6} mol) and Sulfo-NHS (4.14 mg, 19.52×10^{-6} mol) were added to the peptide solution (molar ratio of G4RGDSP:EDC:NHS = 1:1.2:0.6). The solution was incubated at room temperature for 30 min to activate the terminal carboxyl

group of proline. Then, this solution was added to a chitosan solution dissolved in 0.1 mol/L of MES buffer (100 mL, 1 wt%). The coupling reaction was performed for 24 h at room temperature. The products were dialyzed against distilled water using a Dialysis Cassettes (MWCO = 3.5 kDa) (Thermo Fisher) for 3 d to remove uncoupled peptides by changing water 3 times daily. Finally, the products were freeze-dried to obtain the RGD-grafted chitosan [37,39,40].

A gas-foaming method was used to fabricate macroporous CPC scaffold. Following a previous study [24], sodium hydrogen carbonate (NaHCO_3) and citric acid monohydrate ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$) were added as porogen into CPC. The acid-base reaction of $\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$ with NaHCO_3 produced CO_2 bubbles in CPC, resulting in macropores [41]. NaHCO_3 was added to the CPC powder, at a $\text{NaHCO}_3/(\text{NaHCO}_3 + \text{CPC powder})$ mass fraction of 15%, based on a previous study [24]. A corresponding amount of $\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$ was added to the CPC liquid, to maintain a $\text{NaHCO}_3/(\text{NaHCO}_3 + \text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O})$ mass fraction of 54.52% [41].

CPC paste was formed by mixing the CPC-porogen powder with the RGD-grafted chitosan liquid at a powder:liquid mass ratio of 2:1. The paste was placed in molds of 8 mm in diameter and 1 mm in thickness to fabricate CPC disks. The disks were incubated in a humidior with 100% relative humidity for 2 d at 37 °C, sterilized in an ethylene oxide sterilizer (Andersen, Haw River, NC) for 12 h and degassed for 7 d prior to cell seeding.

2.2 Cell culture

The use of hUCMSCs (ScienCell, Carlsbad, CA) and hBMSCs (Lonza, Allendale, NJ) was approved by University of Maryland. hUCMSCs were obtained from umbilical cords of healthy babies [18,42] and cultured in a low-glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) (Invitrogen, Carlsbad, CA) (hUCMSC growth medium). The osteogenic medium for hUCMSCs consisted of the growth medium plus 100 nM dexamethasone, 10 mM β -glycerophosphate, 0.05 mM ascorbic acid, and 10 nM $1\alpha,25\text{-Dihydroxyvitamin}$ (Sigma, St. Louis, MO) [16,18,43].

The hBMSC growth medium consisted of DMEM plus 10% FBS, 1% PS, 0.25% gentamicin and 0.25% fungizone (Invitrogen). The osteogenic medium for hBMSCs consisted of the hBMSC growth medium plus 100 nM dexamethasone, 10 mM β -glycerophosphate, and 0.05 mM ascorbic acid [43].

After culturing in growth medium till 80% to 90% confluence, cells were detached and passaged. Passage 4 cells were used for the experiments of this study. A seeding density of 3×10^5 cells diluted in 2 mL of osteogenic medium was seeded drop-wise onto each macroporous CPC disk, which was placed in a 24-well plate. CPC disks with osteogenic medium but without cells served as control. Medium was changed every 2 d.

2.3 hUCMSC and hBMSC viability after seeding on CPC scaffold

After 1, 4, 7 or 14 d, the medium was removed and the CPC disks were washed two times with 2 mL of phosphate buffered saline. Cells were stained with a live/dead viability and

cytotoxicity kit (Molecular Probes, Eugene, OR) and viewed using epifluorescence microscopy (TE2000-S, Nikon, Melville, NY). The percentage of live cells was measured as $P = \text{number of live cells} / (\text{number of live cells} + \text{number of dead cells})$. The live cell density was measured as $D = \text{number of live cells in the image} / \text{the image area}$ [37]. Three randomly-chosen fields of view were photographed for each specimen. Five specimens of each group ($n = 5$) yielded 15 images for each time point.

To determine the morphology of cell growth, the cell-scaffold constructs at 14 d were examined under scanning electron microscopy (SEM, Quanta 200, FEI, Hillsboro, OR). Samples were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4, dehydrated with gradient ethanol, and rinsed with hexamethyldisilazane. Samples were then dried overnight and sputter-coated with gold for SEM observation.

2.4 qRT-PCR measurement of osteogenic differentiation of cells on CPC

Osteogenic differentiation of hUCMSCs and hBMSCs on RGD-grafted CPC was measured via quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR, 7900HT, Applied Biosystems, Foster City, CA). At 1, 4, 7 and 14 d, the total cellular RNA on the scaffolds was extracted with TRIzol reagent and PureLink RNA Mini Kit (Invitrogen), and reverse-transcribed into cDNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) in a thermal cycler (GenAmp PCR 2720, Applied Biosystems). TaqMan gene expression assay kits, including two pre-designed specific primers and probes, were used to measure the transcript levels of the proposed genes on human alkaline phosphatase (ALP, Hs00758162_m1), osteocalcin (OC, Hs00609452_g1), collagen type I (Coll I, Hs00164004), Runx2 (Hs00231692_ml), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Hs99999905). Relative expression level for each target gene was evaluated using the 2^{-C_t} method [44]. The C_t values of target genes were normalized by the C_t values of the human housekeeping gene GAPDH to obtain the C_t values. The C_t value of hUCMSCs or hBMSCs cultured on tissue culture polystyrene in growth medium for 1 d served as the calibrator [39,43].

2.5 Mineral synthesis by cells in vitro

Mineral synthesis by hUCMSCs and hBMSCs *in vitro* was investigated. RGD-grafted CPC disks seeded with hUCMSCs or hBMSCs cultured in osteogenic medium for 7, 14 and 21 d were fixed with 10% formaldehyde and stained with Alizarin Red S (ARS, Millipore, Billerica, MA). ARS stained calcium-rich deposits by cells into a red color. An osteogenesis assay (Millipore) was used to extract the stained minerals and measure the ARS concentration, following the manufacturer's instructions [37]. CPC control disks without cells were treated in the same manner and measured at the same time periods. The control's ARS concentration was subtracted from that of disks with cells to yield the net mineral concentration synthesized by cells [37].

2.6 In vivo animal experiment and surgical procedures

Three groups of materials were tested in rats: hUCMSC-seeded RGD-grafted CPC scaffold; hBMSC-seeded RGD-grafted CPC scaffold; and RGD-grafted CPC scaffold alone without cells. All specimens were cultured in osteogenic medium for 14 d, and then used for

implantation. The critical-sized cranial defect model in rats was approved by the University of Maryland Baltimore (IACUC # 0909014). All procedures involving animals were performed in accordance with NIH animal care guidelines [2]. Athymic nude rats (200-250 g, 7-8 weeks old) (Harlan, Indianapolis, IN) were anesthetized by intraperitoneal injection with a combination of 75 mg/kg body weight of ketamine and 10 mg/kg of xylazine. The cranium was shaved and iodinated before fixation. Surgery was performed under aseptic conditions. A mid-longitudinal incision of approximately 2 cm was made on the dorsal surface of the cranium, and the periosteum was completely cleared from the surface of the cranium by scraping. A trephine bur was used to create a circular defect with a diameter of 8 mm in the cranium [2]. The full thickness of the cranial bone in the defect was removed under constant irrigation with sterile saline. A CPC disk was placed in the defect and the defect was closed by suturing the soft tissue. For the hUCMSC-CPC and hBMSC-CPC groups, the constructs were implanted with the cell-seeded surface contacting the dura of the rats. Three implantation periods were tested: 4, 12 and 24 weeks (w). At each time, five rats from each of the three groups were euthanized with carbon monoxide, and the implants embedded in the surrounding native bone were retrieved. The implants were fixed for 24 h at 4 °C in 10% zinc-buffered formalin and then analyzed.

2.7 Micro-CT analysis

All the specimens were horizontally placed in a sample holder filled with water and examined using a micro-CT scanner (SkyScan 1172 X-ray microtomograph, Antwerp, Belgium). Acquisition parameters for the scanning were: 59 kV, 167 μ A, rotation step of 0.4° for 180° of rotation, and medium camera pixel resolution. The resulting two dimensional shadow/transmission images were used to reconstruct axial cross-sections by SkyScan's cluster reconstruction software (NRecon/NRecon Server). The data were further analyzed by the software CT Analyzer (Skyscan). The regions of interest (ROI) were defined as a cylindrical area covering the created defect. The micro-CT measurements included the bone mineral density (BMD) and the trabecular thickness (Tb.Th) in the bone defects, following previous studies [45]. All the specimens from the three groups were analyzed and the average value of each group (n = 5) at each time point was obtained.

2.8 Histological and histomorphological analysis

After micro-CT scanning, the specimens were decalcified in 30% buffered formic acid for 7 d at room temperature. After dehydration and clearing, the specimens were embedded in paraffin and the central part of the implant and defect was cut into 5 μ m thick sections. Paraffin sections were stained with hematoxylin/eosin (H&E), and the histologic images were analyzed by Image Pro Plus Software (Media Cybernetics, Carlsbad, CA). The perimeter around the new bone was traced, and the area of the new bone was measured by the software. New bone area fraction was calculated as the new bone area in the defect divided by the entire defect area. Blood vessels were identified by their luminal structure and the presence of red blood cells within their boundaries. The new vessel density was determined by the number of new blood vessels in the defect area divided by the entire defect area. One section from the central part of the implant for each rat was analyzed and the average value of each group (n = 5) was obtained for each group at each time point.

2.9 Statistical analyses

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS 16.0, Chicago, IL). Statistical significance was assessed by using one- or two-way analyses of variance (ANOVA) 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.

3. Results

Live/dead assay results of hUCMSCs and hBMSCs seeded on macroporous CPC are shown in Fig. 1A-D. Live cells (green) appeared to have adhered to scaffolds with a normal spindle morphology and were numerous for both cell types. Dead cells (red/orange) were relatively few. The percentage of live cells (Fig. 1C) was near 90% and had only minor variations between the groups. Live cell density (Fig. 1D) increased over time due to cell proliferation, showing a 6-fold increase from 1 to 14 d ($p < 0.05$). SEM (Figs. 1E and 1F) revealed that both hUCMSCs and hBMSCs attached well to the RGD-grafted CPC and exhibited a healthy spreading morphology.

The expressions of four osteogenic differentiation genes (ALP, OC, collagen I, and Runx2) measured by RT-PCR are plotted in Fig. 2. Both hUCMSCs and hBMSCs successfully underwent osteogenic differentiation, indicated by the high ALP peaks at 4 d, and the OC, Coll I and Runx2 peaks at 7 d. For each gene marker, hUCMSCs had a similar trend and peak values to those of hBMSCs.

Results of mineral synthesis by hUCMSCs and hBMSCs *in vitro* on CPC are shown in Fig. 3. Fig. 3A shows typical mineral staining photos. Mineral synthesis increased from 7 d to 21 d for both cells. By 21 d, there was a dark red staining of minerals synthesized by the cells, which accumulated on the surfaces of CPC disks. Data from the osteogenesis assay are plotted in Fig. 3B. At each time point, there was no significant difference between hUCMSCs and hBMSCs ($p > 0.1$). At 21 d, the mineral amounts synthesized by hUCMSCs and hBMSCs were nearly an order of magnitude greater than that at 7 d.

The results of micro-CT were shown in Fig. 4. Micro-CT images indicated that new bone formation increased with time (example for control in Fig. 4A). For each group, the BMD (Fig. 4B) increased with time ($p < 0.05$). Significantly greater BMD was obtained in the hUCMSCs and hBMSCs groups than in CPC control group at each implantation time period ($p < 0.05$). The Tb.Th value (Fig. 4C) also increased with time. Tb.Th of hUCMSC and hBMSC groups were higher than that of control. However, only the difference in Tb.Th between control and hBMSC group was significant ($p < 0.05$). Tb.Th values of hUCMSC and hBMSC groups were not significantly different from each other at 24 w ($p > 0.1$).

The results of histological examination were shown in Fig 5. Tissues and cells were visible in the images by H&E staining. Signs of inflammation or immunologic response were not noticed in any group at any time period. Extracellular matrix (ECM) deposits and connective tissue ingrowth filled the scaffold pores as early as 4 w. New bone formation was observed when ECM was calcification over time. New bone formation mainly started from the

peripherals of the defect which were the interfaces between the implant and native bone. Then, more new bone gradually generated along CPC surfaces, especially at the dura side. With increasing time of 12 and 24 w, new bone formation was visible not only in the surface regions of the CPC implant, but also inside the CPC implant and throughout the entire defect (Fig. 5). Examinations of all the specimens indicated that there was substantially more new bone in hBMSC-CPC and hUCMSC-CPC than CPC control.

Active new bone formation could be seen by the appearance of osteoid with osteocytes and blood vessels, and with newly formed bone lined by osteoblasts. Typical examples of these features are shown in Fig. 6A. CPC material loss and resorption could be noticed in defects with CPC volume being gradually replaced by new bone. This is consistent with osteoclast-like multinuclear giant cells observed at the internal surfaces of the macropores in CPC, with examples shown in Fig. 6B.

The new bone area fraction results are plotted in Fig. 7A. The amount of new bone increased from 4 to 24 w for all groups ($p < 0.05$). The amount of new bone was smallest in the CPC control defects at each time period ($p < 0.05$). More new bone formed in hUCMSC and hBMSC groups. The difference between hUCMSC and hBMSC groups was not significant at each implantation period ($p > 0.1$).

Fig. 7B showed the new blood vessel density as a function of implantation time. The new vessel density increased with time for all groups ($p < 0.05$). Compared to CPC control, the presence of hBMSCs in CPC scaffolds resulted in approximately 20% increase in blood vessel density. The CPC containing hUCMSCs also had higher new vessel densities at 4, 12 and 24 w than CPC control. There was no significant difference in blood vessel density between hUCMSC and hBMSC groups at each time period ($p > 0.1$).

4. Discussion

This study investigated stem cell seeding on macroporous CPC-RGD scaffolds in an animal model and determined whether hUCMSCs could achieve similar bone regeneration efficacy to hBMSCs *in vivo* for the first time. hBMSCs are frequently studied for bone tissue engineering applications. However, hBMSCs require an invasive procedure to harvest, and their potency may be lost due to aging and diseases of the patients. In the present study, hUCMSCs were shown to yield similar cell viability, osteogenic gene expressions and bone mineral synthesis *in vitro* when seeded on macroporous CPC, compared to hBMSCs harvested from young adults. Furthermore, for the first time, hUCMSCs were shown to generate *in vivo* BMD, Tb.Th, new bone amounts and new blood vessel density similar to hBMSCs in critical-sized cranial defects in rats. Therefore, hUCMSCs are highly promising as a potential alternative to the gold-standard hBMSCs for bone regeneration.

Due to its injectability and bioactivity, CPC is a promising carrier for delivery of stem cells for bone regeneration. The incorporation of chitosan into CPC improved the load-bearing capability of CPC [46,47]. The incorporation of G4RGDSP sequence into the chitosan in CPC was shown to further increase the strength and toughness of CPC scaffold [37]. Furthermore, the incorporation of RGD sequence into CPC enhanced the biological

behaviors of cells [37]. RGD can promote the attachment of cells because it is recognized by the adhesion receptors on the cell membrane. Thus, RGD could improve the affinity between cells and biomaterials [48,49]. A previous study showed that RGD in CPC increased cell attachment and proliferation [37]. In the present study, both hUCMSCs and hBMSCs achieved good cell attachment and proliferation on RGD-grafted CPC. In addition, cells on CPC differentiated into the osteogenic lineage, showing high expressions of osteogenic markers and mineral synthesis by both hUCMSCs and hBMSCs. It is well known that ALP, OC, Coll I and Runx2 play key roles in the osteogenic differentiation of MSCs [50]. The present study showed that these osteogenic markers were all expressed in both hUCMSCs and hBMSCs on CPC. While ALP expression peaked at 4 d, OC, Coll I and Runx2 peaked at 7 d. The earlier peak of ALP was consistent with previous studies about the cascade events for osteogenic differentiation [37,51,52]. In addition, ARS staining and mineral concentration measurement showed that both hUCMSCs and hBMSCs synthesized bone minerals on CPC. Therefore, both hUCMSCs and hBMSCs achieved similarly good attachment, proliferation, osteogenic differentiation and mineralization on CPC.

The transplantation of hUCMSCs and hBMSCs improved ossification in the calvarial model. BMD analysis by micro-CT revealed that the amount of mineralized tissues in the stem cell groups was much higher than that in CPC control group without stem cells. Tb.Th analysis only revealed significant difference between control and hBMSC group. Though hBMSC group achieved the greatest BMD and Tb.Th among three groups, the differences between hUCMSCs and hBMSCs were significant only at early implantation period (BMD at 4 w, and Tb.Th at 4 and 12 w). These results indicate that the contribution of hUCMSCs to bone formation may be comparable to hBMSCs in the long-term.

Histological analysis demonstrated that new bone formation was clearly seen in macroporous CPC throughout the observation period. A previous study reported that CPC alone could achieve $13.89 \pm 2.95\%$ of new bone at 8 weeks [53], while the present study reports $12.79 \pm 1.06\%$ new bone for CPC control at 12 weeks. Considering the differences in CPC scaffold microstructure, animal models and time periods between these two studies, these results were quite consistent. Another previous study showed that seeding rat BMSCs in CPC could achieve $32.60 \pm 6.41\%$ of new bone at 8 weeks [53], while the present study reported $24.86 \pm 3.94\%$ new bone at 12 weeks using hBMSCs. This difference may be due to rat BMSCs having higher osteogenic potential for allograft than hBMSCs for xenotransplantation.

In the present study, the stem cell groups achieved more new bone formation than the control group without stem cells. In addition, more new bone formation was observed on the dura side of the scaffold on which stem cells had been seeded before implantation. These results suggest that the transplanted stem cells had participated in and contributed to new bone formation. Approximately 132%, 94% and 88% increases of new bone were achieved when hBMSCs were transplanted at 4, 12 and 24 weeks, respectively. The cell's contribution agrees with a previous report which showed a 134% increase for rat BMSCs at 8 weeks [53]. hUCMSCs in macroporous CPC generated similar amounts of new bone in the critical-sized defects of rat cranium, compared to hBMSCs. These results suggest that hUCMSCs could have a comparable osteogenic potential *in vivo*, compared to hBMSCs.

Angiogenesis is the process of new blood vessels sprouting from established vessels, which plays a key role in regenerative processes. The regenerating new tissues have low oxygen tension and high metabolic needs which promote angiogenesis [54]. Thus, a dense capillary network during repair is needed to deliver oxygen and nutrients, and to clear away cellular debris [55]. Bone regeneration needs good revascularization in the differentiating tissues. Indeed, blood vessel number was found to be positively correlated to bone formation rate [56], and a temporal and spatial coupling of angiogenesis to bone formation and resorption was noted [57]. Therefore, the sprouting of vessels from surrounding host bone into a defect is important for osteogenesis [58]. One method to evaluate the presence of blood vessels in a tissue is to count the microvessels to calculate the new vessel density [58,59]. In the present study, the mean new vessel density in CPC control group at 4 w was about 20 vessels/mm², similar to previous reports (23.3 vessels/mm²) [60]. The stem cells in the defect resulted in low oxygen tension and high metabolic needs thus resulted in higher new vessel density than CPC control without stem cells. The application of stem cells also induced more new bone formation thus requiring angiogenesis to also increase accordingly. For example, a previous study showed that in the regions of poor vascularity, the undifferentiated pluripotent cells were shunted into a chondrogenic, rather than an osteogenic, pathway [54]. In the present study, no chondro-like tissues were found in the defects. Good vascularization was achieved for bone regeneration in the athymic nude rat model with macroporous CPC, reaching 31-33 vessels/mm² at 24 w for both hUCMSC and hBMSC groups.

In vivo data on the osteogenic potential of hUCMSCs to heal bone defects remain scarce. These cells are enormously attractive for clinical use because of their low cost, inexhaustible cell source, easy accessibility without an additional invasive procedure, high plasticity and developmental flexibility, great expansion capability, minimal immunorejection, and non-tumorigenesis [21]. Prior to clinical use, however, it is necessary to prove that their osteogenic potential is comparable to the gold-standard hBMSCs. A previous *in vitro* study showed that hUCMSCs may have better osteogenic potential than hBMSCs when cells were encapsulated in CPC [43]. With low oxygen pressure in the stem cell-encapsulating CPC construct, hUCMSCs may have better biological performance *in vitro*, because undifferentiated stem cells from baby umbilical cords are much younger than MSCs isolated from adult bone marrow. In the present study, hUCMSCs showed similar osteogenic potential to hBMSCs *in vitro*, likely because macroporous RGD-grafted CPC was cell-friendly and hence both cells grew similarly well. On the other hand, hBMSCs were shown to be more similar to osteoblasts than hUCMSCs, suggesting a better osteogenic potential for hBMSCs [61]. Thus, it remains possible that hUCMSCs do not differentiate spontaneously toward any cell type without additional exogenous inductive factors, while hBMSCs would be more destined to generate bone. This may explain why hUCMSCs showed a slightly lower osteogenic potential than hBMSCs *in vivo* in the present study. However, the new bone area fraction and blood vessel density were generally not significantly different between hUCMSC and hBMSC groups. In addition, while the hBMSCs in the present study came from young adults, the self-renewal and proliferative ability of hBMSCs will decrease with aging and diseases of the patients [9-11]. Therefore, hUCMSCs are promising to offer good osteogenic performance and serve as a viable alternative to hBMSCs, especially for patients in old age and/or with certain diseases.

5. Conclusions

Macroporous RGD-grafted CPC was seeded with stem cells to repair cranial defects in rats, and hUCMSCs and hBMSCs on CPC were compared for bone regeneration *in vivo* for the first time. Both hUCMSCs and hBMSCs seeded with CPC showed excellent attachment, osteogenic differentiation and mineralization *in vitro*. New bone and blood vessels were generated, which increased with time from 4 to 24 weeks. Seeding stem cells with CPC increased new bone and new blood vessel density, compared to CPC without cells. Comparing between CPC groups with and without cells, bone mineral density at 24 weeks was increased by 9% to 19% due to stem cell seeding; new bone area fraction was increased by 57% to 88%; and new blood vessel density was increased by 15% to 20%. In general, hUCMSCs in CPC yielded statistically similar new bone and new blood vessel density to hBMSCs. While hBMSCs require an invasive procedure to harvest and will lose their potency due to aging/diseases, hUCMSCs can be harvested at a low cost, are inexhaustible and have a high plasticity and developmental capability. Therefore, hUCMSCs appeared to be useful for bone tissue engineering, and RGD-grafted macroporous CPC is promising for delivering both hUCMSCs and hBMSCs for craniofacial and orthopedic applications.

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References

- [1]. Mikos AG, Herring SW, Ochareon P, Elisseeff J, Lu HH, Kandel R, et al. Engineering complex tissues. *Tissue Eng.* 2006; 12:3307–39. [PubMed: 17518671]
- [2]. Kaigler D, Krebsbach PH, Wang Z, West ER, Horger K, Mooney DJ. Transplanted endothelial cells enhance orthotopic bone regeneration. *J Dent Res.* 2006; 85:633–637. [PubMed: 16798864]
- [3]. Deville S, Saiz E, Nalla RK, Tomsia AP. Freezing as a path to build complex composites. *Science.* 2006; 311:515–8. [PubMed: 16439659]
- [4]. Johnson PC, Mikos AG, Fisher JP, Jansen JA. Strategic directions in tissue engineering. *Tissue Eng.* 2007; 13:2827–37. [PubMed: 18052823]
- [5]. Sundelacruz S, Kaplan DL. Stem cell- and scaffold-based tissue engineering approaches to osteochondral regenerative medicine. *Semin Cell Dev Biol.* 2009; 20:646–55. [PubMed: 19508851]
- [6]. Varghese S, Hwang NS, Ferran A, Hillel A, Theprungsirikul P, Canver AC, et al. Engineering musculoskeletal tissues with human embryonic germ cell derivatives. *Stem Cells.* 2010; 28:765–74. [PubMed: 20178108]
- [7]. Mao, JJ.; Vunjak-Novakovic, G.; Mikos, AG.; Atala, A. *Translational Approaches in Tissue Engineering and Regenerative Medicine*, Chapters 1-3. Artech House; Boston, MA: 2007.
- [8]. Quarto R, Thomas D, Liang CT. Bone progenitor cell deficits and the age-associated decline in bone repair capacity. *Calcif Tissue Int.* 1995; 56:123–9. [PubMed: 7736320]
- [9]. Mueller SM, Glowacki J. Age-related decline in the osteogenic potential of human bone marrow cells cultured in three-dimensional collagen sponges. *J Cell Biochem.* 2001; 82:583–90. [PubMed: 11500936]

- [10]. Mendes SC, Tibbe JM, Veenhof M, Bakker K, Both S, Platenburg PP, et al. Bone tissue-engineered implants using human bone marrow stromal cells: effect of culture conditions and donor age. *Tissue Eng.* 2002; 8:911–20. [PubMed: 12542937]
- [11]. Stenderup K, Justesen J, Clausen C, Kassem M. Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. *Bone.* 2003; 33:919–26. [PubMed: 14678851]
- [12]. Rodriguez JP, Montecinos L, Rios S, Reyes P, Martinez J. Mesenchymal stem cells from osteoporotic patients produce a type I collagen-deficient extracellular matrix favoring adipogenic differentiation. *J Cell Biochem.* 2000; 79:557–65. [PubMed: 10996846]
- [13]. Suzuki Y, Kim KJ, Kotake S, Itoh T. Stromal cell activity in bone marrow from the tibia and iliac crest of patients with rheumatoid arthritis. *J Bone Miner Metab.* 2001; 19:56–60. [PubMed: 11156475]
- [14]. Wang HS, Hung SC, Peng ST, Huang CC, Wei HM, Guo YJ, et al. Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. *Stem Cells.* 2004; 22:1330–7. [PubMed: 15579650]
- [15]. Zebardast N, Lickorish D, Davies JE. Human umbilical cord perivascular cells (HUCPVC): A mesenchymal cell source for dermal wound healing. *Organogenesis.* 2010; 6:197–203. [PubMed: 21220956]
- [16]. Baksh D, Yao R, Tuan RS. Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. *Stem Cells.* 2007; 25:1384–92. [PubMed: 17332507]
- [17]. Bailey MM, Wang L, Bode CJ, Mitchell KE, Detamore MS. A comparison of human umbilical cord matrix stem cells and temporomandibular joint condylar chondrocytes for tissue engineering temporomandibular joint condylar cartilage. *Tissue Eng.* 2007; 13:2003–10. [PubMed: 17518722]
- [18]. Wang L, Singh M, Bonewald LF, Detamore MS. Signalling strategies for osteogenic differentiation of human umbilical cord mesenchymal stromal cells for 3D bone tissue engineering. *J Tissue Eng Regen Med.* 2009; 3:398–404. [PubMed: 19434662]
- [19]. Can A, Karahuseyinoglu S. Concise review: human umbilical cord stroma with regard to the source of fetus-derived stem cells. *Stem Cells.* 2007; 25:2886–95. [PubMed: 17690177]
- [20]. Karahuseyinoglu S, Kocaefe C, Balci D, Erdemli E, Can A. Functional structure of adipocytes differentiated from human umbilical cord stroma-derived stem cells. *Stem Cells.* 2008; 26:682–91. [PubMed: 18192234]
- [21]. Zhao L, Burguera EF, Xu HHK, Amin N, Ryou H, Arola DD. Fatigue and human umbilical cord stem cell seeding characteristics of calcium phosphate-chitosan-biodegradable fiber scaffolds. *Biomaterials.* 2010; 31:840–7. [PubMed: 19850337]
- [22]. Wang L, Dormer NH, Bonewald LF, Detamore MS. Osteogenic differentiation of human umbilical cord mesenchymal stromal cells in polyglycolic acid scaffolds. *Tissue Eng Part A.* 2010; 16:1937–48. [PubMed: 20070186]
- [23]. Zhao L, Weir MD, Xu HHK. Human umbilical cord stem cell encapsulation in calcium phosphate scaffolds for bone engineering. *Biomaterials.* 2010; 31:3848–57. [PubMed: 20149437]
- [24]. Chen WC, Zhou HZ, Tang MH, Weir MD, Bao CY, Xu HHK. Gas-foaming calcium phosphate cement scaffold encapsulating human umbilical cord stem cells. *Tissue Eng Part A.* 2012; 18:816–27. [PubMed: 22011243]
- [25]. Diao Y, Ma Q, Cui F, Zhong Y. Human umbilical cord mesenchymal stem cells: osteogenesis in vivo as seed cells for bone tissue engineering. *J Biomed Mater Res A.* 2009; 91:123–31. [PubMed: 18767055]
- [26]. Ruhe PQ, Hedberg-Dirk EL, Padron NT, Spauwen PH, Jansen JA, Mikos AG. Porous poly(DL-lactic-co-glycolic acid)/calcium phosphate cement composite for reconstruction of bone defects. *Tissue Eng.* 2006; 12:789–800. [PubMed: 16674292]
- [27]. 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; 25:3453–62. [PubMed: 15020119]

- [28]. Habib M, Baroud G, Gitzhofer F, Böhner M. Mechanisms underlying the limited injectability of hydraulic calcium phosphate paste. *Acta Biomater.* 2008; 4:1465–71. [PubMed: 18445539]
- [29]. Zuo Y, Yang F, Wolke JG, Li Y, Jansen JA. Incorporation of biodegradable electrospun fibers into calcium phosphate cement for bone regeneration. *Acta Biomater.* 2010; 6:1238–47. [PubMed: 19861181]
- [30]. 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; 43:428–32. [PubMed: 9855201]
- [31]. Jansen JA, Veho JW, Ruhe PQ, Kroeze-Deutman H, Kuboki Y, Takita H, et al. Growth factor-loaded scaffolds for bone engineering. *J Control Release.* 2005; 101:127–36. [PubMed: 15588899]
- [32]. Kasten P, Beyen I, Niemeyer P, Luginbuhl R, Böhner M, Richter W. Porosity and pore size of beta-tricalcium phosphate scaffold can influence protein production and osteogenic differentiation of human mesenchymal stem cells: an in vitro and in vivo study. *Acta Biomater.* 2008; 4:1904–15. [PubMed: 18571999]
- [33]. Böhner M. Design of ceramic-based cements and putties for bone graft substitution. *Eur Cell Mater.* 2010; 20:1–12. [PubMed: 20574942]
- [34]. Böhner M, Baroud G. Injectability of calcium phosphate pastes. *Biomaterials.* 2005; 26:1553–63. [PubMed: 15522757]
- [35]. Brown, WE.; Chow, LC. A new calcium phosphate water setting cement. In: Brown, PW., editor. *Cements Research Progress.* Am Ceram Soc; Westerville, OH: 1986. p. 352-79.
- [36]. Schneiders W, Reinstorf A, Pompe W, Grass R, Biewener A, Holch M, et al. Effect of modification of hydroxyapatite/collagen composites with sodium citrate, phosphoserine, phosphoserine/RGD-peptide and calcium carbonate on bone remodelling. *Bone.* 2007; 40:1048–59. [PubMed: 17223400]
- [37]. Chen WC, Zhou HZ, Weir MD, Tang MH, Bao CY, Xu HHK. Human embryonic stem cell-derived mesenchymal stem cell seeding on calcium phosphate cement-chitosan-RGD scaffold for bone repair. *Tissue Eng Part A.* 2013; 19:915–27. [PubMed: 23092172]
- [38]. Chen WC, Zhou HZ, Weir MD, Bao CY, Xu HHK. Umbilical cord stem cells released from alginate-fibrin microbeads inside macroporous and biofunctionalized calcium phosphate cement for bone regeneration. *Acta Biomater.* 2012; 8:2297–306. [PubMed: 22391411]
- [39]. Park KM, Joung KJ, Park KD, Lee SY, Lee MC. RGD-conjugated chitosan-pluronic hydrogels as a cell supported scaffold for articular cartilage regeneration. *Macromol Res.* 2008; 16:517–23.
- [40]. Zhou HZ, Chen WC, Weir MD, Xu HHK. Biofunctionalized calcium phosphate cement to enhance the attachment and osteodifferentiation of stem cells released from fast-degradable alginate-fibrin microbeads. *Tissue Eng Part A.* 2012; 18:1583–95. [PubMed: 22435653]
- [41]. Hesarakis S, Zamanian A, Moztarzadeh F. The influence of the acidic component of the gas-foaming porogen used in preparing an injectable porous calcium phosphate cement on its properties: acetic acid versus citric acid. *J Biomed Mater Res B Appl Biomater.* 2008; 86:208–16. [PubMed: 18161816]
- [42]. Cicuttini FM, Begley CG, Boyd AW. The effect of recombinant stem cell factor (SCF) on purified CD34-positive human umbilical cord blood progenitor cells. *Growth Factors.* 1992; 6:31–9. [PubMed: 1375477]
- [43]. Xu HHK, Zhao L, Weir MD. Stem cell-calcium phosphate constructs for bone engineering. *J Dent Res.* 2010; 89:1482–8. [PubMed: 20929721]
- [44]. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} Method. *Methods.* 2001; 25:402–8. [PubMed: 11846609]
- [45]. Zou D, Zhang Z, He J, Zhu S, Wang S, Zhang W, et al. Repairing critical-sized calvarial defects with BMSCs modified by a constitutively active form of hypoxia-inducible factor-1alpha and a phosphate cement scaffold. *Biomaterials.* 2011; 32:9707–18. [PubMed: 21975460]
- [46]. Xu HHK, Simon CG Jr. Fast setting calcium phosphate-chitosan scaffold: mechanical properties and biocompatibility. *Biomaterials.* 2005; 26:1337–48. [PubMed: 15482821]

- [47]. Weir MD, Xu HHK. Culture human mesenchymal stem cells with calcium phosphate cement scaffolds for bone repair. *J Biomed Mater Res B Appl Biomater.* 2010; 93:93–105. [PubMed: 20091907]
- [48]. El-Ghannam AR, Ducheyne P, Risbud M, Adams CS, Shapiro IM, Castner D, et al. Model surfaces engineered with nanoscale roughness and RGD tripeptides promote osteoblast activity. *J Biomed Mater Res A.* 2004; 68:615–27. [PubMed: 14986317]
- [49]. Secchi AG, Grigoriou V, Shapiro IM, Cavalcanti-Adam EA, Composto RJ, Ducheyne P, et al. RGD peptides immobilized on titanium alloy stimulate bone cell attachment, differentiation and confer resistance to apoptosis. *J Biomed Mater Res A.* 2007; 83:577–84. [PubMed: 17503524]
- [50]. 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; 10:1810–7. [PubMed: 19469498]
- [51]. 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; 28:4091–7. [PubMed: 17586040]
- [52]. Benoit DS, Collins SD, Anseth KS. Multifunctional hydrogels that promote osteogenic hMSC differentiation through stimulation and sequestering of BMP2. *Adv Funct Mater.* 2007; 17:2085–93. [PubMed: 18688288]
- [53]. Zhu C, Chang Q, Zou D, Zhang W, Wang S, Zhao J, et al. LvBMP-2 gene-modified BMSCs combined with calcium phosphate cement scaffolds for the repair of calvarial defects in rats. *J Mater Sci Mater Med.* 2011; 22:1965–73. [PubMed: 21681654]
- [54]. Carter, DR.; Loba, PEG.; Beaupre, GS. Mechanical influences on skeletal regeneration and bone resorption. In: Davies, JE., editor. *Bone engineering.* Em Squared Inc; Toronto: 2000. p. 358-68.
- [55]. Lakey, LA.; Akella, R.; Ranieri, JP. Angiogenesis: implications for tissue repair. In: Davies, JE., editor. *Bone Engineering.* Em Squared Inc; Toronto, Canada: 2000. p. 137-142.
- [56]. Yao Z, Lafage-Proust MH, Plouet J, Bloomfield S, Alexandre C, Vico L. Increase of both angiogenesis and bone mass in response to exercise depends on VEGF. *J Bone Miner Res.* 2004; 19:1471–80. [PubMed: 15312247]
- [57]. Veillette CJ, von Schroeder HP. Endothelin-1 down-regulates the expression of vascular endothelial growth factor-A associated with osteoprogenitor proliferation and differentiation. *Bone.* 2004; 34:288–96. [PubMed: 14962807]
- [58]. Kleinheinz J, Stratmann U, Joos U, Wiesmann HP. VEGF-activated angiogenesis during bone regeneration. *J Oral Maxillofac Surg.* 2005; 63:1310–6. [PubMed: 16122595]
- [59]. Piattelli A, Degidi M, Di Stefano DA, Rubini C, Fioroni M, Strocchi R. Microvessel density in alveolar ridge regeneration with autologous and alloplastic bone. *Implant Dent.* 2002; 11:370–5. [PubMed: 12518705]
- [60]. Degidi M, Artese L, Rubini C, Perrotti V, Iezzi G, Piattelli A. Microvessel density and vascular endothelial growth factor expression in sinus augmentation using Bio-Oss. *Oral Dis.* 2006; 12:469–75. [PubMed: 16910917]
- [61]. Hsieh JY, Fu YS, Chang SJ, Tsuang YH, Wang HW. Functional module analysis reveals differential osteogenic and stemness potentials in human mesenchymal stem cells from bone marrow and Wharton's jelly of umbilical cord. *Stem Cells Dev.* 2010; 19:1895–910. [PubMed: 20367285]

1.

Live/dead assay and scanning electron microscopy (SEM) of hUCMSCs and hBMSCs on macroporous CPC scaffolds. (A, B) Representative photos of cells on scaffolds at 14 d. (C) Percentage of live cells. (D) Live cell density (number of live cells per mm²), which increased with time due to cell proliferation. Bars with dissimilar letters indicate significantly different values ($p < 0.05$). Each value is mean $\pm$ sd; $n = 5$. (E) and (F) Representative SEM micrographs of cells attaching to CPC at 14 d. Both hUCMSCs and hBMSCs attached and proliferated well on CPC scaffolds.

2.

RT-PCR results for osteogenic differentiation of hUCMSCs and hBMSCs on macroporous CPC: (A) ALP, (B) OC, (C) Coll I, and (D) Runx2 gene expressions. Both hUCMSCs and hBMSCs attaching to CPC scaffolds showed osteogenic differentiation. ALP peaked at 4 d. OC, Coll I and Runx2 peaked at 7 d. Bars with dissimilar letters indicate significantly different values ($p < 0.05$). Each value is mean $\pm$ sd; $n = 5$.

3.

Cell mineralization on CPC scaffolds. (A) ARS staining of minerals synthesized by hUCMSCs and hBMSCs on RGD-grafted CPC from 7 d to 21 d. The upper labels indicate the cell type. (B) Cell-synthesized mineral concentration was measured by the osteogenesis assay (mean $\pm$ sd; n = 5). Bars with dissimilar letters indicate significantly different values ($p < 0.05$).

4.

Micro-CT analysis of the repaired rat cranium at 4, 12 and 24 weeks (w) after implantation.

(A) Representative sagittal micro-CT images of CPC control as examples. All other constructs had similar qualitative appearances. (B) Quantitative morphometric analysis of bone mineral density (BMD) in the repaired cranial defect area. (C) Trabecular bone thickness (Tb.Th). In each plot, values indicated with dissimilar letters are significantly different from each other ($p < 0.05$). Each value is mean $\pm$ sd; $n = 5$.

5.

Representative H&E staining histological images. The group names are listed on the upper left corner of each image. The time periods are listed on the right side of each image. The periosteal side is on the top of the image, and the dura side is on the bottom of the image. New bone formation (arrows) was observed for all groups at each implantation period. Examination of all images from all the defects indicated that there was more new bone with increasing time from 4 to 24 weeks, and there was more new bone in constructs with stem cells than those without stem cells.

6.

High magnification images showing typical details in defects. (A) High magnification image of the solid-line rectangle in the last image of Fig. 5. Osteoblasts with a spindle morphology were found around new bone. Osteocytes were found inside new bone. New blood vessels were found both within and around the new bone area. (B) High magnification image of the dotted-line rectangle in the last image of Fig. 5. Osteoclast-like multinuclear giant cells (encircled by yellow lines) surrounded the CPC surface in the resorption lacunae. New vessels were found in defects.

7.

Histomorphometry analysis for the rat cranial defect model. (A) Percentage of new bone area. (B) New blood vessel density. Scaffolds containing hBMSCs and hUCMSCs showed higher new bone area and more new vessels than CPC control. There was no significant difference between hUCMSC and hBMSC group at each implantation period. Bars with dissimilar letters indicate significantly different values ($p < 0.05$). Each value is mean $\pm$ sd; $n = 5$.