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# Bone tissue engineering via human induced pluripotent, umbilical cord and bone marrow mesenchymal stem cells in rat cranium

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ABSTRACT

Human induced pluripotent stem cells (hiPSCs) are an exciting cell source with great potential for tissue engineering. Human bone marrow mesenchymal stem cells (hBMSCs) have been used in clinics but are limited by several disadvantages, hence alternative sources of MSCs such as umbilical cord MSCs (hUCMSCs) are being investigated. However, there has been no report comparing hiPSCs, hUCMSCs and hBMSCs for bone regeneration. The objectives of this pilot study were to investigate hiPSCs, hUCMSCs and hBMSCs for bone tissue engineering, and compare their bone regeneration via seeding on biofunctionalized macroporous calcium phosphate cement (CPC) in rat cranial defects. For all three types of cells, approximately 90% of the cells remained alive on CPC scaffolds. Osteogenic genes were up-regulated, and mineral synthesis by cells increased with time *in vitro* for all three types of cells. The new bone area fractions at 12 weeks (mean ± sd; $n = 6$) were (30.4 ± 5.8)%, (27.4 ± 9.7)% and (22.6 ± 4.7)% in hiPSC–MSC–CPC, hUCMSC–CPC and hBMSC–CPC respectively, compared to (11.0 ± 6.3)% for control ($p < 0.05$). No significant differences were detected among the three types of stem cells ($p > 0.1$). New blood vessel density was higher in cell-seeded groups than control ($p < 0.05$). *De novo* bone formation and participation by implanted cells was confirmed via immunohistochemical staining. In conclusion, (1) hiPSCs, hUCMSCs and hBMSCs greatly enhanced bone regeneration, more than doubling the new bone amount of cell-free CPC control; (2) hiPSC–MSCs and hUCMSCs represented viable alternatives to hBMSCs; (3) biofunctionalized macroporous CPC-stem cell constructs had a robust capacity for bone regeneration.

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## 1. Introduction

In the field of bone tissue engineering and regenerative medicine, generating patient-specific stem cells has been a long-standing goal [1–3]. Human bone marrow mesenchymal stem cells (hBMSCs) are the current gold-standard cell source for many tissue-engineering therapies and have been successfully applied in clinics [4,5]. However, hBMSCs are hampered by an invasive harvesting procedure, limited availability, and loss of potency in seniors or patients with certain diseases and disorders [6]. Human umbilical cord MSCs (hUCMSCs) are capable of differentiating into mesenchymal lineages. They are easily and abundantly available, with robust proliferation and self-renewal capability due to their origin in neonatal tissues [7]. The main obstacle of hUCMSCs is immunogenic concerns when used heterologously. For autologous applications, the umbilical cord has to be properly cryopreserved from childbirth for an extended period of time.

The breakthrough discovery of induced pluripotent stem cells (iPSCs) by Takahashi et al. and Yu et al. offers the possibility of generating a high yield of custom-tailored stem cells [8,9]. This provides the excitement of turning back somatic cells' developmental clock into pluripotent stem cells. hiPSCs represent an enormous source of patient-specific stem cells derived from

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easily accessible cells like fibroblasts [10], keratinocytes [11], blood cells [12], etc. Much progress has been achieved in the investigation of iPSCs, from the use of viral integration [8,9] to virus-free or vector-free non-integrating strategies [13,14], and a better understanding of its similarities and differences to embryonic stem cells (ESCs) [15]. With similar pluripotency as ESCs, yet without the ethical or immunogenic concerns related to ESCs, iPSCs are a potentially new frontier for cell-based regenerative medicine [16]. To date, the application of hiPSCs in osteo-regenerative medicine is a relatively new research field. An extensive literature search revealed only 16 papers on the osteogenic differentiation of hiPSCs and potential applications in bone tissue engineering [17–32], including two for periodontal tissue engineering [28,29]. These findings offered evidence that hiPSCs could be induced into mesenchymal or osteoblastic lineages, presenting alkaline phosphatase (ALP) activity, osteogenic gene expression and mineral synthesis. *In vivo* studies demonstrated *de novo* bone formation or mineral deposition in hiPSC-implanted scaffolds and direct involvement of transplanted cells in bone regeneration [17,20–22,25,27–30]. Thus, hiPSCs or their progeny (hiPSC-derived cells) seeded in appropriate scaffolds could provide a promising strategy for bone tissue engineering.

Calcium phosphate cements have excellent biocompatibility, osteoconductivity, in situ-hardening and molding capabilities and injectability, and can be resorbed and replaced by new bone *in vivo* [33–38]. The first such cement was developed in 1986 and consisted of a mixture of tetracalcium phosphate (TTCP) and dicalcium phosphate anhydrous (DCPA) (referred to as CPC) [39]. CPC was approved in 1996 by the Food and Drug Administration (FDA) for repairing craniofacial defects. Our previous studies enhanced the mechanical, physical and biological properties of CPC through the introduction of absorbable fibers [40], chitosan [41], mannitol porogen [42], gas-foaming agents [43], alginate microbeads [44], and biofunctionalization [45]. These approaches improved the CPC's mechanical strength, setting time, degradability, macroporosity, cell attachment, and delivery of cells and growth factors. Thus, CPC has great potential for bone repair and augmentation. In the present study, water-soluble mannitol porogens were incorporated into CPC to induce macroporosity [46]. Arg-Gly-Asp (RGD), a short integrin-recognition sequence, was also incorporated into CPC to promote cell attachment to scaffold [45,47]. To date, there has been no report on the comparison of hiPSCs, hUCMSCs and hBMSCs seeded on CPC scaffolds for bone regeneration *in vivo*.

Therefore, the objectives of this study were to: (1) investigate hiPSCs, hUCMSCs and hBMSCs for bone tissue engineering; and (2) seed hiPSCs, hUCMSCs and hBMSCs on biofunctionalized macroporous CPC and compare their bone regeneration efficacy in cranial defects in rats for the first time. The following hypotheses were tested: (1) hiPSCs and hUCMSCs would be viable alternative cell sources for bone tissue engineering, matching the bone regeneration of the gold-standard hBMSCs *in vivo*; (2) all stem cell groups (hiPSCs, hUCMSCs and hBMSCs) would greatly enhance bone regeneration *in vivo*, compared to CPC control without cell seeding; (3) biofunctionalized macroporous CPC would be suitable scaffolds to deliver and support stem cells with a robust bone regeneration capacity.

## 2. Materials and methods

### 2.1. Fabrication of RGD-coated macroporous CPC

CPC powder was prepared following previous studies [40–45]. Briefly, TTCP [$Ca_4(PO_4)_2O$] was synthesized using DCPA ($CaHPO_4$) and calcium carbonate (both from J.T. Baker, Philipsburg, 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 in a desiccator, ground in a ball mill (Retsch PM4, Brinkman, NY) and sieved to obtain TTCP powder with a median particle size of 5 μm. The commercial DCPA powder was ground for 24 h in the ball mill in 95% ethanol and sieved to obtain a median particle size of approximately 1 μm. Then the TTCP and DCPA powders at 1:3 M ratio were thoroughly mixed in a micromill (Bel-Alert Products, Pequannock, NJ) to form the CPC powder [45,46].

Water-soluble mannitol ($CH_2OH[CHOH]_4CH_2OH$, Sigma, St. Louis, MO) particles were used as a porogen to create macropores in CPC. Mannitol was recrystallized in an ethanol/water solution at 50/50 by volume, dried, ground, and sieved through openings of 500 μm (top sieve) and 300 μm (bottom sieve). The mannitol particles were mixed with CPC powder at a mannitol/(mannitol + CPC powder) mass fraction of 40% [46]. The previous study showed that the CPC paste containing 40% of mannitol was fully injectable under a syringe force of 10 N, and the set CPC scaffold had a macroporosity of (50.9 ± 6.7)% and a total porosity of (82.6 ± 2.4)% by volume [46]. The CPC liquid contained 0.2 M $Na_2HPO_4$ in distilled water to accelerate the setting reaction [46]. A powder:liquid mass ratio of 2:1 was used to form a flowable CPC paste. The paste was placed in molds with a diameter of 8 mm and a thickness of 1 mm. After incubation in a humidor for 1 day at 37 °C, the disks were demolded and immersed in distilled water at 37 °C for 3 days to dissolve the mannitol. CPC disks were autoclaved, and then placed into 24-well plates with one disk per well. RGD, a well-known integrin recognition sequence, was used to promote cell adhesion on synthetic surfaces [45,47]. Recent studies showed that RGD-biofunctionalized scaffolds exhibited better cell attachment, proliferation, osteogenic differentiation and mineral deposition, than controls [45,47]. Thus, RGD was used in this study to promote cell attachment to CPC. RGD (Sigma) was reconstituted in 0.1 M acetic acid, diluted in phosphate-buffered saline (PBS) to 1000 μg/mL. Each CPC disk was immersed in 100 μL RGD solution for 1 h at 37 °C. When CPC was soaked in the RGD solution, RGD was adsorbed onto the surface and into the pores of the scaffold [48]. In our preliminary study, SEM examination showed that 3 h after cell-seeding, most hiPSC-MSCs on CPC scaffold without RGD were still spherical or fusiform, while cells on CPC scaffold with RGD exhibited a healthy polygonal morphology with spreading cytoplasmic extensions. One day after cell seeding, live/dead staining of hiPSC-MSCs on CPC further confirmed the significant enhancement of cell attachment by soaking CPC scaffold with RGD. Based on the promising preliminary study, the simple method of soaking CPC scaffold in RGD solution was used.

### 2.2. hiPSC culture and derivation of hiPSC-MSCs

The culture of hiPSCs was approved by the University of Maryland Institutional Review Board (HP-00046649). Only one cell line per cell type was used in this pilot study. hiPSC BC1 line was derived from adult bone marrow CD34+ cells which were programed by a single episomal vector pEB-C5 as previously described [49]. Undifferentiated hiPSCs were maintained on mitotically-inactivated murine embryonic fibroblasts (MEF) feeder cells and fed with the hiPSCs culture medium. The hiPSCs culture medium consisted of 80% Dulbecco's modified Eagle medium (DMEM)/F12 (Invitrogen, Carlsbad, CA), 20% Knockout Serum Replacement (a serum-free formulation, Invitrogen), 1% MEM non-essential amino acids solution, 10 ng/mL basic fibroblast growth factor (β-FGF, Invitrogen), 1 mM L-glutamine (Sigma) and 0.1 mM β-mercaptoethanol (Sigma). hiPSCs were detached from a feeder layer and dissociated into clumps through treatment with 1 mg/mL collagenase type IV in DMEM/F12 at 37 °C for 6 min, followed by

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