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Novel hiPSC-based tri-culture for pre-vascularization of calcium phosphate scaffold to enhance bone and vessel formation

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ABSTRACT

Vascularization of tissue-engineered bone is a critical step in maintaining cell viability and advancing cell performance *in vivo*. In this study, a novel tri-culture system was developed to elicit pre-vascularization of calcium phosphate cement (CPC) scaffold in which human induced pluripotent stem cell-derived mesenchymal stem cells (hiPSMSCs) were seeded together with human umbilical vein endothelial cells (HUVECs) and pericytes. In a two-step methodology design, we first performed osteoinduction of the seeded hiPSMSCs on the CPC scaffold and then incorporated HUVECs and pericytes to the hiPSMSC-colonized CPC scaffold under a favorable culturing condition, with an objective to form a stable and functional capillary-like vascular network that sustained the engineered osseous tissue. The angiogenic and osteogenic effects of various culture strategies were studied and compared in nude rat model with cranial bone defects: (1) CPC scaffold alone (CPC control); (2) Pericyte-seeded CPC (CPC-pericytes); (3) HUVEC-seeded CPC (CPC-HUVECs); (4) hiPSMSC-seeded CPC (CPC-hiPSMSCs); (5) HUVECs co-cultured with hiPSMSCs on CPC (bi-culture group) and (6) HUVECs and pericytes co-cultured with hiPSMSCs on CPC (tri-culture group). After 12 weeks, tri-culture group showed the highest amount of new bone (new bone area fraction of (45.3 ± 2.7) %, $p < 0.05$) and vessel formation (new blood vessel density of (50.7 ± 3.8) vessels/mm^2, $p < 0.05$) in all groups. Our results demonstrated that the tri-culture strategy was effective in promoting angiogenesis and osteogenesis for bone tissue engineering.

1. Introduction

Recent advances in bone tissue engineering have shown the exciting potential therapeutic value of stem-cell based bone regenerative therapy for treating bony defects resulted by trauma, extensive orthopedic surgery, cleft palates, and other osteo-degenerative diseases [1,2]. The typical tissue engineering procedure involves culturing stem cells in a porous scaffold along with specific growth factors that trigger osteoinduction and osteogenesis. The engineered osseous tissue is then grafted to repair the bony defect [3,4]. In recent years, more and more hybrid materials are used as both the scaffold and the carrier for the delivery of cells, drugs, genes and biosensors [5–8]. Animal studies have shown that stem-cell based regenerative therapy is likely to produce more reliable and predictable results than scaffold alone in the management of bone defects [9]. This is because grafted osseous tissue not only provides the direct cell source required for tissue regeneration, but also triggers the endogenous bone repair mechanism through the recruitment of host stem/progenitor cells to the lesion site [9]. As the most recent addition to stem cell engineering, induced pluripotent stem cells (iPSCs) have become a promising candidate for bone tissue engineering [10,11]. Our recent study showed that human iPSC derived mesenchymal stem cells (hiPSMSCs) exhibited a comparable *in vivo* bone regeneration capability to human bone marrow stem cells (hBMSCs) and human umbilical cord MSCs (hUCMSCs) when seeded on calcium phosphate cement (CPC) scaffolds to repair the bone defect of critical size defects in rat calvaria cultures [12].

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Adequate and quick vascularization is essential for optimal bone regeneration [13,14]. Bone itself is a highly vascularized organ. The maintenance of the normal cell function, active remodeling and skeletal integrity strongly depends on vascular supplies [15,16]. The viability of the seeded cells in the scaffold will be significantly compromised if their distance to the nearest capillary network is >100–200 μm, which exceeds the diffusion or perfusion limit of nutrients and oxygen [16]. The success of bone grafts has been heavily dependent on post-implant vascularization. However, the spontaneous angiogenesis may occur at a rate as slow as only ~5 μm/h with possible retraction and complete regression of entire neovessels [17]. This in turn may lead to ischemiac damage to the grafted tissue and potential failure [18,19]. Therefore, the establishment of fast and functional vascularization represents one of the major challenges for successful bone regeneration.

Pre-vascularization has emerged as a promising solution in this regard. In practice *in vivo* prevascularization can be achieved by surgically implanting a scaffold into certain parts of body where is abundant in blood vessels and easy-operating, such as a subcutaneous pocket or a muscle pouch [20,21]. Microvascular structures form a network within the engineered tissue construct as a result of invasion and outgrowth of the surrounding host microvasculature [22,23]. After the completion of prevascularization, the tissue construct is harvested and grafted to the defect site, the pre-built micro-capillary inside the construct inosculate and anastomose with the host blood vessels [22]. The disadvantages of this approach include invasive surgery, higher cost, and a relatively longer treatment process.

In vitro pre-vascularization represents a less invasive alternative in which vessel-forming cells are seeded on the engineered tissue construct-scaffold to form microvascular structures without the surgical insertion of tissue construct into a second surgical site. The vessel-forming cells primarily consist of endothelial cells (ECs), the main cellular component of the capillary walls [24–26]. The most widely used ECs are the human umbilical vein endothelial cells (HUVECs). Once seeded on the scaffold, these cells proliferate to form nascent microcapilliary-like structures which eventually interconnect into a microvascular network. However, the tubular structures formed by HUVECs are unstable and prone to regression in the absence of associated peri-vascular cells [27,28]. Perivascular cells such as pericytes contribute to the remodeling and maturation of the primitive vascular network [29,30]. Therefore, co-implantation of HUVECs with pericytes is essential for stable and durable engineered vasculature [9,28].

The purposes and significance of this study was to investigate the establishment of a functional and stable neovasculature in rats with a novel tri-culture of hiPSMSCs, HUVECs and pericytes on CPC scaffolds to promote the new bone formation for the first time. The triad of hiPSMSCs, HUVECs and pericytes targets at initiating a primary tubular network *via* HUVECs, transitioning it to mature vascularization through the involvement of pericytes, and then achieving osteogenesis by hiPSMSCs. The present study investigated the effectiveness of this tri-culture strategy both *in vitro* and *in vivo*. The following hypotheses were tested: (1) hiPSMSCs co-cultured with HUVECs and pericytes on macroporous CPC scaffolds could form pre-vascular networks *in vitro*; (2) Co-culturing with HUVECs and pericytes could promote osteogenic differentiation of hiPSMSCs on CPC scaffolds; (3) the pre-vascularized CPC scaffolds would significantly enhance angiogenesis and osteogenesis *in vivo* after the tissue construct was grafted. Our findings are expected to provide directions on the future development of prevascularized osseous tissues for clinical bone defect repairing and advancement of iPSC-based regenerative medicine.

2. Materials and methods

2.1. Manufacture of macroporous CPC scaffolds

CPC scaffolds were made according to our previous protocol [12,31–36]. The CPC powder was made up of TTCP ($Ca_4(PO_4)_2O$) and DCPA ($CaHPO_4$) powders and mixed at 1:1 M ratio. Water-soluble mannitol (Sigma, MO) crystals (around 125–250 μm) were chosen as a porogen to create macropores in CPC scaffolds. The mannitol particles were mixed with CPC powder at 40% mass fraction [37]. The CPC liquid consisted of 0.2 M Na_2HPO_4 in distilled water to accelerate the speed of the reaction and reduce the setting time [37]. The CPC powder and CPC liquid were mixed to create a flowable CPC paste at 2:1 mass ratio and set in molds (5 mm-diameter and 1 mm-thickness) to form the CPC disks. One-day incubation in a 37 °C humidor was required for complete setting reaction. The disks were then de-molded and soaked in distilled water bath at 37 °C for three days to dissolve the mannitol. The macropores and micropores of each CPC disk were 100–300 μm and 1–50 μm respectively [37]. CPC disks were autoclaved before using.

2.2. Cell culture and derivation of hiPSMSCs

Human iPSCs BC1 (hiPSCs BC1) cell line were derived from adult bone marrow CD34+ cells. In brief, hiPSCs were cultured on top of the mitotically-inactivated murine embryonic fibroblasts (MEF) feeder in hiPSCs culture medium [38]. After proliferation, hiPSC clones were detached from MEF and dissociated into clumps by 1 mg/mL collagenase type IV at 37 °C for 6 min. Then the sedimentation of the cell clumps was collected and transferred to ultra-low-attachment tissue culture flasks to form embryoid body (EB). After 10 days of EB suspension culture, EBs were transferred onto 0.1% gelatin coated cell culture dishes for re-attachment. Cells gradually migrated out from EBs and were sub-cultured in MSC growth medium which consisted of low glucose DMEM (Gibco), 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin and 100 mg/mL streptomycin (PS, Gibco). It was confirmed by our previous study that the derived cells expressed MSC surface markers (CD29, CD44, CD166, CD73), but were negative for typical hematopoietic (CD34), endothelial (CD31) and pluripotent markers (TRA-1-81 and OCT 3/4) [12]. The iPSC-derived cells were capable to differentiate into osteoblasts, chondrocytes and adipocytes [39]. Thus, the cells were named as hiPSC-derived MSCs (hiPSMSCs). Passage 3–4 hiPSMSCs were used in the following experiments.

HUVECs were commercially obtained from Lonza (Walkersville) and maintained in endothelial cell culture medium (EGM-2; Lonza). Pericytes were purchased from PromoCell (PromoCell) and cultured in PromoCell Cell Growth Medium. The 3rd passage of HUVECs and pericytes were used.

2.3. Cell seeding on macroporous CPC scaffolds

For the tri-culture (hiPSMSCs-HUVECs-pericytes) group and the bi-culture (hiPSMSCs-HUVECs) group, a two-step seeding protocol was adopted in this study in order to optimize the conditions for both osteogenesis and angiogenesis [9]. hiPSMSCs were first seeded on CPC scaffolds (1.5×10^5/scaffold) and cultured in osteogenic media (OS media, consisted of MSC growth media supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate, 0.05 mM ascorbic acid and 10 nM 1α,25-dihydroxyvitaminD3 (Sigma)) for 7 days. Our previous study showed that the expression of osteogenic markers in hiPSMSCs such as Runt-related transcription factor (Runx2), alkaline phosphatase (ALP) and collagen I (COL-1) significantly elevated 7 days after osteogenic induction on CPC scaffolds [12]. Thus, the 7-day pre-osteogenic protocol was applied here to commit these stem cells to the bone lineage. In addition, in order to provide a favorable extra-cellular matrix for HUVECs and/or pericytes, 1×10^5 HUVECs and 2.5×10^4 pericytes (HUVECs: pericytes = 4:1) for the tri-culture group or 1.25×10^5 HUVECs for the bi-culture group were suspended in 100 μL liquid Growth Factor Reduced Matrigel Matrix (BD Biosciences, San Diego, CA), and was added over the CPC scaffold pre-cultured with hiPSMSCs. The new construct was maintained in EGM-2 medium for the duration of the *in vitro* experiments or 14 days prior to implantation *in vivo*. For the mono-culture cell group, 2.75×10^5 cells/scaffold were seeded on