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Calcium phosphate cement scaffold with stem cell co-culture and prevascularization for dental and craniofacial bone tissue engineering

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ABSTRACT

Objective. Calcium phosphate cements (CPCs) mimic nanostructured bone minerals and are promising for dental, craniofacial and orthopedic applications. Vascularization plays a critical role in bone regeneration. This article represents the first review on cutting-edge research on prevascularization of CPC scaffolds to enhance bone regeneration.

Methods. This article first presented the prevascularization of CPC scaffolds. Then the co-culture of two cell types in CPC scaffolds was discussed. Subsequently, to further enhance the prevascularization efficacy, tri-culture of three different cell types in CPC scaffolds was presented.

Results. (1) Arg–Gly–Asp (RGD) incorporation in CPC bone cement scaffold greatly enhanced cell affinity and bone prevascularization; (2) By introducing endothelial cells into the culture of osteogenic cells (co-culture of two different cell types, or bi-culture) in CPC scaffold, the bone defect area underwent much better angiogenic and osteogenic processes when

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compared to mono-culture; (3) Tri-culture with an additional cell type of perivascular cells (such as pericytes) resulted in a substantially enhanced prevascularization of CPC scaffolds in vitro and more new bone and blood vessels in vivo, compared to bi-culture. Furthermore, biological cell crosstalk and capillary-like structure formation made critical contributions to the bi-culture system. In addition, the pericytes in the tri-culture system substantially promoted stability and maturation of the primary vascular network.

Significance. The novel approach of CPC scaffolds with stem cell bi-culture and tri-culture is of great significance in the regeneration of dental, craniofacial and orthopedic defects in clinical practice.

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Contents

1. Introduction

The need for dental, craniofacial and orthopedic repairs and regeneration is increasing rapidly as the world population ages. Bone tissue engineering strategies employ scaffolds, growth factors and stem cells for bone regeneration [1,2]. Scaffolds provide a special environment for cell migration and proliferation, and serve as vehicles to deliver growth factor [3,4]. The criteria for a suitable scaffold include: (1) biocompatibility to avoid immune response in the implant area; (2) biodegradability to facilitate bone remodeling; (3) mechanical properties to support defect reconstruction; (4) microarchitecture for stress distribution; (5) osteoinductivity for osteogenic differentiation; (6) porosity for neovascularization and osteogenesis; and (7) surface properties that induce cell migration, proliferation and differentiation [5].

Calcium phosphate cements are bone mineral-mimicking scaffolds that are injectable, load-bearing, biocompatible, bioactive, and resorbable [6,7]. They are promising for dental, craniofacial and orthopedic applications due to their moldability to achieve esthetics, which is especially important for dental and maxillofacial applications. The first calcium phosphate cement consisted of tetracalcium phosphate [$Ca_4(PO_4)_2O$] and dicalcium phosphate anhydrous ($CaHPO_4$), and was referred to as CPC. Since then, other calcium phosphate cements were developed [8,9]. CPC can adapt to the defect shapes and possess better mechanical strength when compared with hydrogels and other injectable polymers

[10,11]. Moreover, CPC can be fabricated as a tailored structure at the micro- and nano-scale, and can allow protein adsorption and cell adhesion to enhance the bone repair process [8].

Besides orthopedic applications, potential dental and craniofacial applications of CPC scaffolds with improved cell adhesion and prevascularization include mandibular and maxillary ridge augmentation, because the CPC paste could be easily molded and contoured to achieve an esthetic shape and then harden in situ. In addition, major reconstructions of the maxilla or mandible after trauma or tumor resection would greatly benefit from a moldable CPC with rapid osteoconduction and prevascularization. Furthermore, periodontal regeneration, the support of metal dental implants and the augmentation of deficient implant sites could all benefit from injectable and moldable CPC scaffolds. However, currently, there are several major challenges facing the use of scaffolds in clinical applications, including: (1) inadequate vascularization, especially in large defects; (2) poor osseointegration; (3) infection; (4) uncertain degradability; and (5) low stiffness and strength [5]. Among them, vessel formation is the most pressing challenge in bone regeneration. Insufficient vascularization leads to inadequate oxygen and nutrition supply, thus causing hypoxia and cell death [12]. This article reviews various novel strategies for the vascularization of CPC scaffolds for bone tissue engineering, including reviewing for the first time the bi-culture and tri-culture with CPC scaffolds for bone regeneration in dental, craniofacial and orthopedic applications.

2. Vascularization in bone tissue engineering

Vascularization is one of the main challenges that must be overcome in bone tissue engineering. The current strategies are mainly characterized in two aspects: the use of angiogenic growth factors; and prevascularization of scaffolds in vitro.

2.1. *Use of angiogenic growth factors in scaffolds*

This strategy was based on growth factor delivery by the incorporation of biological molecules into scaffolds to promote angiogenesis. Physical encapsulation of growth factors and chemical immobilization of growth factors were two distinct approaches applied in the fabrication of smart scaffolds [13,14]. The former approach was realized by the pre-encapsulation of growth factors in scaffolds and their release in a set manner. The latter approach allowed the interaction between a growth factor and a cell or tissue in scaffolds. Indeed, a sustained delivery of vascular endothelial growth factor (VEGF) encapsulated in poly (lactide-co-glycolide) (PLGA) scaffolds promoted angiogenesis [15]. In addition, an engineered variant of VEGF bound to a fibrin network successfully induced local angiogenesis [16]. However, the release of angiogenic growth factors in the process of vascularization is short-term with burst release profiles. Meanwhile, the vascular network was constructed by host cells after implantation, which could reduce the survival rate of stem cells since the activation of vascularization was delayed [13].

2.2. *Prevascularization of scaffolds in vitro*

Prevascularization involved the vascularization of the scaffold in vitro and was a promising method to enhance the vascularization of tissue constructs upon implantation. The strategy consisted of introducing endothelial cells and osteogenic cells together in a co-culture system in vitro to induce prevascularization of the scaffold and generate vascularization in vivo [17]. With the pre-formed vessels, an effective vascularization in vivo could be obtained in a short time, and bone regeneration could start immediately [13]. Several studies explored the various strategies of prevascularization of different types of scaffolds. Ruchi Mishra et al. performed a prevascularization of poly (propylene fumarate)/fibrin composite scaffold by seeding human mesenchymal stem cells (hMSCs) and human umbilical vein endothelial cells (hUVECs) in vitro [18]. After implantation in a severe immunodeficient mice model, the vascular network formed, resulting in an in vitro prevascularization in the scaffolds to support in vivo vascularization. A similar strategy was demonstrated using hollow channel-modified porous silk scaffolds [19]. In another investigation, three-dimensional (3D) printing technique was applied to fabricate a hollow-pipe-patched silicate bioceramic (BRT-H) scaffold [20]. The novel scaffold not only produced bioactive ions, but also provided microcapillary structures for cell migration and vessel formation in a cell co-culture system. When implanted in rabbit radius segmental defects, the BRT-H scaffolds enhanced the early vascularization and later bone regeneration and remodeling. Therefore, scaffold prevascularization is a promising approach to promoting angiogenesis and bone regeneration.

2.3. *Prevascularization of CPC scaffolds*

In order to optimize the affinity of cells to scaffolds thus promoting prevascularization, various bioactive signals such as proteins or peptides were applied before cells seeding [21]. CPC scaffolds showed a better performance in cell attachment when biofunctional agents were incorporated [20]. Thein-Han et al. compared the effects of five types of biofunctional agents in promoting the attachment of human umbilical cord mesenchymal stem cells (hUCMSCs) [22]. They included Arg-Gly-Asp (RGD) peptide, human fibronectin, fibronectin-like engineered polymer protein, extracellular matrix Geltrex, and human platelet concentrate. Their study revealed that hUCMSCs resulted in much better cell attachment, proliferation, osteogenic differentiation and mineral synthesis in biofunctional CPC scaffolds compared to CPC control. In addition, Chen et al. performed a further study to confirm the effect of RGD in the prevascularization of CPC by co-culturing human osteoblasts (hOBs) and hUVECs [23] (Fig. 1). The RGD peptide in CPC improved the function of both hUVECs and hOBs due to its stimulatory effect on both osteogenic and angiogenic differentiation. These results are consistent with previous studies using different substrates and various cell types [24]. Yang et al. confirmed the effect of incorporating RGD in polyethylene glycol diacry-

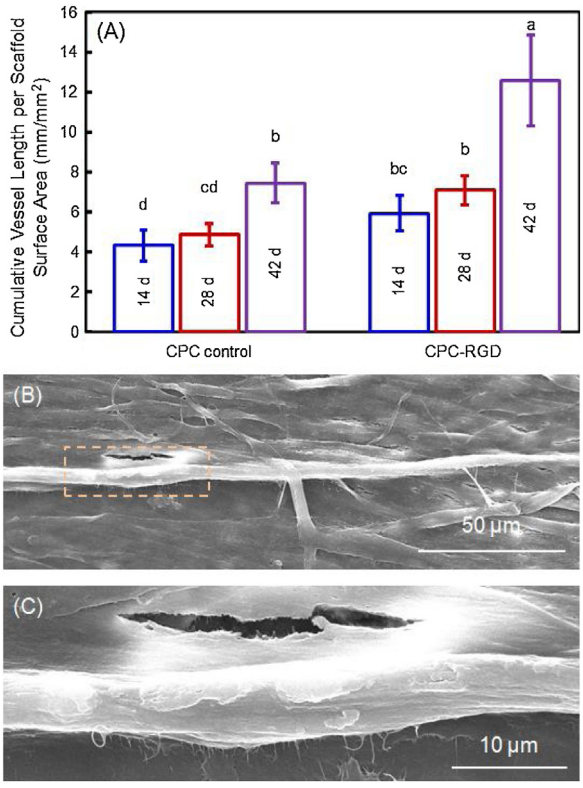

Fig. 1 – Microcapillary-like structure formation on CPC scaffolds: in (A), cumulative length of microcapillary-like structures on CPC scaffolds. In (B, C), representative SEM images of microcapillary-like structures. These images show examples of microcapillary structures on CPC-RGD at 14 d. Image C is a higher magnification of the square area in image B. Adapted with permission from Ref. [23], copyright Elsevier 2014.

late hydrogel on osteogenesis of bone marrow stromal cells [25]. In other studies, CPC incorporated with collagen [26], osteocalcin and O-phospho-L-serine [27] resulted in similar effects on prevascularization. Thus, biofunctionalized CPC scaffolds with greatly enhanced cell affinity and prevascularization are promising materials to facilitate bone regeneration.

3. Cells used in co-culture of CPC scaffolds for vascularization

3.1. *Co-culture strategy for prevascularization*

Early stages of blood vessels are significant for bone regeneration. Capillary-like structures formed in vitro could integrate rapidly after implantation in vivo to become functioning blood vessels. Endothelial cells are able to produce capillary-like structures and are promising for bone vascularization. However, the primary vascular structure fabricated by endothelial cells is usually fragile and unstable [28]. In addition, endothelial cells themselves cannot produce specific pro-angiogenic factors that are critical for vessel maturation [29]. In-depth research demonstrated that there was a cross talk between endothelial cells and hOBs-like cells during bone vascularization, providing new insights regarding the use of endothelial cells together with osteogenic cells for co-culture [30–33] (Fig. 2). Endothelial-osteoblast direct contact and communication were preconditions for both capillary and bone formation [29]. Several different co-culture combinations of two cell types in 2-D or 3-D models were investigated [34–36]. These studies not only demonstrated the superiority of co-culture systems in forming microcapillary-like structures or capillaries, but also revealed the expression of significant genes and proteins relevant for bone vascularization, such as vascular endothelial growth factor (VEGF) [34–36]. They showed that, first, mesenchymal stem cells produced certain soluble factors, including VEGF that is the most identified protein influencing the biological function of neighboring endothelial cells [37]. Second, angiogenesis was promoted as VEGF acted on endothelial cells. Third, the combination of VEGF and VEGF receptors on hOBs induced new bone formation [38,39].

3.2. *The use of several types of osteogenic cells*

3.2.1. *hOB*

hOBs are mature osteogenic cells isolated from bone tissues and they are the first human autologous cell source used for bone tissue engineering. hOB could undergo osteoblastic differentiation when cultured in alpha-modified eagle medium (alpha-MEM) and Dulbecco's modified eagle medium (DMEM) [40,41]. However, obtaining hOB differentiation is usually expensive and the cells tend to undergo de-differentiation after several passages. Therefore, other progenitor cells or stem cells with greater differentiation potential are more promising cells for bone tissue engineering as discussed below.

3.2.2. *Human bone marrow mesenchymal stem cells (hBMSCs)*

hBMSCs possess the potential of differentiating into different cell types, such as hOBs, and they are considered as the gold-standard cells for bone tissue engineering [1]. hBMSCs could adhere to CPC scaffolds and underdo satisfactory osteogenic differentiation [27]. However, the drawbacks of an invasive procedure and inadequate collection of hBMSCs limit their use. Therefore, other sources of stem cells are needed for tissue engineering applications.

3.2.3. *hUCMSCs*

hUCMSCs are collected from human umbilical cords which are inexpensive and non-invasive [42]. hUCMSCs seeded in CPC scaffolds were comparable to the gold-standard hBMSCs in cell proliferation, osteogenic differentiation and mineral synthesis [43]. Hence, hUCMSCs represent a valid stem cell source for bone reconstruction.

3.2.4. *Human umbilical cord perivascular cells (hUCPVCs)*

hUCPVCs are another type of valuable MSCs isolated from human umbilical cord [44]. They showed a higher proliferative potential than BMSCs and were capable of osteogenic, chondrogenic, and adipogenic differentiations [45]. These findings

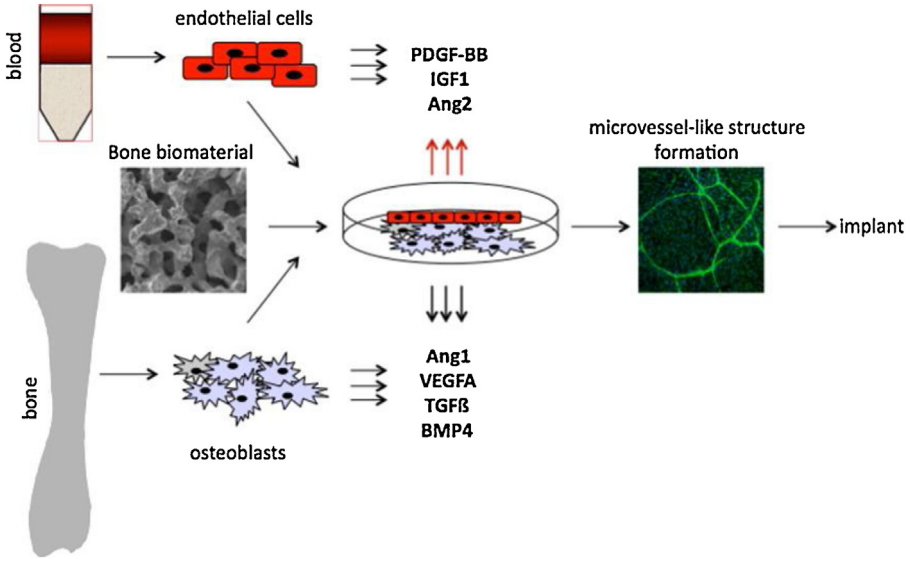

Fig. 2 – Schematic representation of the co-culture system of endothelial cells and osteoblasts in bone tissue engineering. Adapted with permission from Ref. [29], copyright Elsevier 2015.

support the potential utility of hUCPVSs in cell-based bone tissue engineering.

3.2.5. *Human adipose tissue-derived mesenchymal stem cells (hAMSCs)*

hMSCs can also be obtained from the adipose tissue by surgical procedures. Researched showed that hAMSCs possessed the same ability as hBMSCs to differentiate into hOBs and to produce bone matrix [46].

3.2.6. *Human embryonic stem cells (hESCs)*

hESCs are self-renewal cells with the ability of differentiating into many cell types, including hOBs [47]. Their unique characteristics enable them to provide an unlimited source of stem cells for bone engineering. hESCs seeded with CPC scaffolds displayed successful osteogenic differentiation in vitro, and indeed the hESCs differentiated into a osteogenic lineage and expressed osteogenic markers [48]. Similar results were achieved in an animal study [49]. hESCs were seeded onto CPC scaffolds to construct cranial defects in rats, and new bone and blood vessels were much more than those of CPC control group without cells [49].

3.2.7. *Human induced pluripotent stem cells (hiPSCs)*

With the development of a gene reprogramming technique, hiPSCs represented an invaluable resource of stem cells for regenerative medicine [50]. Moreover, hiPSCs could be transduced to MSCs that showed intensive proliferation and osteogenic ability, with less tumorigenic characteristics [51–54]. Recently, hiPSC-MSCs were seeded for the first time onto CPC scaffolds, and both in vitro and in vivo experiments demonstrated an excellent osteogenic and angiogenic ability of hiPSCs in tissue engineering [55].

3.3. *The use of several types of endothelial cells for angiogenesis*

3.3.1. *hUVECs*

hUVECs are isolated from the vein of the umbilical cord. These cells not only participated in angiogenesis [56], but also played an important role in inflammatory response in various tissue regeneration processes [57].

3.3.2. *Human dermal microvascular endothelial cells (hDMECs)*

hDMECs are isolated from human foreskin tissues and are considered as an appropriate alternative to hUVECs to stimulate angiogenesis in a co-culture system. The co-culture of hDMECs with hOBs for 21 days resulted in the formation of microcapillary-like structures on a 3D starch-based scaffold [36].

3.3.3. *Endothelial progenitor cells (EPCs)*

EPCs were first discovered in 1997, when Asahara et al. purified $CD34^+$ hematopoietic progenitor cells that could differentiate into an endothelial phenotype [58]. EPCs are cells with self-renewing ability and they are present in both the bone marrow and blood flow. Studies showed that the infusion of EPCs accelerated the neovascularization of the ischemic tissue and increased the capillary density [59,60].

Endothelial cell-like phenotype of MSCs, ESCs, iPSCs could also be induced due to their superior potential for differentiation [61–63] (Table 1).

3.4. *Co-culture in CPC scaffolds for bone vascularization in vitro*

Thein-Han et al. reported for the first time the prevascularization of CPC scaffolds by co-culturing endothelial cells and hOBs [10]. A gas-foaming method was applied to create macropores in CPC scaffolds. hOBs and hUVECs were seeded onto

Table 1 – Osteogenic and endothelial cells in co-culture for bone vascularization.

	Autologous cell source	MSCs source	ESCs source	iPSCs source
Osteoblast cell source	hOBs	hBMSCs (from bone marrow) hUCMSCs (from peripheral blood) hUCPVCs (from peripheral blood) hAMSCs (from adipose tissue)	hESCs	hiPSCs
Endothelial cell source	hUVECs hDMECs EPCs	hMSCs	hESCs	hiPSCs

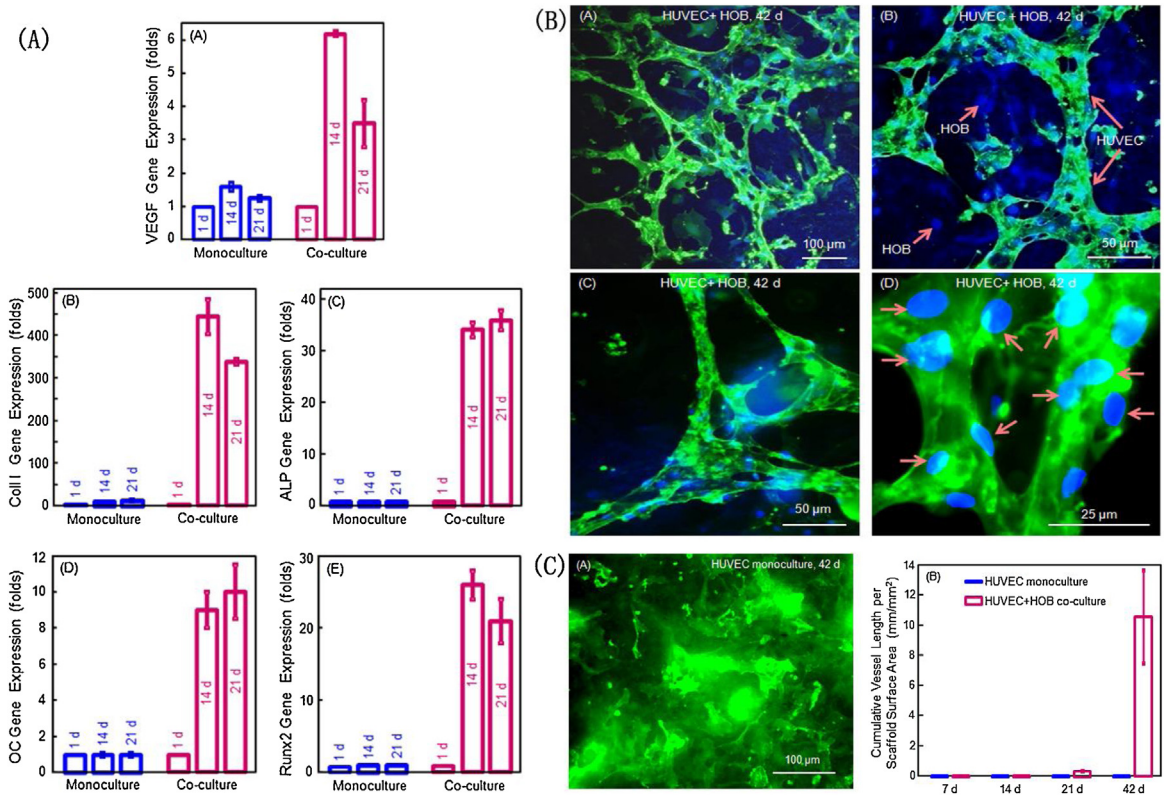

Fig. 3 – (A) Angiogenic and osteogenic gene expression by qRT-PCR. These results indicate that HUVEC + HOB co-culture on CPC had successful angiogenic and osteogenic differentiation; (B) Immunostaining of endothelial-specific PECAM1 on CPC with HUVEC + HOB co-culture; (C) HUVEC monoculture on CPC was immunostained for PECAM1 at 42 days. HUVEC attached well to macroporous CPC and increased the coverage on the CPC surface. HUVEC distributed randomly as monolayers or clumps of cells, with no microcapillary-like structures on CPC. Adapted with permission from Ref. [10], copyright Mary Ann Liebert, Inc. 2013.

CPC scaffolds and incubated for 42 days. The co-culture group expressed much more osteogenic and angiogenic genes than the monoculture group, including VEGF, collagen I, alkaline phosphatase, osteocalcin and runt-related transcription factor 2 (Fig. 3A). Platelet and endothelial cell adhesion molecule 1 (PECAM1), a key marker of endothelial cells, was also highly expressed in the co-culture group (Fig. 3B). At a higher magnification (Fig. 3B), the microcapillary-like structures (long arrows) were seen to extend to form multiple sprouts throughout the hOBs (blue nuclei, with no green stain), while it was rarely expressed in a monoculture group (Fig. 3C). These results demonstrate that microcapillary-like structures on CPC were produced in co-culture, thus showing prevascularization to enhance subsequent angiogenic and osteogenic applications.

3.5. *Co-culture in CPC scaffolds for bone vascularization in vivo*

To further confirm the bone generation and vascularization by co-culture in animals, Liu et al. for the first time seeded hiPSC-MSCs and hUVECs together onto CPC scaffold [64]. Four groups were tested in a cranial bone defect artificially made in nude rats: (1) CPC scaffold alone (CPC control); (2) hUVEC-seeded CPC (CPC-hUVEC); (3) hiPSC-MSC-seeded CPC (CPC-hiPSC-MSC); and (4) hUVECs co-cultured with hiPSC-

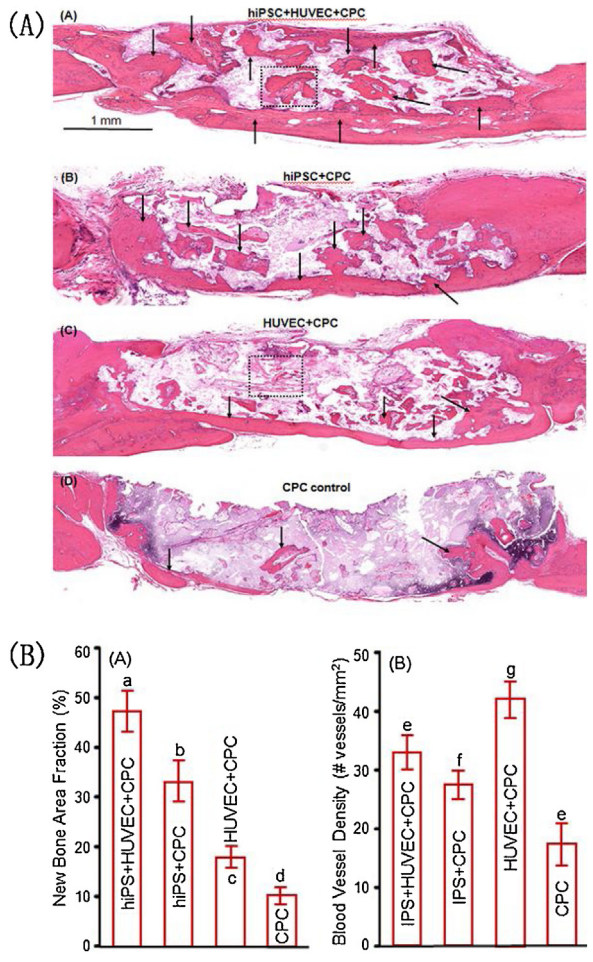

Fig. 4 – (A) H&E staining images of different groups after 3-month implantation in vivo. Mineralized new bone was stained in red (as dark arrow show); (B) Histomorphometric analysis of the new bone area fraction and new blood vessel density. Adapted with permission from Ref. [64], copyright Mary Ann Liebert, Inc. 2017.

MSCs on CPC scaffolds (co-culture group). After 3 months, the co-culture group resulted in the formation of a more extensive new bone area among all groups, with a percentage of 46.38%±3.8% ($p < 0.05$), which was more than four times the 10.61%±1.43% of CPC control (Fig. 4). Therefore, this study provided evidence of the synergistic effects of hUVECs and hiPSC-MSCs in bone tissue regeneration in vivo.

Chen et al. compared the angiogenic and osteogenic effects of hUVECs co-cultured with different mesenchymal stem cells in CPC scaffold [65]. Six groups were tested in cranial bone defects in nude rats: (1) CPC without cells (control 1); (2) CPC with hBMSCs (control 2); (3) hUCMSCs + hUVECs; (4) hiPSC-MSCs + hUVECs; (5) hESC-MSCs + hUVECs; (6) hBMSCs + hUVECs [67]. New bone formation and blood vessel density in the co-cultured groups were much more than in the controls ($p < 0.05$). hUVECs co-cultured with hUCMSCs, hiPSC-MSCs and hESC-MSCs resulted in new bone formation and vessel density similar to hUVECs co-cultured with hBMSCs. Therefore, hUCMSCs, hiPSC-MSCs and hESC-MSCs could all serve as alternative cell sources to the gold-standard hBMSCs.

4. Cells used in tri-culture of CPC scaffolds for vascularization

4.1. *Tri-culture strategy for prevascularization*

Despite the encouraging results regarding bone-forming cells and vessel-forming cells [66], the co-culture approach could have problems such as the stability and maturation of the primary vascular network during bone vascularization [67]. Perivascular cells such as pericytes could act in an important role during tissue engineering by providing physical support for the endothelial cells and by the expression of key angiogenic factors [68,69]. The vascularization started with the recruitment of pericytes by the endothelial cells, which was regulated by an endothelial cell factor such as platelet-derived growth factor-beta [70]. As a result of the interaction between pericytes and endothelial cells, the vascular basement membrane matrix was gradually assembled and regulated by fibronectins, laminins and integrins [71]. Pericytes surrounded the abluminal surface of the endothelial tube with a close distance to endothelial cells [72]. In addition, tissue inhibitor of metalloproteinase-2 and 3 were secreted by endothelial cells and pericytes, respectively, to suppress tube regression through the inhibition of proteolysis [73]. Moreover, molecules such as VEGF, angiopoietin-1 and 2 and transforming growth factor-β, signaling pathways involving Notch and Ephrins, played important roles in vessel maturation [74–77].

Therefore, the tri-culturing strategy involved the pericytes. A recent study generated a pre-vascularized equivalent buccal mucosa in tri-culture of primary buccal epithelial cells, fibroblasts and microvascular endothelial cells, using a native collagen membrane as a scaffold [78]. The results provided a promising approach for the reconstruction of urethral defects. For bone reconstruction, Olga Tsigkou et al. were the first to report the tri-culture of hMSCs, hUVECs and an additional cell type induced by hMSCs in a fibronectin-containing collagen gel for vascularized bone tissue engineering [79] (Fig. 5). The grafted tissue not only provided the direct cell source, but also triggered an endogenous bone repair mechanism.

4.2. *Tri-culture in CPC scaffolds for bone vascularization*

The co-culture of hiPS-MSCs and hUVECs in CPC scaffold showed satisfactory bone vascularization [64]. However, more critical requirements were needed for a stable and long-lasting vascular network. The survival rate of the seeded cells would be compromised because of limited nutrients and oxygen if their distance to the nearest capillary network is > 100-200 μm [80]. Therefore, Zhang et al. proposed for the first time a novel pre-vascularization strategy using tri-culture of hiPS-MSCs, hUVECs and pericytes on CPC [81]. CPC scaffolds were designed to possess 100–300 μm macropores. Six groups were tested: (1) CPC control; (2) CPC-pericytes; (3)

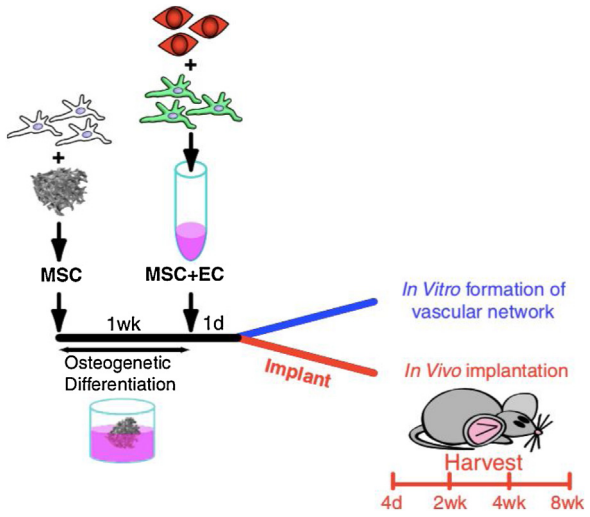

Fig. 5 – Schematic representation of the tri-culture system in bone tissue engineering. The cell/scaffold constructs were then either grown in vitro or implanted subcutaneously in mice. Adapted with permission from Ref. [79], copyright National Academy of Sciences 2010.

CPC-hUVECs; (4) CPC-hiPSMSCs; (5) CPC-hUVECs-hiPSMSCs (co-culture or bi-culture); (6) CPC-hUVECs-hiPSMSCs-pericytes (tri-culture). For tri-culture, first, hiPS-MSCs were seeded on CPC scaffold to obtain osteo-induction. hUVECs and pericytes were subsequently added to the scaffold in sequence. After implantation in rats, much better angiogenesis and osteogenesis were detected in the tri-culture group than in the other groups ($p < 0.05$) (Fig. 6A and B). The results demonstrate that the tri-culture strategy produced the greatest new bone amount and vessel density. Further studies are needed to investigate the CPC scaffold-hUVECs-hiPSMSCs-pericytes tri-culture method in larger animal models such as rabbits and minipigs to evaluate its new bone and blood vessel regeneration efficacy in comparison with bi-culture and mono-culture.

5. Conclusions

This article represents the first review on bi-culture and tri-culture of CPC scaffolds with stem cells to promote vascularization and enhance bone regeneration for dental, craniofacial and orthopedic applications. Macroporous CPC scaffolds are nano-mineral bone cements that are promising for bone tissue engineering due to their load-bearing ability, bone mineral-mimicking ability, bioactivity, and affin-

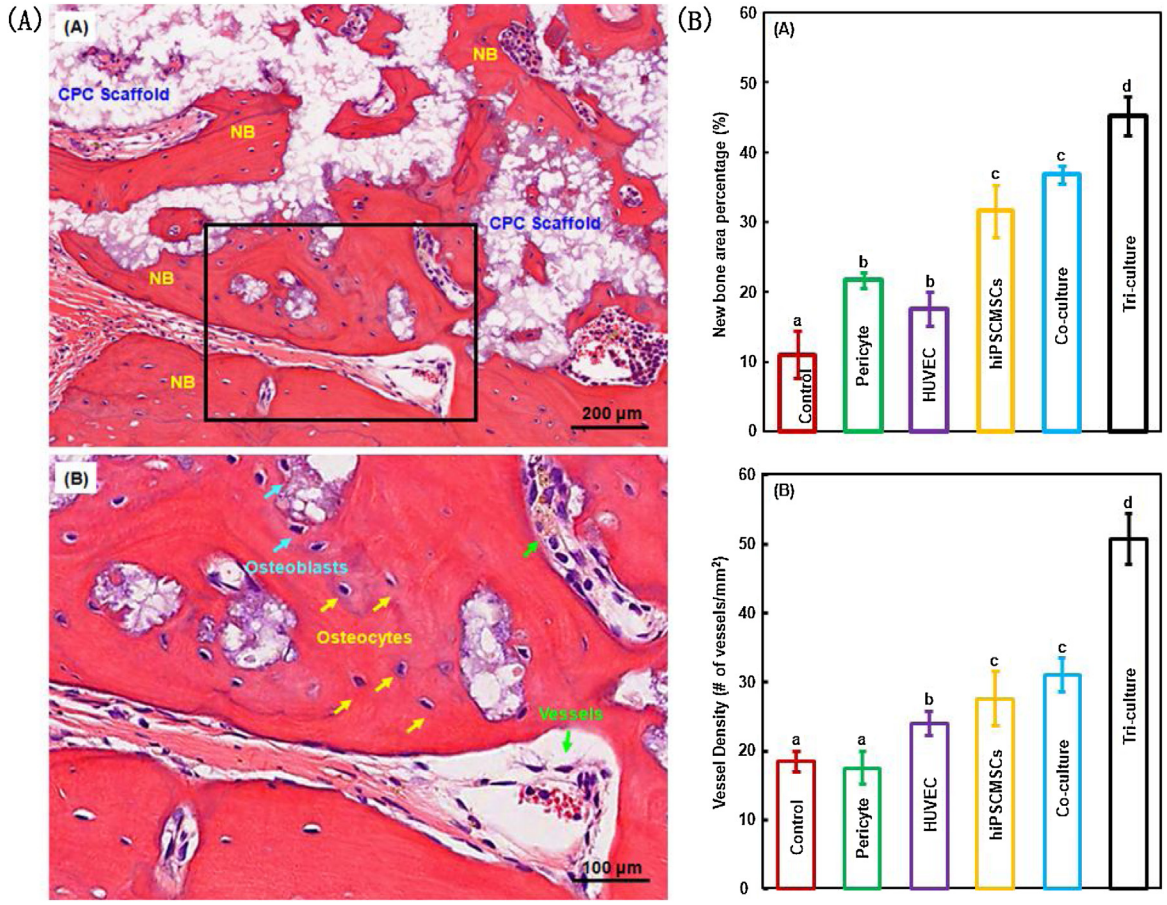

Fig. 6 – (A) New bone and blood vessels were found in the tri-culture group after implantation; (B) The tri-culture group had the greatest amount of new bone and new blood vessel among all the groups tested ($p < 0.05$). Adapted with permission from Ref. [80], copyright Elsevier 2017.

ity for cell seeding. RGD-CPC scaffolds exhibited better performance in enhancing cell attachment and bone prevascularization when compared to CPC control. hBMSCs, hUCMSCs, hUCPVCs, hAMSCs, hESCs, and hiPSCs on CPC all showed excellent angiogenic and osteogenic abilities. The introduction of endothelial cells such as hUVECs into the co-culture system with osteoblast cells or MSCs in CPC scaffolds greatly enhanced the bone defect regeneration through better angiogenic and osteogenic processes, compared to the mono-culture group. Furthermore, the tri-culture strategy with an additional perivascular cell type (such as pericytes) further substantially enhanced the bone regeneration and blood vessel formation in vivo. Therefore, the present review provided an overview of the cutting-edge research on CPC scaffolds with stem cell co-culture and tri-culture. These results provided novel approaches to promoting prevascularization and enhancing bone tissue engineering efficacy, which may have wide applicability to other tissue engineering and regenerative medicine applications.

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