Microengineered Platforms for Co-Cultured Mesenchymal Stem Cells towards Vascularized Bone Tissue Engineering

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Bone defects are common disease requiring thorough treatments since the bone is a complex vascularized tissue that is composed of multiple cell types embedded within an intricate extracellular matrix (ECM). For past decades, tissue engineering using cells, proteins, and scaffolds has been suggested as one of the promising approaches for effective bone regeneration. Recently, many researchers have been interested in designing effective platform for tissue regeneration by orchestrating factors involved in microenvironment around tissues. Among factors affecting bone formation, vascularization during bone development and after minor insults via endochondral and intramembranous ossification is especially critical for the long-term support for functional bone. In order to create vascularized bone constructs, the interactions between human mesenchymal stem cells (MSCs) and endothelial cells (ECs) have been investigated using both direct and indirect co-culture studies. Recently, various culture methods including micropatterning techniques, three dimensional scaffolds, and microfluidics have been developed to create micro-engineered platforms that mimic the nature of vascularized bone formation, leading to the creation of functional bone structures. This review focuses on MSCs co-cultured with endothelial cells and micro-engineered platforms to determine the underlying interplay between co-cultured MSCs and vascularized bone constructs, which is ultimately necessary for adequate regeneration of bone defects.

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INTRODUCTION

Healing of bone defects through the use of tissue engineering strategies by providing scaffolds conducive to osteogenic differentiation is one of the largest fields of tissue engineering [1,2]. Although many researchers have generated successful bone grafts, critical challenges remain. The most daunting challenge in the area of bone tissue engineering is the need to create sufficient vascularization to support the health and long-term functions of the repaired bone. Vascularization is important as a precondition for healing of bone defects [3-6]. Tissue engineering

techniques are often unsuccessful to reconstruct these defects due to irrelevant vascularization. The lack of vascularization in biomaterial scaffolds is the primary cause of inferior bone regeneration, and contributes to reduced mechanical and functional properties. In an attempt to rectify this problem and create functional bones with vascular networks, the delivery of growth factors into bone constructs has been studied [3-5]. However vascular networks induced by this method are likely to be immature, and might not be able to provide sufficient blood perfusion [7]. Like soft tissue organs such as the heart, liver, and kidney, the bone also needs mature vascular networks of stable and long-lasting blood vessels to function properly [3,8]. Vascularization of the bone is a critical step for both intramembranous and endochondral ossification, as vascular invasion is needed for the development of bone marrow and other structures within the bone [9]. Since heavy vascularization is critical for successful bone regeneration, this review focuses on strategies developed for the induction and creation of vascularized bone constructs via regeneration of bones from stem cells using blood

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vessel-inducing cells such as human umbilical vein endothelial cells (HUVECs) and endothelial progenitor cells (EPCs).

Among the several adult stem cell types involved in bone regeneration, mesenchymal stem cells (MSCs) have been reported to directly differentiate into osteoblasts at the interface between existing bones and the periosteum (intramembranous or appositional ossification) [10]. MSCs in the adult bone marrow are multipotent, and have the capacity to differentiate into bones, cartilages, muscles, ligaments, tendons, adipose tissues, and stroma, and contribute to the formation of blood vessels [11-13]. The osteogenic potential of MSCs is critical for bone formation; MSCs migrate into bone fractures and directly contribute to bone regeneration by differentiating towards an osteogenic lineage [14]. When hMSCs are co-implanted with endothelial cells (EC) *in vivo*, they can also develop into perivascular cells that stabilize nascent blood vessels [15,16]. Therefore, co-culturing MSCs with endothelial cells can be an effective strategy to enhance vascularization in bone regeneration, and to improve bone functionality [17,18].

VASCULARIZED BONE BIOLOGY

Microstructural aspect of bone formation

The bone is a specialized connective tissue that provides internal support, movement, and attachment site for muscles and

protection to the body [19,20]. Within the extracellular matrix (ECM), which is composed of various types of materials, a vascular network exists in order to support the cellular components of bone. Therefore, both the structural and cellular aspects of the functional bone need to be mimicked during the creation of engineered bone grafts. In terms of its structural composition, bone ECM has two main compositional phases [21-24]. The organic phase is made up of ECM components such as collagen I and osteopontin, which form a composite material with hydroxyapatite, the major inorganic component of the bone. In the ECM, mineralized collagen fibrils are formed from collagen and hydroxyapatite nanocrystal deposits, which scale up to create the lamellar bone nanostructure (Fig. 1).

The organic component of the ECM, which constitutes almost 20% of bone by weight [24], is composed of proteins and polysaccharides capable that modulates the functions of bone cells. Hydroxyapatite participates in forming the inorganic ECM components, and provides structural support that is comparable to steel by weight [25,26].

To create the structural network found in bones, three main bone cell types known as osteoblasts, osteoclasts, and osteocytes, are required [27,28]. Osteoblasts a specialized form of fibroblasts that stimulate bone formation by producing collagen, bone sialoprotein, and hydroxyapatite on demand, whereas osteoclasts act to reconstruct bone structures by breaking down the miner-

Figure 1. Illustration of macroscopic bone composition and structure. Adapted from Bonzani IC, et al. Novel materials for bone and cartilage regeneration. *Curr Opin Chem Biol* 2006;10:568-575, with permission from Elsevier [97].

alized matrix. The cells of the third type, the osteocytes, are quiescent cells that are capable of regulating bone mineralization in response to mechanical stimuli. Matrix-embedded osteocytes are mechanosensory detectors that coordinate the bone formation and resorption [27,29,30].

In addition to the three main types of bone cells, MSCs and hematopoietic stem cells (HSCs) are also found within the bone marrow and contribute to formation of bone cells [31]. It is known that these stem cells line the exterior of bone underneath the periosteum. While MSCs are working as osteoblast progenitors, HSCs are working as osteoclast progenitors [32,33]. They can also be found in other connective tissues within the body such as fat tissues [34].

Macrostructural aspect of bone formation

The macroscopic bone structure is pervaded by an intricate vascular network that is well developed within both the compact and trabecular bone. Compact bone is the dense exterior bone, while the less dense interior segment is called the trabecular (or spongy) bone [35]. Blood vessels pervade the bone through the Haversian and Volkmann's canals. Compact bone is made up of osteons, which are long, cylindrical structures oriented parallel to the long axis of the bone. These structures are essential for responses to compression stresses. Functionally, osteons can be viewed as miniature weight bearing pillars made up of layers of concentric tubes known as lamellae. In between each layer of tubes are lacunae, where osteocytes reside (Fig. 2).

Figure 2. Illustration of macroscopic bone composition and structure. From: Marieb EN, et al. Human Anatomy. San Francisco, CA: Pearson Benjamin Cummings; 2005 [98]. Copyright © 2005 Pearson Benjamin Cummings. All rights reserved.

Endochondral ossification and intramembranous ossification

It is known that bone forms through two pathways: endochondral ossification and intramembranous ossification [36,37]. Endochondral ossification primarily involves the development, growth, and repair of long bones [38]. Intramembranous ossification is reserved for the development, growth, and repair of bones found in the head. The main difference between the two processes is that during endochondral ossification, osteoprogenitors first differentiate into chondrocytes and deposit cartilage before becoming hypertrophic, recruit osteoblasts, and subsequently mineralizing the matrix. The converted action of various cell types is a distinct process in endochondral ossification. In contrast, intramembranous ossification does not involve this intermediate step, and directly forms mineralized matrix without prior chondrogenic differentiation [36,39].

Biology of bone vasculature

The bone marrow is composed of both red marrow, made up primarily of hematopoietic cells, and yellow marrow, made up primarily of fat cells. Red marrow is predominantly involved in hematopoiesis, and yellow marrow constitutes the majority of the stroma that provides the bone microenvironment. Endothelial cells in the bone maintain vascular integrity, contribute to bone formation, and directly stimulate osteoblasts/osteoclasts [40].

Long bones are generally supplied by a nutrient artery, periosteal vessels, and epiphyseal vessels. These vessels nourish different parts of the bone, but also provide collateral sources of nutrient supply in the case of a blockage. The nutrient artery primarily supplies blood to the cortex of the diaphysis and metaphysis. The periosteal vessels supply the periosteum and the superficial layers of the cortex. The nutrient artery and periosteal vessels converge, allowing one system to take over for both in case an obstruction occurs. The epiphyseal vessels are a ring of arteries that enter the bone between the growth plate and the joint capsule to supply the epiphyses or ends of the bone. While the epiphyseal vessels are joined to the nutrient artery and periosteal vessels in adults, in children, the epiphyseal vessels are the sole blood supply for developing cartilages and bones at the growth plate [41].

Physiologically, when a bone is fractured, bone is capable of recovering to its previous healthy state. First, the surrounding tissue ruptures, causing the tissues to bleed. Blood will then coagulate to form a large clot around the fracture within a week. This large clot is known as a fracture hematoma. Anywhere from two to three weeks following the fracture, blood vessels will penetrate the matrix of the hematoma, recruiting phagocytes to remove non-viable material. In addition, recruited fibroblasts

will remodel the ECM and form collagen fibers, resulting in the development of a rubbery callus consisting of hyaline cartilage. At the beginning of week four, osteoblasts are recruited to mineralize this collagen matrix with HA nanocrystals, leading to the formation of immature bone within 16 weeks [42-47]. Over time, this immature bone matrix is remodeled to create mature, lamellar bone. The structural remodeled bone is the result of the constant deposition and reabsorption of the bone matrix by osteoblasts and osteoclasts, respectively. It is known that weight bearing loads applied through daily routines, such as walking or exercising drives this phenomenon, which is termed Wolff's Law [48-50].

CO-CULTURE TECHNIQUES

Co-culture techniques have been widely used to study cell-cell communication, and has recently been utilized intensively for tissue engineering purposes [51-53]. Since the pioneering works done by Lawrence et al. [54], where gap junction-mediated cellular communication was studied between combined rat ovarian granulosa cells and mouse myocardial cells, co-culture techniques have become popular for investigating cell-cell communications. Gap junction-mediated cellular communication is critical in development and differentiation of cells [55]. At tissues are composed of multiple cell types interconnected within complex spatial structures, co-culture enables us to mimic the tissue microenvironments that need to be restored or replaced. The presence of another cells is elevated the culturing success as well [52]. Moreover, incorporation of stem cells such as embryonic stem cells, induced pluripotent stem cells, and MSCs within the co-culture system is essential for the creation of complex regenerative tissues or organs. For example, co-culture with osteoclast simulated the differentiation of MSCs into bone forming cells [56].

Indirect modes

Indirect co-cultures enable us to observe the role of paracrine factors, which are released by cells, or from the ECM. Paracrine interactions between MSCs and angiogenic cells can be simulated and analyzed. Indirect cellular interactions have long been studied in semi-permeable transwell membrane inserts or conditioned media created from specific cells. Joensuu et al. [57] used transwells for paracrine interaction between MSCs and mononuclear cells (MNCs). Osteoblastic differentiation of MSCs was improved by co-culturing with MNCs in vascular endothelial growth factor (VEGF) supplements that was soluble factors of bone formation and angiogenesis. In addition to these *in vitro* culture methods, applying a specific media for homogeneous cell populations can be considered a type of indirect co-culture,

as each type of cell responds differently to the cell-culture media. For example, one study used osteogenic differentiation medium and endothelial cell growth medium to co-culture MSCs with outgrowth endothelial cells (OECs) [58]. Using endothelial cell growth media, the authors found that the angiogenic properties of OECs were enhanced, while the osteogenic differentiation of MSCs was reduced. Additionally, the authors showed that endothelial cell media supplement with osteogenic factors did not lead to the improved osteogenic differentiation of MSCs. This suggests that the angiogenic potential of MSCs, when co-cultured with OECs, depends on the media type and its paracrine effects exerted by other cells. Overall, these findings indicated that soluble factors present in the media exert differential influences on each cell type in a co-culture environment. In another study, a mixed media consisting of osteogenic differentiation and endothelial growth media was utilized to evaluate the effect of the supportive media on MSCs and ECs, as well as on the growth characteristics of human mesenchymal stem cells (hMSCs). The potential of hMSCs to differentiate towards different lineages was reduced in the mixed media, and there was no preference towards endothelial cell differentiation [59]. In contrast, Pedersen et al. [60] reported that an osteogenic stimulatory media, enriched with dexamethasone (Dex), ascorbic acid, and β -glycerolphosphate, can support growth and maturation of endothelial microvascular networks, as well as perivascular and osteogenic differentiation of MSCs.

Direct modes

In direct co-culture systems, cell interaction occurs via direct adhesion between distinct cell types and cell-ECM [61]. Osteogenesis is a bone healing process that needs the support of vessel-forming cells, and requires cellular communication via direct contact through gap junctions [62]. To evaluate the effect of EC on the differentiation of MSCs toward smooth muscle cells (SMCs), Ball et al. [63] demonstrated that direct contact between MSCs and ECs resulted in the disorganization of smooth muscle α -actin (SM α -actin), while indirect co-culture maintained well organized SM α -actin filaments. This suggests that MSCs phenotypes are influenced by ECs, which determines the fate of MSCs in vascularized bone constructs. In another study, spheroidal co-cultures of HUVECs and hMSCs were used to create three-dimensional perivascular networks *in vitro* [64]. In order to determine the extent of the perivascular network within a HUVEC/hMSC spheroid, different percentages of HUVECs were incorporated. A high seeding density of HUVECs resulted in a reduction of CD31-positive cells in HUVEC/hMSC spheroids after 10 days of *in vitro* cultivation, while 5% of the cells were CD31-positive when only 1% of HUVECs were seeded in a HUVEC/hMSC spheroid. This indicated that the

perivascular network created HUVECs and hMSC co-cultures were the result of direct HUVEC-MSC communications. Subsequent subcutaneous *in vivo* implantation exhibited vessel-like structures within 14 days of subcutaneous implantation. In another study, a spheroid co-culture system consisting of HUVECs and MSCs was studied. The co-cultured MSCs and HUVECs self-assembled into a MSC-centered spheroid surrounded by HUVECs, which resulted in a primitive network of CD31-positive capillary-like structures. The co-cultured spheroid had increased alkaline phosphatase (ALP) activity, indicating that ECs supported osteogenesis of MSCs. One interesting finding was that osteogenic differentiation occurred near the periphery of organoids, where HUVECs and MSCs were co-localized [65]. In the other study, MSCs and EPCs that derived from peripheral blood were co-cultured in highly interconnected three dimensional (3D) calcium phosphate polymer scaffolds with strontium. MSCs and EPCs grew together and interacted with each other in these scaffolds. It was supported that increase of EPCs proportion led to high expression of the endothelial cell marker CD31 and stimulated proliferation and differentiation of MSCs. It was also found strontium in 3D scaffold resulted in cell proliferation and expression of the ALP and VEGF [66].

Other considerations in co-culture modes

In co-culture, several parameters can be manipulated. The number of cell types, the degree of the resemblance, contact of each cell type within the co-culture, culture volumes, and media composition can determine the final outcomes [67]. In a co-culture consisting of hMSCs and HUVECs, the ratio of osteogenic supplement medium and endothelial supplement medium can affect the differentiation potential cells. Hence, the performance of supporting media for HUVECs and hMSCs needs to be assessed prior to usage. Interestingly, with regard to choosing the culture media, osteogenic media can improve the angiogenic potential of HUVECs when co-culture with hMSCs. Additionally, the interplay of co-cultured cells within the biomaterial should be considered as well. In EC-based co-cultures, various materials were utilized: poly (ϵ -caprolactone) (PCL), PCL-starch, porous hydroxyapatite and β -TCP [68-71]. When attached to the biomaterials, each cell type is differentially affected by the substrates, which induce specific interactions between the cells. The co-culture system is not a simple culture system. In fact, delicate manipulation of all the parameters in the co-culture system allows the creation of a vascularized bone structure *in vivo*, such as induction of osteogenesis of hMSCs before seeding of MSCs and HUVECs in a fibrin hydrogel [72].

MICROPATTERNING IN CO-CULTURES

Two dimensional micropatterning technologies

Micropatterning technologies are enabling platform strategies that explore the relationship of cells cultured on substrates. These delicate techniques, which include micro-contact, photolithographic, and microfluidic micro-patterning, can replicate complex architectural and boundary conditions of *in vivo* tissues, which are essential for the *in vivo* vascularization of the bone. Recapitulating the interactions between bone forming cells helps us to create vascularized bone constructs. By manipulating cellular behaviors such as attachment, shape, and spreading (cell size), micro-patterning approaches enable us to study cellular interactions between target and support cells [73-75]. Photolithography and soft lithography are the most common methods for micro-patterns fabrication. In photolithography, a mask with micro-patterns is used to create desired patterns onto a photo-resistant coated silicon wafer. The mask prevents UV-light penetration onto the coated silicon wafer, and leaves the unmasked area to be further processed in subsequent steps. Although a clean room facility is required, photolithography allows for any pattern with 1–2 μm resolution [76]. Another technique that can engrave sophisticated patterns onto wafers is soft lithography, which can create patterned stamps onto polymeric elastomers without the need for a clean room. The patterned micro-stamp can be utilized for micro-contact printing and micro-molding. For example, poly(dimethyl siloxane) (PDMS) stamps can be prepared, and further treated with alkanethiols to contact with gold substrates in micro-contact printing [74]. In the case of micro-molding, photolithography is utilized for creating the micro-molds onto a silicon wafer [77]. For conventional micro-patterning without using lithography techniques, parafilm inserts and liquid tilting can be utilized [78,79]. For the parafilm insert approach, the parafilm is cut and attached onto the cell adhesive surfaces to create a blocking area for cell attachment, while the liquid tilting method creates patterns by tilting volumes of surface blocking solution such as bovine serum albumin.

Three dimensional micropatterning technologies

To create a 3D patterned hydrogel, layer-by-layer stereolithography (SLA) can be employed [80]. The patterned layers can be created precisely, after which, the un-cross-linked layers can be washed away. Because this technique allows for the creation of patterns in individual layers using a stereophotolithographic approach, it is easier to control than post-gelation patterning. Maskless SLA is another technique used to create patterned hydrogels. In this technique, a cross-linkable polymer is cross-linked just after being printed onto a substrate. Maskless SLA is

a layer-by-layer fabrication technique, which allows the seeding of other cell populations onto a patterned structure, and is important for elucidating cellular interactions in co-cultures. The other patterning technique is mask-based photolithography that forms 3D hydrogel [81,82]. In the mask-based SLA approach, a photomask allows for the selective of photoinhibitor-containing pre-polymer gelatin in each layer, so that multi-layered and patterned structures can be constructed.

Co-cultured MSCs with HUVECs using micro-patterning technologies

On micrometer-scale islands of ECM, self-assembled alkanethiolate monolayers were printed on gold using a micro-contact printing technique, following which bovine capillary ECs and human microvascular ECs were cultured on the micropatterned surfaces. It was found that a small adhesive area induced apoptosis while large adhesive areas led to overall cell growth [74]. Nikkhah et al. [83] also developed micro-patterned hydrogels to create highly organized endothelial cord structures. Using photo-crosslinkable gelatin methacrylate hydrogels, they were able to fabricate micro-patterned constructs with different height spacers (50, 100, 150 μm) through a photomask-assisted patterning technique. They loaded HUVECs within the micropatterned hydrogel in order to find the optimal microenvironment for circular and stable cord formation. Among those micropatterned hydrogels, 50- and 100 μm -high constructs showed better aligned structures with highly organized and oriented actin fibers as compared with un-patterned and 150 μm -high constructs. This suggests that geometrical confinement of HUVECs within micro-patterned hydrogels lead to organized cells. Such findings would be useful for increasing angiogenic potential, thereby developing better organized vasculatures. Moreover, differentiation of MSCs has been reported to change along with micro-patterned surfaces. The shape of hMSCs also affected cell differentiation as single hMSCs become adipocytes on small (1024 μm^2) fibronectin islands, while large islands (10000 μm^2) support osteogenic differentiation. It was determined that cytoskeletal tension and RhoA activity was responsible for this change [84].

In addition to modulating the cellular behavior of single cell types on micro-patterned structures, recent studies have shifted towards investigating micro-patterning effects on co-cultured MSCs with HUVECs. Based on the fact that the osteogenic potential of MSCs can be improved by endothelial-derived cytokines such as bone morphogenetic proteins and VEGF, Kim et al. [85] created an aligned nanostructure mimicking human cancellous bone tissue *ex vivo* via ultraviolet (UV)-assisted capillary force lithography with polyurethane acrylate (PUA), and co-cultured MSCs with ECs (Fig. 3). The authors created nano-

topographic PUA matrices with regularly-spaced nanogrooves with a width of 550 nm and three different gaps of 550, 1650, and 2750 nm. hMSCs co-cultured with HUVECs on the PUA matrices showed highly aligned orientations in both cell types on the PUA matrices, even after 7 days of cultivation. Moreover, the nano-grooves with 2750 nm gaps produced more elongated cells than those with 550 nm gaps. Compared to mono-layered cultures on flat substrates, hMSCs co-cultured with HUVECs at half the initial seeding density (0.5×10^4 cells/sample, respectively) showed better osteogenic potential than hMSC monocultures. Instead of using a surface lithographical technique, Trkov et al. [86] used microfluidic channels to create spacing distances from 500 to 2000 μm , and co-cultured MSCs with HUVECs to demonstrate distance-dependent migration of MSCs toward HUVECs. Both cell types were encapsulated in fibrin gels and loaded into two parallel channels spaced 500, 1000, 2000 μm apart. The author found that the migration of MSCs toward HUVECs was higher when they were spaced closer together. This distance-dependent migration of MSCs reflects the concentration-dependent migration of MSCs through endothelial-derived cytokines. Moreover, MSCs that migrated towards HUVECs ex-

pressed alpha-smooth muscle actin, which is a biomarker for SMCs and pericytes, indicating that direct contact of MSCs with endothelial tubes induced the differentiation of MSCs towards the SMC/pericyte lineages.

OTHER THREE DIMENSIONAL TECHNOLOGIES IN CO-CULTURES

Microfluidics

Microfluidic platforms has created more physiological cellular environments than those achievable with traditional techniques. These platforms can simplify the application of various stimuli and improve *in vitro* culture models [87,88]. Microfluidics has a potential as provide significant opportunities for bone physiology and regeneration [88]. It is well known that microfluidics overcomes the difficulties such as sufficient supply of oxygen and nutrition in large structures by using microfabrication to create an entire blood supply [89].

For bone tissue regeneration, it was demonstrated that microfluidic platform can be used to conduct mechano-transduction of bone cell under bone tissue mimicking flow condition [88].

Figure 3. Human mesenchymal stem cells (hMSCs) and human umbilical vein endothelial cells (HUVECs) on nanopatterned matrices. (A) Representative phase-contrast images. The black arrows indicate hMSC while the white arrows represent HUVECs. Representative SEM images of (B) hMSCs and (C) HUVECs on the substrata are shown. (D) Representative immunostaining images of F-actin (red) in co-culture systems, showing aligned cytoskeletal structure of cells on the nanopatterned matrices [56].

Recently, Hasani-Sadrabadi et al. [90] fabricated PDMS microfluidic system with polymeric nanoparticles (NPs) through standard micromolding process. In their system, NPs can control release Dex molecules and regulate osteogenic differentiation of MSCs, suggesting an improved culture system for a bone tissue repair.

Scaffold based three dimensional technologies

Recently, various co-culture studies have been performed in 3D culture system with biofunctional scaffolds [92-94]. In a study with collagen based pseudo-periosteum membrane mimicking native structure of periosteum, cells grown in micropatterned membranes had more proliferation than flat collagen membranes. Then, micropatterned pseudo-periosteum membrane covered 3D porous scaffolds consisting of 70% inorganic nanoparticles and 30% collagen type 1 were tested for co-cultures of MSCs and EC to investigate the effect of a scaffold structure on bone regeneration. The results demonstrated that 3D scaffolds mimicking bone composition promoted osteogenic differentiation as compared to the periosteum-free scaffolds [95].

In another study, MSCs and EPCs or MSCs and HUVECs were co-cultured in the elastomeric polyurethane (PU) scaffolds containing nanoparticles of hydroxyapatite to fabricate 3D PU scaffolds having prevascularized bone implants and thus enabling a faster blood supply. The pre-vascular network was shown as early as 7 days. Both co-culture systems showed earlier osteogenic differentiation than MSCs alone. It was also observed that MSCs and EPCs co-cultures increased tubular structure as compared with MSCs alone. The formation of pre-vascular structure was more mature and abundant in a co-culture with MSCs and HUVECs [96].

CONCLUSION

The bone is a complex tissue that is composed of multiple cell types and an extensive ECM that consists of both organic and inorganic components. While the body is able to form robust and well-vascularized bone structures during development and after minor injuries, it struggles to fully repair major injuries on biomaterial-based replacements. Bone is a complex structure that is designed to be light and yet is able to resist stresses from multiple directions. Furthermore, it also provides cells that make up the immune system. To generate functional bones within large defects, co-cultures containing both MSCs and ECs have been investigated in order to enhance the creation of vascularized bone constructs. Multiple micro-patterning techniques have been utilized to allow the creation of high resolution 2- and 3D patterns of cells and ECM components. In conclusion,

new approaches for the creation of functional bones are necessary for the adequate regeneration of large bone defects.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

There are no animal experiments carried out for this article.

REFERENCES

- Sharma B, Elisseeff JH. Engineering structurally organized cartilage and bone tissues. *Ann Biomed Eng* 2004;32:148-159.
- Laurencin CT, Ambrosio AM, Borden MD, Cooper JA Jr. Tissue engineering: orthopedic applications. *Annu Rev Biomed Eng* 1999;1:19-46.
- Santos MI, Reis RL. Vascularization in bone tissue engineering: physiology, current strategies, major hurdles and future challenges. *Macromol Biosci* 2010;10:12-27.
- Simmons CA, Alsberg E, Hsiong S, Kim WJ, Mooney DJ. Dual growth factor delivery and controlled scaffold degradation enhance in vivo bone formation by transplanted bone marrow stromal cells. *Bone* 2004;35:562-569.
- Vo TN, Kasper FK, Mikos AG. Strategies for controlled delivery of growth factors and cells for bone regeneration. *Adv Drug Deliv Rev* 2012;64:1292-1309.
- Klenke FM, Liu Y, Yuan H, Hunziker EB, Siebenrock KA, Hofstetter W. Impact of pore size on the vascularization and osseointegration of ceramic bone substitutes in vivo. *J Biomed Mater Res A* 2008;85:777-786.
- Benjamin LE, Golijanin D, Itin A, Podes D, Keshet E. Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. *J Clin Invest* 1999;103:159-165.
- Koike N, Fukumura D, Gralla O, Au P, Schechner JS, Jain RK. Tissue engineering: creation of long-lasting blood vessels. *Nature* 2004;428:138-139.
- Saran U, Gemini Piperni S, Chatterjee S. Role of angiogenesis in bone repair. *Arch Biochem Biophys* 2014;561:109-117.
- Marie PJ, Fromiguet O. Osteogenic differentiation of human marrow-derived mesenchymal stem cells. *Regen Med* 2006;1:539-548.
- Phinney DG, Prockop DJ. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views. *Stem Cells* 2007;25:2896-2902.
- Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;284:143-147.
- McFadden TM, Duffy GP, Allen AB, Stevens HY, Schwarzmaier SM, Plesnila N, et al. The delayed addition of human mesenchymal stem cells to pre-formed endothelial cell networks results in functional vascularization of a collagen-glycosaminoglycan scaffold in vivo. *Acta Biomater* 2013;9:303-316.
- Dimitriou R, Tsiridis E, Giannoudis PV. Current concepts of molecular aspects of bone healing. *Injury* 2005;36:1392-1404.
- Oswald J, Boxberger S, Jørgensen B, Feldmann S, Ehninger G, Bornhäuser M, et al. Mesenchymal stem cells can be differentiated into endothelial cells in vitro. *Stem Cells* 2004;22:377-384.

16. Au P, Tam J, Fukumura D, Jain RK. Bone marrow-derived mesenchymal stem cells facilitate engineering of long-lasting functional vasculature. *Blood* 2008;111:4551-4558.
17. Kirkpatrick CJ, Fuchs S, Unger RE. Co-culture systems for vascularization--learning from nature. *Adv Drug Deliv Rev* 2011;63:291-299.
18. Liu Y, Chan JK, Teoh SH. Review of vascularised bone tissue-engineering strategies with a focus on co-culture systems. *J Tissue Eng Regen Med* 2015;9:85-105.
19. Weatherholt AM, Fuchs RK, Warden SJ. Specialized connective tissue: bone, the structural framework of the upper extremity. *J Hand Ther* 2012;25:123-131; quiz 132.
20. Meghji S. Bone remodelling. *Br Dent J* 1992;172:235-242.
21. Rey C, Combes C, Drouet C, Glimcher MJ. Bone mineral: update on chemical composition and structure. *Osteoporos Int* 2009;20:1013-1021.
22. Robinson RA. Bone tissue: composition and function. *Johns Hopkins Med J* 1979;145:10-24.
23. Eastoe JE. The chemical composition of bone and tooth. *Adv Fluorine Res* 1965;21:5-17.
24. Ashman RF, Buckwalter JA, Devane P, Dobbs MB, Ferguson PJ, Flatow EL, et al. *Turek's Orthopaedics: Principles and Their Application*. 6th ed. Iowa City: Lippincott Williams and Wilkins; 2005.
25. Yang L, Perez-Amodio S, Barrère-de Groot FY, Everts V, van Blitterswijk CA, Habibovic P. The effects of inorganic additives to calcium phosphate on in vitro behavior of osteoblasts and osteoclasts. *Biomaterials* 2010;31:2976-2989.
26. Fung YC. *Biomechanics: Mechanical Properties of Living Tissues*. 2nd ed. New York: Springer-Verlag; 1993.
27. Alford AI, Kozloff KM, Hankenson KD. Extracellular matrix networks in bone remodeling. *Int J Biochem Cell Biol* 2015;65:20-31.
28. Nijweide PJ, Burger EH, Feyen JH. Cells of bone: proliferation, differentiation, and hormonal regulation. *Physiol Rev* 1986;66:855-886.
29. Heino TJ, Kurata K, Higaki H, Väänänen HK. Evidence for the role of osteocytes in the initiation of targeted remodeling. *Technol Health Care* 2009;17:49-56.
30. Vrahnas C, Sims NA. EphrinB2 signalling in osteoblast differentiation, bone formation and endochondral ossification. *Curr Mol Bio Rep* 2015;1:148-156.
31. Ehninger A, Trumpp A. The bone marrow stem cell niche grows up: mesenchymal stem cells and macrophages move in. *J Exp Med* 2011;208:421-428.
32. Yin T, Li L. The stem cell niches in bone. *J Clin Invest* 2006;116:1195-1201.
33. Caplan AI. Why are MSCs therapeutic? New data: new insight. *J Pathol* 2009;217:318-324.
34. Müller-Sieburg CE, Cho RH, Thoman M, Adkins B, Sieburg HB. Deterministic regulation of hematopoietic stem cell self-renewal and differentiation. *Blood* 2002;100:1302-1309.
35. Marieb EN, Hoehn k. *Human anatomy and physiology*. 7th ed. San Francisco, CA: Pearson Benjamin Cummings; 2007. xxvii. 1159. A1 -I51 p.
36. Mackie EJ, Tatarczuch L, Mirams M. The skeleton: a multi-functional complex organ: the growth plate chondrocyte and endochondral ossification. *J Endocrinol* 2011;211:109-121.
37. Thompson Z, Miclau T, Hu D, Helms JA. A model for intramembranous ossification during fracture healing. *J Orthop Res* 2002;20:1091-1098.
38. Ortega N, Behonick DJ, Werb Z. Matrix remodeling during endochondral ossification. *Trends Cell Biol* 2004;14:86-93.
39. Schindeler A, McDonald MM, Bokko P, Little DG. Bone remodeling during fracture repair: the cellular picture. *Semin Cell Dev Biol* 2008;19:459-466.
40. Streeten EA, Brandi ML. Biology of bone endothelial cells. *Bone Miner* 1990;10:85-94.
41. Fujihara K, Kotaki M, Ramakrishna S. Guided bone regeneration membrane made of polycaprolactone/calcium carbonate composite nano-fibers. *Biomaterials* 2005;26:4139-4147.
42. Lu Y, Lekszycki T. Modeling of an initial stage of bone fracture healing. *Continuum Mech Thermodyn* 2015;27:851-859.
43. Cruess RL, Dumont J. Fracture healing. *Can J Surg* 1975;18:403-413.
44. Ketenjian AY, Jafri AM, Arsenis C. Studies on the mechanism of callus cartilage differentiation and calcification during fracture healing. *Orthop Clin North Am* 1978;9:43-65.
45. Tsunoda M, Mizuno K, Matsubara T. The osteogenic potential of fracture hematoma and its mechanism on bone formation--through fracture hematoma culture and transplantation of freeze-dried hematoma. *Kobe J Med Sci* 1993;39:35-50.
46. Macmahon P, Eustace SJ. General principles. *Semin Musculoskelet Radiol* 2006;10:243-248.
47. Sfeir C, Ho L, Doll BA, Azari K, Hollinger JO. Fracture Repair. In: Lieberman JR, Friedlander GE, editors. *Bone Regeneration and Repair: Biology and Clinical Applications*. Totowa, NJ: Humana Press Inc.; 2005.
48. Singh MAF. Exercise and bone health. *Nutrition and Bone Health*. New York, NY: Springer; 2015. p. 505-542.
49. Cowin SC. Wolff's law of trabecular architecture at remodeling equilibrium. *J Biomech Eng* 1986;108:83-88.
50. Greer RB 3rd. Wolff's Law. *Orthop Rev* 1993;22:1087-1088.
51. Battiston KG, Cheung JW, Jain D, Santerre JP. Biomaterials in co-culture systems: towards optimizing tissue integration and cell signaling within scaffolds. *Biomaterials* 2014;35:4465-4476.
52. Jin GZ, Han CM, Kim HW. In vitro co-culture strategies to prevascularization for bone regeneration: a brief update. *Tissue Eng Regen Med* 2015;12:69-79.
53. Suh JD, Lim KT, Jin H, Kim J, Choung PH, Chung JH. Effects of co-culture of dental pulp stem cells and periodontal ligament stem cells on assembled dual disc scaffolds. *Tissue Eng Regen Med* 2014;11:47-58.
54. Lawrence TS, Beers WH, Gilula NB. Transmission of hormonal stimulation by cell-to-cell communication. *Nature* 1978;272:501-506.
55. El-Sabban ME, Sfeir AJ, Daher MH, Kalaany NY, Bassam RA, Talhouk RS. ECM-induced gap junctional communication enhances mammary epithelial cell differentiation. *J Cell Sci* 2003;116:3531-3541.
56. Sinclair SS, Burg KJ. Effect of osteoclast co-culture on the differentiation of human mesenchymal stem cells grown on bone graft granules. *J Biomater Sci Polym Ed* 2011;22:789-808.
57. Joensuu K, Uusitalo L, Alm JJ, Aro HT, Hentunen TA, Heino TJ. Enhanced osteoblastic differentiation and bone formation in co-culture of human bone marrow mesenchymal stromal cells and peripheral blood mononuclear cells with exogenous VEGF. *Orthop Traumatol Surg Res* 2015;101:381-386.
58. Kolbe M, Xiang Z, Dohle E, Tonak M, Kirkpatrick CJ, Fuchs S. Paracrine effects influenced by cell culture medium and consequences on microvessel-like structures in cocultures of mesenchymal stem cells and outgrowth endothelial cells. *Tissue Eng Part A* 2011;17:2199-2212.
59. Ern C, Krump-Konvalinkova V, Docheva D, Schindler S, Rossmann O, Böcker W, et al. Interactions of human endothelial and multipotent mesenchymal stem cells in cocultures. *Open Biomed Eng J* 2010;4:190-198.
60. Pedersen TO, Blois AL, Xue Y, Xing Z, Cottler-Fox M, Fristad I, et al. Osteogenic stimulatory conditions enhance growth and maturation of endothelial cell microvascular networks in culture with mesenchymal stem cells. *J Tissue Eng* 2012;3:2041731412443236.
61. Paschos NK, Brown WE, Eswaramoorthy R, Hu JC, Athanasiou KA. Advances in tissue engineering through stem cell-based co-culture. *J Tissue Eng Regen Med* 2015;9:488-503.
62. Melchiorri AJ, Nguyen BN, Fisher JP. Mesenchymal stem cells: roles and relationships in vascularization. *Tissue Eng Part B Rev* 2014;20:218-228.
63. Ball SG, Shuttleworth AC, Kielty CM. Direct cell contact influences bone marrow mesenchymal stem cell fate. *Int J Biochem Cell Biol* 2004;36:714-727.
64. Rouwkema J, de Boer J, Van Blitterswijk CA. Endothelial cells assemble into a 3-dimensional prevascular network in a bone tissue engineering

- construct. *Tissue Eng* 2006;12:2685-2693.
65. Saleh FA, Whyte M, Genever PG. Effects of endothelial cells on human mesenchymal stem cell activity in a three-dimensional in vitro model. *Eur Cell Mater* 2011;22:242-257.
 66. Fu WL, Xiang Z, Huang FG, Gu ZP, Yu XX, Cen SQ, et al. Coculture of peripheral blood-derived mesenchymal stem cells and endothelial progenitor cells on strontium-doped calcium polyphosphate scaffolds to generate vascularized engineered bone. *Tissue Eng Part A* 2015;21:948-959.
 67. Goers L, Freemont P, Polizzi KM. Co-culture systems and technologies: taking synthetic biology to the next level. *J R Soc Interface* 2014;11.
 68. Fuchs S, Ghanaati S, Orth C, Barbeck M, Kolbe M, Hofmann A, et al. Contribution of outgrowth endothelial cells from human peripheral blood on in vivo vascularization of bone tissue engineered constructs based on starch polycaprolactone scaffolds. *Biomaterials* 2009;30:526-534.
 69. Santos MI, Unger RE, Sousa RA, Reis RL, Kirkpatrick CJ. Crosstalk between osteoblasts and endothelial cells co-cultured on a polycaprolactone-starch scaffold and the in vitro development of vascularization. *Biomaterials* 2009;30:4407-4415.
 70. Unger RE, Sartoris A, Peters K, Motta A, Migliaresi C, Kunkel M, et al. Tissue-like self-assembly in cocultures of endothelial cells and osteoblasts and the formation of microcapillary-like structures on three-dimensional porous biomaterials. *Biomaterials* 2007;28:3965-3976.
 71. Usami K, Mizuno H, Okada K, Narita Y, Aoki M, Kondo T, et al. Composite implantation of mesenchymal stem cells with endothelial progenitor cells enhances tissue-engineered bone formation. *J Biomed Mater Res A* 2009;90:730-741.
 72. Correia C, Grayson WL, Park M, Hutton D, Zhou B, Guo XE, et al. In vitro model of vascularized bone: synergizing vascular development and osteogenesis. *PLoS One* 2011;6:e28352.
 73. Peng R, Yao X, Cao B, Tang J, Ding J. The effect of culture conditions on the adipogenic and osteogenic inductions of mesenchymal stem cells on micropatterned surfaces. *Biomaterials* 2012;33:6008-6019.
 74. Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE. Micropatterned surfaces for control of cell shape, position, and function. *Biotechnol Prog* 1998;14:356-363.
 75. Kim S, Kim BS. Control of adult stem cell behavior with biomaterials. *Tissue Eng Regen Med* 2014;11:423-430.
 76. Nakanishi J, Takarada T, Yamaguchi K, Maeda M. Recent advances in cell micropatterning techniques for bioanalytical and biomedical sciences. *Anal Sci* 2008;24:67-72.
 77. Tang MD, Golden AP, Tien J. Molding of three-dimensional microstructures of gels. *J Am Chem Soc* 2003;125:12988-12989.
 78. Javaherian S, O'Donnell KA, McGuigan AP. A fast and accessible methodology for micro-patterning cells on standard culture substrates using Parafilm™ inserts. *PLoS One* 2011;6:e20909.
 79. Javaherian S, Li KJ, McGuigan AP. A simple and rapid method for generating patterned co-cultures with stable interfaces. *Biotechniques* 2013;55:21-26.
 80. Khetan S, Burdick JA. Patterning hydrogels in three dimensions towards controlling cellular interactions. *Soft Matter* 2011;7:830-838.
 81. Hasan A, Paul A, Vrana NE, Zhao X, Memic A, Hwang YS, et al. Microfluidic techniques for development of 3D vascularized tissue. *Biomaterials* 2014;35:7308-7325.
 82. Ji D, Jiang L, Jiang L, Fu X, Dong H, Yu J, et al. A novel method for photolithographic polymer shadow masking: toward high-resolution high-performance top-contact organic field effect transistors. *Chem Commun (Camb)* 2014;50:8328-8330.
 83. Nikkhah M, Eshak N, Zorlutuna P, Annabi N, Castello M, Kim K, et al. Directed endothelial cell morphogenesis in micropatterned gelatin methacrylate hydrogels. *Biomaterials* 2012;33:9009-9018.
 84. McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev Cell* 2004;6:483-495.
 85. Kim J, Kim HN, Lim KT, Kim Y, Pandey S, Garg P, et al. Synergistic effects of nanotopography and co-culture with endothelial cells on osteogenesis of mesenchymal stem cells. *Biomaterials* 2013;34:7257-7268.
 86. Trkov S, Eng G, Di Liddo R, Parnigotto PP, Vunjak-Novakovic G. Micropatterned three-dimensional hydrogel system to study human endothelial-mesenchymal stem cell interactions. *J Tissue Eng Regen Med* 2010;4:205-215.
 87. Tourovskaia A, Figueroa-Masot X, Folch A. Long-term micropatterned cell cultures in heterogeneous microfluidic environments. *Conf Proc IEEE Eng Med Biol Soc* 2004;4:2675-2678.
 88. Abbott RD, Kaplan DL. Strategies for improving the physiological relevance of human engineered tissues. *Trends Biotechnol* 2015;33:401-407.
 89. Riehl BD, Lim JY. Macro and microfluidic flows for skeletal regenerative medicine. *Cells* 2012;1:1225-1245.
 90. Andersson H, van den Berg A. Microfabrication and microfluidics for tissue engineering: state of the art and future opportunities. *Lab Chip* 2004;4:98-103.
 91. Hasani-Sadrabadi MM, Hajrezaei SP, Emami SH, Bahlakeh G, Daneshmandi L, Dashtimoghdam E, et al. Enhanced osteogenic differentiation of stem cells via microfluidics synthesized nanoparticles. *Nanomedicine* 2015;11:1809-1819.
 92. Kirkpatrick CJ, Peters K, Hermanns MI, Bittinger F, Krump-Konvalinkova V, Fuchs S, et al. In vitro methodologies to evaluate biocompatibility: status quo and perspective. *ITBM-RBM* 2005;26:192-199.
 93. Yim EK, Wan AC, Le Visage C, Liao IC, Leong KW. Proliferation and differentiation of human mesenchymal stem cell encapsulated in polyelectrolyte complexation fibrous scaffold. *Biomaterials* 2006;27:6111-6122.
 94. Sudo R, Chung S, Zervantonakis IK, Vickerman V, Toshimitsu Y, Griffith LG, et al. Transport-mediated angiogenesis in 3D epithelial coculture. *FASEB J* 2009;23:2155-2164.
 95. Shi X, Chen S, Zhao Y, Lai C, Wu H. Enhanced osteogenesis by a biomimic pseudo-periosteum-involved tissue engineering strategy. *Adv Healthc Mater* 2013;2:1229-1235.
 96. Dutenhoefer F, Lara de Freitas R, Meury T, Loibl M, Benneker LM, Richards RG, et al. 3D scaffolds co-seeded with human endothelial progenitor and mesenchymal stem cells: evidence of prevascularisation within 7 days. *Eur Cell Mater* 2013;26:49-64.
 97. Bonzani IC, George JH, Stevens MM. Novel materials for bone and cartilage regeneration. *Curr Opin Chem Biol* 2006;10:568-575.
 98. Marieb EN, Wilhelm PB, Mallatt JB. *Human Anatomy*. San Francisco, CA: Pearson Benjamin Cummings; 2005.