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# Recent advancement in vascularized tissue-engineered bone based on materials design and modification

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ABSTRACT

Bone is one of the most vascular network-rich tissues in the body and the vascular system is essential for the development, homeostasis, and regeneration of bone. When segmental irreversible damage occurs to the bone, restoring its vascular system by means other than autogenous bone grafts with vascular pedicles is a therapeutic challenge. By pre-generating the vascular network of the scaffold in vivo or in vitro, the pre-vascularization technique enables an abundant blood supply in the scaffold after implantation. However, pre-vascularization techniques are time-consuming, and in vivo pre-vascularization techniques can be damaging to the body. Critical bone deficiencies may be filled quickly with immediate implantation of a supporting bone tissue engineered scaffold. However, bone tissue engineered scaffolds generally lack vascularization, which requires modification of the scaffold to aid in enhancing internal vascularization. In this review, we summarize the relationship between the vascular system and osteogenesis and use it as a basis to further discuss surgical and cytotechnology-based pre-vascularization strategies and to describe the preparation of vascularized bone tissue engineered scaffolds that can be implanted immediately. We anticipate that this study will serve as inspiration for future vascularized bone tissue engineered scaffold construction and will aid in the achievement of clinical vascularized bone.

## 1. Introduction

Bone is a highly vascularized connective tissue that depends on the interaction between blood vessels and bone cells for its development [1]. It has been shown that 10 %–15 % of the mammalian cardiac output goes to the skeletal system [2]. The skeleton's circulatory system performs a crucial supportive function in skeletal development. Intramembranous osteogenesis and endochondral osteogenesis are two types of skeletal development. Blood vessels proliferate where the bone is to be created during intramembranous osteogenesis, and blood vessels invade the cartilage during endochondral osteogenesis, signaling the start of ossification [3]. The processes of osteogenesis and angiogenesis are coupled during bone regeneration, and the communication between osteoblasts and endothelial cells is crucial for the bone remodeling process [4]. Arteries supply not only oxygen and nutrition during fracture healing, but also osteogenic stem cells and the ions required for mineralization in the latter phases of fracture healing.

Bone defects produced by tumors, injuries, and geriatric diseases are widespread in clinical practice [5]. The bone tissue can heal itself when the bone defect is small, such as greenstick fracture or crack fracture [6]; however, a bone substitute is needed to assist in the treatment when the bone defect exceeds the critical value (a defect involving 50 % of the cortical diameter with a minimum length of 1 cm) [7,8]. Autologous bone grafts with vascular pedicles are the gold standard for the treatment of large bone defects caused by bone tumors [9]. Nevertheless, there are significant drawbacks to autologous bone grafting, such as the restricted size and quantity of bone blocks available and the trauma caused by bone extraction to the donor site [10]. Bone tissue engineering techniques have been proposed to address these disadvantages.

Tissue engineered bone is a significant tool in the treatment of bone defects in clinical practice. The creation of new bone and blood vessels, as well as tissue integration and functional bearing, are the characteristics of successful artificial bone implantation [11]. The construction of a functional vascular system is central to the application of tissue

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engineered bone [12]. Whereas the skeletal vascular system is heterogeneous, its characteristic endothelial phenotype is closely related to the formation of functional blood vessels [13]. Functional vascularization must first be induced after bone substitute implantation in order to provide the basis for the subsequent repair of bone defects [14]. Previously, the vascular network formed after implantation of the bone substituted into the recipient site decreases gradually from the periphery of the scaffold to the center [15]. The blood supply to the core of the scaffold is deprived, which will eventually lead to failure of the scaffold implantation [16].

To enable the construction of functional vascularization in bone tissue engineered grafts that rapidly communicate with the host vascular system, researchers have proposed the use of pre-vascularization techniques to enhance blood vessel formation within the implant [17,18]. The pre-fabrication technique allows the scaffold to be enriched with a functional vessel before implantation and enables instantaneous anastomosis of the scaffold vessel to the host vessel after implantation [19]. This will significantly increase the likelihood of scaffold activation, but patients will have to wait an excessive amount of time for a premade donor. Enhancing the angiogenic capability of bone tissue engineered scaffolds will not only shorten the time to the pre-production of scaffolds by pre-vascularization techniques, but will also allow exploration of the construction of immediately implantable tissue engineered scaffolds with vascularization capability.

Biomaterial-derived scaffolds play an important role in promoting vascularized bone regeneration in bone tissue engineering [3,20]. Materials that have excellent biological characteristics, such as calcium phosphate, natural polymers, and synthetic polymers, have been widely used in the construction of vascularized scaffolds for bone tissue engineering [21,22]. Aside from the material, the scaffold's structure influences blood vessel formation and osteogenesis. Porous scaffolds favor cell migration, tissue development, nutrition delivery, and waste elimination more than solid scaffolds [23]. The properties of porous scaffolds, such as pore size [24], porosity [25], porous interconnectivity [26], and internal morphology [27], will determine whether peripheral vessels can successfully grow into the scaffold and induce new tissue formation. Furthermore, fracture repair is closely related to the role of cytokines, with vascular endothelial growth factor (VEGF) and bone morphogenetic protein 2 (BMP-2) having a decisive role in vascular repair after fracture [28,29]. Loading cytokines onto scaffolds has become an effective strategy to induce vascularized bone tissue production [30].

## 2. Skeletal vascular network and bone regeneration

### 2.1. Skeletal vascular network

The skeletal vascular system plays an important role in bone development, regeneration, and remodeling [17]. At the macroscopic level, the skeletal vascular system displays a classical hierarchical arrangement of afferent arteries, capillary networks, and efferent veins [31]. In the long bones, for example, the afferent arteries are mainly composed of the central nutrient artery, the metaphyseal-epiphyseal artery, and the periosteal artery [32]. Its capillaries were categorized into type-H vessels (CD31hiEmcnhi) and type-L vessels (CD31loEmcnlo) due to the differential expression of platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) and salivary glycoproteins (Emcn) [33]. Type-H vessels are highly expressive of CD31 and Emcn markers, mainly in the metaphysis and subperiosteum, and are interconnected by distal vascular rings or arches [34]. In comparison, type-L vessels, which are low in CD31 and Emcn markers, are distributed in the backbone and form a dense, perforated, and highly branched sinusoidal network in the bone marrow lumen [33]. At the microscopic level, the periosteal arteries, the small branches flowing from the central nutrient artery, and the small distal arteries in the epiphysis flow into specialized type-H vessels [35,36]. The type-H vessels are connected to the type-L vessels, which eventually join the outflow vein [37]. These two types of vessels are closely connected at the epiphyseal–diaphyseal junction and form a complete vascular bed in the marrow cavity [38](Fig. 1g). Notably, Langen et al. reported that type-H vessels can transition to type-L vessels, and that this process is accompanied by the rapid developmental expansion of bone and none marrow, suggesting that type-H endothelial cells may be upstream endothelial cells in bone [13,39]. In addition, E-type vessels (CD31hiEmcnlo) also exist, which have high expression of CD31 and low expression of Emcn. These E-type vessels are predominantly found in embryonic and early postnatal bone and have the ability to differentiate into type-H vessels [39]. In addition to the classical vascular system described above, Grüneboom et al. recently reported a transcortical vasculature (TCV), also known as the small periosteal vessels [40]. The heterogeneity and uniqueness of the bone vascular system plays an important role in the regulation of bone metabolism.

### 2.2. Skeletal vascular system influences bone regeneration

In the skeletal system, the vascular system is closely related to bone metabolic activity, i.e., angiogenesis, vascular secretory signaling, and osteogenesis are coupled with each other [41]. Schmidt-Bleek et al. reported that revascularization is essential for the regenerative bone healing process [42]. Immunosuppressive agents with anti-angiogenic properties can inhibit new blood vessel formation in fracture healing tissue and delay fracture healing if used [43]. Vascular invasion is an important step in all modes of osteogenesis, and type-H vessel formation is thought to play a key role in regulating the bone formation and repair processes [44]. During the healing of mammalian bone defects, the proliferation of type-H vessels shows spatiotemporal specificity. On day 3 after bone injury, type-H vessels proliferate and are widely distributed throughout the repair area. At 7 and 14 days after injury, type-H vessels began to shrink and were concentrated around the growing trabeculae in the vicinity of the front growth area [45]. This is mainly due to the close molecular communication between type-H endothelial cells and osteoblasts [46]. Kusumbe et al. reported that type-H endothelial cells mediate local growth in the vascular system and provide ecological niche signaling for Osterix + bone progenitor cells, type 1α+ collagen osteoblasts, Runx2+ bone progenitor cells, and PDGFRβ+ pericytes [33]. In contrast, type-L vessels are barely surrounded by osteoblast progenitor cells [47]. This is closely related to type-H endothelial cells with high expression of platelet-derived growth factor A (PDGF-A), PDGF-B, and fibroblast growth factor 1 (FGF1) [33,39]. The above findings further confirm the critical role of type-H vessels in bone regeneration.

In humans, the type-H vessel content decreases progressively with aging, and improving vascularized bone regeneration by inducing type-H vessel formation is a potential target for tissue engineering [48]. Hypoxia-inducible factor-1 (HIF-1α), VEGF, BMP-2, Notch, and Slit3 signaling pathways can exert control over type-H vessel formation and osteogenesis [31,34,44](Fig. 1f). Hypoxia-inducible factor-1 (HIF-1α) expression and activity are regulated by hypoxia and are important regulators of angiogenesis in physiological or pathological conditions [49]. HIF-1α plays a crucial role in the angiogenic–osteogenic cascade reaction [50]. Kusumbe et al. demonstrated that HIF-1α is an important promoter of metaphyseal type-H vessel formation [33]. High levels of HIF-1α expression in cells in the region of bone defects leads to the significant expansion of type-H endothelial cells and metaphyseal vascular columns [51,52]. Bone tissue engineering scaffolds can activate HIF-1α signaling by carrying hypoxia-inducing cobalt (Co) ions to promote type-H vessel and bone generation [53]. Sun et al. prepared a hydrogel that allows slow-release control of Co2+ release. The hydrogel sustained Co2+ release over 21 days and stably activated HIF-1α signaling, which matched type-H vessel formation during bone repair [54]. VEGF is a classical factor that promotes angiogenesis, and regulates the formation of type-H blood vessels [55]. Liang et al. found that

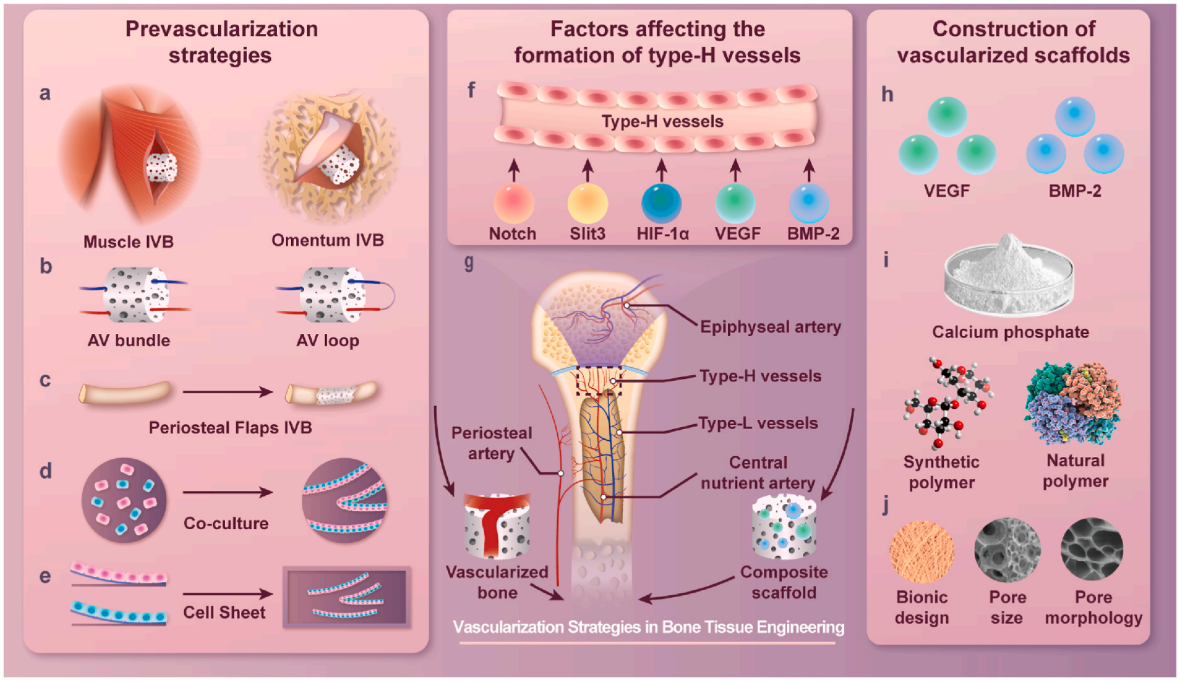


**Fig. 1.** Vascularization strategies in bone tissue engineering include in vivo and in vitro based pre-vascularization strategies and the construction of immediately implantable scaffolds with vascularization capabilities. In vivo pre-vascularization strategies can be divided into tissue flap IVB (a), axial vascular tip IVB (b) and periosteal flap IVB (c). In vitro pre-vascularization strategies can be categorized into co-culture techniques (d) and cell sheet techniques (e). f) Factors affecting the type-H vessels formation. g) Skeletal vasculature system. Immediately implantable scaffolds with vascularization capability are constructed by cytokine loading (h), scaffold materials selection (i), and scaffold structure design (j).

Panax ginseng saponin (PQS) could promote type-H vessel formation by stimulating VEGF expression [56]. In addition, BMP-2, a more widely used growth factor in orthopedic surgery, can also regulate type-H vessel formation [57]. Yao et al. implanted a hydrogel loaded with BMP-2 in a rat mandibular defect model, and animal experiments showed that the BMP-2-triggered region of new bone was rich in type-H blood vessels and associated Osterix + bone progenitor cells [58]. There is evidence that blood flow is also closely related to type-H vessel generation. Ramasamy et al. found that reducing blood flow by ligating the femoral artery or using a blood flow-lowering drug resulted in a significant reduction in the number of type-H blood vessels in the epiphysis of mice, whereas increasing blood flow promoted an increase in the number of type-H vessels and thus promoted bone formation. This is because high blood flow velocity ($0.98 \pm 0.1$ mm $s^{-1}$) stimulates Notch signaling and thus promotes type-H vessel formation [59,60]. In addition, Xu et al. determined, by transcriptome analysis, that SLIT3 is an osteoblast-derived, SHN3-regulated factor that promotes H-type formation. They found that mice with mutations in SLIT3 showed reduced type-H endothelial cells and corresponding defects in fracture repair, whereas mice with mutations in SHN3 showed enhanced fracture repair [47]. Zhai et al. processed polycaprolactone, collagen, and nano-hydroxyapatite into a composite scaffold with vascularized osteogenesis using electrostatic spinning technology. The composite scaffold could significantly promote SLIT3 gene expression in bone marrow mesenchymal stem cells (MSCs) and could effectively repair cranial defects in mice [61].

The vascular system in the skeletal system is more than a passive conduit system; it is intimately and actively involved in the complex processes of bone metabolism. Among the multiple cellular activities and various signaling pathways involved in bone regeneration, type-H vessels have a great capacity to couple osteogenesis with angiogenesis. Currently, the lack of vascularization and functional vasculature in tissue engineered bone has become the biggest obstacle in its clinical application, and the use of type-H vessels as a target for stimulating vascularized bone regeneration holds great promise.

## 3. Pre-vascularization strategy

The scaffold pre-vascularization approach provides the implant with a vascular pedicle before placement. These vascular pedicles can be anastomosed with the blood vessels at the recipient location after implantation, allowing the scaffold to be perfused instantly and bypassing the vascularization phase. As a result, it can be employed to solve the vascularization problem [62]. Pre-vascularization techniques for scaffolds in bone tissue engineering may be separated into in vivo pre-vascularization procedures and in vitro pre-vascularization strategies.

### 3.1. *In vivo pre-vascularization strategies*

The in vivo pre-vascularization strategy for bone tissue engineering takes advantage of the body's self-regenerative capacity by using a part of the body as an in vivo bioreactor (IVB) for in situ or ex situ culturing of bone tissue engineering scaffolds [63]. Depending on the scaffold and implantation location, vascularization of the graft can take weeks or months [64]. Vascularized scaffolds for the reconstruction of bone defects are obtained immediately after the end of culture. The preparation of the scaffold and the selection of the implantation site are two key elements of the in vivo pre-vascularization strategy for bone tissue engineering [65]. Currently, 3D printing technology allows the construction of scaffolds or culture chambers with different shapes according to individual patient variability [66]. Secondly, the choice of scaffold or chamber filling materials, seed cells, and growth factors needs to be determined according to the implantation site [67]. Furthermore, the choice of implantation site determines the subsequent steps and clinical outcomes. Constructing an IVB at the defect site can directly repair bone defects without secondary surgery. If the culture conditions of the defect site are poor, then ectopic construction of the IVB can be chosen. Currently, in bone tissue engineering, in vivo pre-vascularization strategies can be classified into tissue flap-based IVB, axial vascular tip-based IVB, and periosteal flap-based IVB, depending on the

implantation location and approach.

### 3.1.1. IVB construction based on tissue flaps

*3.1.1.1. IVB based on muscle bag.* The muscle pouch preformation technique utilizes the self-regenerative ability and safe induction of differentiation of living muscle bags to create vascularized tissue engineered bone [68]. The technique can be traced back as far as 1995 when Fujimura et al. successfully induced ectopic osteogenesis in rat thigh muscles using BMP-2 and type I collagen [69]. The traditional approach

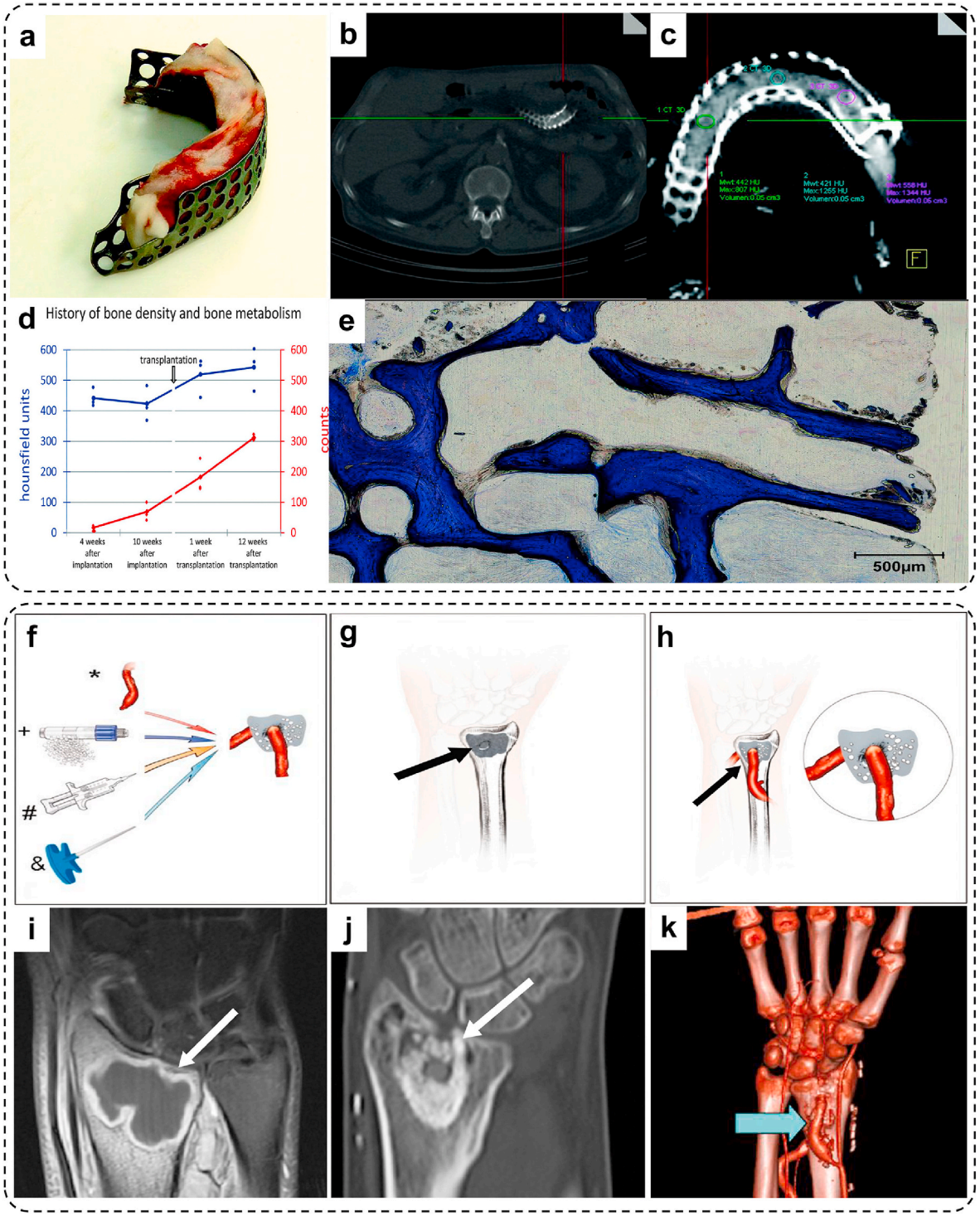


**Fig. 2.** a) Titanium mesh cage filled with bone mineral blocks, recombinant bone morphogenetic protein-2 (rhBMP-2), and human bone marrow aspirate. b) Computed tomography (CT) image of the titanium mesh cage 10 weeks after implantation in the greater omentum. c) CT image of the prefabricated titanium mesh cage 1 week after implantation into the mandible. d) Bone density and bone metabolic activity during transplantation. e) Histological evaluation of bone biopsies 3 months after transplantation [76]. f–h) Schematic diagram of arteriovenous (AV) loop repair of a bone defect. f) After the creation of the bone defect. g) Placement of osteogenic material into the defect. h) Anastomosis of the radial artery and cephalic vein forms an AV loop and penetrates the osteoid structure. i) Preoperative magnetic resonance imaging (MRI) showed a cystic lesion of the distal radius. j–k) Angiography at 14 months after surgery showed AV loop patency and complete healing of the bone defect [81].

is to create a non-degradable chamber based on the shape of the bone defect and fill the chamber with osteoinductive material. The chamber is then surgically implanted into a blood-rich muscle pocket for culture. A few weeks later, the personalized vascularized bone is transferred to the recipient site for microvascular anastomosis [70]. The technique enables the vascular pedicle to be transferred along with the vascularized bone mass, which allows for better repair in areas where the vascular bed is significantly damaged [71]. Importantly, the role of BMP-2 in ectopic preformation is to induce ectopic osteogenesis, which is, therefore, an essential cytokine in the preformation process. Cao et al. prefabricated 3D printed β-tricalcium phosphate (TCP) scaffolds (with or without a recombinant bone morphogenetic protein-2 (rhBMP-2) coating) using monkey latissimus dorsi. After prefabrication, they found that only the TCP scaffold with the rhBMP-2 coating would have new bone generation. They then implanted the prefabricated rhBMP-2/TCP scaffold together with the myocutaneous flap into the rhesus mandibular defect. The scaffold demonstrated strong vascularized osteogenesis compared to the unprepared control group [72]. In addition,muscle bag prefabrication techniques have been reported to be successfully applied in clinical practice. Kokemueller et al. used the patient's latissimus dorsi muscle for ectopic vascularization cultures to obtain artificial bone blocks rich in blood vessels. A personalized titanium mesh filled with the vascularized bone block was then implanted into the patient's segmental mandibular defect. After a period of recovery, the patient's mandibular defect healed well and the temporomandibular joint was stabilized [73].

*3.1.1.2. IVB based on omentum.* In comparison to muscle tissue, the gastrocolic omentum is thin, flexible, rich in vascular tissues, and has precursor cells that support osteogenic differentiation [74]. The porcine omentum was employed as a bioreactor for prefabricated vascularized scaffolds by Naujokat et al. [75]. Custom-made titanium chambers containing pig bone marrow aspirate, bone mineral blocks, and BMP-2 were implanted into the porcine omentum for culture, and customized bone blocks with vascular pedicles were obtained after 8 weeks. Additionally, the omental prefabrication technique has been successfully shown in clinical situations. Wiltfang et al. [76] inserted titanium mesh cages containing human bone marrow aspirate, rhBMP-2, and bone mineral blocks into the patient's gastrocolic omentum for prefabrication. Three months later, the team removed the prefabricated vascularized bone block from the titanium cage and reconstructed the patient's mandibular defect. Grafts increased in density and metabolic activity both before and after transplantation, suggesting adequate vascular supply and survival of induced bone tissue. Three months after implantation of the defect, histological evaluation showed that most of the graft was covered by a bone-like matrix, and the patient's quality of life was significantly improved (Fig. 2a–e). The large amount of space in the omentum makes it possible to prefabricate vascularized scaffolds of different sizes, including large bone substitutes of various shapes. Moreover, the omentum is rich and regenerative, and removal of part of it does not affect the body. Therefore, the application of omentum for the prefabrication of vascularized bone has a relatively broad application prospect.

Bone marrow aspirates used for muscle pouch and greater omentum pre-vascularization may contain CD31hiEmcnhi subpopulations, bone progenitor cells, and bioactive factors. The addition of BMP-2 during preparation can promote type-H vessel formation and ectopic osteogenesis [57,58]. In addition, scaffolding raw materials, such as bone mineral blocks and calcium phosphate, also have osteoinductive and conductive properties [77,78]. Furthermore, the specially designed culture chambers provide a hypoxic microenvironment that facilitates the secretion of HIF-1α from the surrounding tissues.

Moreover, the muscle pouch and peritoneal environment in which the culture chambers are located can provide the appropriate stress conditions for the growth of vascularized scaffolds [79]. These conditions provide a favorable microenvironment for the ectopic culture of vascularized bone. Evidence suggests that neonatal bone tissue obtained by pre-vascularization in culture is more capable of restoring bone defects than autologous bone. Dai et al. obtained highly vascularized juvenile bone within 3–5 weeks after subcutaneous implantation of gelatin scaffolds loaded with BMP-2 in mice. Compared with autologous bone, this vascularized juvenile small bone showed fewer senescent MSCs, and abundant type-H vessels and bone progenitor cells. Compared with the non-pre-vascularized treatment group, the juvenile small bone could completely repair critical-sized cranial defects in young and old mice 2 weeks after transplantation [80].

### *3.1.2. IVB construction based on axial vascular tip*

*3.1.2.1. Arteriovenous (AV) loop model.* The arteriovenous (AV) loop model is also an IVB that induces the generation of axially vascularized tissue. In animal models, superficial arteries and veins are usually anastomosed to form an AV loop, after which the AV loop is placed in an implantation chamber with bioactive material, and vascularized bone tissue is obtained over the period of culture [82]. AV loop cultured grafts feature more vasculature and are more densely packed than the muscle pocket and omentum, making them safer and more promising for clinical use [83]. Horch et al. [81] applied the AV loop model to successfully treat a 3 × 9 × 4 cm bone defect in the distal radius of one patient. Vascularization of this defect was achieved by inserting a segment of a lower arm vein graft into the arteriovenous loop between the palmar radial artery and the dorsal cephalic vein. The filler in the defect was composed of clinically approved β-tricalcium phosphate/HA, fibronectin, and direct autograft bone marrow taken from the right iliac crest. At the postoperative follow-up, imaging showed the presence of an open arteriovenous loop as well as a fully healed radial bone defect (Fig. 2f–k). This clinical example fully demonstrates the feasibility of the AV loop technique for clinical application.

The immune response, hemodynamic alterations, and hypoxia may be involved in the process of the AV loop model supporting vascular development and bone replacement remodeling. Hessenauer et al. [84] observed the presence of rolling and firmly adherent leukocytes at the site of the vein graft and microsurgical anastomosis in the rat AV loop model. White blood cell recruitment is the first step in the integration of any biomaterial tissue. Leukocytes at these sites of inflammation produce angiogenic factors, including VEGF, angiopoietin-1, PDGF, transforming growth factor (TGF), and epidermal growth factor (EGF) [85]. VEGF and PDGF can significantly promote the expression of type-H vasculature [31,34]. Furthermore, the venous segment in the AV loop is critical for initiating flow-mediated angiogenesis [86]. Researchers discovered that the venous side of the AV loop generated more vascularized tissue than the arterial side in the mouse AV loop model built by Wong et al. [87]. They further discovered that as the culture period increased, the vascularized tissue in the lumen infiltrated the matrix around the chamber and spread radially from the arteriovenous anastomosis to the surrounding region [87]. This is primarily due to the hemodynamic effects of the AV loop's specific structure, namely the high flow rate and shear stress on the thin vein wall [88]. This high shear stress and blood flow effect promote vascular Notch signaling, thereby promoting type-H vessel formation [60]. In addition, there is a direct correlation between the high blood flow state and the expression of the Cx gene in hemodynamics. Connexin (CXS) is a four-helix transmembrane protein. Myoendothelial cells (ECs) and vascular smooth muscle cells (VSMCs) can communicate through CXS. This exchange of information between cells enables vascular networks to adapt to changes, such as short-term changes in vascular tension to regulate blood flow [89], and long-term adaptation processes, including angiogenesis and wound repair [90]. Schmidt et al. investigated the hemodynamic effects on AV loop formation in axially vascularized tissue using a rat arteriovenous ring model. They discovered that significant hemodynamic alterations resulted in a significant increase in connexin

Cx43 expression in venous segments [91]. Furthermore, in vitro studies show that high shear stress and blood flow acting on ECs can increase Cx40-linked protein expression via the PI3K/Akt pathway [92]. Cx40 is thought to be essential for vascular arterial homogeneity and plays a role in angiogenesis [93]. Hypoxia is also thought to be an important cause of AV loop formation. A previous study discovered that AV loops in isolation chambers experience hypoxia, which was characterized by the upregulation of HIF [94]. Yuan et al. observed, through a constructed rat AV loop model, that vessels began to grow rapidly with increasing HIF-1α ratios in an AV loop model [95]. This suggests that HIF-1α promotes vascularized bone formation by facilitating type-H vessel formation [50,94].

*3.1.2.2. AV bundle model.* The AV bundle model requires a section of the unbranched AV bundle to pass through a custom chamber filled with osteoinductive material to obtain vascularized artificial bone. Compared to the AV loop model, the AV bundle model does not require anastomosis of the vessels. This reduces the risk of thrombosis and angioma formation. The customized chamber filled with biomaterials can then be prefabricated through the AV bundle [96]. This technique simplifies the AV loop technique by eliminating the need to graft additional vein segments; however, it produces less vascularized tissue [97]. There are currently successful applications of vascular bundle technology. Ismail et al. placed chambers filled with allogeneic inactivated bone matrix and autologous stromal vascular component (SVF) cells and BMP-2 within the patient's latissimus dorsi muscle and cultured AV bundles from the thoracic dorsal vasculature through the chambers. After 32 weeks of pre-culture, the mature vascularized bone was successfully transplanted into the patient's maxillary defect and enabled the functional reconstruction of the patient's maxilla [98]. The addition of AV bundles within prefabricated chambers provides a well-defined vascular axis and improves vascularization and bone formation in prefabricated structures [96]. In addition, Charbonnier et al. reported the application of a single vein as a vascular axis to successfully induce vascularized osteogenesis in a porous bioceramic scaffold loaded with autologous bone marrow. The team applied avascular control to induce bone levels of 9 %–26.6 %, whereas osteogenic levels of up to 66 ± 6 % were achieved after the application of a single venous shaft for induction [99]. This osteogenic effect, which is no less than that of the AV bundle, may be related to the fact that thin-walled veins are subjected to pressures that are an order of magnitude higher than physiological pressures, resulting in the germination of the official lumen [100,101].

### 3.1.3. IVB construction based on periosteal flaps

When inducing ectopic osteogenesis, all the above approaches require the injection of cytokines to stimulate osteogenic differentiation, whereas periosteal osteogenesis does not. Periosteal induction is a method that uses the periosteum's intrinsic osteogenic and angiogenic properties as an IVB to induce vascularized bone growth in a specified shape [102]. This approach works by removing a portion of the prefabricated object's rib and leaving the empty periosteum, followed by implanting a reaction chamber filled with bone induction material into the rib periosteum for internal culture. After the prefabrication is finished, it can be removed and implanted into the problem location [103]. This is a newer model, and its viability has been demonstrated in successful animal models. Tataraa et al. [66] successfully repaired the model of a large mandibular defect in sheep using periosteal reactor technology. In their periosteal bioreactor, engineered bone with good vascularization can be obtained by using calcium phosphate cement or crushed sheep autogenous bone as bone induction material. In contrast to the above methods, the periosteal bioreactor can induce ectopic osteogenesis without exogenous BMP-2. However, this technique requires surgical osteotomy to obtain the empty periosteum and is more traumatic to the prefabricated area. In the future, if in vitro bionic construction of the periosteum is possible, the treatment of defective areas can be personalized.

These in vivo pre-vascularization methods face some problems, such as the need for multiple operations, possible infection, hemangioma, and the risk of thrombosis, compared with autogenous bone transplantation. In addition, the in vivo prefabrication procedure may be interfered with by soft tissues when the rate of proliferation of soft tissues is faster than the rate at which inducers stimulate new bone formation [104]. The soft tissue contained in prefabricated scaffolds can be counterproductive to bone regeneration and can even lead to serious problems with soft tissue entrapment at the defect site after implantation. We can interfere with soft tissue generation by the following methods. Firstly, the formation of functionalized bone blocks can be controlled by controlling the number of inducers, such as BMP-2, thus avoiding soft tissue seating in the implant [80]. Secondly, cytokines that inhibit the growth of soft tissues such as muscle can be added to the prefabricated cells during prefabrication [105]. Moreover, the period of the ectopic culture of vascularized bone in the human body must be investigated further. When the culture period is too short, the bone does not have adequate vascular pedicle and volume, and when the culture time is too long, bone resorption might occur. According to Wolf's law, if the stress on bone tissue is insufficient, the bone will collapse [106]. In IVB, engineered bone is subjected to only limited mechanical loading. The prefabricated vascularized bone may have a better therapeutic effect if some stress is applied to the bone mass during the ectopic culture process.

## 3.2. In vitro pre-vascularization strategies

In vitro pre-vascularization procedures are less dangerous than in vivo pre-vascularization strategies and can prevent subsequent injury to patients. In recent years, there has been an increase in the sophistication of in vitro pre-vascularization techniques, which focus on pre-vascularization prior to graft implantation in vitro. These techniques primarily involve the use of tissue-specific cells in culture to generate vascularized tissue structures that can be implanted into the defect with improved vascular connectivity and osseointegration [107]. The co-culture technique and the cell sheet technique are the two main types of cell-based in vitro pre-vascularization strategies.

### 3.2.1. Co-culture techniques

The co-culture of MSCs and ECs is one of the most straightforward approaches to bone tissue engineering co-culture. Co-culture techniques can better mimic the in vivo situation because signals between cells are transmitted via connections between different cell types, exosomes, and paracrine activity [108]. Bo et al. co-cultured dental follicle-derived stem cells (DFSCs) with human umbilical vein endothelial cells (HUVECs). They discovered that the cultures had a significantly higher expression of angiogenesis-related genes and proteins, as well as improved osteogenic potential [109]. This co-culture system is kept going for a long time to produce tissue with a vascular structure, but this vascular structure is only suitable for a 2D culture environment and the vasculature does not function [19]. The solution to this problem is the use of scaffolds, which can be tailored to the shape of the defect so that grafts with a 3D vascular network can be formed in co-culture. By covering hydrogels packed with human adipose-derived MSCs (ADMSCs) and HUVECs on 3D printed polycaprolactone/hydroxyapatite scaffolds, Kuss et al. successfully created composite scaffolds with vascularized osteogenic potential [110]. In vitro experiments with immunohistochemistry and qPCR analysis showed that the co-culture system promoted capillary network formation and vascularization gene expression. Microvessel formation was visible on the scaffold in the overall view and histological analysis 4 weeks after subcutaneous implantation. The immunohistochemical analysis further showed that these official structures were formed by HUVECs (Fig. 3a–c). Furthermore, Smirani et al. discovered that pre-vascularized grafts had more vascular network connections to the recipient site, as well as a higher proportion of erythrocytes in the lumen and greater blood flow when

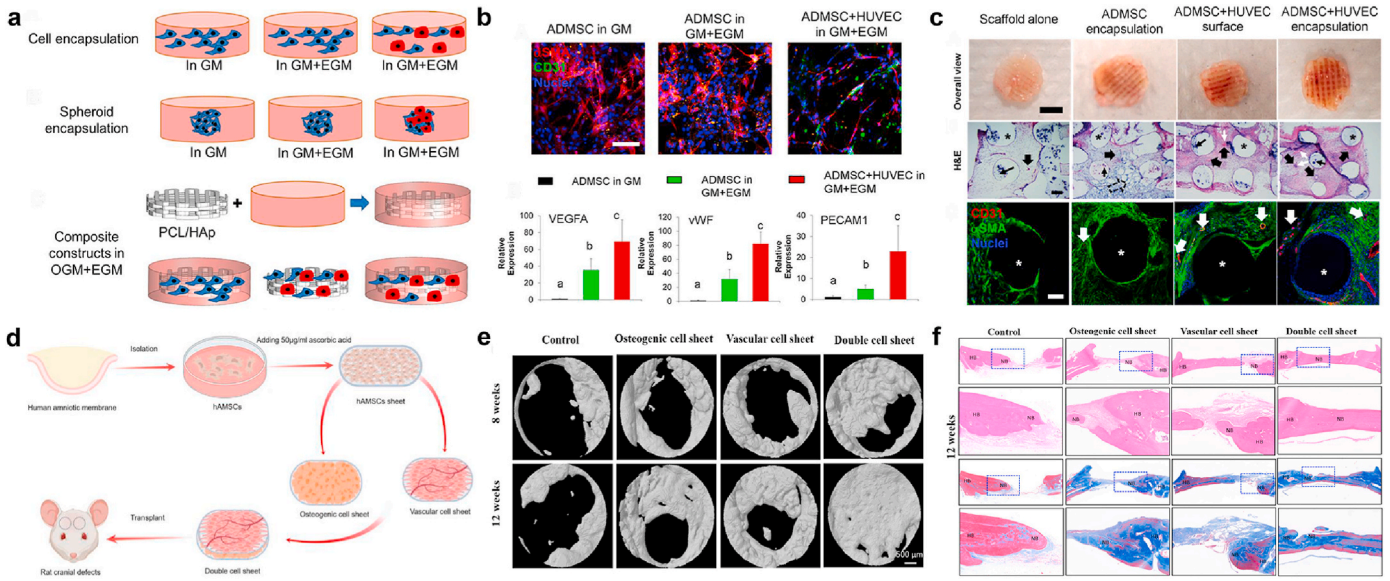


**Fig. 3.** a) Preparation of polycaprolactone/hydroxyapatite (PCL/HAp) scaffolds loaded with encapsulated adipose-derived mesenchymal stem cell (ADMSC) and human umbilical vein endothelial cell (HUVEC) hydrogels. b) The co-culture system promotes capillary network formation and vascularization gene expression. c) In vivo angiogenesis within the composite scaffold [110]. d) Experimental procedure of different cell sheets for bone defects. e) Micro-computed tomography (CT) analysis of in vivo performance at 8 weeks and 12 weeks. f) Hematoxylin and eosin staining and Masson staining at 12 weeks [112].

compared to the control group [111]. These findings demonstrate the potential of pre-vascularized scaffolds in healing large bone defects.

### 3.2.2. *Cell sheet technology*

Cell sheet technology typically involves growing unitary tissue-specific cells on temperature-sensitive polymers that allow cells to adhere and proliferate at 37 °C. After lowering the temperature, cell slices can be separated without the use of trypsin. Controlling the culture temperature can result in a single cell sheet with a complete cell connections and an established extracellular matrix [113]. EC sheet layers can be included in a multilayer cell sheet to create vascularized tissue structures using this technique [114]. Xu and colleagues seeded bone marrow-derived stem cell (BMSC)-derived ECs on BMSC cell sheets. Lumen-like structures and osteoblast slices formed on the flake tissue after co-culture. The vascularized tissue was then implanted into a major skull defect in rats, and the results revealed that the flake tissue developed functioning perfusion arteries and new bone tissue [115]. Similarly, Zhang et al. [112] used human amniotic mesenchymal stem cell (hAMSC) sheets as the basis for constructing vascular cell sheets, osteoblast sheets, and bicellular sheets, respectively. They then used a rat cranial defect model to validate the regeneration of bone defects treated with different cell sheets. Micro-competed tomography (CT) at 8 and 12 weeks postoperatively showed that the bicellular sheets exhibited significantly better bone repair. Histological analysis showed that bone defects covered by bicellular sheets formed more regularly arranged bone covered with cuboidal osteoblast-like cells compared to the other groups (Fig. 3d–f). In addition, cell sheet technology can be combined with metal scaffolds, enhancing the vascularization ability of metal scaffolds. MSC sheets were adhered to the surface of titanium implants by Yan et al. to form a tightly connected MSC sheet-titanium composite. In the experiment, the composite scaffold promoted osteogenesis and angiogenesis very well [116]. The cell sheet technique is predicted to improve metal transplant biocompatibility and the ability to generate vascularized bone. Although the cell sheet technology has excellent advantages, the number of layers accumulated by the cell sheet can never break through the limit of 12 layers. This may be due to the limited diffusion distance of oxygen and nutrients, thus, EC slices will not form more complete blood vessels [117]. Nonetheless, this technology has promise, and some researchers may be able to push it beyond the 12-layer limit in the future by combining cell sheets with other cytokines and materials for better application in bone tissue engineering. In addition, no researchers have reported that functional type-H vessels can be obtained using co-culture techniques and cell sheet techniques, so the exploration of targeting type-H vessels may be a potential direction for in vitro pre-vascularization studies.

The two pre-vascularization techniques discussed above appear to be a promising approach for improving graft integration into the host bone defect, particularly when the graft is large. It has a positive impact on ongoing neovascularization after implantation because it allows for pre-vascularization of the graft, enabling good integration with the host receptor site microenvironment. Nevertheless, the pre-vascularization procedure takes a long time to prefabricate a prosthesis and cannot be employed immediately in fresh bone defects. If the preformation period could be greatly decreased, this approach would offer tremendous potential for the correction of segmental bone defects.

## 4. Vascularization scaffold materials in bone tissue engineering

Given the extended duration needed for the prefabrication process of the pre-vascularization technique, the emergence of filling tissue engineering scaffolds that possess immediate vascularization potential presents a promising alternative for managing segmental bone defects. A scaffold is a biomaterials-based 3D structure that offers a surface milieu for cell adhesion, development, reproduction, and function, as well as structural and mechanical support for cellular interactions [118]. It has been demonstrated that the chemical makeup of the scaffold material influences the angiogenic process at the implantation site [119]. Therefore, the choice of scaffold material is crucial to vascularized bone tissue engineering. The following requirements should be carefully considered while creating a bone scaffold with vascularization potential. Various natural and synthetic materials, biodegradable and non-biodegradable, have been employed in the fabrication of bone scaffolds through various techniques. Each of these materials has distinct features. Based on these requirements, we outlined two types of materials for vascularized bone tissue engineering applications: calcium phosphate and polymers.

### 4.1. Calcium phosphate

#### 4.1.1. Advantages of calcium phosphate in vascularized bone tissue engineering

The biocompatibility, osteogenic induction, and osteoconductivity of calcium phosphate materials make them one of the important biomaterials in the field of bone tissue engineering [120]. The chemical composition and hierarchical multilevel structure are generally considered to be factors in osteogenesis induced by calcium phosphate materials [121]. Recently, with the deeper exploration of calcium phosphate materials, researchers have suggested that osteogenesis induced by calcium phosphate materials may be related to the activation of BMP/Smads, Wnt, and Notch signaling pathways. Tang et al. [122] examined the expression of BMSC signaling molecules on calcium phosphate ceramics and culture plates, respectively. They found that Smad1, 4, 5, and Dlx5, the major molecules in the BMP/Smads signaling pathway, could be significantly upregulated by the calcium phosphate ceramic plate. In addition, Wang et al. [123] evaluated the expression of Wnt, Notch signaling pathway, and osteogenesis-related genes on CaP ceramics in BMSCs with and without the Wnt pathway inhibitor DKK1. The expression of Wnt, Notch signaling pathway, and osteogenesis-related genes increased and then decreased without the addition of the inhibitor, whereas the overall expression showed a decreasing trend after the addition of the inhibitor. The above studies suggest that BMP/Smads, Wnt, and Notch signaling pathways play important roles in calcium phosphate-induced osteogenic differentiation, but the mechanism of the synergistic effects needs to be further explored.

In addition, the effect of calcium phosphate materials on angiogenesis is manifested in several ways. First, the solid-phase calcium phosphate material implanted at the trauma site is degraded to liquid-phase calcium and phosphate by solution-, protein-, and cell-mediated mechanisms [124]. In vivo, these increased calcium and phosphate levels were negatively correlated with apoptosis of VSMCs, oxidative stress, and EC apoptosis [125]. Angiogenesis is tightly regulated by pro-angiogenic factors, and the expression of VEGF-promoting signaling pathways correlates with increased intracellular calcium concentrations [126]. Nadège et al. demonstrated that a calcium-rich environment facilitates the generation of vascular tissue in the initial phase [127]. Second, the physical properties of the calcium phosphate material can have an impact on angiogenesis. The process of calcium phosphate scaffold degradation produces stress changes in the surrounding tissues, and the ECs respond to the mechanical pulling of the surrounding tissues [128]. Thus, changes in ambient stress may affect angiogenesis [129]. Furthermore, the porous nature of calcium phosphate materials facilitates the invasion of vascular tissues [130]. The ability of calcium phosphate materials to induce angiogenesis and osteogenesis varies with their physicochemical properties [131]. Therefore, it is important to control these properties and select the right calcium phosphate for a specific application. The most commonly used calcium phosphate materials in bone tissue engineering today are hydroxyapatite (HAp), β-tricalcium phosphate (β-TCP), and bidirectional calcium phosphate (BCP).

#### 4.1.2. HAp, β-TCP, and BCP

Natural bone contains HAp as a component of calcium hydrate. It is the most stable form of calcium phosphate known [132]. HAp is commonly employed in bone tissue engineering because of its great biocompatibility and osteoinductivity [133,134]. Burgio et al. described a miniature scaffold made of Hap, which displayed typical expression of angiogenic marker genes in the experiment, indicating that HAp had excellent angiogenic effects [135]. Because of its distinctive hard and brittle qualities, HAp has mostly been used as a HAp coating and nano-HAp delivery system, rather than in situations where high stresses are applied [136,137].

HAp implants synthesized by high temperature sintering or hydrothermal conversion have a much higher crystallinity than bone minerals, making HAp non-degradable after implantation [138]. In contrast to HAp, β-TCP is biodegradable and can be completely replaced by new bone minerals [139,140]. Under physiological conditions, the solubility of β-TCP is similar to that of bone minerals and is normally dissolved by osteoclasts and forms an apatite layer on the surface [141]. On the one hand, the newly formed surface apatite layer absorbs proteins from the surrounding tissue and encourages osteoblasts and angiogenic cells to adhere, proliferate, and differentiate, leading to the healing of bone defects [142]. On the other hand, calcium released by β-TCP lysis is closely related to the process of neovascularization at the fracture site. Among them, free calcium ions may affect the structure of the blood clot at the fracture site, thus affecting fracture healing [143]. In addition, β-TCP significantly promoted neovascularization at the defect, as demonstrated by Anghelescu et al. by establishing a tibial healing model [144].

Calcium phosphate ceramics composed of β-TCP and HAp are known as BCP, which is highly similar to the composition of bone minerals [145]. The Hap/β-TCP ratio is the primary determinant of the solubility of BCP ceramics, and the absorbability and stability of BCP can be managed by adjusting the material ratio [146]. Shao et al. constructed BCP scaffolds with various HAp/β-TCP ratios (HAp30/β-TCP70, HAp50/β-TCP50, and HAp70/β-TCP30) and examined their biological and degrading characteristics. The findings demonstrated that BCP scaffolds with a HAp/β-TCP ratio of 30/70 were capable of regenerating bone with excellent efficiency and a degradation rate that was consistent with bone formation [147]. BCP scaffolds have been demonstrated to induce osteogenesis, increase cell adhesion, and absorb growth hormones in BMSCs [148]. In addition, BCP has been shown to promote angiogenesis [149]. Chen et al. evaluated the possibility of BCP ceramics to stimulate the differentiation of BMSCs to ECs in a real physiological environment by modeling an in vivo diffusion chamber. They prepared diffusion chambers containing BCP ceramics inoculated with BMSCs and implanted the chambers into subcutaneous pockets on the backs of New Zealand rabbits for culture. Results of in vitro and in vivo experiments show that at the genetic level BCP ceramics significantly stimulates the differentiation of BMSCs towards ECs [150](Fig. 4a–c). Additionally, to investigate the angiogenic and osteogenic potential of BCP scaffolds, Zhang et al. implanted them into mandibular abnormalities in miniature pigs. An osseointegration transcriptional study indicated that genes associated with angiogenesis were increased in the BCP group [151]. The preceding research establishes the vascularized osteogenic capacity of BCP ceramics.

#### 4.1.3. The role of calcium phosphate in vascularized bone tissue engineering

The hard and brittle qualities of calcium phosphate materials currently limit their usage in load-bearing applications. They are primarily employed in drug delivery systems, biological coatings, and doping with other materials to generate composite scaffolds in bone tissue engineering [153]. The nano calcium phosphate drug delivery system is an advanced drug delivery method. The significant surface area to volume ratio of calcium phosphate nanoparticles allows for greater diffusion drive and particle solubility. This high surface-to-volume ratio can influence the adhesion of specific proteins, making it particularly suitable for the delivery of therapeutic factors [154]. Calcium phosphate nanoparticles have now been successfully employed to provide bone repair therapeutic components. Kim et al. assembled BMP-2 and VEGF into large (300–500 μm) and small (100–200 μm) microcarriers, respectively. Scanning electron microscopy revealed that both sizes of microcarriers contained hollow internal structures and no differences in surface morphology were observed. Histological results of critical-size cranial defects in rats showed that a calcium phosphate delivery system loaded with growth factors can lead to substantial new bone formation compared with controls [152] (Fig. 4d–f). Calcium phosphate nano-delivery systems have also shown promise in combination with gene therapy for bone repair [155].

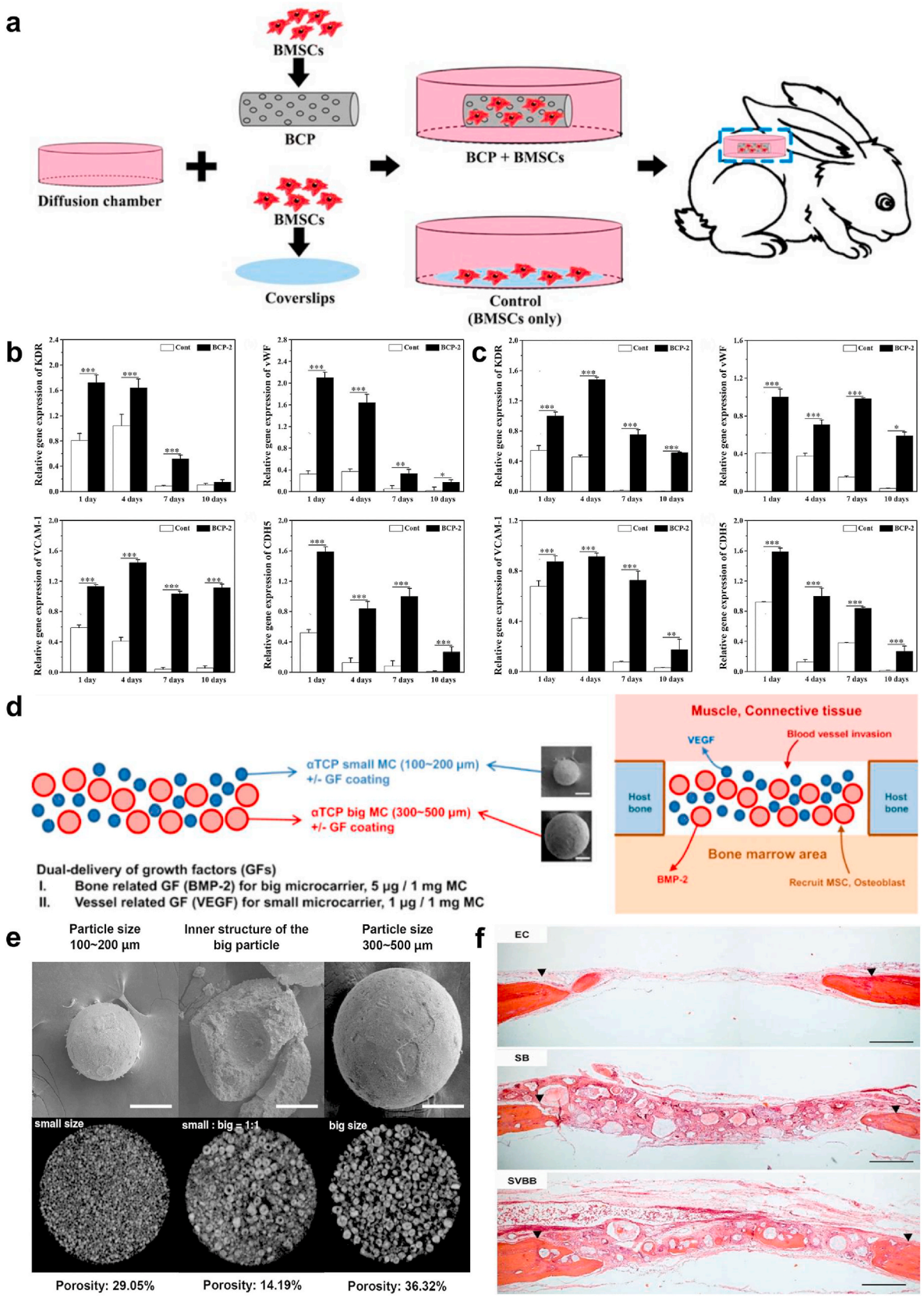


**Fig. 4.** a) Schematic diagram of the in vivo model of bidirectional calcium phosphate (BCP)-induced differentiation of bone marrow-derived mesenchymal stem cells (BMSCs). b) Expression of angiogenic genes in BMSCs when cultured on BCP and coverslips in vitro. c) Expression of angiogenic genes in BMSCs when implanted on BCP and coverslips [150]. d) Schematic plane of the application with two bioactive factors incorporated in the calcium phosphate microcarrier for bone regeneration. e) Scanning electron microscopy (SEM) micrographs of calcium phosphate microcarriers. f) Implantation of dual growth factor-loaded calcium phosphate microcarriers into a mouse cranial bone model resulted in substantial new bone formation [152].

Schlickewei and colleagues developed an injectable DNA-loaded nano calcium phosphate paste. They loaded the paste with transfected BMP-7 and VEGF-A DNA and implanted it into critical-size bone lesions in rabbits. The transfected DNA group healed bone more quickly and for a longer period of time than the control group [156].

The use of calcium phosphate coating on scaffolds is intended to increase biocompatibility and vascularization [157]. Research on a titanium plate with a calcium phosphate coating was published by Khlusov et al. [158], who reported microvascular invasion of the calcium phosphate layer after implanting the scaffold subcutaneously in mice for 3 weeks. The calcium phosphate coating modification considerably improved the vascularization of titanium grafts. This is a relatively new way of drug delivery. Prosolov et al. describe a method for fabricating a drug delivery system based on a calcium phosphate coating that can carry several different drugs and can appropriately regulate their release [159]. Drug delivery systems based on calcium phosphate coatings are currently considered to be beneficial drug delivery systems. In complex bone disease cases, calcium phosphate coatings can release Ca, P, and drugs available for bone growth in a localized area and can induce new bone production under multiple effects. However, there are some limitations in its native province, such as the loose manner in which the coating adsorbs the drug and the limited drug-loading capacity of the coating [160]. Secondly, the burst release of calcium phosphate carrier drugs is a major problem [161]. Therefore, continuous improvement is still needed in terms of more controlled drug release and precise administration of high concentrations of drugs for topical application.

Calcium phosphate can be combined with other materials to create composite scaffolds. These scaffolds can be modified to attain the right mechanical strength and elastic modulus by modifying the material ratio and structure. They also have strong biological qualities because they are made of calcium phosphate. A polycaprolactone-poly (lactic-ethanolic acid)-tricalcium phosphate composite scaffold was described by Kumar et al. [162]. This scaffold is biocompatible and has the proper porosity to encourage vascularized bone tissue to grow inward. Furthermore, throughout its resorption by the body, the scaffold can maintain acceptable mechanical characteristic. As previously stated, using calcium phosphate in conjunction with other biomaterials allows for greater control and improvement of their characteristics, hence better fulfilling the duty of stimulating vascularized bone tissue formation.

### 4.2. Polymers

Natural biopolymers and synthetic polymers are the most common organic materials used in bone tissue engineering [163]. Proteins (collagen, elastin, fibronectin, filamentous protein) and polysaccharides are among the natural polymers that can be employed as scaffold materials (chitosan and alginate). They offer good biocompatibility, biodegradability, cell adhesion, and growth-promoting qualities [164]. However, they also have some disadvantages such as immunogenic reactions, poor mechanical properties, and uncontrollable degradation rates [165]. Synthetic polymers have lesser bioactivity and fewer cell recognition sites than natural polymers, but they degrade at a controlled rate [166]. Polycaprolactone (PCL), polylactic acid (PLA), and polylactic acid-glycolic acid copolymers are the most utilized synthetic polymers in bone tissue engineering scaffold fabrication (PLGA).

#### 4.2.1. Natural polymers

*4.2.1.1. Collagen.* Collagen is a key component of normal bone formation. It is abundant in the bone matrix secreted by osteoblasts and plays a significant role in matrix mineralization [164]. More than 20 different types of collagen have been identified, of which the most common is type I collagen [167]. Patients with type I collagen defects develop osteogenesis imperfecta, which is characterized by bone fragility and skeletal deformities [168,169]. Mizuno et al. found that when BMSCs were cultured in contact with type I collagen, BMSCs differentiated towards osteoblasts and expressed an osteoblastic phenotype [170]. Furthermore, type VII collagen enhances the osteogenic potential of human MSCs via the ERK-dependent pathway [171]. Collagen has been reported to have the potential to induce the differentiation of BMSCs into ECs. Kulakov et al. co-injected collagen and MSCs into the subcutis of rats and discovered considerable vascularization in the newly produced tissue after 7 days [172]. This study found that collagen can promote the formation of vascularized tissue. Researchers have attempted to overcome collagen's poor mechanical qualities to fully use the material's osteoinductivity and angiogenesis capabilities. One approach would be to use collagen as the soft matrix component of the hard scaffold. For example, Culla et al. first prepared calcium phosphate cement (CPC) scaffolds and then injected mineralized collagen matrix (MCM) into the CPC scaffolds, resulting in composite MCM–CPC scaffolds. The team used a mouse model of a critical bone defect in the femur to validate the scaffold's osteogenic properties. Histological analysis showed more new bone tissue growing deeper within the hybrid scaffold compared to the CPC group alone. The combination of CPC and MCM accelerates the growth of new bone in the scaffold while enhancing the biomechanical stability [173] (Fig. 5a and b). Another approach is to combine collagen in a low ratio with other inorganic materials to create a hybrid scaffold with a certain mechanical strength. Baheiraei et al. [174] constructed a collagen/β-TCP scaffold by freeze-drying β-TCP powder into a porous collagen matrix with a β-TCP/collagen weight ratio of 4. Compression tests showed that the collagen scaffold had a compression modulus of 0.8 ± 1.82 KPa, while the composite scaffold had a compression modulus of 970 ± 1.20 KPa. In addition, the composite scaffold exhibited good angiogenic effects. A mixture of collagen and elastin has been shown to enhance the proliferative potential of ECs. Scaffolds built from a combination of these two proteins with low porosity are ideally suited for the production of tiny-diameter vessels and are predicted to be employed in vascularized bone tissue engineering.

*4.2.1.2. Fibrin.* Fibrin is a type of extracellular matrix protein that is similar to collagen [177]. It is a protein with high density and the potential to keep transplanted cells alive. Oh et al. found that fibrin promotes the proliferation of larger MC3T3-E1 preosteoblasts and promotes intracellular osteogenic gene expression and calcium deposition under low cell density conditions [178]. Due to the lack of substantial mechanical properties of fibrin scaffolds [179], fibrin is mainly used as an auxiliary component in bone tissue engineering [180]. In order to more effectively encourage bone tissue regeneration, Siddiqui et al. modified the surface of genipin cross-linked chitosan/nano-TCP composite scaffolds with fibrin [181]. In the process of wound healing, the blood clot formed by fibrin can interact specifically with VEGF, in addition to providing attachment sites for ECs, which can effectively promote angiogenesis [182,183]. Dohle et al. combined ECs and osteoblasts with platelet fibrin-rich (PRF) medium for mixed culture. Immunohistochemical analysis after 7 days showed the formation of lumen and microvessel-like structures in this culture system. In addition, the genes and proteins related to angiogenesis also showed a high expression status [175](Fig. 5c and d). These favorable biological properties result in fibronectin being one of the most promising cofactors in the field of vascularized bone tissue engineering.

*4.2.1.3. Silk protein.* Collagen and fibrin are both proteins found naturally in the human body, but silk protein (SF) is a biological substance generated by farmed silkworms, spiders, and scorpions [184]. SF is a biocompatible protein with an RGD cell-binding domain that allows cells to connect and proliferate [185]. It has excellent mechanical properties; for example, the tensile strength of SF is in the range of

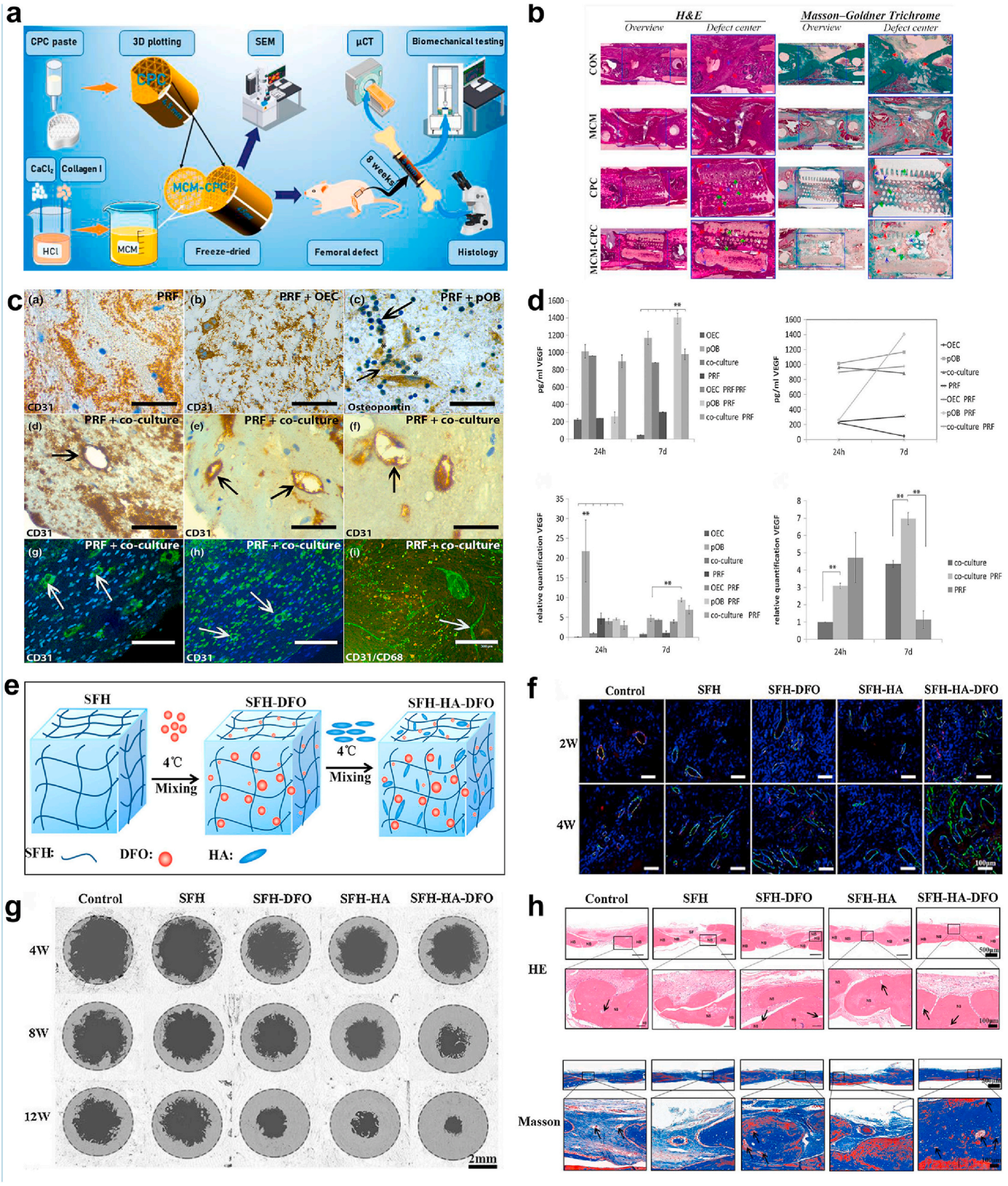


**Fig. 5.** a) Preparation process of the calcium phosphate collagen (CPC) composite scaffold. b) Tissue sections 8 weeks after in vivo implantation show a tight pincer-like structure between the receptor site and the scaffold [173]. c) Lumina and microvessel-like structure formation in co-cultures mixed with platelet fibrin-rich (PRF) medium. d) Effect of PRF medium on angiogenic gene and protein expression in two cell types [175]. e) The preparation process of the deferoxamine (DFO)-loaded SFH-HA composite hydrogel. f–g) Neovascularization and bone regeneration of the defects treated with different hydrogels. h) Bone and vascular neogenesis in the histological analysis at week 12 after implantation [176].

360–530 MPa, and the elastic modulus is in the range of 10–15 GPa, which is similar to that of cortical bone [164]. Furthermore, the degradation rate of SF in vivo matches the repair cycle of bone defects [186]. Based on the above advantages, SF has been widely used in the field of bone tissue engineering. SF scaffolds (ET scaffolds) were prepared by mixing Eri (Philosamia ricini) and Tasar (Antheraea mylitta) silk in a 70:30 ratio by Panda et al. The osteogenic properties of ET scaffolds were verified using scaffolds derived from gelatin and *Bombyx mori* (BM) sericin as a control group. The experimental results showed that the ET scaffolds significantly promoted the expression of osteogenic markers in human MSCs compared with the control group [187]. In addition, SF can promote angiogenesis. Fan et al. [188] reported that after cultivating BMSCs on an SF scaffold, the BMSCs had a proclivity to develop into ECs. When the SF scaffold was implanted subcutaneously in rats, they observed high-density angiogenesis in the scaffold region. SF proteins with pro-angiogenic effects can also be combined with other materials to prepare composite scaffolds with even better performance. Liu et al. prepared four SF/BCP scaffolds with different SF contents (0, 20, 40, and 60 % SF). The 40 % SF group had the highest compressive strength (40.80 ± 0.68 MPa) and showed good integrated bone-building ability in the rat model [189].

*4.2.1.4. Chitosan.* In addition to proteins, natural polymers of polysaccharides are among the candidates for vascularized bone tissue engineering. Chitosan, derived from chitin, is a unique natural polysaccharide with excellent biodegradability, biocompatibility, non-antigenicity, and cellular affinity [190]. Chitosan and its derived materials enhance the differentiation of bone progenitor cells and promote new bone formation [191]. Liu et al. compared the biological performance of titanium rod prostheses coated and uncoated with carboxymethyl chitosan (CMC) in New Zealand rabbits after total knee arthroplasty. It was found that CMC could reduce the inflammatory response around the rabbit knee prosthesis and promote osteogenesis by affecting the OPG/RANKL/RANK signaling pathway [192]. In addition, chitosan-based materials can also promote angiogenesis. When Han et al. examined the capacity of three sulfated chitosan to differentiate on HUVECs, they discovered that all SCSs stimulated the development and proliferation of HUVECs. In particular, 2,6-SCS could encourage capillary development and intracellular nitric oxide release [193]. Gniesme et al. demonstrated that compounding chitosan with other materials can significantly enhance the angiogenic performance of composite scaffolds. The team prepared PCL–chitosan scaffolds with more significant pro-angiogenic properties than PCL scaffolds alone [194]. In addition, chitosan and its derivatives have good anti-fungal and bacteriostatic effects and are often used as antibacterial materials, carrier materials, and film-forming materials [195]. Based on the above examples, chitosan is considered to be one of the excellent materials for vascularized bone tissue engineering. Due to its poor mechanical stability and bone conductivity, it is usually used in combination with other natural polymers or bioceramics to create more stable scaffolds [166]. Maji et al. reported a chitosan composite scaffold with a matched mechanical strength and porosity by adjusting the ratio of gelatin, chitosan, and β-TCP scaffold components [196].

*4.2.1.5. Alginate.* Alginate is a polysaccharide polymer similar to chitosan that is commonly utilized in vascularized bone tissue engineering. Alginate is a natural polymer of algal origin that forms gels with divalent cations under normal physiological circumstances, which is one of the most essential features, along with its biocompatibility and biodegradability [197]. Although alginate is non-toxic to host tissues and cells, it lacks cell adhesion qualities [198]. It can be modified by incorporating adhesion ligands and growth factors that promote cell attachment to enhance osteogenesis [199]. By combining BMP-2 and recombinant peptide (RCP) microspheres in alginate gel, Fahmy-Garcia and colleagues created a new natural polymer gel sustained release system. This system balanced inflammatory cell infiltration, BMP-2 release, and angiogenesis in the experiment, resulting in more fully vascularized new bone tissue [200].

Although natural polymers have good biological properties, their mechanical properties are poor, and they cannot withstand the stress transmitted by bones when used as scaffolds alone [201]. As a result, they can be combined with other materials to improve the mechanical properties while also exerting bone inductivity and cellular affinity [202].

### 4.2.2. Synthetic polymers

Synthetic polymers are often used as scaffolds for vascularized bone tissue engineering because of their good mechanical properties. At present, the commonly used synthetic polymers are polycaprolactone (PCL), polylactic acid (PLA), and polylactic acid–glycolic acid copolymer (PLGA) [203].

*4.2.2.1. PCL.* PCL has non-toxicity, absorbability, and cost-effectiveness, and the Food and Drug Administration (FDA) has authorized it for biomedical engineering applications [204]. PCL's toughness and mechanical stiffness under physiological conditions make it ideal for bone tissue engineering [205]. Based on these various advantages, PCL has been explored as a potential delivery scaffold for stem cells to support bone regeneration research. Xue et al. found that PCL nanofiber scaffolds were able to promote osteogenic differentiation of human MSCs through activation of the Wnt/β-linker protein signaling and Smad3-related signaling pathways [206]. Ji et al. further found that PCL scaffolds can regulate cell proliferation and differentiation by promoting cell senescence, cell cycle, and deoxyribonucleic acid (DNA) replication pathways, accelerating endochondral ossification and healing tissue formation [105]. PCL could also induce angiogenesis; Sekula et al. found that PCL can stimulate human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) to differentiate toward angiogenesis in the absence of additional chemical stimulation [207]. Electrospun nanofibers of PCL are cytocompatible by virtue of their nanometer size and facilitate the rapid growth of vascularized bone tissue [208]. Different diameter PCL nanofibers have different pro-vascular differentiation potentials. According to Reid et al. PCL nanofibers with a diameter of 4.83 m were better at promoting the expression of the angiogenic marker CD31 in HUVECs [209].

*4.2.2.2. PLA.* PLA, like PCL, is a low-cost, biodegradable polyester [197]. The degradability and mechanical properties of PLA are related to the molecular weight of the polymer. Low molecular weight PLA has a faster degradation rate and mechanical properties that better match the tissue growth rate during degradation than higher molecular weight PLA [210]. Therefore, low molecular weight PLA is the preferred choice for constructing vascularized bone tissue engineering. Skua et al. investigated the impact of an FDA-approved PLA on the biological characteristics of hUC-MSCs. PLA increases the angiogenic differentiation capability of hUC-MSCs, according to genetic analyses [207]. The degradation product of PLA is lactic acid, which can be metabolized by the human body. However, during the rapid degradation of PLA, a large number of lactic acid by-products will form a local acidic environment, leading to tissue inflammation and cell death [211]. To deal with this issue, calcium phosphate can be employed as a buffer to keep the pH steady. Barbeck and colleagues created a biphasic scaffold by combining PLA with biodegradable calcium phosphate glass. The experimental results revealed that the biphasic scaffold not only has a greater compression modulus and more lasting mechanical qualities than the basic PLA scaffold, but it also has a better capacity to stimulate vascularized bone growth [212].

*4.2.2.3. PLGA.* PLGA is a copolymer created by the polymerization of the lactic acid monomer and ethanoic acid monomer. It is one of the

most extensively used biodegradable polymers [213]. By controlling the ratio of lactic acid to glycolic acid, the mechanical properties and degradability can be flexibly controlled [214]. The metabolites of PLGA can be eliminated safely in vivo [215]. Regarding the relationship between cells and PLGA in osteogenic differentiation, Calvert et al. found that in the presence of osteoinductive factors BMSCs attach to PLGA scaffolds and are secreted to form a mineralized matrix [216,217]. Additionally, PLGA scaffolds can induce blood vessel growth. When Jehn et al. implanted PLGA scaffolds into the dorsal skin folds of mice, they discovered that the scaffold's peripheral area had a significant branching vascular network.

The above-mentioned scaffold materials can mimic the natural bone tissue composition and can induce limited vascularized bone tissue. However, the use of these single materials does not meet the need to construct a perfect bone substitute. No single material possesses good biocompatibility, biodegradability, porous 3D structure, osteoconductivity, osteoconductive, and angiogenesis at the same time, except for autologous bone with limited bone volume [218]. Combining the benefits of several materials to create a composite scaffold with a variety of good qualities is another way to induce scaffold vascularization. Wang et al. [176] created an injectable high-performance composite hydrogel by combining deferoxamine (DFO), silk fibroin nanofibers, and HAp. The composite scaffold can achieve the stable release of pro-angiogenic substances at the critical defect of the rat skull for more than 2 months. Immunohistochemical results at 2 and 4 weeks after implantation showed that the DFO silk nanofiber-HAp composite hydrogels (SFH-HA-DFO) group achieved optimal angiogenesis. In addition, micro-CT and histological analysis of the defect showed more and denser bone tissue formation in the SFH-HA-DFO group. The composite scaffold provides a stable stimulation ecological niche for vascularized bone tissue regeneration (Fig. 5e–h). In addition, composite scaffolds designed to target type-H vessel formation may be more promising. He et al. created a PCL/fibronectin/human umbilical vein endothelial cell-derived decellularised extracellular matrix (HdECM) (PFE) composite scaffold, which showed early vascularization infiltration and enhanced bone regeneration following implantation of PFE into a femoral defect in rats. Immunofluorescence analyses revealed that the PFE was able to regenerate the bone through a variety of channels, including PFE-mediated endogenous angiogenesis and osteogenesis, through a large number of type-H vessels and bone progenitor cells [219]. Composite scaffolds may be the best alternative material for scaffolding major bone lesions in the future [220]. Kumar et al. constructed a composite scaffold out of PCL and β-TCP. During the degrading phase, the composite scaffold could preserve correct porosity and mechanical characteristics [221]. This time-graded porosity structure is predicted to aid in the growth of blood vessels into the scaffold, facilitating the development of vascularized new bone.

## 5. Structural design of vascularized bone tissue engineered scaffolds

Currently, orthopedic scaffolds are mainly divided into solid scaffolds and porous scaffolds. Compared to solid scaffolds, porous scaffolds can significantly accelerate cell and protein penetration and are the preferred scaffolds for promoting the growth of vascularized bone tissue [222]. Kuboki et al. implanted solid, porous, and lamellar structured hydroxyapatite scaffolds subcutaneously into rats for comparison of the angiogenic and osteogenic abilities. Significant bone and angiogenesis were observed within the porous and lamellar structure of HAp scaffolds with a BMP mixture supplied to all scaffold groups [223]. Appropriate pore size, pore distribution, and connectivity between pores provide the microenvironment for cell infiltration, migration, blood vessel formation, and metabolism [224]. Therefore, when manufacturing porous bone scaffolds, the pore size, porosity, internal porous morphology, and overall bionic design of the scaffold are critical criteria to consider.

### 5.1. Pore size and porosity

The point-to-point distance in normal bone tissue between adjacent osteocyte centers is 24.1 ± 2.8 μm [225]. The interior pore size of porous scaffolds is often bigger than this distance to permit osteoblastic, endothelial, and inflammatory cell infiltration and proliferation [224]. Scaffolds with various hole diameters affect vascularization differently. Gupte et al. [24] discovered that poly (*l*-lactic acid) PLLA scaffolds with pore sizes ranging from 60 to 125 μm hindered endochondral ossification in BMSCs by blocking inward vascular development. The 125–250 μm hole size facilitated inward capillary development, which dramatically improved cartilage differentiation in human BMSCs but had little effect on mineralization. The 425–600 μm pore size allowed microvessels to develop in the scaffold, promoting bone tissue vascularization. Similarly, Swanson et al. demonstrated that a PLLA scaffold with pore diameters less than 125 μm prevented vessel penetration into the scaffold, while pore diameters greater than 250 μm promoted vessel formation [226]. It is clear that a scaffold with a pore larger than 250 μm will grow develop arteries. As the pore size increases, the vascularization effect is better. Wang et al. [227] examined the angiogenic and osteogenic properties of titanium scaffolds with pore sizes of 350 μm, 450 μm, and 550 μm, respectively. They discovered that as the pore size of the titanium scaffold increased, so did the number of vessels within the tissue section, with the 550 μm pore size scaffold having the most vessels. However, Feng et al. compared the angiogenic capacity of four different pore sizes (300–400, 400–500, 500–600, and 600–700 μm) of β-TCP scaffolds and found no significant difference in the neovascularization area when the scaffold pore size was bigger than 400 μm [228]. There is an ongoing debate regarding the optimal pore size that affects angiogenesis. The two studies above have different angiogenic performances at the same pore size. We suspect that the primary cause of this may be the fact that the various scaffold materials have varying levels of vascular productivity. The biodegradable scaffold's internal pore size enlarges and becomes more favorable to vascular development over time following implantation. Non-degradable scaffolds have constant pore sizes and consistent vascularization-affecting variables. Therefore, studies on the effect of pore size on vascularization should be conducted using scaffolds of the same material and systematically controlling for other variables.

Porosity, which is the proportion of void space in the scaffold, is a significant element influencing the growth of arteries into porous scaffolds. Increasing the porosity of the porous scaffold within a particular range promotes cell migration and value addition, allowing blood vessels to extend into the porous interior [229]. However, excessive porosity will lower the scaffold's mechanical strength, making it unsuitable for load bearing [230]. Therefore, while constructing scaffolds, the porosity should be adjusted to both encourage vessel growth and provide the scaffold with a robust enough support base.

### 5.2. Porous morphology

Cells can adapt the cytoskeleton to the surrounding geometry during tissue growth [231]. Therefore, the porous morphology inside the scaffold affects the growth of vascular and bone tissues [232]. The morphology of porous scaffolds that have been widely studied can be broadly classified into two categories. The first category is the branched rod-like structures represented by cubic morphology, which is the conventional porous morphology in 3D printed scaffolds [233]. The second category is the Triply periodic minimal surface (TPMS) with spherical surface morphology, which has excellent potential for clinical applications [234]. The interior surface of the TPMS structure is very smooth and well connected between the porous holes, with no sharp edges or knots, as in the case of cubes and hexagonal supports [235]. In addition, the average curvature of this TPMS structure is zero, and the average curvature of the bone trabeculae is also zero, which gives the TPMS structural scaffold a natural bionic advantage [236]. Wu et al. used a

rabbit dorsal muscle embedding system to investigate the in vivo angiogenic potential of three scaffold structures (cube, gyroid, and hexagon). SEM images of the scaffolds show that the pore geometries are in perfect agreement with the designed pore models, with no obvious cellular defects or deformations, and the pore walls exhibit similar microstructure and densification. They discovered that compared to the cuboidal and hexagonal structures, the TPMS structure promoted a denser and thicker vascular network. The team analyzed the results and found that scaffolds with curved and less angular pore morphology were more suitable for mediating angiogenesis [27] (Fig. 6a–c). This conclusion is consistent with previous studies, demonstrating that curvature affects tissue growth [237].

The roughness of the scaffold surface can also affect angiogenesis. Duan et al. observed that tricalcium phosphate with submicron surface morphology induced macrophages to polarize toward M2. M2-polarized macrophages then enhanced tube formation in HUVECs. In contrast, tricalcium phosphate with a micrometer shape does not have this effect [131]. In addition, Hou et al. demonstrated that human MSC adhesion is

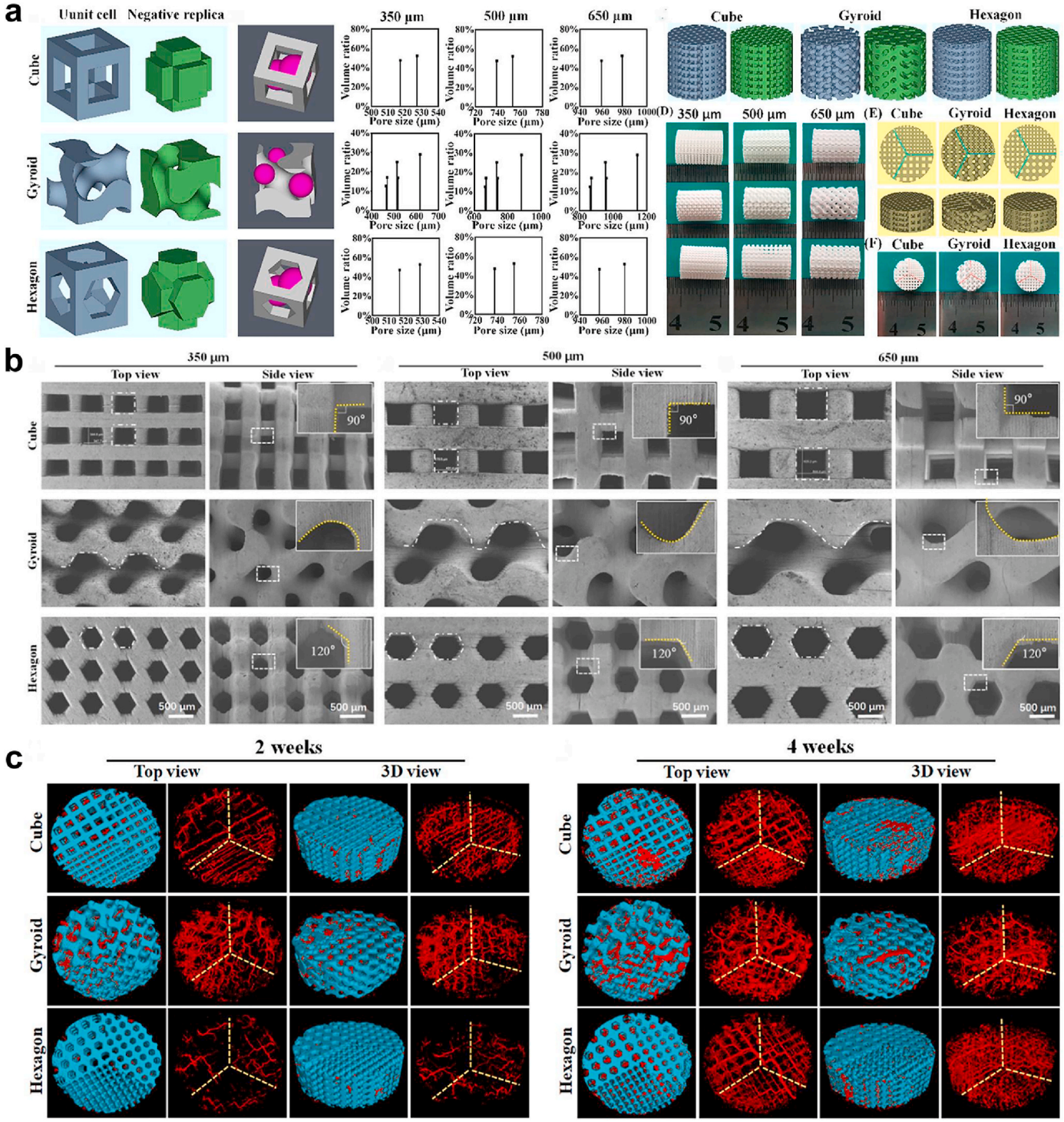


**Fig. 6.** a) Three-dimensional modeling and preliminary characterization of scaffolds with different hole geometries. b) Scanning electron microscopy (SEM) images of the surface morphology of bioceramic scaffolds with different pore geometries. c) Microangiographic images at 2 and 4 weeks after implantation [27].

bi-directionally regulated by interfacial roughness by setting up a gradient roughness interface [238]. At moderate roughness, cells have better adhesion properties [239], and adhesion is necessary for angiogenesis [240,241]. Importantly, Partida et al. demonstrated that rough-modified titanium scaffolds have better angiogenic properties than smooth titanium scaffolds [242].

Several studies mentioned above have shown that pore morphology is an important factor affecting the growth of blood vessels into the scaffold. An effective porous structure design can promote the growth of vascularized bone tissue inside the scaffold.

### 5.3. Bionic design

Mimicking bone structure and bone function is one of the important vascularization strategies for bone tissue engineering. Barati et al. constructed a bionic cortical bone scaffold with a microtubule-like porous interconnected structure similar to natural cortical bone [243]. The bionic scaffold promotes human MSC and endothelial colony-forming cells to form neovascularization and bone tissue without the addition of cytokines. Constructing structures in the scaffold that mimic the natural microvascular network is an important means of promoting vessel growth. The bionic hollow tube structure scaffold prepared by Duan et al. using polycaprolactone has the advantage of being highly interconnected and permeable [244]. In comparison to non-hollow-tube structured scaffolds, the specially integrated hollow-tube scaffold dramatically improved cell adhesion, spreading, and proliferation, as well as osteogenic differentiation and angiogenesis in vitro studies. In vivo experimentation further substantiated that the bionic scaffold conferred a marked improvement in both bone regeneration and angiogenesis within rabbit femoral defects.

The periosteum is a highly vascularized thin tissue with excellent osteogenic capacity [245]. Constructing bionic bone by mimicking periosteal tissue has become a new strategy for bone defect repair and regeneration. Dai et al. induced periosteum-like tissue by implanting gelatin scaffolds loaded with BMP-2 and chondroitin sulphate (CS) into the skin of mice. The composite scaffold induced a bionic periosteum with a structure similar to that of natural periosteum and was rich in functional periosteum-like tissue-derived cells (PTDC), type-H vessels, and osteochondral progenitor cells. The bionic periosteum showed strong osteogenic repair ability in a cranial defect model [246].

Furthermore, mimicking plant structures in nature is the focus of the combination of bionics and vascularized bone tissue engineering. Inspired by the lotus structure, Han et al. prepared hydrogel microspheres encapsulating DFO liposomes as “lotus seeds” using microfluidics, and combined them with 3D-printed bioceramic scaffolds with a biomimetic structure to prepare scaffolds with a biomimetic lotus structure (TGL). The team investigated the bone repair ability of the bionic scaffold in a rat distal femur defect model. In vivo micro-CT and quantitative protein analysis showed that the TGL group integrated new bone faster and better than the other groups and had high expression of osteogenic and angiogenic proteins [247] (Fig. 7a–c). These bionic scaffolds take advantage of complementary materials and technologies to exhibit excellent osteogenic and angiogenic capabilities and are highly instructive for exploring multifunctional vascularized scaffolds that promote the regeneration of adventitial bone tissue.

## 6. Cytokines in the vascularization of bone tissue engineering

Sufficient angiogenesis at the fracture site is a prerequisite for efficient bone regeneration [248], and the release of a number of angiogenic factors plays a vital role in early angiogenesis [249]. One of the hottest study areas in bone tissue engineering is the creation of bone substitutes by mixing artificial scaffolds with cytokines. The two most commonly employed cytokines in bone tissue engineering are VEGF and BMP.

### 6.1. VEGF

The VEGF family is the most specific promoter of angiogenesis and the most important activator, which is widely used in bone tissue engineering [250]. VEGF-A is the most abundant type in the organism [251]. During bone formation, VEGF can mediate osteogenesis by regulating H-type formation [55]. Inhibition of VEGF has been shown in several investigations to form bone discontinuity models [252]. VEGF expression reaches its peak in the early stage of fracture healing, which can promote the migration and proliferation of ECs to form tubular vessels [248]. Importantly, VEGF-loaded artificial scaffolds for bone tissue engineering can efficiently stimulate the fast creation of new blood vessels and offer metabolic support for the production of new bone [253]. Liu et al. created a VEGF-loaded PCL/HAp scaffold, which could significantly induce the formation of microvessels and promote the rapid bone regeneration of the rat skull defect model [254]. Similarly, Li et al. used thermosensitive collagen hydrogel as a carrier for VEGF, which was compounded with a porous titanium alloy scaffold. This complex system was shown to strongly promote angiogenesis-mediated bone regeneration and significantly promote more osseointegration in a rabbit lateral femoral condyle bone defect model [255].

However, VEGF seems to have a dose-dependent range of action. Therefore, if the dose of VEGF is too high, it may lead to vascular malformation, and if too low, it will be ineffective [256,257]. Wang et al. developed a mechano–chemical coupling model of a biodegradable polymer scaffold loaded with VEGF, which showed that there is an optimal range of VEGF doses that can promote the efficiency of bone regeneration [256]. Indeed, Dreyer et al. evaluated the literature from the previous 10 years, indicating that the lowest single dosage of VEGF used was 0.2 μg, the highest was 24 μg, and the highest single dose with a consistently positive response was 2.6 μg [252]. This interval can be used as a reference for future studies of single VEGF dose loading. Furthermore, the dosing interval of the two-factor loading system needs to be investigated further. Walsh et al. loaded a collagen/HAp scaffold with 2.5 μg of VEGF and 2.5 μg of BMP-2; experimental models of bone defects showed that this dose of the two-factor system significantly promoted vascularized bone production [258].

Furthermore, loading VEGF by direct adsorption may lead to the burst release of VEGF, which may have adverse effects [259]. Inducing the expression of VEGF by indirect means is an alternative way of sustained release. Pekozer et al. reported a study of VEGF-inducing agents loaded onto PLGA scaffolds for vascularized osteogenesis. The inducer-loaded scaffold has been shown to support EC recruitment, vascularization, and bone repair in vivo [260]. In addition, genetic engineering-based delivery strategies can achieve the precise release of cytokines without worrying about the short half-life of exogenous cytokines. Moreira et al. delivered the gene encoding VEGF to human dermal fibroblasts; this strategy allows cells to produce functional VEGF, which induces the formation of capillary-like structures faster and for longer periods of time in experiments [261]. Similar to this, Yao et al. [262] created a composite scaffold that could regulate VEGF regeneration and release by combining exosomes containing VEGF genes with PCL porous scaffolds using the exosome anchor peptide CP05. Engineered exosome-transfected rat BMSCs exhibit significant angiogenic and osteogenic differentiation in vitro experiments. Histological evaluation and immunofluorescence staining analysis of healed radial defects in rats further confirmed that the composite scaffold was effective in inducing massive vascularized bone regeneration (Fig. 8a–d). VEGF is also closely associated with bone growth factors (e.g., BMP) and exhibits synergistic stimulation in the formation of bone and angiogenesis signaling pathways. Dashtimoghadam et al. encapsulated VEGF and BMP-2 into microcarriers; the dual-factor microcarrier device was discovered to have significant osteogenic and angiogenic potential as well as a sustained growth factor release profile with prolonged bioavailability [263].

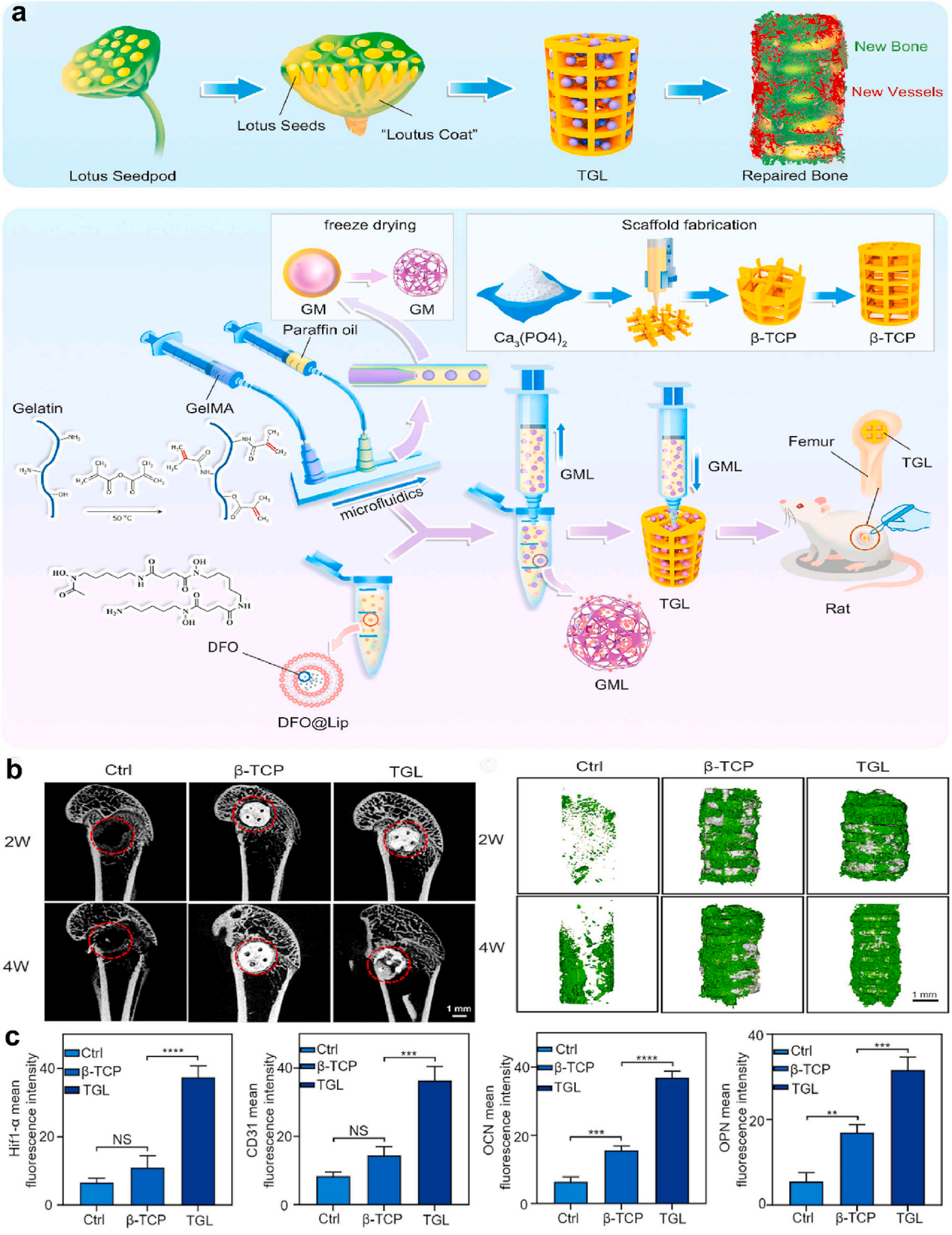


**Fig. 7.** a) Schematic diagram of a vascularized bionic scaffold and its biomechanical role in the repair of bone defects in a rat model. b) Three-dimensional reconstruction of micro-computed tomography images of the implant site showing the regenerative effect. c) Differential expression of relevant proteins in de novo tissues after implantation of bionic scaffolds into defects [247].

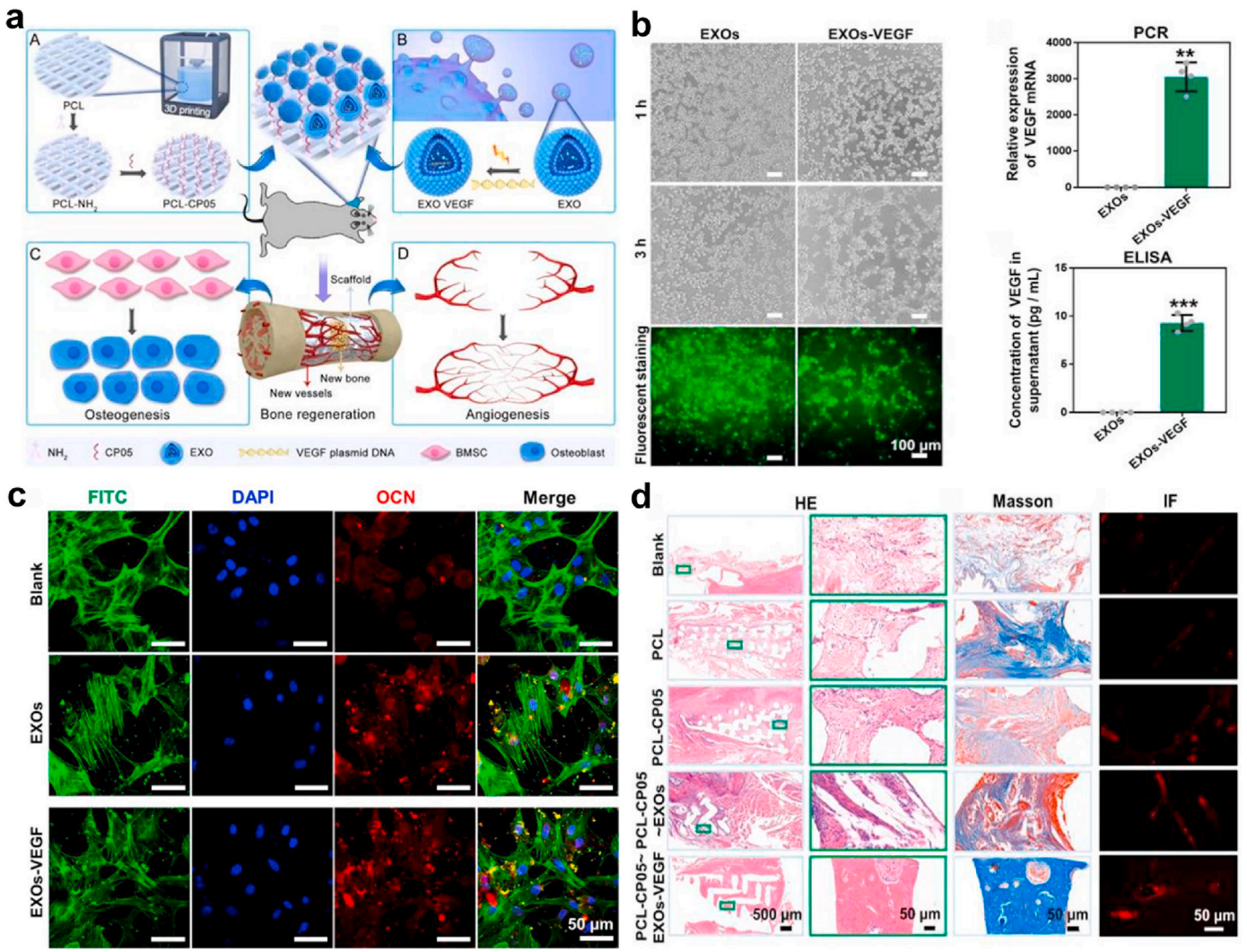


**Fig. 8.** a) General idea of engineered exosome-enhanced therapies on osteogenesis and angiogenesis. b) Expression of VEGF and its pro-angiogenic effect after transfection of engineered exosomes with rat bone-marrow derived mesenchymal stem cell (BMSCs). c) Immunofluorescence staining of osteogenic markers, OCN, nucleus, and cytoskeleton. D) Histological evaluation of healed radial defects and immunofluorescence staining for the angiogenesis marker CD31 in rats [262].

The above results suggest that scaffold-released VEGF can induce vascularized new bone regeneration. However, the translational application of VEGF for the treatment of bone defects currently needs to be further explored. VEGF administered in direct form is susceptible to biodegradation. Various techniques for cytokine transport and release are constantly evolving. Cytokine control strategies based on genetic engineering and microcarrier technology show great promise. However, the ethical implications of therapeutic gene therapy techniques must also be considered.

### 6.2. *BMP-2*

BMP-2 is one of the most popular growth factors in vascularized bone tissue engineering. It regulates the repair and regeneration of segmental bones by promoting the formation of bone, cartilage, angiogenesis, and fibrotic tissue [264]. Among the BMP family, BMP-2 plays an integral role in fracture healing. Mice with defective Bmp-2 expression in the extremities may have normal skeletal development throughout growth but poor fracture repair. Moreover, other forms of BMPs cannot fill this crucial role [29]. Due to its critical nature, BMP-2 has been used clinically for the treatment of open tibial fractures, non-healing bone injuries, and spinal fusion, and is included in the FDA-approved bone regeneration system [265,266]. BMP-2 is a trigger/signal molecule in fracture repair. It primarily aids bone regeneration by causing bone progenitor cells to develop into osteoblasts and attracting BMSCs to the wounded area [267]. Furthermore, BMP-2 is the cytokine that promotes the production of alkaline phosphatase and osteocalcin, which is important for fracture healing [268]. Importantly, BMP-2 can promote angiogenesis via chemotaxis of circulating endothelial progenitor cells in peripheral blood and increased secretion of paracrine angiogenic growth factor by MSCs [269,270]. This may be related to BMP-regulated type-H vessels formation [57,58]. As a result, applying BMP-2 alone at the site of bone defects may have a pro-angiogenic effect. In addition, BMP-2 can synergize with VEGF to further enhance vascularized bone tissue production [271].

The dual-factor loading system with VEGF and BMP-2 has more angiogenic and osteogenic capabilities than the system loaded with VEGF alone. Jie et al. investigated the effects of calcium phosphate scaffolds loaded with VEGF and BMP-2 on osteogenesis. They discovered that combining VEGF and BMP-2 was more efficient than either VEGF or BMP-2 alone in inducing osteogenesis and vascularization of the composite scaffold [272]. Similarly, the HAp/PLGAs scaffold, prepared by Wang et al. and equipped with dual VEGF and BMP-2 factors, also showed a stronger ability to promote bone tissue maturation than the control group. They further investigated that the bifactor may promote vascularized osteogenesis by activating the p38 MAPK pathway to

promote the nuclear translocation of osterix proteins [273].

Despite its benefits, systemic BMP-2 administration has been linked to side effects such as prevertebral swelling, airway edema, carcinogenesis, and ectopic bone growth [274,275], whereas local BMP-2 injections are not successful in promoting local bone production [276]. To manage the osteogenic impact of BMP on the target region, it is vital to employ a good delivery vehicle and an optimum dosage to optimize the effect of BMP-2. Datta et al. encapsulated BMP-2 into a chitosan microsphere system to achieve spatiotemporal control and sustained release of BMP-2 and demonstrated good osteogenic and angiogenic effects in the experiments [277]. In addition to using microcarriers to achieve a rational release of BMP-2, gene transfection techniques can be used to better control the optimal dose of BMP-2 in bone repair [278]. Geng et al. modified BMSCs with mRNAs encoding the human BMP-2 and VEGF-A genes and inoculated them onto a collagen scaffold. They used a rat cranial defect model to validate the bone healing ability of the two-factor system. Reconstructed images of 3D micro-CT scans at 4 and 12 weeks after treatment show outstanding bone regeneration in the bifactorial group. In addition, quantitative assessment of OCN and CD-31-positive cells at the site of bone regeneration showed more osteoblastic and angiogenic cells clustered around the bifactor group. This system exhibited excellent cytokine precision control and the ability to synergistically drive osteogenesis and angiogenesis in a murine cranial defect model [279] (Fig. 9a–c).

One of the most promising methods for creating vascularized bone tissue engineered bone is the development of a cascade system of spatiotemporally adjustable multifactor inside tissue engineered grafts. Direct adsorption, multifactor adsorption, hydrogel delivery, microcarrier transport, and gene editing approaches are all being used by researchers to further this concept. Importantly, the ethical and practical concerns associated with the aforementioned solutions must also be taken into account.

## 7. Conclusions and prospects for vascularized bone engineering

The skeletal vascular system plays a crucial role in the process of bone regeneration and healing. The blood vessels in and around bone tissue provide oxygen, nutrients, growth factors, and cells for the survival, proliferation, and differentiation of bone formation. Currently, the lack of functional vasculature in tissue engineered bone has become the biggest obstacle in its clinical application. Among all cellular and signaling pathways, type-H vessel formation is a key factor in coupling

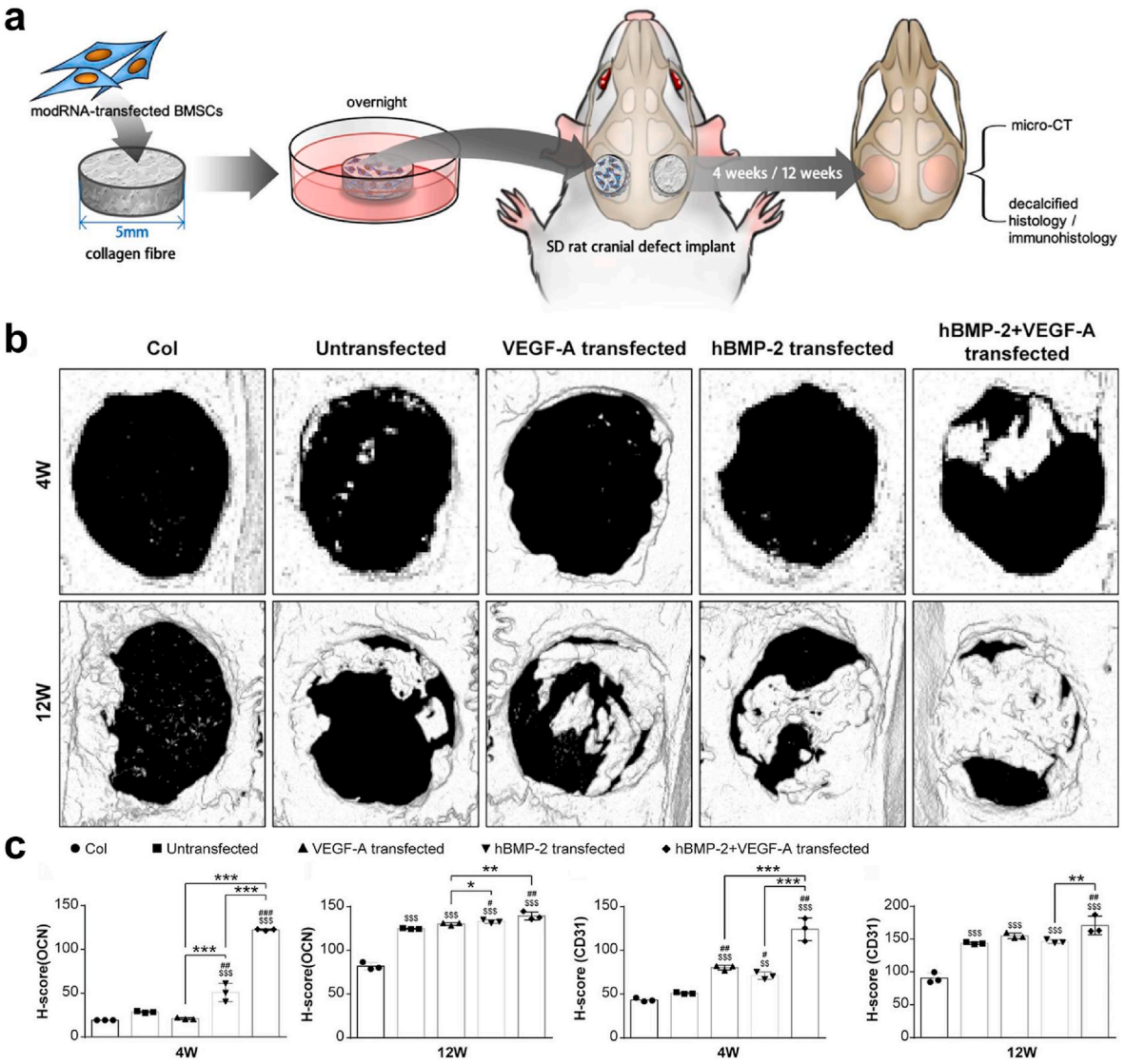


**Fig. 9.** a) Experimental procedure of modified mRNA-treated bone marrow-derived mesenchymal stem cell (BMSC) composite biomaterials to promote bone healing in a rat cranial defect model. b) Micro-computed tomography (CT) reconstruction of bone regeneration after treatment. c) Quantification of OCN and CD-31-positive cells at the sites of bone regeneration [279].

angiogenesis to osteogenesis. Therefore, the use of type-H vessels as a target for stimulating vascularized bone regeneration in bone tissue engineering may be a promising therapeutic approach. However, there are some challenges to research on type-H vessels. Deletion of the gene encoding integrin β1, a cell-surface transmembrane glycoprotein, results in a loss of normal morphological and functional properties of H-type ECs. However, the expression of CD31 and Emcn, markers characteristic of H-type ECs, is unaffected. This mutation leads to the formation of dysfunctional H-type vessels, which can impair normal bone metabolism and lead to bone loss [39]. In addition, abnormal accumulation of type-H vessels may lead to osteoarthritis [280]. We believe that because the coupling of type-H vessel formation and osteogenesis involves multiple factors, inducing functional type-H vessels while avoiding aberrant type-H vessel formation is necessary for vascularized bone tissue engineering.

In vivo pre-vascularization strategies have been a hot topic of research in the field of regeneration. Different pre-vascularization sites will exhibit different rates of bone formation. In general, an increased blood supply means faster bone formation. The use of muscle pouches and the greater omentum as bioreactors to pre-vascularize bone has achieved initial clinical results. However, there are a number of challenges that are currently being faced. Firstly, achieving proper coordination between blood vessel formation and bone tissue regeneration is crucial. If the culture process is delayed or too fast, it may lead to insufficient bone or more soft tissue in the prefabricated scaffolds, which can ultimately lead to suboptimal tissue integration. The current solution is to control the prefabrication process by adjusting the dose of inducers, such as BMP-2 [80]. However, individual patient variability, such as age, health status, and underlying medical conditions, can affect the determination of the optimal rhBMP-2 dose. Therefore, the optimal rhBMP-2 dose for humans needs to be explored and demonstrated. Secondly, the optimal stage of implantation of pre-vascularized bone should be determined. Pre-vascularized bone at different developmental stages will show different proportions of type-H vessels and bone progenitor cells, which is important for the regenerative capacity of the determined pre-vascularized bone. Thirdly, the stability and longevity of pre-vascularized bone should be guaranteed prior to clinical application, and the stability and long-term function of the vascularized network after implantation is crucial for sustained tissue regeneration and functional rehabilitation. In addition, regulatory requirements and safety issues need to be carefully considered before translating pre-vascularization strategies into clinical applications. Scaffold design, material selection, and loading drugs have been the focus of research in vascularized bone tissue engineering.

Currently, single materials do not fully meet the conditions for scaffolds to promote vascularized bone formation, and composite scaffolds prepared from multiple materials may be one of the solutions to the difficulties in vascularizing scaffolds. Each material of a composite scaffold has unique properties, resulting in scaffolds with improved structural, mechanical, and biological properties. Composite scaffolds achieve mechanical strength and stability by combining biodegradable polymers and ceramics to match the defect area. This allows for better weight-bearing capacity and support during bone regeneration. Composite scaffolds can also incorporate osteoconductive materials (e.g., HAp) that provide scaffolding for bone growth, osteoinductive materials (e.g., BMPs) that induce the differentiation of stem cells into bone-forming cells, and vasoinductive materials (e.g., VEGF) that induce angiogenesis. Although the current status of composite scaffolds in vascularized bone tissue engineering is promising, there are still a number of challenges that need to be addressed. The primary challenge is to ensure compatibility between composite scaffold materials and overall scaffold biocompatibility. It is desirable to design composite scaffolds in which each component synergizes with each other to promote vascularized osteogenesis. Secondly, the optimal ratio and distribution of the different materials within the composite scaffold must be determined. This is necessary for the composite scaffold to achieve matched mechanical properties, excellent vascularization induction, and osteogenesis. In addition, it is important to consider whether the composite scaffold can achieve long-term stability after implantation, which is essential for subsequent vascularized bone regeneration and functional recovery.

In conclusion, future work needs to further explore the interactions and mechanisms between the skeletal vascular system and bone regeneration. Based on this, research into safe and effective pre-vascularization strategies and exploration of new materials, fabrication techniques, and optimized composite scaffold designs would be effective solutions to the challenges of vascularization in bone tissue engineering.

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## Authorship contribution statement

**Hao Liu:** Conceptualization, Data curation, Methodology, Writing – original draft, Formal analysis, Investigation, Visualization; **Hao Chen:** Conceptualization, Methodology, Writing – original draft, Writing - Review & Editing; **Qin Han:** Conceptualization, Methodology, Supervision, Project administration, Writing - Review & Editing, Funding acquisition; **Sun Bin:** Conceptualization, Visualization, Writing – original draft; **Yang Liu:** Writing – review & editing, Visualization; **Aobo Zhang:** Data curation, Writing – review & editing; **Danyang Fan:** Formal analysis, Writing – review & editing; **Peng Xia:** Conceptualization, Methodology, Resources, Writing–review & editing, Supervision, Project administration, Funding acquisition; **Jincheng Wang:** Supervision, Project administration, Funding acquisition.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

No data was used for the research described in the article.

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