Strategies for *in situ* tissue engineering of vascularized bone regeneration (Review)

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Abstract. Numerous physiological processes occur following bone fracture, including inflammatory cell recruitment, vascularization, and callus formation and remodeling. In particular circumstances, such as critical bone defects or osteonecrosis, the regenerative microenvironment is compromised, rendering endogenous stem/progenitor cells incapable of fully manifesting their reparative potential. Consequently, external interventions, such as grafting or augmentation, are frequently necessary. *In situ* bone tissue engineering (iBTE) employs cell-free scaffolds that possess microenvironmental cues, which, upon implantation, redirect the behavior of endogenous stem/progenitor cells towards a pro-regenerative inflammatory response and reestablish angiogenesis-osteogenesis coupling. This process ultimately results in vascularized bone regeneration (VBR). In this context, a comprehensive review of the current techniques and modalities in VBR-targeted iBTE technology is provided.

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1. Introduction

Bone is a highly vascularized tissue whose vascular supply strictly limits its development, remodeling and regeneration. In specific pathologies/conditions, such as critical bone defects due to trauma, osteonecrosis and tumor resection, the limited ability of bone to heal itself requires external regenerative bone procedures, where tissue engineering and biomaterials come on stage. During bone regeneration, sufficient vascular supply provides the bone tissue with essential nutrients, oxygen, growth factors (GFs) and hormones (1). Therefore, while developing artificial bone substitutes that provide temporary mechanical support and boost bone regeneration, the necessary condition of neovascularization must also be taken into account. Traditional tissue engineering (TE) techniques treat bone defects by introducing osteoblasts or osteogenic-differentiated mesenchymal stem cells (MSCs) onto/into a scaffold and undergo a period of *in vitro* culture followed by implantation. However, the cell-loaded bone substitute is initially avascular. In circumstances where the defect exceeds a thickness of 200 μm, hypoxic conditions occur immediately after implantation, resulting in the death of the seeded cells (2). To avoid necrosis, alternative cell-free *in situ* TE (iTE) techniques were developed with the fundamental recognition that mammals have self-regenerative potential and may be manipulated by the provided microenvironmental cues. *In situ* bone TE (iBTE) scaffolds may be engineered

to contain biologically instructive/microenvironmental cues that, when implanted, may modulate the endogenous stem/progenitor cells' behavior, such as angiogenesis-osteogenesis coupling and inflammation, eventually leading to tissue repair (Fig. 1) (3,4). Studies at the cellular and molecular levels have revealed the interaction between endothelial cells (ECs) and osteoblasts (OBs), and by synchronously modulating the two, facilitated the achievement of vascularized bone regeneration (VBR) (5,6). In addition, a range of studies has found that promoting angiogenesis alone was also able to enhance bone regeneration (Fig. 2) (7-9). Based on the current understanding, the development of biomaterial scaffolds for iBTE has been upgraded by combining proangiogenic factors with osteoinductive/osteoinductive biomaterials. In the present narrative review, iTE strategies, particularly those targeting VBR, are summarized.

2. iBTE from the conventional cell-seeded concept to cell-free scaffolds

BTE has undergone significant advancements over the years, transitioning from conventional methods to more sophisticated approaches. One such development is the iBTE, which has progressed from the traditional concept to the utilization of cell-free scaffolds possessing microenvironmental cues.

Traditional concept to iBTE. TE traditionally emphasizes the importance of three key components: Cells, scaffolds and signaling molecules (10). Cells generate the extracellular matrix (ECM) and other factors crucial for tissue growth and repair. Scaffolds offer a structural framework for cells to attach and migrate, while signaling molecules modulate cell behavior and differentiation. By utilizing endogenous cells, engineered scaffolds and bioactive cues/signaling molecules, the iBTE method remains consistent with the traditional concept while advancing its application. It builds upon them by leveraging advanced biomaterials and strategies to enhance the body's regenerative capabilities.

Responsiveness of bone to iBTE. In contrast to cartilage and nerve tissues, which exhibit limited endogenous cellularity near defects and necessitate the use of conventional cell-seeded TE scaffolds, bone tissue presents a highly suitable target for iTE strategies. This suitability arises from bone's innate characteristics, including its abundant endogenous cell population, intrinsic structural support, remarkable self-healing capacity and sensitivity to microenvironmental cues (3).

iBTE from past to present. The concept of iBTE originated from the observation that the body's natural bone healing process may be harnessed and enhanced by providing a suitable scaffold-microenvironment. Early cell-free scaffolds were composed of natural or synthetic biomaterials designed to mimic the structure and properties of native bone tissue, such as calcium phosphate bioceramics, collagen, hydroxyapatite and various biodegradable polymers (11). Over time, researchers have developed more advanced cell-free scaffolds, incorporating bioactive materials and functional modifications to promote bone regeneration (12). In the subsequent sections, current perspectives on iTE approaches for VBR will be explored.

3. Understanding *in situ* VBR and its evaluation methods

The definition of VBR in the current literature broadly consists of several terms: VBR, vascularized osteogenesis, and angiogenesis and osteogenesis. Being familiar with the terminology facilitates the search and summary of the relevant literature. The cellular basis behind VBR is closely linked to the coupling of angiogenesis and osteogenesis; therefore, to evaluate the potential impact of iBTE scaffolds on the *in situ* VBR, ECs and OBs or MSCs have been widely used (13,14). However, it is suggested that numerous biological agents that may promote angiogenesis also act on osteogenesis directly or indirectly. Evolution has provided the physiological necessity that the two processes are paired. Proliferation assays, such as the Cell Counting Kit-8 and 5-bromo-2-deoxyuridine assay, are the foremost modality for evaluating the cytotoxicity of different bioactive agents (15). For angiogenesis evaluation, at a cellular level, the effect of the biomaterials on EC migration and morphogenesis is usually assessed by scratch-healing assay and tube-formation assay (16). At the molecular level, biomarkers related to angiogenesis, such as hypoxia-inducible factor (HIF)-1α, VEGF, basic fibroblast GF (bFGF), platelet-derived GF (PDGF) and angiopoietin 1, are usually detected by fluorescence quantitative PCR and western blot analysis (16). As for bone regeneration, alkaline phosphatase (ALP) and alizarin red are usually detected qualitatively and quantitatively by co-incubating OBs or MSCs with the biomaterials or supplemented with their extracts to the culture medium. The expression of molecular markers related to bone formation, such as ALP, bone morphogenetic protein (BMP), RUNX family transcription factor 2 (Runx2) and collagen type I (Col1), is further detected. With the advancement of high-throughput technology, proteomics, transcriptome sequencing and enrichment analysis have also been applied to evaluate the effect of scaffold materials on endogenous cells and screen for their potential mechanisms (17).

Ex vivo models, such as the aortic ring assay and fetal mouse metatarsal assay, have also gained popularity in evaluating angiogenic activities (18,19). iBTE scaffold in a hydrogel form is advantageous, as the aortic ring may be directly embedded. By contrast, scaffold extracts may be readily added to the culture medium of the *ex vivo* metatarsal bone. The choice usually depends on the properties of the biomaterial, feasibility and the perception of researchers. However, the two assays carry individual drawbacks. For instance, the two assays are composed of different cells, such as ECs and fibroblasts, macrophages and smooth muscle cells, which do not closely resemble the *in vivo* angiogenesis of bone (16).

For *in vivo* evaluation, appropriate animal models, such as the rabbit or rat femoral epicondylar bone defect model, calvarial defect model or segmental bone defect model, are widely used. New bone formation and vascularization at the defect site may be examined qualitatively and quantitatively by microCT and angiography imaging at various time-points after implantation. Furthermore, calcein staining is also advocated, given its high affinity to calcium ions within the newly formed bone. Fluorescent signals in the tissue section signify the new mineralized bone matrix. Furthermore, tissue immunohistopathology may also investigate osteogenesis-related molecular markers, such as osteocalcin, Runx2, Col1, osteopontin and

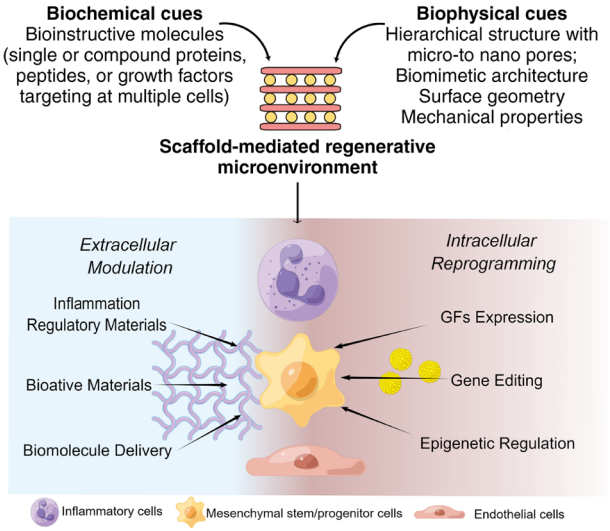

Figure 1. Schematic demonstration of the concept of *in situ* tissue engineering. GF, growth factor.

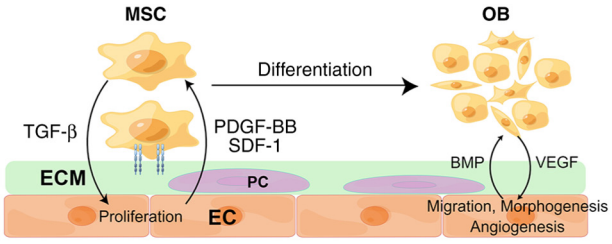

Figure 2. Coupled osteogenesis-angiogenesis mediated by paracrine effect, cell-matrix interaction and cell-cell interaction. MSC, mesenchymal stem cell; OB, osteoblasts; EC, endothelial cells; PC, perivascular cells; ECM, extracellular matrix; PDGF, platelet-derived growth factor; BMP, bone morphogenetic protein; SDF, stromal cell-derived factor.

biomarkers of vascular infiltration such as CD31 and α-smooth muscle actin (20,21). Recently, a novel H-type vessel, characterized by the high expression of surface markers CD31 and endomucin, was considered an essential type of capillary in mediating the *in situ* VBR. The detection of colocalizing type H vessels is becoming a popular evaluation entity in evaluating whether novel iBTE scaffolds may induce coupled angiogenesis and osteogenesis (2,22-24). Furthermore, the assay of chick chorioallantoic membrane, to which the test biomaterial is attached, is another mainstay for testing their proangiogenic capacity. After being correctly placed, neovascular infiltration of the biomaterial may be observed and quantitatively studied, such as vascular volume and connectivity (25). However, the abovementioned choice differs across various literatures and biomaterials, and it should be noted that there is no one-size-fits-all modality.

4. Fundamental biology of the iBTE scaffold for VBR

The most appropriate model for scrutinizing *in situ* VBR may be ascertained through the investigation of the biological mechanisms governing fracture repair (Fig. 3). Following a fracture, local blood vessel damage leads to the immediate formation of a hematoma (blood clot). This blood clot serves as a temporary scaffold for the subsequent infiltration of cells, including inflammatory cells, ECs and osteoprogenitors, which are important for bone regeneration. Initially, it comprises platelets, leukocytes, macrophages, bioactive GFs and cytokines. The destructive phase, characterized by local inflammation and low oxygen levels, lasts for 1-3 days before transitioning into the reconstructive phase. During the reconstructive phase, ECs migrate and capillary infiltration ensues. Under local hypoxia, tissue damage and cytokines secreted by inflammatory cells are stimulants for the neo-capillaries. Soon after the neovascularization, providing sufficient oxygen and nutrients, MSCs are recruited to the area and differentiated into chondrocytes (CCs) and OBs. The CCs are responsible for the preliminary cartilage matrix and are replaced by the mineralized bone matrix produced by OBs. In the remodeling phase, OCs are recruited to catabolize bone to reach a dynamic balance with the osteogenesis produced by the OBs. Based on the current body of literature (8,26,27), achieving VBR usually takes the first three steps of intervention. These steps require complex interactions between multiple cell types, mediated by either soluble or insoluble cues, which have yet to be elucidated.

This did not hamper the development of the current iBTE strategy, which emphasizes 'biomimicry', which describes the designing of materials or structures that mimic the natural properties of living organisms. In the context of iTE for VBR, biomimicry involves creating a microenvironment at the implanted site that closely resembles the sequence of events that occur during natural bone healing (3). This may involve using biomaterials that have similar mechanical properties to bone, as well as incorporating GFs and other signaling molecules that are known to have a role in bone regeneration (28). By mimicking the natural healing process, researchers hope to promote more efficient and effective bone regeneration *in vivo*. Much effort has been focused on two technical routes toward the common goal: Directly endowing the scaffold with angiogenesis-osteogenesis coupling factors or modulating the early inflammatory microenvironment towards a proangiogenic and proosteogenic state (29). The two routes share certain commons by providing biophysical/biochemical cues through extracellular or intracellular mechanisms.

5. iBTE strategy for VBR via extracellular biophysical signals

Biophysical cues are physical properties of biomaterials proven to have roles in directing cell function and stem cell differentiation commitment (30). They are frequently regarded as primary elements in biomaterial design. iBTE scaffolds are designed for the common purpose of promoting *in situ* VBR. Their forms and types may be broadly classified into e.g. mesoporous scaffolds, hydrogel networks, nanoparticles, electrospun fiber and 3D printing constructs (3).

Architectures. Although different scaffolds are prepared in a diversity of means, there is a consensus that scaffolds should have a porous structure. Pores in the scaffold allow cells to penetrate, attach, migrate and proliferate. At the same time, the infiltrating neo-capillaries may deliver oxygen and nutrients and remove metabolites (31). It has been reported that the pore structure has

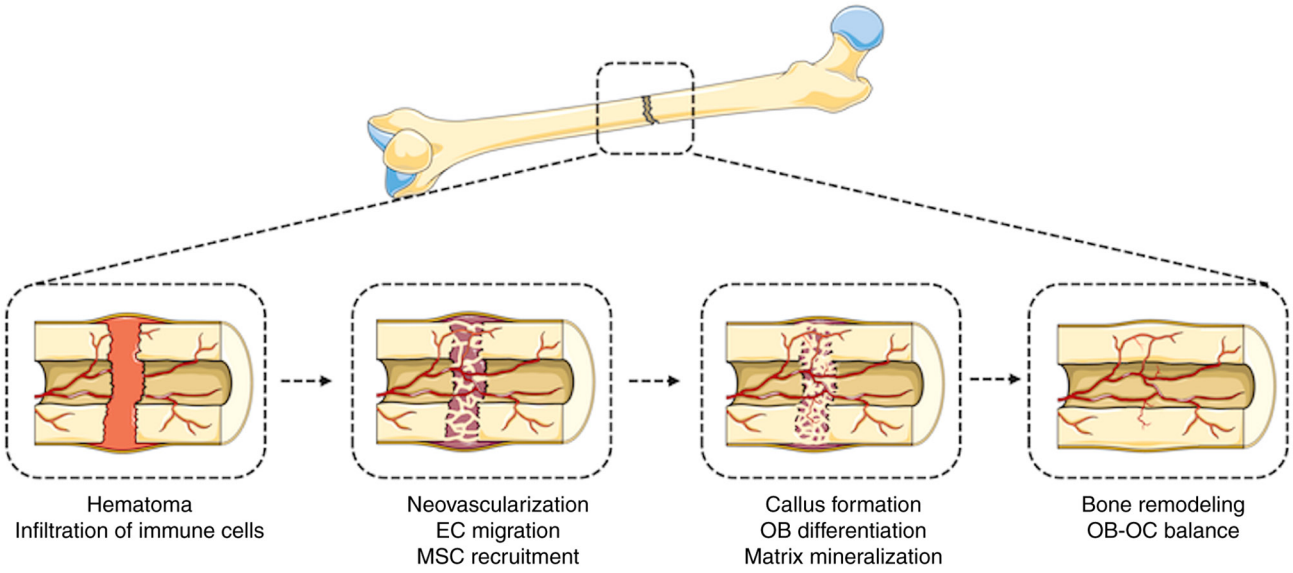

Figure 3. Typical processes in natural bone healing following a fracture. EC, endothelial cell; MSC, mesenchymal stem cell; OB, osteoblast; OC, osteoclast.

important effects on cell inoculation efficiency, viability, migration, morphology, differentiation and angiogenesis (32-34). The porous structure is characterized by pore size, geometry, inter-pore connectivity and porosity. In terms of size, hundred-micron, micron and nanoscale pore structures are referred to as macropore, micropore and nanopore, respectively. Hayashi *et al* (35) designed honeycomb scaffolds (HCS) of different sizes (100, 200 and 300 μm) to investigate the threshold of the most effective macropore size of iBTE scaffolds. At four weeks after implantation into rabbit femoral defects, it was observed that the HCS with 300 μm pore size were extensively filled with new bone and vascular tissue, demonstrating that scaffolds with a high degree of inter-pore connectivity and homogeneity at 300 μm are more conducive to *in situ* VBR. Studies further revealed that the progressive hierarchy of pore size indicated that the multi-scaled pore distribution is more advantageous than the single-scaled one. Wang *et al* (36) fabricated an apatite-collagen-polycaprolactone (PCL) scaffold by filling cross-linked collagen into the pores of a 3D-printed PCL scaffold, which was then mineralized *in vitro* by simulated body fluid immersion. The scaffold possesses a macro-micro-nanoporous hierarchy, which favors the host bone ingrowth and biomaterial osseointegration. Another scaffold with a macro-medium-microporous architecture was fabricated based on poly(3-hydroxybutyrate-poly-hydroxyhexanoate) (37). The scaffold on which ECs were cultured exhibited increased migration and metabolic activity, suggesting proangiogenic potential. After being loaded with the pro-osteogenic BMP, the multi-level porous scaffold achieved a significant increase in bone regeneration and revascularization after being implanted in a segmental bone defect model. Based on the findings on porosity, the advancement of 3D printing technology has further enabled the readiness of processing bone scaffolds with gradient porosity, as well as customized architecture, shape and mechanical strength. In addition, different bioactive molecules may be loaded and precisely immobilized within specific regions (38-40). Lian *et al* (41) used a low-temperature deposition model to prepare a spongy PCL scaffold with the same hierarchical and interconnected pores, which was able to promote the paracrine effects of MSCs via focal adherent kinase, its downstream AKT and yes-associated protein (YAP) mechanical signaling pathways, leading to a pro-regenerative macrophage phenotype, neovascularization and eventually the VBR.

Stiffness. Other biophysical properties of iBTE scaffolds, such as stiffness, surface geometry and mechanical stimulation, have also been proven to alter the local tissue microenvironment through intracellular and intercellular signaling (3,42). MSC differentiation is influenced by the stiffness of the biomaterial, in which rigid material induces the osteogenic differentiation of MSCs and softer matrices promote their adipogenic differentiation (43-45). ECs also exhibit different morphology and transcriptomic profiles when cultured on various substrates with varying stiffness. A shift from round to elongated morphology was observed as the stiffness of the culture surface increased from soft to hard (46,47). For instance, Santos *et al* (48) incubated ECs onto collagen-coated polyacrylamide (PAAm) hydrogels with different stiffnesses. They observed that ECs on high-stiffness PAAm hydrogels had downregulated expression of VEGF receptor-2 (VEGFR2) protein and an upregulated expression of caveolin-1, wingless-type 2, BMP-2 and bFGF, indicating that hydrogel rigidity has a particular effect to promote both angiogenesis and bone formation (48).

Geometry. The design of the surface geometry of the iBTE scaffold has also been the focus of research in recent years. ECs may sense the modification in the micro- and nano-texture of the culture surface and regulate *per se* the actin polymerization and migration via Rac family small GTPase 1 and cell division cycle 42 (49). Abagnale *et al* (50) compared the behavior of MSCs on polyimide fabricated with different groove morphologies and found that a 15-μm groove promoted adipogenic differentiation and rendered cells with a rounded appearance, whereas a 2-μm groove promoted osteogenic differentiation and led to elongated cell morphology. Of note, MSCs were cultured on nanosheets with 600 nm diameter, 650 nm spacing and 200 nm groove depth and exhibited an elongated shape without any tendency to differentiate. MSCs

were able to express the corresponding genes for osteogenesis and adipogenesis when treated with osteogenic and adipogenic media. It has been postulated that the nanoscale surface structure resembles cell receptors and the guidance by contact may influence and regulate the fate of stem/progenitor cells (51). Although studies of material surface morphology have focused on *in vitro* studies of EC, MSC or macrophage behavior and *in vivo* studies are lacking, the results provide a sound theoretical basis for designing VBR-targeted iBTE scaffolds.

Mechanical stimulation. In addition to the surface topology, the mechanical force generated by the scaffold is also a biophysical microenvironmental cue affecting cells. The iBTE fibrous scaffolds doped with magnetic nanoparticles undergo minor deformation when an external magnetic field is applied and therefore, they were able to produce bending and stretching effects on the cells to which they are attached (52,53). Hao *et al* (54) discovered that their superparamagnetic scaffolds inhibited the activation of macrophage Toll-like receptor 2/4 and enhance VEGFR2 activity, inhibiting the expression of downstream pro-inflammatory cytokines and upregulating VEGF and PDGF expression. This discovery indicated the possibility of mechanomodulation of macrophages to indirectly achieve VBR.

Piezoelectric properties. Nanomaterials with piezoelectric properties have also been investigated in iBTE scaffolds. Upon external stress, the dipoles in crystallized poly(hydroxybutyrate-co-hydroxypentanoic acid) (PHBHV) internally rotate and eventually generate electricity or electrodeposition. It has been indicated that MSCs cultured on PHBHV fibers improved the vascularization of engineered bone tissue (55). Similarly, the GaN/AlGaN scaffold developed by Zhang *et al* (56) was found to promote osteogenic differentiation of MSCs and *in vivo* bone regeneration by modulating the intensity and direction of the piezoelectric polarization.

Possible underlying mechanisms. The biophysical cues, such as porosity and surface geometry endowed by the iBTE scaffolds, acting on endogenous stem/progenitor cells are under investigation (31). However, the explanation may also be attributed to the rearrangement of cytoskeletal networks after cell-receptor recognition and aggregation. For instance, integrin, once bound to the surface of biomaterials, may, in turn, activate the downstream Wnt, YAP and c-Jun N-terminal kinase signaling, leading to changes in gene expression (57). The design of iBTE scaffolds aims to create a bioinstructive microenvironment to regulate the behavior of endogenous cells through materials, which requires a comprehensive understanding of organismal physiopathology, cellular function and material science. Previous studies have focused on the effect of a single biochemical cue on cells. Still, as research advances the understanding of biophysical signatures, the design of iBTE scaffolds in the future will be able to integrate multiple factors to achieve *in situ* VBR.

6. iBTE strategy for VBR through extracellular delivery of biochemical cues

Compared to relatively recent times, when researchers began to realize the role of physical factors in biological processes, studies on biochemical molecules have a far longer history. Biochemical cues refer to chemical signals that are involved in regulating cellular behavior and communication (30). Biochemical cues may be broadly classified as GFs, bioactive protein molecules, metallic ions, Traditional Chinese Medicine (TCM) and compound biologics, such as decellularized ECM (dECM), platelet-rich plasma (PRP) and exosomes (58), which act on other cells in the extracellular environment. These cues may be incorporated into/onto the iBTE scaffolds via various processing methods and mechanisms, most of which have been examined previously both *in vitro* and *in vivo* to elucidate a relatively precise mechanism of action and therapeutic effects. Therefore, the iBTE scaffolds are more likely to have a role not just as structural support but also as carriers for cues. iBTE scaffolds for *in situ* VBR were designed to deliver biochemical cues via extracellular signaling mechanisms. This approach is considered safer and more straightforward. This extracellular mechanism avoids the need to directly manipulate the genetic material of cells, which can be more complex and potentially riskier. Therefore, they are more widely studied and gradually translated into clinical practice.

GFs. The broad studies of GFs are an ideal arsenal for bioengineering researchers to selectively choose their armors from. The most widely studied GFs targeting VBR are the BMPs, members of the TGF-β superfamily. BMP-2 and BMP-7 have been reported to have dual functions in osteogenic differentiation and angiogenesis (59-61). Two products loaded with human recombinant BMP-2 and BMP-7, INFUSE™ and OP-1™, respectively, have completed clinical trials and are approved for use (62-64). BMP-2 promotes the osteogenic commitment of MSCs and osteoprogenitors (OP) and indirectly enhances neovascularization through the paracrine effects of Ops (65). Similarly, BMP-7 promotes neovascularization by upregulating VEGF expression in ECs (66). However, applying BMPs has an uncontrollable risk of ectopic bone formation (67). Due to these undesirable effects, the OP-1™ was removed from the market globally. Therefore, the latest BMP-based iBTE strategy focuses extensively on developing novel biomaterials with optimal controlled and spatiotemporal delivery properties (68).

Other GFs, such as VEGF, FGF and PDGF-BB, were also found to be involved in the process of VBR. VEGF is the primary GF controlling blood-vessel formation and osteogenesis (69). Various iBTE scaffolds delivering VEGF have demonstrated a beneficial effect on the *in situ* VBR (9,70-73). As the spatial and temporal arrangement and the emergence of GFs and their mechanism of action in the microenvironment of bone regeneration were clarified, studies are more inclined to investigate different fabrication modalities, such as 3D printing, frozen microgels and nanomaterials, to achieve a precise spatial and temporal delivery (74-77). Lee *et al* (74) developed a dual cryogel system consisting of gelatin/chitosan cryogel (GC) and gelatin/heparin cryogel (GH) to achieve the sequential release of two GFs: The outer GH releases VEGF to induce early angiogenesis to provide blood supply in the defect area, while the inner GH releases BMP-4 for the continuous osteogenic induction. In another system, Zhou *et al* (76) loaded bFGF in a gelatin methacrylate hydrogel to mimic the angiogenic signal from soft callus during early bone healing, while

BMP-2 was incorporated into the mineral-coated microparticles to simulate the osteogenic signal during hard callus formation and bone remodeling. The biomimetic strategy has achieved an early bFGF release accompanied by sustained release of BMP-2, mimicking the typical GFs presentation in the natural bone healing process. Of note, *in vitro* and *in vivo* studies indicated that PDGF-BB, secreted by osteoclast (OC) precursors, was able to promote bone marrow-derived MSC (BMSC)-based VBR by enhancing the osteogenic and angiogenic capacity (78). On top of this, the scaffold GEM21S™, loaded with human recombinant PDGF-BB, was approved by the Food and Drug Administration for periodontal bone regeneration procedures. In addition, PDGF-BB was indicated to induce the formation of type H vessels that have recently been identified as a critical process coupling angiogenesis and osteogenesis (79-81). Therefore, it is reasonable to conjecture that the modulation of OC precursors and PDGF-BB secretion to promote type-H vessel formation may provide an additional path for building iBTE scaffolds. However, there is also an alternative path to achieve *in situ* VBR: To use the osteoimmune-related cytokines to modulate the osteoimmune microenvironment. For instance, Zheng *et al* (4) implanted demineralized bone matrix scaffolds into bone defects, while providing interleukin-4 (IL-4), which shifted the macrophages from a pro-inflammatory M1 to an anti-inflammatory M2 phenotype. Enhanced host bone ingrowth and neovascular infiltration were overserved in this pro-reparative inflammatory microenvironment.

Bioinorganic ions. As the indispensable component, the bioinorganic ions are included in the spectrum of biochemical cues in the extracellular environment (82), most of which function as cofactors for enzymes or coenzymes in different physiological activities and participate in signal transduction indirectly and directly (83). For instance, numerous studies have confirmed that magnesium (Mg^{2+}), copper (Cu^{2+}), cobalt (Co^{2+}), silicon (Si^{4+}) and also the ion-doped iBTE scaffolds promote the angiogenesis-osteogenesis coupling or have an immunomodulatory effect (38,84-92). Mg^{2+} is a critical ion involved in bone metabolism, as verified by OBs and OCs exhibiting functional abnormalities in the absence of Mg^{2+} (93-95). The Mg^{2+}-rich microenvironment stimulates MSC osteogenic differentiation and promotes neovascularization (96,97). *In vitro* experiments have demonstrated that Mg^{2+} promotes the proliferation of OB and the expression of related molecular markers. Furthermore, it also has immunomodulatory effects, including the inhibition of the expression of RANKL-induced cytokines, such as c-Src, MMP-9, and OC activity-related genes such as tartrate-resistant acid phosphatase, proteinase K and calcitonin receptor gene (92). Hu *et al* (98) found that Mg^{2+} reversed the phenotype of M1-macrophages activated by lipopolysaccharide/IFN-γ and upregulate the percentage of M2-macrophages (98). Wang *et al* (99) found that the magnesium-containing calcium phosphate cement (MCPC) down-regulated pro-inflammatory cytokines (TNF-α, IL-6) and upregulated bone-repair cytokine (TGF-β1) (99). At the same time, it was indicated that the osteogenic capacity of BMSCs and the angiogenic potential of ECs were enhanced in the MCPC-induced immune microenvironment. Another two ions, Cu^{2+} and Co^{2+}, are elements that may mimic hypoxia and stabilize HIF-1α, thereby promoting downstream VEGF expression and angiogenesis (89,100-102). The multifunctional Cu^{2+}-doped bioactive glass-collagen scaffold exerted osteogenic and angiogenic effects *in vitro* (103). Similarly, it was found that the addition of low doses of Co^{2+} (<5%) to mesoporous bioactive glass scaffolds promoted the expression of VEGF, HIF-1α and osteogenesis-related genes in BMSCs. In similar studies, by doping Co^{2+} with β-tricalcium phosphate (β-TCP), 45S5 bioglass scaffolds induced a coupling effect of osteogenesis and angiogenesis. As a similar element to carbon in the periodic table, silicon is a significant component of colloids and bioceramics. Si^{4+} may promote osteogenesis of MSCs and enhance angiogenesis of ECs, and it has also been widely used in the preparation of iBTE scaffolds (104,105). Cell studies have reported that Si^{4+} effectively promoted the proliferation, migration and tube formation of ECs and upregulated the expression of angiogenesis-related genes (VEGF, HIF1-α) (106-109). The definite mechanism by which ions are pushed toward VBR is yet to be defined at the moment but likely involves changes in various signaling pathways and gene expression. However, based on these preliminary results and their relatively safe profile, it is clear that the above ions have become popular candidates for developing iBTE scaffolds. However, studies have identified the appropriate ion concentration ranges. Questions related to each bioinorganic ion's possible mechanism of action and its dose-dependent and time-dependent effects have not yet been fully answered. For instance, a recent study has identified a bidirectional mode of action of Mg^{2+} in bone repair (110). Mg^{2+} promotes the upregulation of transient receptor potential cation channel member 7 during the early inflammatory phase, thus creating a favorable osteoimmune microenvironment. By contrast, during the subsequent bone remodeling phase, sustained high-dose exposure to Mg^{2+} leads to excessive activation of NF-κB signaling in macrophages and an increase in the number of OCs, which may have a negative impact on osteogenesis that outweighs the initial osteogenic effect. Although doping iBTE scaffolds with bioinorganic ions is safer and more cost-effective than adding GFs, more persuasive evidence is required.

Other biochemical molecules. In addition to ions, numerous biochemical molecules were found to promote VBR. Due to the limitation in article length and the diversity of molecules, only brief examples are provided. Several studies have indicated that the activation or stabilization of the HIF-1α transcriptional factor leads to the expression of downstream genes, some of which couple angiogenesis and osteogenesis (5,6,111). Therefore, several trials targeting the HIF-1α were performed. Deferoxamine (DFO), a medication approved for the treatment of iron toxicity, was found to stabilize HIF-1α and maintain its activity by inhibiting the prolyl hydroxylase (24). Yan *et al* (14) loaded the DFO into a 3D-printed PCL scaffold using high-temperature melt-printing technology and achieved *in situ* VBR by activating the HIF-1α signaling pathway (112). Furthermore, inspired by the structure of 'lotus', a 3D printed porous bioceramic scaffold was used as the strut of the lotus, and the DFO-releasing liposomes were combined with hydrogel microspheres as 'lotus seeds'. The scaffold exhibited the potential to induce *in situ* vascularization and MSC osteogenic differentiation *in vivo*. Other molecules affecting the HIF-α were also studied. An MBG (mesoporous bioactive

glass)-poly(lactide-co-glycolide) (PLGA) scaffold loaded with the bioactive lipid FTY720 achieved type H vessel-related *in situ* VBR by upregulating HIF-1α expression via the Erk1/2 pathway (113). Ha *et al* (114) filled a gelatin-silica nanofiber (GSN) network into a porous PCL scaffold, followed by embedding the mesoporous silica nanoparticles (MSNs) loaded with bone-forming peptide-1 within the GSN scaffold. The outer surface of the scaffold was then anchored with MSNs loaded with the angiogenic molecule dimethyl oxalyl glycine. The scaffold achieved a spatial distribution and sequential release of the two biochemical molecules targeting the respective angiogenesis and osteogenesis processes. The following subcutaneous and cranial defect implantation verified that the dual-drug delivery model with hierarchical microstructure successfully facilitated vascularization and bone regeneration. Another molecule, calcitonin gene-related peptide (CGRP), is a neuropeptide worth mentioning, as ongoing studies have revealed that factors secreted by peripheral nerves in close proximity to the defect site take a role in neovascularization and bone regeneration (115-119). The physiological doses of CGRP coordinate the interaction of osteoblasts with other cells and affect angiogenesis in addition to osteogenesis, osteolysis and lipogenesis (120). *In vitro* experiments have demonstrated that CGRP promotes osteogenesis in several cell types, such as OB, MSC and periosteal-derived stem cells. Its osteogenic effects are associated with the typical Wnt/β-catenin signaling pathway and the cyclic AMP response element binding protein signaling pathway (117,118,121). CGRP also activates adenylate cyclase and the downstream protein kinase A upon binding to its receptor, CGRPR, resulting in the efflux of nitric oxide from EC and Ca^{2+} from smooth muscle to exert vasodilatory effects (119). *In vitro* experiments also revealed that CGRP promoted EC proliferation and tubule formation by enhancing VEGF expression (115,116,120,122). CGRP was released in the fracture site upon electrical stimulation applied in the dorsal ganglion root and type H vessels were also found along with high expression of CGRP (122). Similarly, unpublished data by our research team also indicated that upregulated CGRP expression colocalizes with the type H vessel-related *in situ* VBR. These findings suggest that CGRP is essential in coupling angiogenesis and osteogenesis. On top of these findings, CGRP-loaded gelatin microspheres demonstrated enhanced bone regeneration in osteoporotic rabbits, as indicated by increased trabeculae and reduced trabecular separation (123). Continuous research on iBTE scaffolds employing CGRP is being conducted (124).

Biologics. Composite biologics such as PRP, dECM and exosomes are also worth discussing. The therapeutic mechanisms of these compounds are observed to be multifactorial and although the effective molecule of these biologics is yet to be elucidated, their efficacy in both preclinical and clinical settings has attracted much attention. PRP is a mixed agent enriched with multiple autologous GFs derived from the donors' blood. Numerous studies on iBTE scaffolds incorporating PRP are being investigated because of their inherent high safety and convenience. It was found that PRP was also able to induce angiogenic-osteogenic coupling (125-127), which may be attributed to the various GFs, such as PDGF-BB, IGF and FGF. However, varieties of PRP resulted from numerous factors, including donor variability and preparation methods, leading to relatively inconsistent effectiveness results. Another composite biologics agent is the dECM, a low-immunogenic natural biomaterial that retains multiple biochemical molecules simulating the tissue-specific regenerative microenvironment. A periosteal decellularized matrix (PEM) hydrogel was prepared using the decellularized periosteal matrix by Qiu *et al* (128). The PEM hydrogels rapidly recruited inflammatory cells and shifted macrophages from the M1 pro-inflammatory phenotype to the M2 reparative phenotype in the early stage after implantation. In addition, the PEM hydrogels had a positive role in promoting angiogenesis, osteogenesis and subsequent mineralization in the later stage. He *et al* (2) fabricated the human umbilical vein endothelial cell-derived decellularized matrix/fibrin/PCL scaffold, exhibiting accelerated VBR after implantation into rat femoral defects, and revealed that the underlying mechanism may be related to the formation of type H vessels. Other cell-derived biologics, exosomes or extracellular vesicles (EV) are membrane-like natural nanoparticles released by cells. Exosomes and EVs may carry mRNA, micro (mi)RNA and bioactive proteins, and have multiple potential biological functions, such as reducing the inflammatory response, promoting angiogenesis and facilitating bone formation (129-131). Fan *et al* (132) developed a bone marrow MSC-derived exosome-functionalized polyetheretherketone implant (SPEEK). SPEEK promotes macrophage polarization toward M2 by inhibiting the NF-κB signaling pathway, enhancing the osteogenic differentiation of BMSCs. Also, SPEEK exhibited superior osseointegration. Although angiogenesis was not solely investigated in this study, the result demonstrated that the proangiogenic role was ineligible. This study also suggested that exosomes may be used as a surface-modified biochemical cue to prepare iBTE scaffolds.

TCM compounds. In addition, TCM has a deep historical background and is being gradually used as an alternative therapy. Herbal medicine has sparked the enthusiasm of numerous researchers due to its diverse therapeutic effects and mechanisms of action. In-depth research found that the active ingredients in various TCM formulations promote osteogenesis and angiogenesis (133,134). Lin *et al* (133) used a low-temperature rapid prototyping technique to prepare a PLGA/β-TCP composite scaffold incorporating low, medium and high doses of salvianolic acid B. It was found that salvianolic acid B promoted osteogenesis and angiogenesis in a dose-dependent manner *in vitro*. Animal experiments also confirmed the scaffold's dose-dependent effects on new bone formation, mineralization and angiogenesis. It was indicated that the PLGA/β-TCP composite scaffold doped with salvianolic acid B increased the bony fusion of vertebral bodies by contributing to bone and blood vessel formation. Wu *et al* (134) developed novel micro/nanostructured hydroxyapatite particles to construct a delivery system for icariin. The scaffold exhibited enhanced osteogenesis and angiogenesis in a rat femoral defect model. *In vitro* experiments revealed that the delivery of icariin promoted osteogenic differentiation and expression of angiogenesis-related factors in MSCs via the Akt signaling pathway. Although certain studies have proven the proangiogenic and osteogenic activities of naringin and ginsenoside *in vitro* (135-138), their application

in constructing iBTE scaffolds has rarely been reported. A wide range of active components of TCM requires further exploration to provide alternative solutions for the fabrication of iBTE scaffolds.

7. VBR through intracellular gene delivery iBTE strategy

The field strives to develop innovative strategies to enhance VBR. A critical aspect of this process involves the spatiotemporal delivery of GFs, which may be challenging to achieve through conventional methods involving the use of biochemical molecules. To overcome these limitations, researchers have turned to genetic manipulation, exploring the potential of GF vectors, gene activation matrix (GAM) and engineered exosomes as alternative means to promote angiogenesis-osteogenesis coupling. By harnessing the power of genetic manipulation, it is possible to create more precise and cost-effective treatments that may mimic the natural phases of VBR while minimizing unwanted side effects.

GF vectors. As previously mentioned, the delivery of biochemical molecules through iBTE scaffolds, in most cases, cannot fulfil a satisfying spatiotemporal release mimicking the natural phase of VBR. For instance, excessive VEGF may lead to vascular leakage and OC activation, and high concentrations of BMP result in ectopic bone formation (139). In addition, even with the appropriate dose and release kinetics, the half-life of these biochemical molecules limits their effectiveness within a short period. The GFs required in the regenerative process may not be of therapeutic value if released too early. Fortunately, genetic manipulation is more cost-effective than high-dose GF delivery and with a more precise control (140). Furthermore, it is technically achievable to deliver multiple customized genes (141). Researchers have verified the strategies to maintain a sustained expression of target proteins through direct gene delivery. As mentioned earlier, BMP and VEGF are major GFs in angiogenesis-osteogenesis coupling and iBTE strategies using genetic manipulation have been reported in several pieces of literature (142-145). Despite the fact that virus-based gene delivery is more effective in certain animal studies, the safety issue remains a critical question to be answered in human experiments (146,147). Non-viral vectors have lower transfection efficiency than viral ones but are safer in consensus. Therefore, the following section focuses on the intracellular gene delivery iBTE strategies with non-viral vectors.

GAM. GAM is an iBTE scaffold containing a gene delivery vector (148). After the biomaterial successfully delivered genes, which were internalized and translated, the recombinant proteins were able to be expressed *in situ* by endogenous cells. Meanwhile, the framework of the GAM temporarily serves as a support for tissue formation. It directs the growth of new functional tissues, and despite the small amount of target protein secreted, as compensation that the prolonged expression could also promote VBR (149). Bozo *et al* (150) developed a GAM bone implant based on octacalcium phosphate and naked VEGF plasmid DNA. *In vitro* experiments revealed that the GAM scaffold did not produce cytotoxicity but slightly decreased the doubling time of MSCs. In a luciferase bioimaging assay, the scaffold continued to express the signal for 28 days, suggesting that GAM as a vector may sustain the expression of the target gene *in vivo*. In a rabbit cranial defect model, GAM increased bone formation by directly inducing angiogenesis. Subsequently, the team conducted a non-randomized human clinical trial (NCT03076138). The GAM was implanted in the socket after tooth extraction. CT was used to measure the proportion of newly formed bone tissue in the surgical area at 6 months after surgery. The primary and secondary outcomes were the frequency of adverse events (AEs), serious AEs (SAEs) and surgical failure rate. After completing the clinical trial, each patient had the teeth implanted in the graft area before a biopsy was taken. No AE or SAE had been reported during the clinical trial and within the follow-up period (30 months). In all of the cases, newly formed tissue was detected in the grafted area, with no significant differences between the subgroups of patients with alveolar atrophy and jaw defects. Histological analysis indicated that the grafted area consisted of the newly formed bone tissue and the fragments of the GAM scaffold were partially resorbed and integrated with the host new bone, with no intervening spacing of fibrous tissue. The present study claimed to be the first to translate GAM bone scaffolds from the laboratory to the clinic.

Engineered exosomes. In addition to the naked plasmid described previously, exosomes are among the ideal candidates for gene delivery due to their excellent biocompatibility, low immunogenicity and efficient cellular internalization. Exosome-delivered mRNA and miRNA have also participated in VBR by different mechanisms (130). For instance, early healing of rat cranial defects was observed after MSC-derived exosome administration, which may be associated with the exosomal miRNA-196a that promotes osteoblast proliferation and differentiation (151). Besides, the miR-129, miR-136 and miR-17-92 clusters enriched in exosomes were found to promote EC proliferation and angiogenesis (152). Exosomes have been explored as biomimetic and safe cargo carriers, and exosome-based engineering modifications have also been investigated. Zha *et al* (153) constructed gene-activated exosomes carrying the VEGF gene and then loaded them onto 3D-printed scaffolds with nanoparticles via the CP05. Subsequently, the *in vivo* experiments verified that this gene-activated exosome iBTE scaffold effectively induced a substantial amount of neovascularization and new bone. Based on the above, the team prepared a novel exosome analog (EM) encapsulated with VEGF165 plasmid DNA, aiming to improve the current shortcomings of exosome-based therapeutics, such as low exosome yield and unstable efficiency (154). Compared with the traditional method of obtaining exosomes, the EM method has a higher yield of exosomes with similar characteristics. The EM encapsulated with VEGF165 plasmid DNA was attached to the GAM composed of electrospun nanofiber membrane via the biotin-avidin system and achieved the local release of the VEGF165 plasmid and exhibited enhanced VBR *in vitro* and *in vivo*.

Future perspectives. Upon examining the available evidence, it becomes evident that the GAM approach constitutes a viable iBTE strategy for accomplishing *in situ* VBR. Through the

prolonged local delivery of genes, endogenous cells undergo reprogramming and persistently produce GFs. This process emulates the stepwise presentation of GFs, thereby simulating the physiological process of bone repair. While gene therapy has experienced significant advancements across various fields, the current utilization of gene editing and epigenetic modulation, particularly concerning VBR, has not been thoroughly investigated. Consequently, further research is necessary to ensure the safe and effective implementation of these techniques in the context of VBR.

8. Summary and outlook

Evidence has indicated that the expeditious establishment of vascular networks is crucial for successful bone regeneration. In recent years, iTE strategies aimed at VBR have garnered considerable interest due to their capacity to promote angiogenesis and hasten the establishment of vascular supply. These strategies encompass the employment of biophysical and biochemical cues to facilitate the differentiation and proliferation of bone-forming cells, stimulate angiogenesis and blood vessel formation, and modulate the inflammatory response. Biophysical cues, including mechanical forces, electrical and magnetic fields, porosity and topography, may be utilized to direct the fate of endogenous progenitor cells, a vital component in achieving functional tissue regeneration. Furthermore, biochemical cues may be delivered through extracellular signaling mechanisms or regulation of intracellular genetic material. The former approach offers a safer and more straightforward method than the latter, which entails more intricate genetic manipulation. Both strategies have exhibited promise in preclinical investigations and are progressively being translated into clinical practice.

In light of the increasing diversity and sophistication of biomaterials, drawing comparisons between individual materials may be challenging. The advancement of biomaterials research is intimately connected to the exploration of host biology, as these two fields exhibit a reciprocal relationship that fosters innovation and discovery in both areas. For instance, recent developments in high-throughput sequencing have unveiled the striking heterogeneity of host cells and their varied responses to different biomaterials. This knowledge subsequently informs the design and optimization of biomaterials, customizing them to elicit specific biological responses and enhance their integration with host tissues.

As increasingly sophisticated implanted biomaterials are being developed and implemented, the understanding of the complex biological reactions they induce within the body deepens. This bidirectional relationship between biomaterials research and host biology not only encourages the creation of advanced materials with improved biocompatibility and functionality but also clarifies the underlying mechanisms governing tissue regeneration and repair.

As the field of iTE of VBR continues to progress, several areas of potential growth and improvement emerge. First, as the heterogeneity of host cells and biological responses becomes apparent, it would be prudent to develop novel biomaterials with tunable properties, enabling precise spatiotemporal control over the biophysical and biochemical cues provided to cells in the regenerative environment. In addition, future integrated strategies combining biophysical and biochemical approaches may result in synergistic effects that promote more efficient and robust VBR. These advancements would not only be applicable to critical-sized bone defects but may also extend to various bone diseases, such as osteonecrosis and osteoporosis.

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Authors' contributions

YH drafted the manuscript and performed critical analyses of the literature. LL and CL collected the raw data for analysis. JH and ZZ organized the framework of this paper, supervised the work and revised the manuscript. All authors have read and approved the final manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

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Not applicable.

Competing interests

The authors declare that they have no competing interests.

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