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Biomimetic Approaches for Bone Tissue Engineering

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Abstract

Although autologous bone grafts are considered a gold standard for the treatment of bone defects, they are limited by donor site morbidities and geometric requirements. We propose that tissue engineering technology can overcome such limitations by recreating fully viable and biological bone grafts. Specifically, we will discuss the use of bone scaffolds and autologous cells with bioreactor culture systems as a tissue engineering paradigm to grow bone *in vitro*. We will also discuss emergent vascularization strategies to promote graft survival *in vivo*, as well as the role of inflammation during bone repair. Finally, we will highlight some recent advances and discuss new solutions to bone repair inspired by endochondral ossification.

Keywords: : bone, tissue engineering, biomimetics

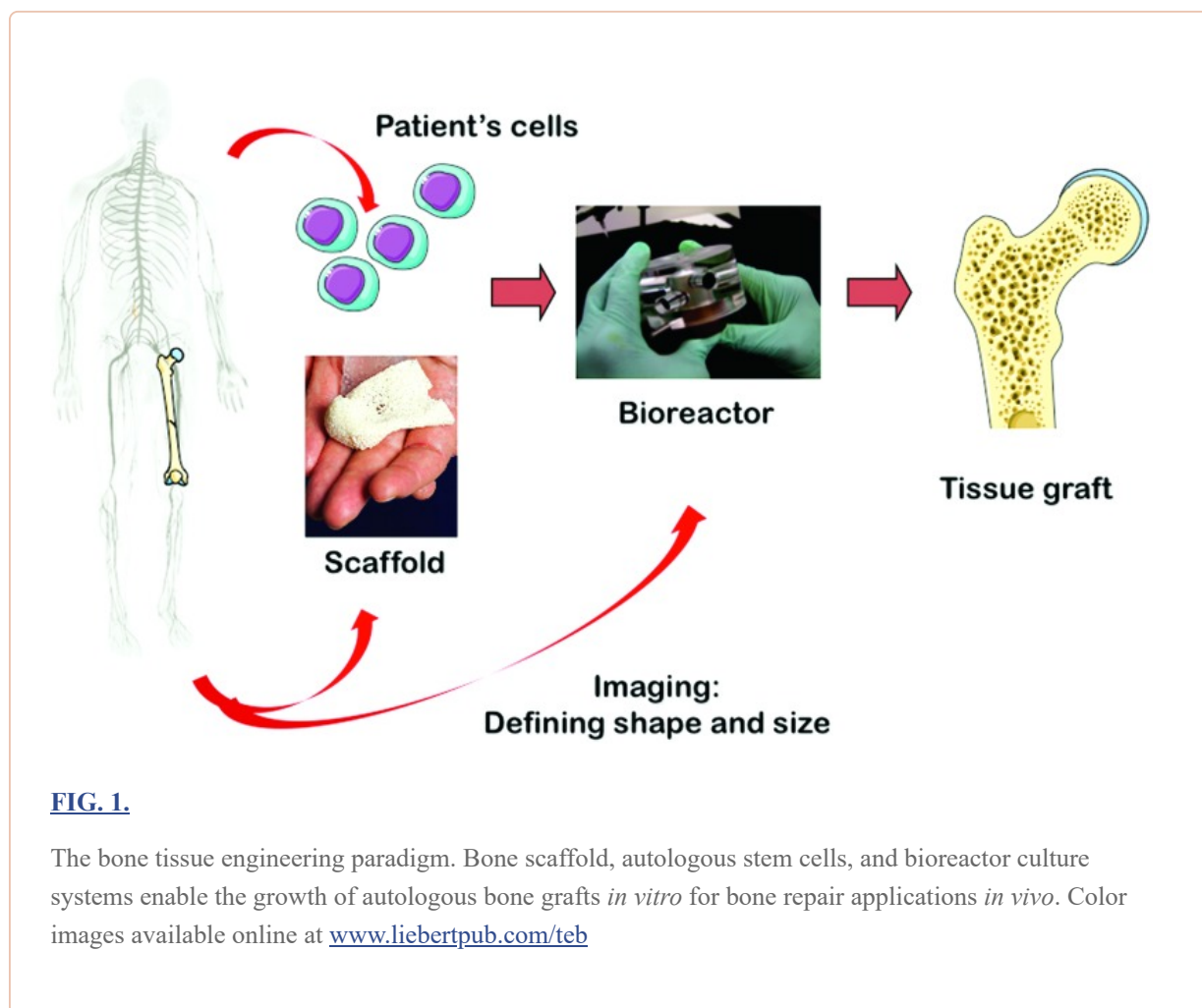
Introduction

IN THE UNITED STATES alone, there are 6 million bone fractures per year, and 5–10% of these fractures suffer incomplete healing due to bone loss, failed fixation, infection, and inadequate vascularization.¹ Current treatment that involves autografts harvested from other locations in the body has drawbacks, including limited tissue supply, donor site morbidity, infections, and poor integration. Allografts are also widely used, but have been associated with disease transmission and host rejection.²

Tissue engineering provides an important treatment alternative using the patient's own cells in combination with a scaffold for bone repair. Common scaffolding materials include natural and synthetic polymers such as poly (lactic-co-glycolic acid) (PLGA), poly (caprolactone) (PCL), and silk,^{3–7} and bioceramics such as tricalcium phosphate (TCP) and hydroxyapatite (HAp) resembling the mineral phase in bone.^{8,9} Decellularized bone (DCB), used in the clinic for bone repair, has also gained interest as bone scaffold due to its native matrix and unique osteoinductive properties.^{10,11} Biomimicry

can guide the development of bone grafts *in vitro* to promote safe and effective repair. Biomimetic approaches to engineer bone have taken cues from the native bone structure and milieu and involve the use of specialized scaffolds.

In this review, we examine the status of biomimetic approaches for bone tissue engineering (Fig. 1). We first discuss the fabrication of synthetic scaffolds mimicking the native bone, the use of decellularized tissue scaffolds, the cell sources, and bioreactor systems for cultivation of bone grafts *in vitro*. Next, we discuss the importance of angiogenesis in bone repair and current strategies to promote scaffold vascularization that is critical for establishing blood supply to the bone graft upon implantation. Finally, we discuss how endochondral ossification of engineered cartilaginous template is inspiring a new generation of bone grafts.



The Bone Tissue Engineering Paradigm: Scaffold, Cells, and Bioreactor

Developments in fabrication of synthetic bone scaffolds

Scaffold design to support bone formation largely determines the success of bone regeneration. Scaffold material and design in tissue engineering play key roles by providing a suitable structure, mechanical function, and delivery of bioactive factors. In an excellent review of scaffold design for tissue engineering, Hollister said, "The art of scaffolding is where to put the holes and the biofactors," paraphrasing architect Robert le Ricolais who said, "The art of structure is where to put the holes."¹² Thus, scaffold design must entail the creation of hierarchical porous structures resembling the native tissue to provide mechanical support, mass transport, and incorporation of biomolecules for recruiting cells and directing bone formation.

Synthetic and natural polymers such as PCL and type I collagen, as well as bioceramics such as calcium phosphates (CaPs) and bioactive glasses, have been used to fabricate scaffolds for bone tissue engineering.^{7,13-15} These materials recapitulate the organic and inorganic phases of the native bone that confer toughness, strength, and osteoconductivity. Although synthetic polymers can form scaffolds with interconnected pores, they lack the mechanical property and osteoconductivity of native bone.

In contrast, scaffolds fabricated from ceramics such as Hap and TCP are osteoconductive and have compressive strengths that are comparable to that of the native bone.^{14,16} However, ceramic scaffolds lack interconnected pores and are prone to fracture. For these reasons, some investigators have attempted to reinforce ceramic scaffolds with polymers such as PCL and collagen,^{17,18} whereas others have used natural composite material such as the coral exoskeleton with an inorganic CaP phase growing onto an organic template.¹⁹ Ceramic scaffolds are slow to be resorbed *in vivo* and lack biological factors present in the native bone to promote robust osteogenic differentiation.

Conventional methods to create pores in scaffolds include salt leaching, gas forming, phase separation, and freeze-drying.²⁰ However, these methods offer limited control over the scaffold microarchitecture. Recent advances in additive manufacturing have enabled the creation of scaffolds with precise features and pore structures by 3D printing.^{18,21,22} Bioceramics have also been successfully incorporated into ink mixtures that are compatible with 3D printing by photopolymerization or extrusion. However, many 3D printed ceramic composite scaffolds require high temperature processing, which then precludes incorporation of biological factors. Still, some noteworthy progress has been made recently in bridging the gap between additive manufacturing and clinical translation.

In one study, a bone scaffold mixture comprising PCL/TCP and a cell-laden hydrogel were alternately printed by extrusion to form a calvarial bone construct that supported bone regeneration in a rat model.²³ In another study, HA combined with PCL and PLGA binders and other solvents to form a mixture that printed into a hyperelastic bone scaffold by extrusion. The hyperelastic bone scaffold resisted fracture and permanent deformation under high-impact loads, supported osteogenesis *in vitro*, and promoted spinal fusion in a rat model, as well as calvarial regeneration in a macaque model.²²

Unique benefits of DCB as a scaffold

Despite the many advances in fabrication of synthetic scaffold, DCB remains an attractive and popular choice of scaffold for bone regeneration. The many qualities of DCB include its hierarchical porous structure, mechanical competence, osteoinductivity, and osteoconductivity. Interestingly, most modern research into the use of DCB for bone repair dates back to the seminal work by Urist in the 1960s, who first demonstrated the osteoinductive nature of bone matrix. The inductive agents were subsequently identified as potent trophic factors called bone morphogenetic proteins (BMP), which enhance and regulate cartilage and bone formation.^{24,25} BMP-2 has since been isolated and become widely used in research and clinic for bone regeneration.²⁶ Scaffold mineralization of DCB was later shown to significantly affect the osteogenesis *in vitro*.²⁷ Importantly, bone matrix derived from DCB has also been shown to impart osteoconductivity to collagen matrix.²⁸ Together, the endogenous trophic factors and mineral and matrix components within the DCB support bone formation.

Currently, multiple companies are using proprietary methods to test, sterilize, and decellularize bone allografts for clinical use. Notable FDA-approved DCB products include GrafTech[®], GraftCage[®], BTB Select[®], BioCAP Select[™], MatriGRAFT[®], and ReadiGRAFT[®]. Proprietary mixes comprising matrices derived from demineralized DCB have also been approved and such products include Grafton[®], Osteofil[®], ALLOMATRIX[®], AlleGro[®], Optium DBM[®], and OsteoBiologics I/C Graft Chamber[®]. The large number of DCB products suggests that DCB can be derived safely from animal or cadaveric sources.²⁹

Still, cellular grafts were shown to result in better bone regeneration than acellular controls in several autologous implantation models.³⁰ It is likely that the constituent cells and biological factors are necessary to enhance osteogenesis at the site of bone defect. Thus, personalized bone grafts generated from DCB and autologous cells could provide an effective alternative to autografts for bone reconstruction.

Sources of autologous cells for bone formation

Osteoprogenitor cells have been isolated from adult bone tissue and periosteum, by preparation of explant cultures from dissected tissues, or enzymatic release of progenitor cells from endosteal and periosteal layers.^{31,32} Such primary human bone and periosteal cells formed bone-like tissues when cultured on porous scaffolds, confirming their osteogenicity.³³

Due to their multipotency and proliferative ability, adult mesenchymal stem cells present a more attractive cell source and have been widely used for tissue engineering. Found in a variety of tissues, including the bone marrow, adipose tissue, synovium, and dental pulp, they can reach up to 50 population doublings and are capable of differentiating into bone, cartilage, tendon, ligament, muscle, and adipose cells.

Among the different adult stem cell sources, bone marrow mesenchymal stem cells (BMSCs) isolated from bone marrow stroma are the most studied source for bone regeneration. These cells are commonly isolated based on adherence and growth on tissue culture plastics or immunoselected by characteristic markers. The number of BMSCs within each bone marrow aspirate varies among patients (0.001–0.01% of the nucleated marrow cells), and there is a need for expansion in culture to reach clinically relevant numbers for therapeutic purposes.^{34,35} Many groups have demonstrated the osteogenic potential of BMSCs and their usefulness toward bone tissue engineering.^{6,10,36} When differentiated in porous scaffolds under osteoinductive conditions, these cells can form bone-like tissues.

Adipose-derived stem cells (ASCs) were discovered more recently and quickly became an attractive cell source for bone tissue engineering due to their abundance in readily accessible lipoaspirates and their ability to differentiate into multiple lineages.^{37,38} Depending on patients, several liters of lipoaspirate containing a relatively high frequency of ASCs (1–5% of isolated nucleated cells) can be obtained. Isolation protocols involve density gradient centrifugation of collagenase-digested tissue (lipoaspirate or minced adipose) followed by selection and culture of adherent cell populations. The formation of bone-like tissue from ASCs cultured on scaffolds has already been reported separately.^{39–42}

Bioreactor systems for bone tissue engineering

A key limitation in the development of bone tissues of clinically relevant sizes *in vitro* is the insufficient transport of nutrients and oxygen. To overcome such transport limitations, bioreactor systems have been developed to support the culture of large engineered bone tissues *in vitro*.^{6,10,36,39,41–51} These systems are summarized in [Table 1](#). Rotating wall vessels, spinner flasks, perfusion bioreactors, and compression bioreactors have been used to culture engineered bone *in vitro* to different effects. In general, rotating wall vessels and spinner flasks support the formation of smaller bone constructs and are limited by suboptimal transport inside the construct core.⁵² It is also unclear whether these systems promote robust osteogenic differentiation or improvement in bone formation over static culture.

TABLE 1.

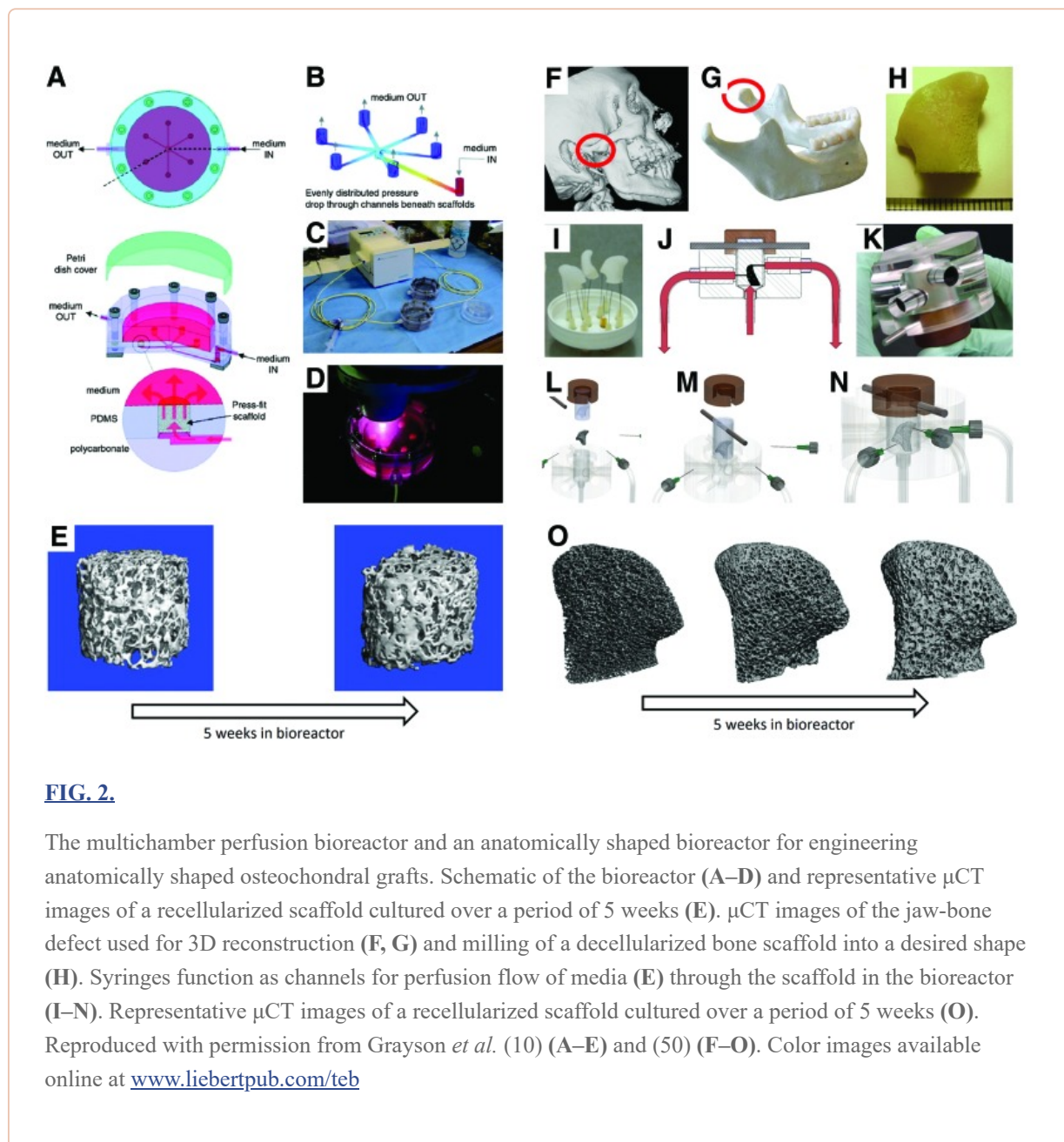
SUMMARY OF BIOREACTOR SYSTEMS FOR BONE TISSUE ENGINEERING

<i>System</i>	<i>Cells</i>	<i>Scaffold</i>	<i>Outcomes (vs. static)</i>	<i>Source</i>
Rotating wall vessel	BMSCs	Gelatin-HA	↑Proliferation	(43)
	ASCs	ZrO ₂ based ceramic/HAp	↑Proliferation, Differentiation	(41)
	BMSCs	Gelatin-Coral/Hap	N.A.	(44)
Spinner flasks	ASCs	PET	↑Proliferation	(42)
	BMSCs	Collagen	↑Differentiation	(45)
	BMSCs	Silk	↑Proliferation, Differentiation	(46)
	BMSCs	Silk	N.A.	(6)
	BMSCs	Gelatin-HA	↑Proliferation, Differentiation	(43)
	BMSCs	Chitosan	↑Proliferation, Differentiation	(47)
	Perfusion	Osteoblasts	Titanium	↑Proliferation, Differentiation
BMSCs		Titanium/MSC-ECM	N.A.	(36)
BMSCs		Si-TCP/HAp-TCP	↑Proliferation, Differentiation	(49)
BMSCs		DCB	↑Proliferation, Differentiation	(10)
BMSCs		DCB	↑Proliferation, Differentiation	(50)
ASCs		DCB	↑Proliferation, Differentiation	(39)
ASCs		DCB	N.A.	(51)

ASC, Adipose-derived stem cell; BMSC, bone marrow mesenchymal stem cell; DCB, decellularized bone; ECM, extracellular matrix; HA, hyaluronic acid; HAp, hydroxyapatite; PET, polyethylene terephthalate; Si-TCP, silicate substituted TCP; TCP, tricalcium phosphate; ZrO₂, zirconium dioxide; N.A., not applicable; ↑, increase.

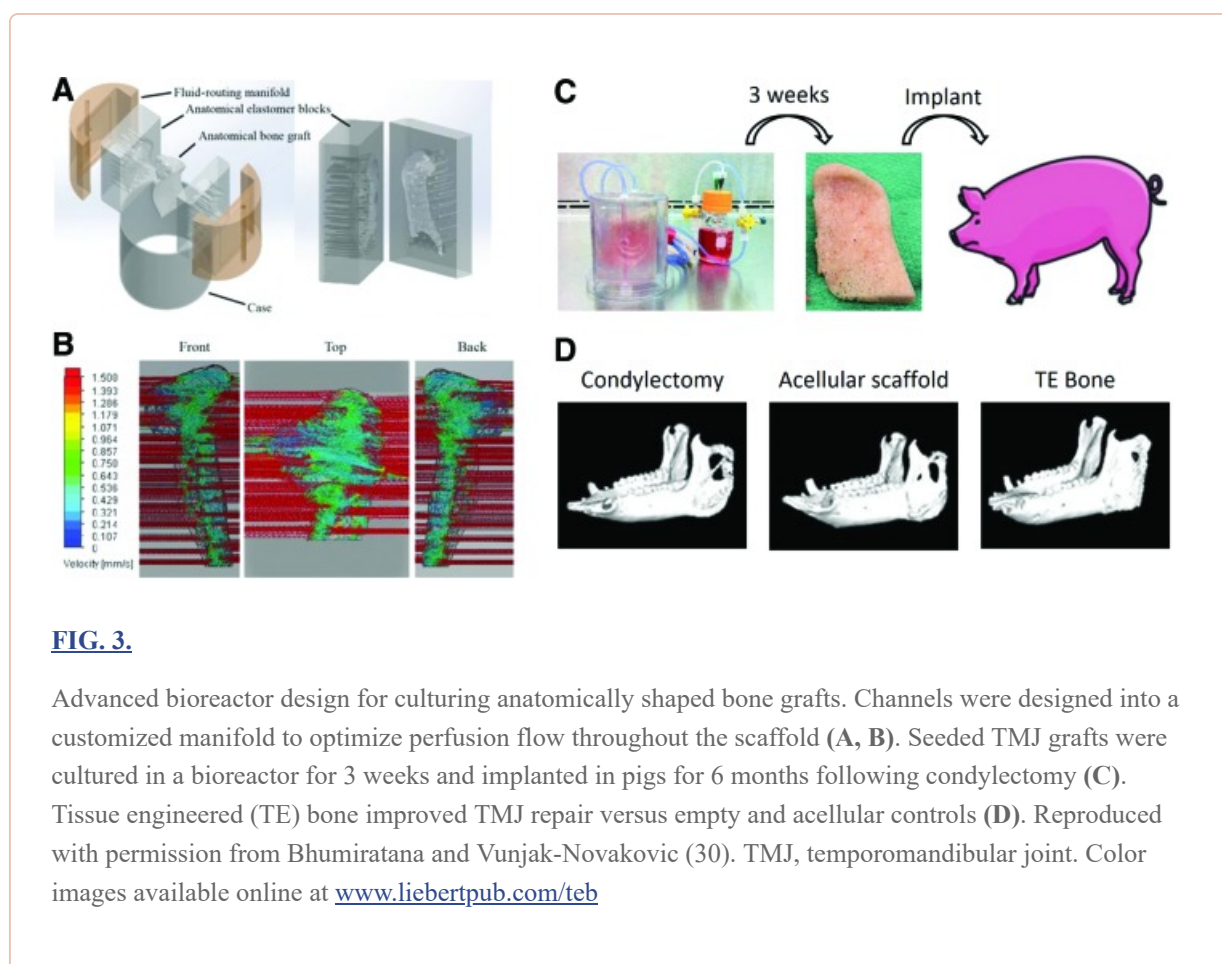
Instead, perfusion bioreactors enable optimal transport throughout the constructs and provide biophysical stimulation to bone-forming cells.^{49,53} In native bone, mechanosensitive osteocytes enable adaptation to mechanical loading. Through the lacuna-canalicular network, osteocytes communicate with osteoblasts, osteoclasts, and osteoprogenitor cells using paracrine signaling to induce bone formation or resorption.⁵³ Fluid shear stress also modulates the release of nitric oxide (NO) and upregulation of prostaglandin (PG) by osteoblasts and mediates bone maintenance.^{54,55} More recently, stem cells have also been shown to be mechanosensitive and capable of undergoing mechanically induced osteogenic differentiation. BMSCs and ASCs cultured under flow stimulation upregulated the expression of osteogenic markers and increased deposition of bone matrix proteins.^{56,57} Mechanotransduction during osteogenic differentiation involve multiple pathways that include calcium signaling and components of the mitogen-activated protein kinase (MAPK)/ERK, Wnt, Hippo, and RhoA/ROCK pathways.⁵⁸ Mechanosensors, in particular, the primary cilia, have been shown to directly mediate flow-induced osteogenic differentiation of BMSCs.⁵⁶

In one study, a perfusion system enabled the cultivation of up to six tissue constructs simultaneously, with equal flow rate in all scaffolds, for a wide range of flow rates and scaffold densities (Fig. 2A–E).¹⁰ When cultured under perfusion, hMSCs and hASCs proliferated and formed spatially uniform bone on DCB scaffolds.^{10,39} Shear forces associated with perfusion resulted in the upregulated expression of osteogenic genes and improved bone formation over static culture. Flow velocities within the cultured tissues ranging from 400 to 800 $\mu\text{m/s}$ resulted in the most dense and homogenous matrix deposition.⁵⁹ Interestingly, flow perfusion synergized with native bone-like matrix to enhance osteogenic differentiation of MSCs on titanium fiber mesh.³⁶ However, the enhancement was abolished upon denaturation of the matrix. Thus, the flow dependence of bone formation within a perfusion bioreactor is complex and could involve the geometry, internal architecture, and cellular maturity of the construct.



Bioreactor for personalized bone reconstruction

To achieve clinical relevance, the grafts also need to match the defect in terms of shape, architecture, and biomechanical properties. The maintenance of cellularity in large, anatomically shaped bone grafts *in vitro* necessitates a biomimetic scaffold-bioreactor system. With such a system, the formation of a clinically sized, anatomically shaped, and viable human temporomandibular joint (TMJ)^{50,51} has been reported. Using computer aided design (CAD) guided by digitized micro computed tomography (μ CT) images, TMJ shaped DCB scaffolds and elastomer blocks with corresponding chambers were generated. The scaffolds were seeded with BMSCs and press fitted in the elastomer blocks fitted with channels for controllable perfusion throughout the constructs (Fig. 2F–O). After 5 weeks of culture, fully viable and anatomically shaped bone grafts with physiologic cell density and uniform bone matrix were formed.⁵⁰ Using a similar system, autologous bone grafts grown from ASCs enhanced regeneration of the ramus–condyle unit (RCU) following condylectomy in a porcine model (Fig. 3).⁵¹ The engineered RCU promoted near-complete bone regeneration after 6 months of implantation, whereas acellular scaffolds and untreated controls resulted in extensive fibrous tissue formation at the site of defect.



Together, the osteoinductive capacity of the DCB, the osteogenic capacity of various adult stem cells (hMSCs, hASCs), and the physiologic benefits of perfusion flow contribute to a comprehensive paradigm of bone tissue engineering (Fig. 1). This paradigm heralds the next generation of autologous bone grafts for bone reconstruction. Major challenges in reconstructing large bone defects remain, such as the ability to prevascularize the graft and establish blood perfusion immediately following implantation to maintain graft viability. In the next section, we discuss the importance of vascularization and current strategies to vascularize bone grafts.

Strategies to Promote Vascularization

Importance of angiogenesis in bone development and repair

Bone development in the limb bud is concomitant with angiogenesis. Flat bones develop by a process called intramembranous ossification, in which MSCs differentiate directly into osteoblasts as capillaries invade from the surrounding tissue.⁶⁰ The osteoblasts secrete bone matrix nodules and eventually fuse to form woven bone. Long bones develop through endochondral ossification, in which MSCs differentiate into chondrocytes to form a cartilaginous anlage to guide vascularization. Infiltrating blood vessels bring osteoprogenitor cells and recruit osteoclasts to degrade the cartilaginous template, which is then replaced by bone.^{60–63}

Cross talk between endothelial cells (ECs) and osteoblasts is essential for bone formation, acting through the secretion of paracrine factors, especially vascular endothelial growth factor (VEGF), platelet-derived growth factor-BB (PDGF-BB), and BMP.^{60,61,64} VEGF stimulates the migration of ECs and the formation of an immature vascular network.⁶⁵ PDGF-BB stabilizes the blood vessels by recruiting pericytes that wrap around the blood vessels and prevent them from regressing.⁶⁶ The rate of ossification depends on vascularization, and only the cells that are near capillaries contribute to bone formation.^{61,67}

Bidirectional signaling between bone cells and cells of the vasculature plays a major role in bone homeostasis and function.^{60,61} Bone is a highly vascularized tissue, with most cells positioned within 100 μm of the nearest capillary.⁶⁵ Blood vessels transport oxygen, nutrients, and cells to bone tissue and are essential for bone viability and health.⁶¹ Obstruction of the blood supply often results in skeletal pathologies such as osteonecrosis and osteoporosis.⁶⁰

Bone repair is also dependent on the blood vessel network.⁶⁸ Upon disruption to the circulation, bone fracture triggers inflammation.⁶⁹ Pro-inflammatory signals, tumor necrosis factor- α (TNF α) and interleukin-1 β (IL1 β), recruit cells for bone formation.⁷⁰ Osteoclasts degrade necrotic tissue, while osteoprogenitor cells form new bone.⁷¹ Clinically, vascular comorbidity is a major risk factor for delayed or nonunion fracture healing.⁷² Experimentally, stimulation of neovascularization was shown to recover bone healing in hind limb ischemia.⁷³

Prevascularization of bone grafts

Given the importance of angiogenesis in bone development and repair, a successful bone tissue engineering strategy should also address the challenge of vascularization. The coupling of osteogenesis and angiogenesis during skeletal development is well documented.^{74,75} Cross talk between ECs and osteoprogenitor cells through paracrine signaling and cell–cell contact also enhanced bone formation *in vitro*.^{76–79} Importantly, prevascularization of grafts could help graft survival by anastomosis with host vasculature and rapid establishment of blood supply within the graft upon implantation. Thus, investigators have been pursuing different strategies to vascularize bone tissues grown *in vitro*.

To prevascularize the bone grafts, some investigators chose to include native vessels and vascular bundles such as the femoral artery and vein, the carotid artery, jugular vein, or saphenous bundle in osteoconductive scaffolds.^{80–82} Following implantation, *de novo* bone deposition and neovascularization within the grafts were observed.

Other investigators instead chose to vascularize grafts by seeding ECs with perivascular cells and allowing the ECs to form microcapillary-like networks within the grafts.^{83,84} However, anastomosis between the preformed microvascular structures and host vasculature was limited in some cases. Interestingly, vascular network formation was observed in bone tissue comprising DCB seeded with osteogenically differentiated MSCs and undifferentiated MSCs and ECs in hydrogels.⁸⁵ The

undifferentiated MSCs in the hydrogel assumed roles of perivascular cells and formed more mature vascular networks with the ECs. Upon implantation, the ECs seeded with MSCs resulting in dense and persistent neovasculature that anastomosed with the host vasculature over time. In another 3D coculture study, Hedgehog signaling modulated the morphology of vasculature *in vitro* and extent of ectopic bone formation *in vivo*.⁸⁶ Activation of Sonic Hedgehog (Shh) in the ECs in aggregate culture with MSCs resulted in more perfused lumens and increased formation of mature bone tissue *in vivo*.

Taken together, modulating the behavior ECs and MSCs *in vitro* could lead to potent graft vascularization strategies. While it is evident that prevascularization of bone grafts could enhance graft survival and *de novo* bone formation *in vivo*, we still need a better understanding of the bioactive factors and cellular responses to control vascularization *in vitro* more effectively.

Delivery of angiogenic factors

The delivery of pro-angiogenic factors for bone tissue engineering has been extensively investigated. Various combinations of VEGF, PDGF-BB, fibroblast growth factor (FGF), and BMP have been shown to enhance both vascularization and bone formation *in vivo*.^{87–92} Notably, growth factors engineered to bind extracellular matrix (ECM) proteins strongly improved tissue repair and reduced unwanted side effects.⁹³ Sequential application recapitulating the temporally defined sequence of VEGF and PDGF-BB activity during angiogenesis could also enhance blood vessel formation.^{87,94} The delivery of PDGF-BB enhanced vascularization and bone formation in an ovariectomized mouse model of postmenopausal osteoporosis.⁹⁵ This finding is important because most early tissue engineering studies are conducted in relatively healthy animals, and the patients are likely to have significant comorbidities that will affect bone regeneration.

Moreover, changes in the concentrations, timing, or spatial distribution of angiogenic growth factors during development can result in vascular abnormalities,⁶⁵ highlighting the importance of careful control over the release of angiogenic factors. Excessive VEGF can lead to leaky vessels that are prone to regression.^{96,97} Instead, VEGF engineered to bind strongly to ECM proteins reduced vascular permeability.⁹³ Still, it is not possible to engineer a system that fully recapitulates the myriad of growth factors that are highly regulated in terms of dose, timing, and localization, during normal angiogenesis. For this reason, strategies that stimulate the angiogenic properties of the host inflammatory response, which would be expected to more faithfully recapitulate the appropriate processes in angiogenesis, are being investigated.

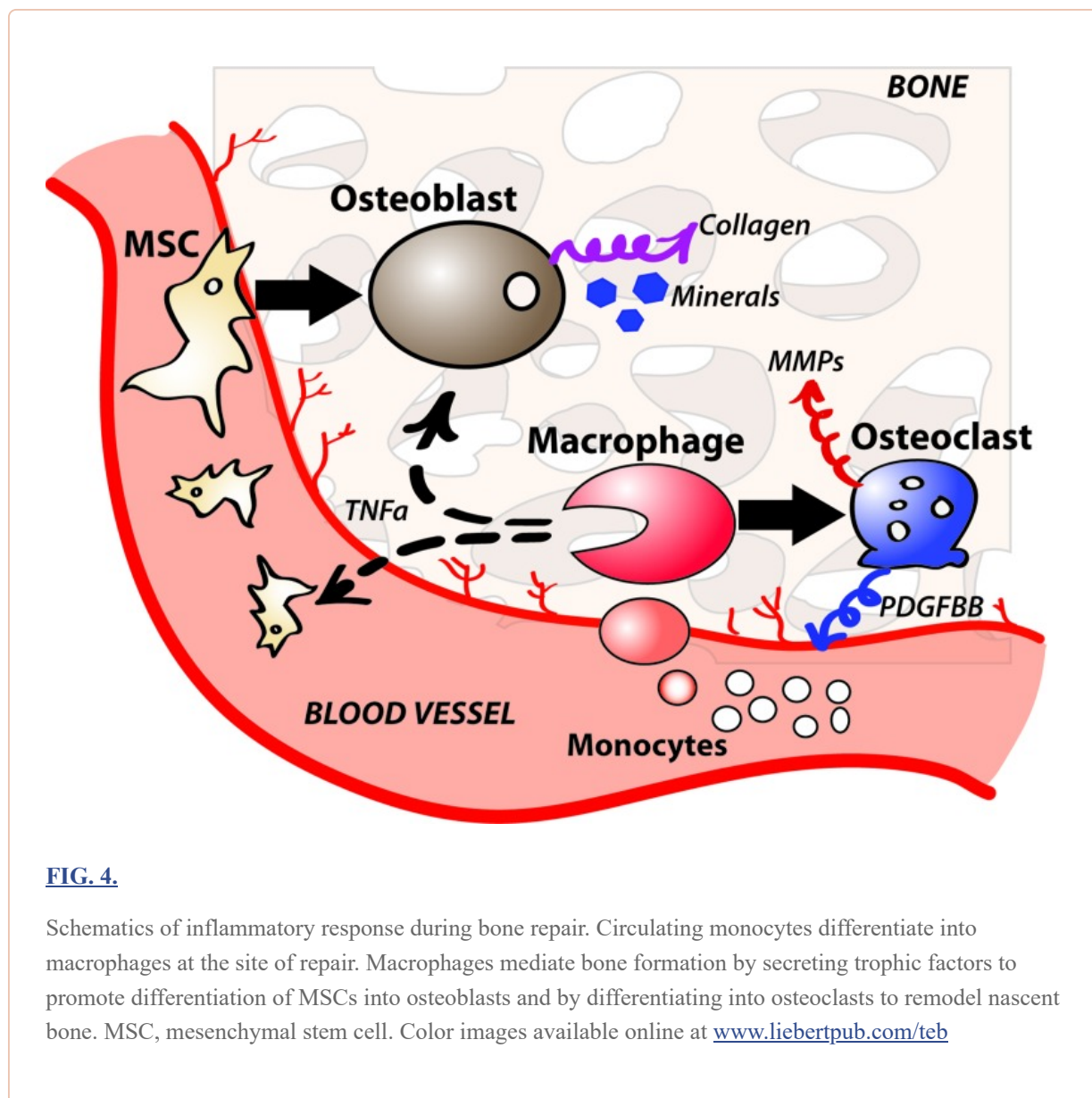
Harnessing the Inflammatory Response

Role of inflammation in bone repair

Bone is unique in that small fractures heal perfectly, without scarring,⁹⁸ while large bone defects remain a challenge.⁹⁹ Therefore, strategies aimed at recapitulation of bone repair represent an attractive approach in tissue engineering.

The process of inflammation is critical for the initiation of bone healing (Fig. 4). Experimentally, removal of the inflammatory milieu contained within the fracture hematoma impairs fracture healing in animal models.^{100,101} Administration of the pro-inflammatory cytokine TNF α to mouse bone fractures within 24 h after injury significantly enhanced fracture healing.¹⁰² Macrophages, the primary cells of the inflammatory response, and their bone-resident cousins, osteoclasts and osteal macrophages, are essential for bone formation during repair.¹⁰³ Mechanical manipulation of bone fractures in mice

significantly altered the behavior of macrophages and modulated bone healing through endochondral or intramembranous ossification.¹⁰⁴ Signals from macrophages have also been shown to directly affect skeletal cell differentiation *in vitro*.^{105–107}



Moreover, prolonged inflammation beyond the initial phase (~4 days) leads to impaired healing in bone^{108,109} and other tissues.^{110–112} Thus, inflammation must be tightly controlled, so that it initiates bone repair at early stages of injury yet subsides in a timely manner.

Role of macrophage phenotype in bone formation and angiogenesis

The importance of macrophages in angiogenesis has been demonstrated by its inhibition in animal models depleted of macrophages^{113–118} or increased angiogenesis when macrophages are transplanted into ischemic tissue.^{119,120} Preosteoclasts in developing bone in mice have been shown to release PDGF-BB, which is essential for bone vascularization and bone formation.⁹⁵ Recently, the ability of macrophages to rapidly and dramatically alter their phenotype in response to changing environmental

stimuli has provided further insight into the mechanisms of macrophage regulation of bone repair and angiogenesis. Classically activated M1 macrophages secrete pro-inflammatory cytokines, while alternatively activated M2 macrophages regulate the balance of ECM synthesis and remodeling.¹²¹

In the normal response to injury, M1 macrophages dominate at early stages (1–3 days) and M2 macrophages control later stages (4–10 days). M1-to-M2 transition was observed during the remodeling phase of long bone repair in a mouse osteotomy model.¹²² A coculture study recapitulating the transition also showed a significant improvement in osteogenesis by preosteoblastic cells *in vitro*.¹²³ Instead, a study on persistent inflammation found that healing in numerous tissues was drastically impaired when the transition was disrupted.¹²⁴ Similarly, elevated levels of pro-inflammatory (i.e., M1 associated) cytokines delayed and impaired healing.^{108,125,126} A persistent presence of M1 macrophages surrounding an implanted biomaterial at later time points (i.e., later than ~3 days following implantation) has been associated with chronic inflammation.^{127–129} M1 polarization of macrophages in response to wear debris from orthopedic implants has also been linked directly to implant loosening.¹³⁰ M1 polarization has also been shown to reduce the osteogenic effects of macrophages on MSC.¹⁰⁵

The roles of different macrophage phenotypes in angiogenesis have also been widely investigated. M2 macrophages are typically described as the angiogenic phenotype,¹³¹ although most studies are focused on the M2-like tumor-associated macrophages.¹³² Macrophages polarized *ex vivo* to the M2 phenotype promoted angiogenesis subcutaneously in mice¹³³ and in a chick chorioallantoic membrane model.¹³⁴ Several studies found that M2 macrophages are associated with greater levels of blood vessel infiltration into porous biomaterial scaffolds.^{128,135} Interestingly, M1 macrophages have also been shown to promote angiogenesis *in vitro* and *in vivo*^{136,137} and affect blood vessel infiltration into scaffolds.^{138,139}

Interestingly, human macrophages polarized to the M1 or M2 phenotypes *in vitro* contributed to angiogenesis in different ways (Fig. 5a).¹³⁷ M1 macrophages expressed and secreted factors that promote the initiation of angiogenesis, especially VEGF. M2 macrophages secreted factors involved in later stages of angiogenesis, especially PDGF-BB, which recruits stabilizing pericytes. This study sheds light on the apparent controversy that surrounds the roles of macrophage phenotypes on angiogenesis (Fig. 5b).

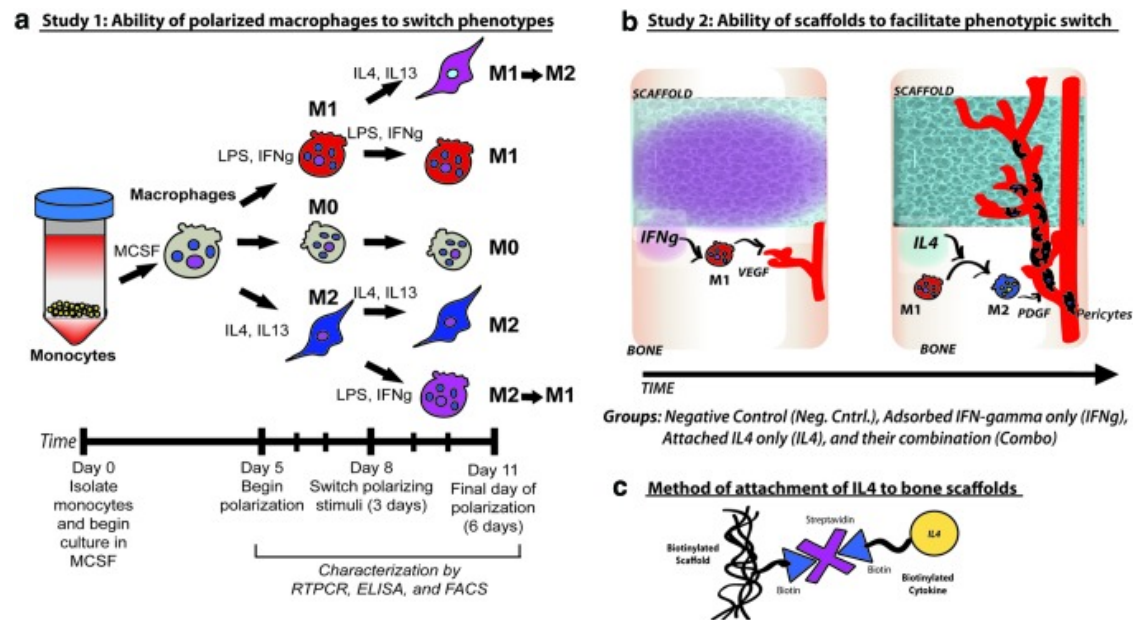


FIG. 5.

Harnessing the inflammatory response for graft vascularization by modulating macrophage phenotype. Our first study provides *in vitro* evidence of the phenotypic plasticity of macrophages differentiated from monocytes in peripheral blood (a). Macrophages can be differentiated into M0, M1, and M2 phenotypes and also be induced to switch between the M1 and M2 phenotypes. Schematics of how macrophages can be induced to promote scaffold vascularization. IFN- γ stimulates M1 differentiation to result in the secretion of VEGF that promotes angiogenesis. IL4 stimulates the transition of M1 to M2, which results in the secretion of PDGF and recruitment of pericytes for vasculature stabilization (b). For sequential release of IFN- γ followed by IL4, IFN- γ is adsorbed to the scaffold, whereas IL4 is attached to the scaffold by biotin–streptavidin interaction (c). Reproduced with permission from reference [156]. PDGF, platelet-derived growth factor; IFN- γ , interferon-gamma; VEGF, vascular endothelial growth factor. Color images available online at www.liebertpub.com/teb

Furthermore, M2 macrophages also expressed high levels of tissue inhibitor of metalloproteinase-3 (TIMP3), which inhibits angiogenesis by blocking the actions of MMP9 and VEGF¹⁴⁰ and preventing the release of the inflammatory cytokine TNF α .^{141,142} TIMP3 also stabilized vasculature formation from ECs *in vitro*.¹⁴³ In line with these results, media conditioned by M2 macrophages inhibited *in vitro* sprouting of human umbilical cord-derived endothelial cells on Matrigel. In addition, human macrophages polarized to the M2c phenotype, a distinct subset of M2, promoted EC sprouting *in vitro*.¹³⁷ M2c macrophages also secrete high levels of MMP9, which promotes angiogenesis through remodeling of the basement membrane of blood vessels and other independent signaling mechanisms.^{144,145} Macrophages that are positive for the M2c marker CD163 have been associated with angiogenesis in humans.^{146,147} Thus, it appears likely that M1 macrophages initiate angiogenesis through the release of VEGF, M2a macrophages stabilize the growing vasculature by recruiting pericytes and regulating the actions of M1 macrophages, and M2c macrophages promote vascularization through remodeling.

Controlling macrophage behavior to promote bone regeneration

Macrophages can also be actively targeted to enhance bone repair. For example, an increased ratio of M1-to-M2 macrophages was found at sites of bisphosphonate-induced osteonecrosis of the jaw in both humans and mice.¹⁴⁸ When *ex vivo* programmed M2 macrophages were infused into the mice, the incidence of osteonecrosis was significantly suppressed. Blocking the pro-inflammatory cytokine IL17 also shifted the macrophage populations toward the M2 phenotype and ameliorated osteonecrosis. In another study, twice weekly administration of IL33 caused M2 polarization of osteal macrophages and inhibited bone loss in a transgenic mouse model of spontaneous joint inflammation.¹⁴⁹ These studies suggest that strategies aimed at controlling macrophage polarization have the potential to be applied clinically for the treatment of bone disorders.

Transplantation of MSCs into bone defects predominantly enhances bone repair through immunomodulatory effects, as opposed to their direct differentiation into bone-forming cells. Interestingly, the transplantation of MSCs into rat femoral defects induced an early increase in M1 macrophage recruitment and ultimately enhanced bone healing relative to implants without MSCs, although later time points were not evaluated.¹⁵⁰

FTY720 is an agonist of sphingosine 1-P (S1P), which stimulates M2 polarization of macrophages.¹⁵¹ Controlled release of FTY720 from PLGA scaffolds promoted the recruitment of macrophages, scaffold vascularization, and new bone formation in rodents.^{152–154} Interestingly, the cytokine profile following implantation of gelatin hydrogels that released another S1P agonist, SEW2871, was shown to be primarily M1 at 3 days following implantation and primarily M2 at 10 days, recapitulating the natural sequence observed in normal repair.¹⁵⁵

Similarly, decellularized scaffolds can be bone to sequentially release the M1-promoting cytokine interferon-gamma (IFN γ) followed by the M2-promoting IL4 (Fig. 5b, c).¹⁵⁶ In a subcutaneous implantation model in mice, IFN γ -releasing scaffolds were more vascularized than control scaffolds, but no effects were observed from the release of IL4 alone or in combination with IFN γ . The lack of vascularization was attributed to the overlapping and conflicting M1 and M2 signals at early time points, as confirmed *in vitro* by studying the responses of human macrophages to the scaffolds. Future aimed at further separating the M1 and M2 phases could improve bone regeneration outcomes *in vivo*. Considering the major roles of macrophages in angiogenesis and bone formation, they represent an attractive target for strategies to engineer vascularized bone.

Recapitulation of Endochondral Ossification as a Paradigm for Long Bone Repair

Another exciting direction in bone tissue engineering is inspired by the process of endochondral ossification that drives natural bone development and long bone fracture repair. Before bone formation, mesenchymal progenitors form a cartilaginous callus that serves as a bone anlage. Crucial to this process are the hypertrophic chondrocytes that trigger the transition from the soft callus to the nascent bone by provoking vascular invasion and initial bone template deposition. The use of hypertrophic chondrocytes is an attractive alternative for engineering bone grafts as they can survive in the hypoxic environments of cartilage and, thereby, withstand the time delay necessary for the vascular development they help orchestrate.

In line with this general theme, an induction regimen has been developed for differentiating MSCs into hypertrophic chondrocytes, and cartilaginous templates comprising hypertrophic chondrocytes induced from hMSCs underwent robust endochondral ossification following ectopic implantation.^{157,158} The usefulness of such a paradigm was confirmed in an orthotopic model, as autografts of cartilaginous callus at bone injury sites resulted in the healing of a separate nonunion bone fracture.¹⁵⁹

Notably, endochondral ossification of engineered cartilaginous templates was accompanied by vascular invasion. The newly formed blood vessels consisted of CD31⁺ ECs and NG2⁺ pericytes, suggesting maturation of the vascular structure.¹⁶⁰ Intriguingly, one study found that hypertrophic chondrocytes reversed differentiation upon their release from the lacunae and assumed the role of pericytes during endochondral ossification.¹⁶¹ Instead, other studies found that hypertrophic chondrocytes underwent transdifferentiation into osteoblasts directly or through a transient pluripotent state.^{159,162} Although our understanding of the underlying mechanisms is still incomplete, these studies showed that engineered cartilaginous templates comprising hypertrophic chondrocytes promote bone regeneration *in vivo*. This challenges previous concepts of bone tissue engineering that are focused on osteogenic induction and graft vascularization.

Putting this proposition to the test, cartilaginous templates formed from MSCs enabled the healing of critical-sized and massive femur defects in a rat model.¹⁶³ Remarkably, the biomechanical strengths of the implant group reached that of a normal femur after 8 weeks, indicative of near complete healing. Although it remains to be seen if such encouraging results can be reproduced in larger animal models, this elegant study shows that a biomimetic strategy recapitulating endochondral ossification is of great interest to bone tissue engineering.

To decouple the roles of the cells and ECM within the cartilaginous template, the *in vivo* outcomes of devitalized cartilaginous templates and cartilage-derived matrix with or without MSCs were investigated. Whereas apoptosis-driven devitalization maintained the capacity of the cartilaginous template to undergo endochondral ossification *in vivo*, devitalization by freeze and thaw cycles abolished bone formation.¹⁶⁴ This revealed the importance of ECM integrity and endogenous bioactive factors for triggering endochondral ossification *in vivo*. Cartilage-derived matrix underwent endochondral ossification *in vivo* only when seeded with MSCs, confirming the role of cells in creating a cartilaginous template suitable for endochondral ossification.¹⁶⁵

However, it remains to be seen if MSCs from other sources can form grafts that undergo endochondral ossification. A recent study found epigenetic differences that underpin an endochondral signature in BMSCs, but a lack thereof in MSCs from other sources. When implanted without *in vitro* conditioning, BMSCs formed bone filled with marrow and cartilaginous remnants, whereas ASCs formed bone without marrow.¹⁶⁶

Evidently, we are only beginning to understand how different tissue engineering components enable grafts to undergo endochondral ossification upon implantation. Still, these studies reveal the tremendous promise of this exciting new direction as they enrich the engineers' toolbox and the clinicians' options for next-generation bone reconstruction.

Conclusions and Future Directions

In summary, the bone tissue engineering paradigm comprises an osteoinductive scaffold, an osteogenic cell source, and a bioreactor to improve transport and provide biophysical stimuli. To enhance graft survival and bone regeneration *in vivo*, the formation of functional blood vessels perfused with blood and a positive interaction with the inflammatory response must be promoted. A particularly promising strategy is to harness the inflammatory response by encouraging the natural sequence of inflammatory and anti-inflammatory signals. Another new direction we discussed is long bone repair by mimicking endochondral ossification.

Most *in vivo* studies still rely on evidence gleaned from small animals that are young and healthy. Whether the implantation successes of tissue engineered bone grafts can be recapitulated in diseased and larger animals remains to be seen. Much like how the osteoinductive capacity of bone matrix led to the discovery of BMP-2, our continuous pursuit of engineering bone grafts could take lessons from the

native bone. We need to refine our understanding of bone development, regeneration, and remodeling to better guide the *in vitro* cellular responses and the *in vivo* outcomes. The coupling between ECs and MSCs leading to bone formation, regulation of macrophage response during inflammation, and the processes by which a cartilaginous template becomes a mature bone are just a few important phenomena we are only beginning to understand.

Tissue engineering has the potential of providing fully biological bone grafts for bone repair, and we are currently on the verge of clinical translation. Although autografts remain the gold standard for bone repair, rapid advances in bone tissue engineering seem to be fostering translation, and the tissue-engineered bone technology could soon become clinical practice.

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Disclosure Statement

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