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Controlled Release Strategies for Bone, Cartilage, and Osteochondral Engineering—Part I: Recapitulation of Native Tissue Healing and Variables for the Design of Delivery Systems

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The potential of growth factors to stimulate tissue healing through the enhancement of cell proliferation, migration, and differentiation is undeniable. However, critical parameters on the design of adequate carriers, such as uncontrolled spatiotemporal presence of bioactive factors, inadequate release profiles, and supraphysiological dosages of growth factors, have impaired the translation of these systems onto clinical practice. This review describes the healing cascades for bone, cartilage, and osteochondral interface, highlighting the role of specific growth factors for triggering the reactions leading to tissue regeneration. Critical criteria on the design of carriers for controlled release of bioactive factors are also reported, focusing on the need to provide a spatiotemporal control over the delivery and presentation of these molecules.

Introduction

The recreation and maintenance of functionally viable tissues using tissue engineering (TE) approaches incorporating controlled release functionalities is quite challenging due to the fact that cells respond to a wide array of structural, biochemical, and temporal cues in a microenvironment, which is difficult to simulate.[1] These stimuli work in cooperation to assemble and organize cells and their respective matrix into tissues. The concept of biomimicry relies on the development of nature-inspired biomaterials aiming for the generation of new tissues and organs. Biomimetic approaches can be applied through different perspectives, including tissue functionality, materials/composition, and biological mechanisms, intervening in tissue formation, remodeling, and healing.[2]

This review will address the increasing level of complexity and functionality in the design of TE approaches through the spatiotemporal controlled delivery of bioactive factors from three-dimensional (3D) constructs and their effect on the skeletal tissue healing. It comprises the recapitulation of native bone, cartilage, and osteochondral interface morphogenesis and healing; specifically, the sequence of events leading to tissue regeneration and the key signaling molecules involved in those processes. Moreover, the variables involved in the design of controlled delivery systems for the desired targeting tissues are also highlighted; specifically, the presentation of the appropriate sequence, rate, and dosage of bioactive factors in a spatiotemporal controlled manner.

Brief Recapitulation of Native Skeletal Tissue Morphogenesis and Healing

There has been considerable interest in understanding the signaling interplay in bone and cartilage due to their limited ability to heal upon serious fracture or trauma. Bone is comprised of a variety of cell populations, extracellular matrix (ECM), and other proteins as well as inorganic components that work synergistically to sustain physical forces, molecular signals, and systemic hormone networks.[3] On the other hand, articular cartilage is a highly resilient connective tissue that covers the surfaces at the ends of long bones.[4] The osteochondral tissue is a gradual transition from cartilage to bone in which the key constituents of each tissue undergo an exchange in predominance.[5,6] Therefore, the treatment of osteochondral lesions is even more problematic because tissue damage involves two different tissues with different intrinsic healing capabilities.[7]

Bone healing

Bone is distinguished from other tissues by the presence of inorganic hydroxyapatite[8] and a wide range of organic components, mostly collagen type I. The mineral part constitutes 65%–70% of the matrix, whereas the organic phase

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comprises the remaining 25%–30% of the matrix. From a materials science perspective, bone can be considered as a truly composite material.[9] The mineralized collagen-based ECM is the defining feature of bone, which provides its unique biomechanical properties.[10–13] Collagen type I can initiate and orientate the growth of carbonated apatite mineral, controlling its size and 3D distribution.[14–16] The complex hierarchical structure, material properties of the constituents, cellular organization, and molecular cues work in concert to perform the function of bone.[17] The hierarchical geometrical structure of bone is critical, not only for the macroscopic mechanical properties, but also for cells, which respond to these by converting mechanical and architectural cues into intracellular signals, which drive activities, such as gene expression, protein production, and general phenotypic behavior.[17–22]

Upon injury, bone tissue presents the ability to self-repair, in contrast to soft tissue that heals by forming scar tissue.[23,24] The vast majority of defects heals on their own or recovers after standard orthopedic procedures.[25,26] Surgical treatments of bone defects typically fall into two groups: the Ilizarov method[27] and the bone graft transplant.[28] Although the Ilizarov method—osteotomy followed by bone distraction—takes advantage of the regeneration potential of bone, it is a highly inconvenient procedure for the patient.[29] Over the last decades, the medical field has advanced dramatically in the understanding of tissue and organ healing and repair.[30] Transplantation of organs or tissues is still a common accepted methodology to treat patients and tissue replacements, such as autografts,[31–33] allografts,[34–36] xenografts,[37–39] and graft substitutes,[40–42] are clinically available therapies to restore the tissue structure and function.[43] However, the current situation is suboptimal at best[44,45] with the current grafting methodologies presenting several and obvious limitations, including lack of donors,[46] donor-site morbidity,[47] complicated surgical procedures,[48] immune rejection,[49] chronic inflammation,[50] and lack of clinical predictability.[51] However, extreme situations, such as compromised wound environment,[44,52] biomechanical instability,[45,53] or insufficient surgical techniques,[54,55] might lead to large defects with limited intrinsic regeneration potential, often designated as critical size defects. In these cases, complete regeneration cannot occur.[25,56]

Bone metabolism is a complex process regulated by a large number of bioactive molecules, and bone repair is, to a large extent, a recapitulation of developmental events.[57,58] During the fracture repair process, cells progress through stages of differentiation reminiscent of those that cells undergo during normal fetal bone development.[26] Four stages of bone repair have been described, as shown in Figure 1: (1) immediate postfracture (day 1–2). Trauma leads to the activation of the local host response, with the activation and influx of inflammatory cells and secretion of various mediators, leading to the formation of hematoma. This process is necessary for the initiation of tissue repair and wound healing[59]; (2) intramembranous ossification (days 3–5). Immune cells stimulate cell division of osteochondrogenitors and fibroblast-like cells in the cambium layer of periosteum[60]; (3) chondrogenesis (days 6–9), through the proliferation of chondrocytes and production of cartilaginous matrix[61] and; (4) endochondral ossification (days 10–20), through the production of mineralized ECM and vascularization of the tissue.[62,63] Although overlapping occurs, there is a temporal sequence of maximum levels of specific growth factors (GFs) and cytokines.[64] One of the critical aspects for bone formation is the formation of an extensive network of blood vessels as both intramembranous and endochondral bone ossification occur in close proximity to vascular ingrowth.[8,65] Endochondral ossification is the process by which mesenchymal stem cells (MSCs) differentiate toward chondrocytes, producing a cartilaginous template, which contributes to longitudinal growth of the majority of bones and is gradually replaced by bone and marrow. During this process, chondrocytes proliferate, undergo hypertrophy, and die. The deposited cartilage ECM is invaded by blood vessels, osteoclasts, bone marrow MSCs (BMSCs) and osteoblasts and deposition of mineralized ECM is initiated.[66,67] On the other hand, intramembranous ossification occurs in the absence of a cartilage template, whereby bone develops directly from mesenchymal progenitors.[67,68]

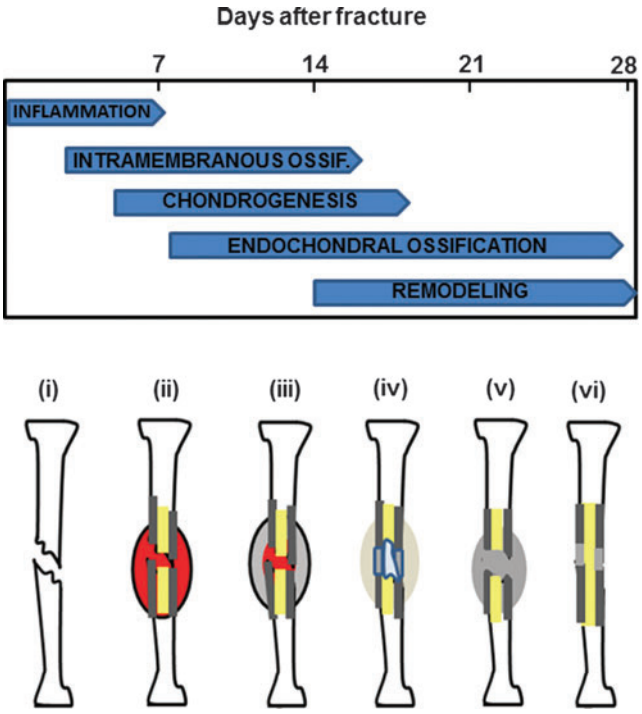

FIG. 1. Stages of bone healing upon fracture/injury, which can be divided in five main steps: inflammation, intramembranous ossification, chondrogenesis, endochondral ossification, and remodeling. In more detail, after injury (i), bone undergoes an induction stage where there is an influx of inflammatory cells and occurs the formation of hematoma, as shown in (ii). Gray areas represent necrotic bone tissue. Afterward, during inflammation, occurs the formation of a cartilage intermediate, as it can be observed by the gray area in (iii). Stage (iv) represents the formation of the soft callus, in which a chondrogenic matrix unites the defect area. From this moment, ossification occurs and woven bone replaces the temporary cartilaginous template through the invasion of blood vessels (stage (v)) and finally, remodeling occurs in step (vi) with lamellar bone promoting the union of the defects and the medullar cavity being restored. Adapted from refs.[68,69] Color images available online at www.liebertpub.com/teb

Bone formation is comprised of series of cellular events, which include (1) chemotaxis of osteoblast precursors to the sites of resorption defects[69,70]; (2) proliferation of these precursors to form a team of osteoblasts capable of filling in the resorption defect[71,72] followed by (3) differentiation of the preosteoblasts to form mature cells, which are responsible for expressing the structural proteins of bone, such as collagen type I and other functional proteins.[67,73–75]

Bone biochemical environment permits and promotes cellular functions that lead to matrix production and ossification.[76] The matrix is an active and dynamic biochemical system, including important regulatory cues to nearby cells, affecting gene expression and changes at the cytostructural level.[76] There are several mobile (GFs, transcription factors, and cytokines)[77–83] and immobilized (collagen type I, fibronectin, decorin, and biglycan)[84–89] macromolecules in the bone ECM directing bone cells behavior. Some of the most important soluble mobile macromolecules include the platelet-derived growth factor (PDGF),[90] Bone morphogenetic protein (BMP),[91] Insulin growth factor (IGF),[92] and transfoming growth factor (TGF).[93] In addition to mineralization, osteoblasts produce a matrix of proteins that not only serve to structurally support cells, but also to provide a variety of chemical cues, which regulate functionality, namely, the ability to promote mineralization.[17,94–96] The most abundant protein is collagen type I, which besides playing the main role in the tensile properties of bone, also contains peptides, which cue bone cells.[97] The most abundant noncollagenous bone ECM protein is osteonectin, known for its multiple calcium and collagen binding sites. Moreover, it has been shown to be a potential nucleator of hydroxyapatite.[17,98,99] The second most abundant noncollagenous protein in bone ECM is osteocalcin, which presents affinity for calcium/hydroxyapatite and has also been involved in the osteoclast migration process.[17,100]

Inflammation is also a key component of the early response to bone injury.[101,102] Inflammatory cells are recruited to the damaged bone and release cytokines, chemokines, and GFs that amplify the process.[103] Inflammation in the early phase of fracture repair is associated with enhanced healing, while chronic inflammation has a deleterious effect on healing.[3] Following implantation of materials, inflammatory responses are also expected. The host initiates a physiological healing reaction, consisting first of an acute inflammatory response followed by repair processes.[104,105] This inflammatory stage provides the appropriate signals for the shift from inflammation to repair and remodeling of the tissue.[106–108] The extent or degree of the inflammatory response is controlled by the extent of injury in the implantation procedure, by the tissue or organ into which the device is implanted and the extent of provisional matrix formation.[107] The initial week-long inflammatory phase of fracture healing is characterized by the influx of inflammatory cells, that is, neutrophils, lymphocytes, and macrophages and the release of various cytokines and GFs.[109] The inflammation process constitutes the fundamental difference between development and regeneration.[26] Therefore, modulation of inflammation has been an increasingly used strategy to control tissue regeneration.[110] When delivering cytokines, precise spatial and temporal control over the delivery profile is required because both prolonging and obliterating signaling might impair bone healing.[109]

There is a close inter-relationship between bone healing and formation of the vascular compartment. The clinical significance of angiogenesis in bone regeneration is of uttermost importance as an appropriate blood supply has been recognized as an essential component of normal fracture healing.[111,112] Angiogenesis, the growth of new capillary blood vessels from pre-existing host vasculature,[113] is also involved in the initiation of fracture healing and promotion of endochondral and intramembranous ossification in bone development, through blood vessel invasion of avascular cartilage[114,115] and ingress of osteoblast progenitors,[111,116,117] respectively. It involves the formation of capillary networks by endothelial cells, thereby enabling the transport of oxygen, nutrients, and waste throughout the tissue.[118] The sufficient supply of nutrients and oxygen to the cells transplanted into the body is indispensable for cell survival and consequent maintenance of their biological function.[119,120] Angiogenesis is a complex process involving endothelial cell activation,[121] recruitment,[122] and migration [123] to sprout the neovessels to the mural cells (pericytes or smooth muscle cells) making up the surrounding vessel wall for stabilization.[8] All vascularization processes involve a series of interactions among cytokines, GFs, various types of cells, and enzymes.[124,125] Osteoblasts and endothelial cells cross-talk and act synergistically toward the formation of a mature vascular network and for the differentiation of osteoprogenitor cells into osteoblast and formation of bone ECM.[3,126,127]

Cartilage healing

Articular cartilage is composed by a unique type of cell, the chondrocyte, embedded within a dense ECM consisting of 80% water, a collagen type II network, and proteoglycans.[128,129] Although cartilage appears to be a simple aneural, avascular, connective tissue, there are many levels of complexity in its composition and structure.[130,131] Both cells and matrix distribute within successive cartilage layers identified as superficial, transitional, radial (deep zone), and calcified zones.[29] Cartilage tissue demonstrates significant differences in cell phenotype, composition, and matrix organization along the depth of the tissue, reflecting different biomechanical and functional requirements of different zones.[132–134]

Hyaline cartilage is characterized by its high content on proteoglycan aggrecan, which exists in the form of proteoglycan aggregates in association with hyaluronan (HA) and link protein (LP). These aggregates are responsible for the turgid nature of cartilage, providing the osmotic properties necessary to resist compressive loads.[135,136] A variety of small leucine-rich repeat proteoglycans (SLRPs), such as decorin,[137] biglycan,[137] fibromodulin,[138] and lumican,[139] are also present and contribute for the maintenance of integrity of the tissue and to modulate its metabolism.[135]

The most basic functions of cartilage include providing near frictionless surface between load bearing in the joints to allow for pain-free motion, shock-absorbance, and load distribution.[140] The avascularity of articular cartilage has led to the assertion that the tissue is immunoprivileged, whereby the body's immune system is limited in its ability to detect and reject implanted tissue.[141]

Numerous GFs work in concert to regulate development and homeostasis of articular cartilage throughout life,

in particular bioactive factors from the TGF-β superfamily,[142–145] Fibroblast growth factor (FGF) family,[146] and IGF-I.[147–149] Architectural design for regenerative medicine and surgery is also an adaptation of the phenomenon observed during development and morphogenesis.[150] To properly restore the zonal structure of cartilage, phenotypical differences between chondrocyte populations and the response of chondrocyte subpopulations to GFs and external stimulus should be fully understood.[151]

Typically, chondrocytes respond to injury caused by mechanical insult, joint instability, and inflammatory cytokines through matrix activation, cell proliferation, apoptosis, and matrix destruction.[152,153] The activation of chondrocytes can result in modulation of gene expression, resulting in different patterns of protein synthesis, fibroblast dedifferentiation, hypertrophy, or regeneration of mature cartilage.[152] The lack of vascular supply in cartilage limits early repair responses upon injury.[150] Consequently, injury to cartilage usually heals through a scar tissue formation mainly composed of fibrocartilage with inferior mechanical properties and a gradually degrading nature.[4] Moreover, cartilage has a low cell:matrix ratio and chondrocytes have a relatively low metabolic activity limiting the tissue remodeling activity.[154] Only when the injury reaches the subchondral bone, self-healing processes are initiated by the release of mesenchymal progenitor cells from bone marrow into the wound site.[2] This observation forms the basis of surgical repair techniques, such as abrasion arthroplasty,[155,156] drilling,[157] and microfracture[158] to penetrate the subchondral bone. Periosteum is also a source of cells that can differentiate into chondrocytes and autogenous or allogeneic cell/tissue transfer via periosteal grafts[159,160] has been used, while other techniques have used chondrocytes or cartilage directly. Autologous chondrocyte transplantation involves harvesting cells from a noninvolved area of the joint, to expand them in culture, and then transplanting them to the area of involvement.[161–163] However, these techniques present several limitations particularly on cell and tissue availability, unwanted fibrocartilage formation and inadequate graft integration.[164–166] Another strategy for the regeneration of articular cartilage is mosaicplasty and it is based on the creation of multiple small osteochondral grafts.[167–170] The limiting factor of mosaicplasty resides in the donor-site availability of grafts.

In degenerative diseases, such as osteoporosis or rheumatoid arthritis, the remodeling equilibrium of cartilage is disrupted and the rate of collagen and proteoglycans loss from the matrix exceeds the rate of deposition of newly synthesized molecules.[152,171,172] The upregulation of cartilage-degrading enzymes can be caused by several factors, such as chemokines, other inflammatory mediators, and mechanical loading.[173]

Although current approaches are reasonably effective in achieving clinical endpoints of symptomatic relief, they have not been successful at preventing future degeneration of the repaired tissue and surrounding host environment.[174] Hence, TE is a promising approach for the regeneration of articular cartilage as it might provide the tools to overcome the limitations observed with the current treatments.[175]

Osteochondral regeneration

Cartilage and bone arise from a common progenitor and exist in apposition at articular surfaces of synovial joints as well as in the epiphyseal growth plate.[176] Structurally, the osteochondral interface is the connection between a layer of hyaline cartilage and underlying bone and it is crucial for load transfer between bone and cartilage.[6] Moreover, it has been shown that the osteochondral interface is critical to maintain the integrity of cartilage by simultaneously limiting vascular ingrowth from the subchondral bed and preventing ectopic mineralization.[177] The interface typically is characterized by a decreased amount of water content and collagen type II in comparison with the more superficial layers of cartilage. Moreover, the collagen fiber diameter is also increased and perpendicular in direction with respect to the articulating surface.[178]

Osteochondral tissue is comprised of osteoblasts, osteoclasts, and chondrocytes, but as these phases merge, there is an overlap in cellular function. Hence, this interface is composed of hypertrophic chondrocytes embedded in a mineralized cartilage matrix.[5,177] While the chondral repair mechanism relies on the intrinsic healing capabilities of chondrocytes, osteochondral defects allow the recruitment of progenitor cells from bone marrow to assist regeneration of cartilage and underlying bone structure.[179]

Osteochondral defects penetrate through the vascularized subchondral bone and spontaneous repair occurs as mesenchymal chondroprogenitor cells invade the lesion and form cartilage. However, full-thickness defect repair is only transient. The neo-formed tissue is fibrous and enriched in collagen type I and fibronectin and does not have the functional properties of native hyaline cartilage.[4,29,180]

For the treatment of large osteochondral defects, one of the options is autologous osteochondral grafts such as those used in mosaicplasty.[170,181,182] However, donor-site morbidity and lack of integration can compromise long-term graft outcomes.[183] Presently, a significant barrier to clinical translation is how to achieve functional integration of tissue-engineered orthopedic grafts.[184]

The concept of osteochondral TE, a hybrid of bone and cartilage regeneration, has attracted considerable attention, particularly as a technique for promoting superior cartilage integration and as a treatment for osteochondral defects.[179,185–196] For osteochondral scaffolds, additional design criteria should be considered to achieve the best possible simultaneous growth of the two independent tissues involved. This may require the use of biphasic constructs with gradients of mechanical, structural, and molecular properties.[154,177,197–200] The most common approach consists in the development of independent layers for each zone, because chondrocytes and bone cells show distinct metabolic and structural functionality, yet communicating and interacting in a unique culturing system.[201] The junction of two layers has been achieved by fibrin sealant, simple press-fitting, suturing, or external fixation.[179] These scaffolds constitute the first generation of stratified scaffolds.[183] A scaffold with a predesigned inhomogeneity can better sustain and transmit the distribution of complex loads inherent at the multitissue interface and several studies have reported new designs to fulfill this request.[202–206] Constructs to be used for regeneration of osteochondral defects may also benefit from the application of hierarchically and structurally organized drug delivery systems. One area of special importance in osteochondral graft design is the regeneration of the bone to cartilage interface or a calcified cartilage layer between bone

and cartilage regions, which is critical for graft integration and for establishing long-term functionality.[183]

Signaling molecules on natural cascades of bone, cartilage, and osteochondral formation/healing

Bone. During bone formation, mobile cues directing cell behavior in bone can be produced by local osteoblasts or delivery via blood stream. These GFs have indisputable roles in osteoblast proliferation, differentiation, and subsequent bone formation and regulation.[17,207–209] These molecules act through autocrine and paracrine signaling mechanisms to induce the migration, proliferation, and differentiation of osteoprogenitor cells and/or synthesis of collagen type I and matrix apposition by mature osteoblasts.[210] Upon matrix destruction, either caused by natural bone remodeling process or bone fracture, these factors are released to initiate osseous healing and to maintain the cyclic anabolic and catabolic processes that continuously remodel bone.[210]

The local concentration of chemical cues that can influence bone cells increases substantially in the event of an injury. Bone tissue injury initiates a cascade of events leading to the migration of neutrophils, macrophages, and fibroblasts, which subsequently express and secrete a variety of cytokines and transcriptional factors, which direct migration of MSCs, neovascularization, and remodeling/healing.[17,211]

Moreover, immobilized bone ECM macromolecules act as primary chemical effectors in cell signaling and functionality. Several bone ECM proteins contain progenitor and osteoblast integrin-binding sites and GF-binding sites, presenting an obvious selection for developing scaffolds for bone.[17]

Many GFs, such as BMPs,[212] basic FGF (bFGF),[213] IGF,[214] TGF-β,[215] PDGF,[216] and vascular endothelial growth factor (VEGF),[217] have been found to induce new bone through their effects on the recruitment, proliferation, and differentiation of bone-forming cells.[218] Their role in bone morphogenesis and regeneration is described in Table 1 and their expression profile is shown in Figure 2. It is likely that multiple factors regulating distinct aspects of the regenerative process can be used in parallel to affect the regeneration of functional tissues.[219]

Cytokines are included in this group of factors and they are mainly involved in systemic processes, such as host defense and homeostasis, including interleukins (ILs), which are proinflammatory molecules involved in bone resorption and remodeling.[76] Cytokines encompass a large family of immunomodulating agents playing key roles in the cross talk between the immune and skeletal systems.[57] Bone fracture is an injury that initiates an inflammatory response that peaks 24 h following the injury and is complete by the first week.[110] During this time, a complex cascade of proinflammatory signals and GFs are released in a temporally and spatially controlled manner.[110]

Bone fracture stimulates expression of several dozen inflammatory cytokines, including several isoforms of ILs. IL-1, IL-6, as well as tumor necrosis factor-alpha (TNF-α) are shown to play a key role in initiating the repair cascade. They have a chemotactic effect on other inflammatory cells and on the recruitment of MSCs.[220]

Levels of most inflammatory mediators return to baseline after the week-long acute inflammatory phase.[109] Other relevant bioactive factors acting on the morphogenesis of these tissues include hormones known for controlling serum calcium concentrations and stimulating osteoblast proliferation and differentiation.[76]

During bone regeneration there is a temporal sequence of GFs and cytokines expression.[63,234,235] Angiogenic GFs are predominantly expressed during the early phases to reestablish vascularity, whereas osteogenic GFs are continuously expressed during bone formation and remodeling.[219] Numerous GFs are involved in angiogenesis, including VEGF,[236] FGF-2,[237] PDGF,[238] Ang-1,[239] and -2,[240] IGF,[241] among others.[124]

During normal bone healing, VEGF expression was shown to peak in early days with high expression from days 5 to 14, while BMP expression peaked at a later time point. Since establishment of a vascular bed is an early event that precedes the formation of bone, a similar temporal release profile should be designed to promote bone regeneration.[242,243] VEGF is likely produced by inflammatory cells as well as mesenchymal progenitors that are recruited to the site of bone injury. VEGF expression can also be driven to hypoxia as VEGF represents a target gene of hypoxia-inducible factor.[111]

Despite extensive studies on the biology and delivery of GFs, only two formulations are currently approved by the Food and Drug Administration (FDA) for clinical use in USA. BMP2 (Infuse™, Medtronic Sofamor Danek, Inc.) and BMP7 (OP-1™, Stryker Biotech) repair injuries by mediating spinal fusion or fracture healing.[244,245]

During fracture repair, BMPs are produced by MSCs, osteoblasts, and chondroblasts, and trigger a cascade of events, such as proliferation and differentiation of MSCs, angiogenesis, and synthesis of the ECM.[246] BMP-2 acts on global cellular mobilization and it is also present during the later stages of osteogenesis and chondrogenesis, whereas BMP-7 acts on osteogenic differentiation.[246] Given that BMPs are not tissue specific, their localized, targeted, and controlled delivery is required to prevent any undesired and uncontrolled bone formation in inadequate tissues of the body.[247]

In human clinical treatments, large doses of BMPs (2–12 mg) have been used,[248–251] which exceed by far the normal physiological concentrations of these proteins (18.8–22 pg/mL)[252] in bone defect areas. As an example, the concentration of BMP-2 approved for application in spine fusion is 1.5 mg/mL.[251] Therefore, it is clear that the collagen carriers used in the FDA-approved formulations mentioned above are less effective in providing structural integrity, effective mechanotransduction in large nonunion sites, and control over release kinetics.[253] Furthermore, BMP-2 is a well-known chemoattractant for lymphocytes, monocytes, and macrophages and it has also been reported that rapid release of BMP-2 in high doses can induce transient osteoclast-mediated resorption before new bone formation occurs in metaphyseal defects.[251,254] The role of the carrier on the outcome of the delivery therapy has been evaluated and the effective and therapeutic doses change according to the carrier, thus highlighting its relevant function.[255,256]

While the current landscape of GFs used for bone regeneration is dominated by BMPs, a number of other GFs are being investigated for their potential to regenerate bone.[245] It has been reported that TGF-β1 promotes osteogenic differentiation in the early and late stages of

Table 1. Growth Factors Roles on Bone and Cartilage Formation and Healing

Growth factor	*Role on bone formation/healing*	*Role on cartilage formation/healing*
BMPs	Most osteoinductive GFs[223] Promotion of migration, proliferation, and osteogenic differentiation of MSCs[57,221–223] Influence on skeletal pattern formation.[222]	Induction of *Sox9* expression Initiation and regulation of embryonic development and morphogenesis of cartilage Proliferation and maturation of chondrocytes Strong induction of chondrogenic differentiation of MSCs by isoform 2
TGF-βs	Chemoattractant for osteoprogenitor cells[57,73,221,223] Promotion of collagen, osteonectin, and osteopontin production[221,223] Stimulation of undifferentiated MSCs proliferation[73,222] Inhibition of mature and progenitor osteoclast cells proliferation[221] Inhibition of matrix metalloproteinases, known for their role on ECM digestion Stabilization of new blood vessels Direction of BMSCs toward resorption sites	Induction of *Sox9* expression Initiation of matrix proteins aggrecan and *COMP* expression Enhancement of chondrocyte proliferation by isoforms 2 and 3 Stimulation of chondrogenic differentiation of MSCs by isoforms 1 and 3 Inhibition of the activity of MMPs by isoform 1[147] Isoform 3 is imperative for *Sox9* expression, but it is not required for continued expression of chondrogenic markers at later stages of chondrogenesis
IGF-I	Stimulation of osteoprogenitors proliferation[73] and bone matrix production[221,222] Stimulatory effects on osteoblast activity and chemotaxis and antiapoptotic effect on preosteoblasts[57] Upregulation of collagen type I Inflammation mediator	Stimulation of anabolic activity and proliferation of chondrocytes[147,151] Chondrogenic differentiation of MSCs Stimulation of ECM production[151] Protection of ECM from IL-1 and TNF-α-mediated degradation during cartilage injury[147]
FGF-2	Stimulation of migration, proliferation, and differentiation of mature and progenitor osteoblasts[57,73,111,221] Differentiation stage-specific effect Stimulation of angiogenesis through activation of capillary endothelial cells and fibroblasts[223]	Activation of pathways ultimately leading to the upregulation of *Sox9* Promotion of cell proliferation[4,147] and inhibition of chondrogenic differentiation
PDGF	Chemotactic and mitogenic effects (precursor and mature endothelial cells and inflammatory cells)[57,73,111,222,223] Maturation of blood vessels by the recruitment of SMCs to the endothelial lining Upregulation of VEGF expression.	Maintenance of hyaline-like cartilage phenotype Enhancement of chondrocyte proliferation and proteoglycan synthesis[147]
VEGF	Essential for endochondral bone formation due to its ability to induce migration and differentiation of osteoblasts[116] Initiatior of angiogenesis. Formation and maintenance of blood vessels[57,111,223] Recruitment of circulating endothelial progenitor cells and inhibition of endothelial cells apoptosis[111,223]	

Information was collected from Refs.[4,57,73,76,111,116,147,151,221–233]

BMSCs, bone marrow mesenchymal stem cells; BMP, bone morphogenetic protein; COMP, cartilage oligomeric matrix protein; ECM, extracellular matrix; FGF, fibroblast growth factor; GFs, growth factors; IL, interleukin; IGF, insulin growth factor; MMPs, matrix metalloproteinases; MSCs, mesenchymal stem cells; SMC, smooth muscle cells; TGF, transfoming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

ectopic bone formation despite its inhibitory effects *in vitro.*[257] FGF-2 displays a dual role, acting both on the stimulation of angiogenesis and osteogenesis.[111] It is suggested that (FGF/FGF receptor) signaling pathways coordinates a bone anabolic effect by simultaneously activating *Runx2* and BMP-2 pathways.[231] The sequential supplementation of FGF-2 followed by BMP-2 tends to enhance bone phenotype markers.[258] While BMP-2 acts mainly on the osteoblastic differentiation, FGF-2 promotes cell proliferation, increasing the cell population that will be influenced by the action of BMP-2.[259]

Factors that drive mobilization of BMSCs have been unclear, but one of the earliest consequences of a bone fracture or injury is local tissue hypoxia. Multipotential MSCs are mobilized into peripheral blood and the Stromal-Derived Factor-α (SDF-α) is known for its role in stem cell homing, mobilization of endothelial progenitor cells, and hematopoietic stem cells to the injury site.[260]

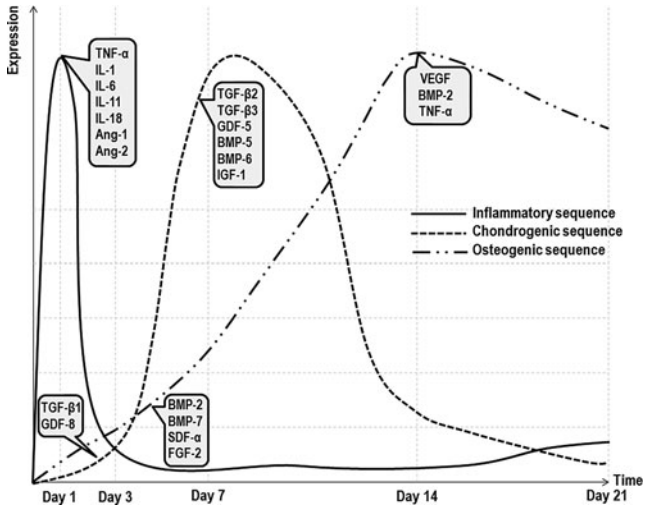

FIG. 2. Temporal sequence of growth factors (GFs) and cytokines expression during bone regeneration. The solid dash line represents the inflammatory sequence of events, peaking early upon the injury with high expression of cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, -6, -11, and -18, and angiogenic molecules Angiopoietin-1 (Ang-1) and Ang-2. These molecules are involved in the stimulation of cell migration, production of cartilaginous callus, and vascular endothelial growth factor (VEGF) production. The square dot line represents the chondrogenic route that leads to the generation of a cartilaginous callus peaking between 7 and 14 days after injury. Transfoming growth factor (TGF)-β1 and Growth differentiation factor-8 (GDF-8) act early in this step, with other isoforms responsible for chondrogenesis and endochondral ossification peaking later in the cycle. The long dash double dot line represents the osteogenic sequence of molecules acting on the formation of a bony callus. Simultaneously to the expression of chondrogenic markers, bone morphogenetic protein (BMP)-2, BMP-7, and stromal cell-derived factor-α (SDF-α) are responsible for inducing the migration of mesenchymal stem cells (MSCs) with fibroblast growth factor (FGF)-2 stimulating their proliferation. As callus chondrocytes proliferate, they become hypertrophic and express high levels of VEGF, promoting the invasion of blood vessels, leading to the tissue vascularization. During this step, TNF-α initiates chondrocyte apoptosis and promotes the recruitment of MSCs with osteogenic potential. Moreover, this cytokine is also involved in bone remodeling, the last step in which the hard callus undergoes a resorptive phase to form the typical lamellar bone structure with a central medullar cavity.[201–203]

Cartilage. In cartilage repair, the most investigated GFs include TGF-β, BMPs, and IGF-I.[147,148,261] Further details on the role of the most significant GFs acting on cartilage can be found further in Table 1 and Figure 3. Despite the amount of publications reporting the effect of specific GFs on chondrocytes and chondrogenic differentiation of stem cells, little is known about GFs regulating cartilage wound healing and, specifically, the spatial and temporal expression of GFs in acute cartilage wound healing.[262] Most of the information regarding TE of cartilage was provided by the study of growth plate of long bones. However, it is admitted that the knowledge of fetal development can provide relevant insights for regenerative medicine purposes as regeneration partially recapitulates several developmental steps.[263,264]

In early embryological development, Sox9 is required for the aggregation of MSCs. Moreover, this transcription factor is also required for the expression of collagen II and aggrecan, two of the most important ECM components in hyaline cartilage.[261]

Active TGF-β1, 2, and 3 are generally considered to be potent stimulators of proteoglycans and collagen type II synthesis.[265] During acute cartilage wound healing (Fig. 3B), TGF-β1 and TGF-β3 are highly expressed, particularly during the first 2 weeks upon injury.[262] Despite the powerful ability of TGF-β1 to repair damaged cartilage, high dose of intra-articular injections of the GF resulted in chemotaxis and activation of inflammatory cells, promoting fibrosis. Additionally, TGF-β1 has been shown to regulate the autocrine/paracrine axis of IGF-I.[266] Hence, drug delivery systems with fine tuning over dosage and rate of delivery are required to fulfill the potential of these bioactive agents.[267]

In fact, intra-articular injections of specific drugs have failed to produce the desired therapeutical outcomes. The high reactivity of synovium and rapid efflux of drugs from the joint cavity have impaired the effect of bioactive agents injected directly into the articular cartilage.[268]

Numerous GFs are needed to properly sequence chondrogenesis and it is unlikely that any single GF will lead to complete cartilage repair or affect the arthritic milieu, but rather a combination in a synergistic approach will be required.[147] There is considerable cross talk between the TGF and BMP signaling pathways and simultaneous activation of both seems to promote chondrogenic maturation.[227]

Combined treatments of TGF-β1 with BMP-2 and IGF-I have led to the enhancement of glycosaminoglycan deposition and mechanical properties.[175] However, the most serious limitations of the use of MSCs for chondrogenic differentiation through the supplementation with bioactive factors reside on the fact that cell differentiation do not stop at the prehypertrophic stage.[263]

Osteochondral interface. Hypertrophic chondrocytes are the resident cell population identified at the native osteochondral interface.[206] As described in Figure 3, after differentiation and maturation of cells toward the chondrocyte phenotype, *Osterix* and *Runx2* become upregulated and chondrocytes achieve their hypertrophic state. At this stage, collagen type X is highly expressed. The osteochondral interface is also composed by a mineralized and vascularized matrix, in which angiogenic factors, such as VEGF and FGF-2, are upregulated.[269,270] However, much interest has been centered on BMPs to promote osteochondral tissue formation as these molecules have a powerful effect in stimulating both new bone and cartilage formation.[271]

In conclusion, natural processes are multifactorial and skeletal morphogenesis and regeneration are typically driven by the concomitant action of multiple factors that can work synergistically on the same process. Release kinetics are dependent on the type of tissue and defect.[210,272]

Besides the presentation of the appropriate factor or combination of factors, the concentration and duration of function are critical parameters involved in promoting neotissue formation. Concentrations of GFs should be used within a therapeutic range, whereas crossing the dosage limits would result either in an inefficient strategy or in the production of an abnormal tissue.[272]

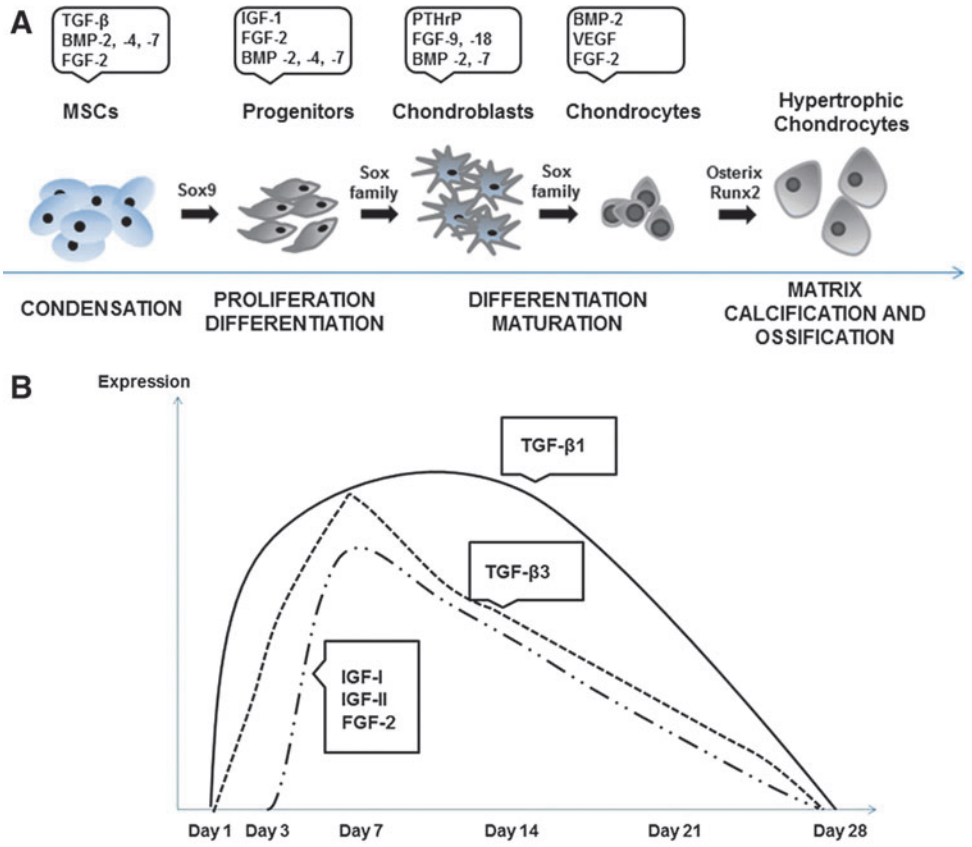

FIG. 3. **(A)** Chondrogenic differentiation of MSCs, which involves three main stages influenced mainly by the Sox family transcription factors, followed by the ossification process. Once chondrocytes reach their mature phenotypic state, osteogenic transcription factors *Osterix* and *Runx 2* are expressed and chondrocytes evolve to a hypertrophic state. Different GFs are expressed by cells, depending on their differentiation state. BMP-2 is expressed throughout the process, while TGF-β family is highly expressed during the MSCs condensation step. The mechanism here described occurs in the growth plate of long bones and it is responsible for the endochondralossification for bone turn-over. Adapted from [236,243] **(B).** Cartilage layers expressing TGF-β1 and TGF-β3 increased between day 1 and 3 and TGF-β1 maintained approximately those levels until day 14. An increase of insulin growth factor (IGF)-I and FGF-2 was observed between day 3 and 7. IGF-I, FGF-2, and TGF-β3 reached a new peak level at day 7 and showed gradual decrease afterward. At day 28, GF expression returned to basal levels. Color images available online at www.liebertpub.com/teb

Controlled Release Strategies: The Need for Spatiotemporal Control

The delivery of multiple GFs has been pursued through application of different methodologies, making use of the great versatility of delivery systems developed over the last decades. However, biological mechanisms of tissue regeneration are more complex and, thus, require more than a particular temporal sequence of therapeutic agents, with some regenerative tasks demanding tight coordination of the spatial and temporal presentation of multiple factors.[219]

GF signaling in tissue healing involves precise regulation of the concentration, temporal gradient, and spatial gradient of factors, which ultimately determines the outcome of the regenerative therapy.[124] Controlling the location of release can create concentration gradients by diffusion of the factors from the release site.[272] The osteochondral interface is a paramount example of this scenario. The regulation of concentration and delivery rates is also particularly relevant as a suboptimal delivery system does not exploit the full potential of the released bioactive factor, thus requiring higher dosages to provide the desired effect.[254]

Systems releasing drugs acting synergistically provide a highly inductive therapeutic option, replicating more accurately developmental osteogeonic and chondrogenic cascades, which will ultimately result in a superior clinical performance using considerably reduced doses of GFs. However, this will be achieved at the cost of greatly increased complexity.[273] Some of the materials used to produce the carriers for GFs may provide additional favorable properties by themselves, such as calcium phosphates or other ions for bone regeneration, which might not be enhanced by the addition of specific bioactive agents.[274] The combination of stimulus is not straightforward and the outcomes of the combination are most of the times unexpected. Hierarchical systems,[275–277] multiple layer systems,[278,279] and intelligent hydrogels[280,281] have been developed aiming for the simultaneous and/or sequential release of multiple signaling molecules.

The spatial organization of the GFs in the matrix is critical because the retention of the molecular bioactivity is also affected by several parameters, including interactions between the biomaterial and the GF, the influence of pH and temperature and porosity.[207,282]

Spatial gradients of factors in scaffolds can be controlled by varying the positioning of polymer vehicles, immobilizing ligands on polymer networks to attract target cell populations, or designing delivery systems to provide spatially distinct cues.[124] Current knowledge of the signals within the microenvironment that regulate cell fate has led to the development of increasingly sophisticated constructs. Scaffolds with precisely controlled architectures regulating spatiotemporal release of GFs and morphogens and responding dynamically to environmental cues have been increasingly developed.[283]

Spatial patterns in tissues are controlled by both the architectural features of the ECM and concentration profiles and gradients of diffusible bioactive factors.[284] The ability to combine topographical and biochemical cues within a single scaffold presents a valuable opportunity to evaluate their synergistic impact.[285]

Angiogenesis is paramount of the relevance of a temporal sequence of bioactive factors expression. Certain factors initiate angiogenesis, while others induce maturation of newly formed vessels.[286] Later on, a third group of molecules act on the maintenance of the integrity of the established vasculature.[287] If the appropriate presence and sequence of bioactive factors are not achieved, poor vascularization occurs.[283]

Scaffolds can perform the dual roles of biomechanical and biochemical support by presenting the appropriate mediators to the surrounding tissue. The ultimate goal is to develop a multifunctional support performing two main roles: (1) acting as a temporary structure for cell attachment and colonization; (2) acting as a delivery platform for multiple GFs to stimulate tissue regeneration.

Many techniques have been developed to regulate the kinetics and distribution of soluble factors, including multiple levels of encapsulation and noncovalent bonding of bioactive factors to peptides with a range of dissociation constants mimicking ECM immobilization of GFs.[283] One of the most used approaches involves the use of the scaffold as a controlled release platform by the simple dispersion of bioactive agents in the matrix or via immobilization to the scaffold by electrostatic interactions or covalent bonding.[284] These monolithic scaffolds, even when combined with GFs and cells, are still far from leading to successful tissue reconstruction in clinical settings, mainly due to the limited control exerted over biodegradation and drug delivery.[288]

Incorporation of GFs into preformed scaffolds has the advantage that the optimized conditions for scaffold processing are not substantially affected by the presence of proteins. Moreover, the biological activity of the protein can be preserved.[43] However, typically, only small amounts of proteins can be attached and their release profile becomes unpredictable. Therefore, scaffold bioactivation is increasingly being accomplished through the incorporation of preformed GF-loaded delivery systems, such as polymeric particles instead of through direct incorporation, as demonstrated in Figure 4.[43]

Microspheres/nanospheres have been widely used as tools for controlled drug delivery due to their small dimensions and the corresponding high surface area, high drug loading efficiency, high reactivity toward surrounding tissues, and high diffusibility and mobility.[288] Moreover, their size allows them to respond quickly to environmental stimulus. Generally, microspheres can be processed into macroscopic constructs as (1) a dispersed phase surrounded by a

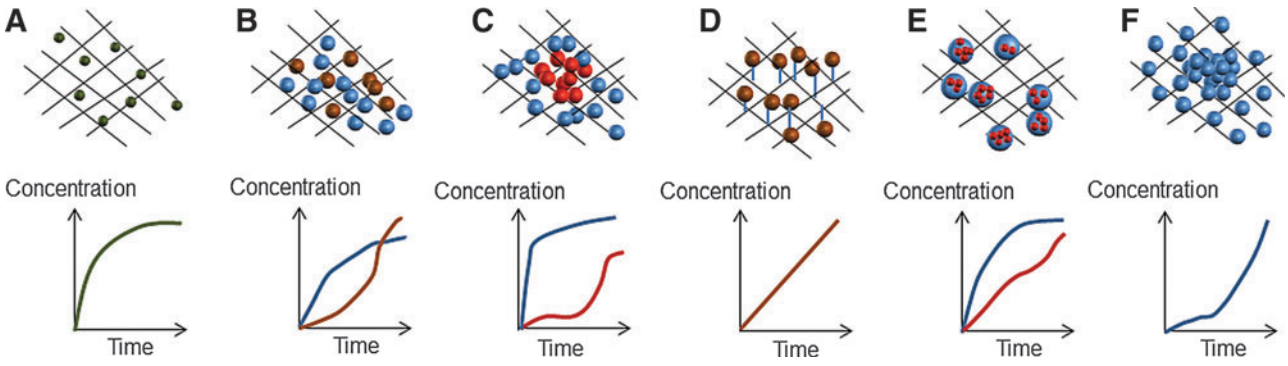

FIG. 4. Strategies to promote the release of multiple bioactive factors from a matrix. Molecules can be physically dispersed inside the matrix by dispersion of the factor before the carrier processing or by impregnation postcarrier preparation **(A)**. Typically, this leads to uncontrolled and fast drug delivery kinetics. One strategy to incorporate multiple bioactive signaling molecules into a matrix is developing hierarchical multiscale systems by the incorporation of nano- and microscale carriers into the constructs. The entrapment of the drugs in these carriers might offer an enhanced protection for degradation and high control over the delivery kinetics. These particulate carriers can be tailored to release bioactive factors in specific kinetics, allowing either a simultaneous **(B)** or sequential **(C)** presentation of signaling molecules. The spatiotemporal controlled release profiles can also be achieved through the conjugation of the drugs with ligands. Bioactive factors or particulate carriers can be covalently bound to the matrix, resulting in a more controlled mechanism of incorporation **(D)**. Affinity-bound systems such as binding through heparin domains are a common approach. These mechanisms can be combined to design even more complex systems. Moreover, the development of multiscale systems is highly promising as nanoparticles can be incorporated inside microparticles within the constructs **(E)**, promoting dual or multiple release systems with distinct delivery rates. The development of gradients of bioactive factors **(F)** is also an increasingly used approach. Besides promoting cell migration and inducing specific cell responses according to the concentration gradients, these systems allow a precise tailoring over the availability of the desired factor, following a biomimetic approach. Color images available online at www.liebertpub.com/teb

continuous matrix[289,290]; or as (2) building blocks to establish integral scaffolds without a surrounding matrix by a bottom-up approach.[288,291,292]

When included in a scaffold, they can also act as reinforcement phase, providing higher mechanical strength and protecting the drug from *in vivo* degradation.[288] Moreover, the incorporation of preformed delivery systems allows the combination of carriers with different release rates, entrapping different drugs, thus showing the potential for tailoring the availability of multiple signaling molecules at different preprogrammed rates (Fig. 4).[288–290]

Some of the advantages of entrapping preformed microparticulate delivery systems in TE constructs include the extra protection of the entrapped drugs to the physical and chemical adversities inherent to scaffold processing techniques and the fact that it avoids a new scaffold design for optimization of processing parameters.[43] Delivery from scaffolds loaded with particulate carriers allows to retain the bioactive factor for an extended time, overcoming the disadvantages of the direct immobilization of GF in scaffolds that have poor control over release rates due to an open pore structure and exposure of the drug to the medium.[43,293] When molded into 3D constructs, the drug delivery capacity of the carriers is coupled with the structural support provided by the scaffold. Although in these cases the release is mostly controlled by the properties of the carriers, the entrapment within the hydrogel/scaffold also influences the delivery.[272]

The integration of controlled release systems such as micro- and nanoparticles within scaffolds leads to the development of hierarchically organized and multifunctional constructs with enhanced ability to control and guide neotissue formation through the recapitulation of spatial and temporal microenvironments presented by the ECM.[284,294] However, it should be noted that the addition of those carriers by themselves into the scaffolding structure might have a surprisingly large impact on the cell response and should be considered when designing these structures.[295]

Following a bottom-up approach, some studies have shown the possibility of designing highly defined geometries by the assembly of micro- and nanocarriers.[296,297] The bottom-up approach for the generation of new materials has become increasingly attractive for developing novel engineering scaffolds with precise combination of cells, biomolecules, and synthetic biomaterials. These particles act as building blocks and can assemble by random packing, while simultaneously entrapping signaling biomolecules, bioactive minerals, or cells seeded on the surface.[288] However, one drawback is their poor integrity resulting from weak particle interactions. To preserve the agglomeration of these formulations, glues and crosslinkers have been used or even multilayered films prepared by layer by layer of polyelectrolytes.[298] Moreover, thermal fusion of the particles into integrated scaffolds has also been used as an alternative. Nanoparticles can also assemble by an electrostatic interaction between oppositely charged spheres—colloidal gels.[299–301]

Summary

There are several GFs and other bioactive molecules involved in the process of bone, cartilage, and osteochondral interface regeneration. These signaling molecules are presented *in situ* with a specific dosage, spatial distribution, and temporal sequence and TE strategies are increasingly trying to mimic these native healing cascades. As expected, this is not an easy task and mixed results have been obtained in this field. Part II of this review will report some of the most relevant studies on the use of single, dual, and multiple GF delivery in direct and indirect approaches for bone, cartilage, and osteochondral TE strategies.

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Disclosure Statement

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