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How does the pathophysiological context influence delivery of bone growth factors?*

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Abstract

“Orthobiologics” represents an important category of therapeutics for the regeneration of bone defects caused by injuries or diseases, and bone growth factors are a particularly rapidly growing sub-category. Clinical application of bone growth factors has accelerated in the last two decades with the introduction of BMPs into clinical bone repair. Optimal use of growth factor-mediated treatments heavily relies on controlled delivery, which can substantially influence the local growth factor dose, release kinetics, and biological activity. The characteristics of the surrounding environment, or “context”, during delivery can dictate growth factor loading efficiency, release and biological activity. This review discusses the influence of the surrounding environment on therapeutic delivery of bone growth factors. We specifically focus on pathophysiological components, including soluble components and cells, and how they can actively influence the therapeutic delivery and perhaps efficacy of bone growth factors.

Keywords

Orthobiologics; Growth factors; Pathophysiological microenvironment; Growth factor delivery; Bone regeneration

1. Introduction

Regeneration of injured or diseased bone tissue represents a tremendous clinical need. With an estimated number of over 1 million fractures each year in the United States at a cost of \$10 billion; the field of bone tissue engineering is aiming at developing new technologies with the goal of meeting the clinical need [1]. Therapeutic strategies often rely on the

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delivery of “orthobiologics”, which include bone growth factors (GFs), small molecules, pro-osteogenic cell types, and polynucleotides (e.g. RNA, DNA). As an emerging class of therapeutic agents, orthobiologics have generated a high level of interest for clinical orthopedic applications [2–4]. In this review we focus on bone growth factors, a subset of orthobiologics, as they have been widely explored in controlled release applications and have a significant recent history in clinical applications. This class of orthobiologics emerged after Urist and co-workers first demonstrated the potential of demineralized bone matrix (DBM) to induce ectopic bone formation in animal muscle pouches [5]. In subsequent studies, the investigators identified a family of bone morphogenetic proteins (BMPs), members of the transforming growth factor- β (TGF- β) superfamily, as the bone growth factors present in this matrix and responsible for inducing bone formation.

1.1. Importance of biologics delivery in orthopedic and related applications

Clinical translation of BMPs for orthopedic applications has progressed substantially over the past 30 years [2–4]. There are an extensive number of preclinical studies that have demonstrated the effectiveness of applying BMPs, and have led to the clinical introduction of rhBMP-2 and rhBMP-7 within absorbable collagen sponges for spinal fusion, open tibial fractures and oral maxillofacial applications since 2002. Specific examples include Medtronic’s INFUSE® product (rhBMP-2 in collagen sponge) (Fig. 1) and Stryker’s OP-1 product (rhBMP-7 in collagen sponge). Several clinical studies demonstrate the pro-osteogenic capabilities of these BMPs. For example, the use of INFUSE® in lumbar spine fusion resulted in higher fusion rates, with results that were superior to autologous bone grafts (Fig. 1-D) [6,7]. During this span, rhBMP-7 (in Stryker’s “OP-1” product) and rhBMP-2 (in Medtronic’s INFUSE® product) represented the most extensively investigated molecules [8–10]. Common bone defect sites where the use of rhBMPs has improved healing in clinical orthopedic applications include diaphyseal tibial nonunions [4], open fractures [11], and lumbar spine fusions [2,4,8,9, 12,13].

1.2. Purpose and scope of review

Several approaches have demonstrated an ability to control bone growth factor delivery in vitro. However, the characteristics of the in vivo environment can substantially influence the delivery kinetics as well as the efficacy of a bone growth factor delivery strategy. The surrounding environment has a significant impact on the ability to load, release and maintain the biological activity of bone growth factors. Ideal delivery systems would not only maintain local biological activity of bone growth factors, but also deliver growth factors in response to physiological requirements, having the capacity to sense changes in the tissue environment and alter protein release accordingly. This review focuses on describing the influence of the surrounding environment on therapeutic delivery of bone growth factors. We introduce the promise and challenges of orthobiologics delivery, then review the characteristics of the in vivo environments that receive bone growth factors. Finally, we describe the impact of dynamic in vivo environments on bone growth factor delivery, and discuss how the environmental “context” may influence the efficacy of bone growth factors and other emerging orthobiologics.

2. Bone growth factors as orthobiologics

Since the original discovery of BMPs, several growth factors have shown potential for bone regeneration. These include multiple members of the TGF β superfamily, insulin like growth factor 1 (IGF-1), fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs) and vascular endothelial growth factor (VEGF). Interest in these growth factors is supported by biological studies of fracture healing [14]. Specifically, fracture examination during the different phases of defect healing has identified the required presence of TGF- β superfamily members, IGF-1, FGFs, PDGFs, and VEGF for proper new bone formation to occur [8,14,15]. Their presence at controlled concentration, timing, and spatial location within defect site suggests they serve an essential role in the fracture healing mechanism [16]. The generalized biological mechanism of bone growth factor-mediated defect repair begins with their secretion from resident cells as an injury response within the fracture. The secreted growth factor can then bind to the cognate receptor on a resident precursor cell. This results in the activation of the intracellular kinase domain, followed by a phosphorylation cascade that ends with translocation of second messenger to the nucleus, where a key transcription factor activation upregulates the expression of pro-osteogenic genes [10,14].

The details of the bone growth factor signal-transduction mechanisms that relate to osteogenesis are an actively studied area, and have been reviewed elsewhere [10,14]. Briefly, pro-osteogenic BMP signal-transduction occurs via the type II serine-threonine kinase family of TGF- β receptors, and the associated downstream SMAD signaling. The lack of tissue specificity of the BMP ligand–receptor interaction, susceptibility of the growth factor to denaturation and proteolytic degradation, and the need to maintain the appropriate therapeutic concentration result in a series of therapeutic delivery challenges. While BMP delivery provides the most well characterized example of the promise and complexity of bone growth factors, it is not a unique example. Other bone growth factors are similarly capable of inducing bone formation, but suffer poor in vivo stability as well as the potential for significant side effects, suggesting a general need to control bone growth factor dosages and delivery kinetics. As a result, many recent studies have focused on developing localized, sustained release systems capable of controlling bone growth factor delivery to bone defect sites [17,18].

2.1. Unique challenges in delivery of bone growth factors

The concept of appropriate local and sustained dosages is particularly challenging for growth factor orthobiologics. Not only do growth factors need to reach the intended site of interest with maintained bioactivity and at an efficacious concentration, but the delivery mechanism must also be appropriate for the growth factor's therapeutic index. The therapeutic index is defined as the ratio of the maximal nontoxic system concentration to the minimal dose that elicits a positive therapeutic response [19]. Unfortunately, for biologically active compounds such as rhBMP-2 this index can be quite small [20,21]. This is due in part to rhBMP-2's poor solubility, short biological half-life, and rapid local clearance under physiological conditions in vivo. As a result, large BMP dosages are required. Indeed, there is more exogenous BMP-2 in a single dose of the INFUSE $\text{\textcircled{R}}$ product than would be present in 1000 human bone defects, raising concerns about safety and cost [22]. High rhBMP-2

dosages have recently been directly associated with serious side effects in clinical studies (including heterotopic bone formation [23,24], edema [25,26], and retrograde ejaculation [27]). In essence, the delivery of bone growth factors poses unique drug delivery challenges due to the high doses required due in part to poor pharmacokinetics, the low doses required to limit off-target effects, and the narrow therapeutic range of efficacy at the actual defect site. Therefore, controlled local delivery is an important property that biomaterial-mediated delivery of growth factors can offer [28].

3. Dynamic microenvironments during bone growth factor delivery

The microenvironment is a highly diverse and complex milieu composed of cells, extracellular matrix (ECM), and soluble factors. The crosstalk between these components provides the surrounding cells with regulatory signals which lead to the development of desirable cells, tissues and organs. More importantly, the microenvironment for resident cells is a highly dynamic niche, in which biophysical and biochemical cues are presented to the cells in a dynamic fashion [29]. The dynamic changes in the ECM can not only influence the immobilization and liberation of affiliated growth factors, but also modulate the physiochemical properties of the local microenvironment, which can further affect growth factor activity [30]. Although cells do not tend to directly affect growth factor release, their interaction with the ECM under certain physiological and pathological conditions could influence growth factor delivery. This effect has been clearly demonstrated by the action of many cell types such as platelets, macrophages and osteoclasts during bone healing. Furthermore, a variety of environmental factors such as pH, drug carrier, mechanical stimuli and serum also tend to change dynamically during the delivery of bone growth factors [31]. Table 1 summarizes the dynamics of the microenvironment during bone healing.

Bone healing is a tightly regulated process accompanied by a series of physicochemical and biological events which can influence growth factor delivery [32]. During bone tissue formation, regular inflammatory cells, including macrophages and neutrophils, are involved in creating the local environment for GF delivery. In addition, bone specific cells including osteoblasts, osteoclasts, and osteocytes actively regulate binding and release of GFs from ECM and other drug carriers [33]. For example, GFs embedded within bone matrix are liberated during osteoclast-mediated bone remodeling [34]. Growth factor bioactivity is also closely associated with local ECM components present during bone healing. For instance, basic FGF (bFGF) sequestering on fibrin fibers within a fracture hematoma stabilized its bioactivity, and augmented its impact on bone healing [35]. Therefore, a variety of environmental factors during bone healing influence the delivery of GFs in multiple ways including GF binding/release, activity (i.e. protein stabilization), and synergistic effects between GFs and the ECM, and all these factors can affect the eventual outcomes of bone healing. While the impact of the dynamic microenvironment on regulation of cell behavior has been extensively reviewed elsewhere [36,37], how it influences the delivery of growth factors has not been systematically reviewed. In view of the importance of bone growth factor delivery, several aspects of the dynamic microenvironment that influence growth factor release and efficacy (Fig. 2) are discussed in the following sections.

3.1. The extracellular matrix

The ECM is an assembled network composed of structural and functional components secreted and deposited by cells, and providing mechanical strength, cell adhesive ligands and growth factor sequestering ligands [38]. The interactions between the ECM, cells and growth factors result in mutual influence and dynamic reciprocity. The ECM can serve as a storage reservoir through its direct interaction with a growth factor. It can also indirectly influence growth factor activity by controlling cell adhesion via the presentation of adhesive ligands [39]. Additionally, the abnormalities caused by various physiological or pathological conditions can alter the interaction between the ECM and growth factors, thereby altering the course of tissue healing [40,41]. The impact of the ECM as an environmental factor on bone growth factor delivery is highlighted in the following sections.

3.1.1. Direct interaction between growth factors and the ECM—The ECM can locally sequester and release growth factors, and therefore serves as a repository. Many ECM components contain growth factor binding sites with high affinity for certain types of growth factors, and are able to bind to these molecules through non-covalent interactions (e.g. H-bonds, Van der Waals interactions) [33]. For example, proteoglycans (PGs), possess extraordinary capacity to bind to a variety of growth factors. PGs harboring glycosaminoglycan (GAGs) such as heparin and heparan sulfate contain high concentrations of anionic sulfate and glucuronic acid, which enable them to non-covalently bind to cationic heparin-binding domains on growth factors [42]. The sequestering of growth factors by the ECM can localize the growth factor and protect a bound growth factor from degradation. For example, it was found that bFGF could only act as mitogen when bound to heparan sulfate at the cell surface [43]. The bound bFGF also stimulated plasminogen activator production by endothelial cells in a more sustained manner over time when compared to free bFGF [44]. ECM proteins such as fibrin, collagen, fibronectin and vitronectin can also bind to a number of growth factors either indirectly via their heparin-binding domains or more directly via their growth factor-binding domains [45–47]. Fibrin is of particular interest for orthobiologics delivery, as it not only provides a scaffold for cell infiltration, but can also concentrate growth factors at wound sites during the inflammatory phase of healing. A set of pro-angiogenic factors such as bFGF, VEGF and interleukin-1 (IL-1) was sequestered in fibrin and used to induce endothelial cell proliferation, which ultimately led to angiogenesis [35,48]. Collagens, the most abundant class of ECM proteins in tissues, can also bind to a variety of growth factors such as VEGF, PDGF, and TGF- β 1 [49]. Reports have found that PDGFs and TGF- β 1 could retain their activity when bound on collagen IV [50,51].

In contrast, certain ECM components can also down-regulate growth factor activity [52]. For instance, the binding between TGF- β and decorin can keep TGF- β away from its cognate receptor and decrease its bioavailability during development and regeneration [52]. Another ECM component, SPARC (secreted protein, acidic, and rich in cysteine), was also shown to impair the mitogenic effect of PDGF-AA and -BB on human smooth muscle cells upon binding. Some inhibitory effects of ECM proteins are dependent on the context in which GFs are sequestered. For example, collagen has the ability to retain activity of GFs bound on its surface, but collagen also reduced the biological activity of VEGF when endothelial cells were adhered to collagen I [53]. A similar inhibitory result was found on

other ECM components such as heparan sulfate and chondroitin sulfate [54,55]. Together, these results have demonstrated that certain types of ECM can inhibit the activity of certain growth factors. This may have particular relevance when choosing a carrier for delivery of bone GFs, as an ECM-derived carrier with inhibitory capabilities would decrease their bioactivity.

3.1.2. Indirect interactions between growth factors and the ECM—The ECM can indirectly regulate the cellular response to growth factors via signaling through a variety of cellular adhesion ligands [56]. This is mainly achieved via supramolecular complexes of integrin receptors and growth factor receptors (GFR), initiated by presentation of cellular adhesion ligands to the cells [57,58]. Thus, the integrin binding domains on ECM proteins such as fibronectin, vitronectin, laminin and collagen not only present as cellular adhesion ligands, but also actively participate in growth factor signaling. For example, VEGF dependent endothelial cell proliferation was significantly faster on substrates coated with fibronectin when compared to bovine serum albumin (BSA) coated substrates. This was mainly attributed to the activation of VEGFR-2 through binding to integrin subunit β_3 , which was elevated by the presence of cellular adhesive ligands [59]. A similar mechanism has been reported for multiple growth factors including VEGF, EGF and TGF- β_1 [60–62]. The synergistic cooperation between integrins and GFRs can sensitize the GFR, which may allow for activation of signal transduction with a lower concentration of the growth factor. Therefore, this synergy may be useful to potentiate the growth factor activity. For example, the cooperation of $\alpha_v\beta_3$ integrin together with TGF- β_1 remarkably enhanced fibroblast proliferation, while blocking of $\alpha_v\beta_3$ integrin abolished the synergistic effect. Interestingly, $\alpha_v\beta_3$ integrin expression was co-localized with TGF β IIR under TGF- β_1 exposure, indicating that these receptors clustered during signal transduction [63]. Similarly, the biological activity of EGF and TGF- β_1 was also enhanced when their corresponding receptor was influenced by ECM components such as collagen, fibronectin and vitronectin [61,64]. Furthermore, the impact of cell adhesion on growth factor signaling influenced cell motility. Griffith and co-workers reported that various aspects of fibroblast migration, such as locomotion speed, membrane extension and retraction activity were actively regulated by either fibronectin or nanoscale RGD clustering under EGF stimulation [65,66]. Taken together, these studies highlight the importance of cell-ECM adhesion as a regulator of GF signaling; and may provide further information to instruct design of GF carriers in orthopedic applications.

3.1.3. ECM under various conditions—ECM-growth factor interactions can be disrupted by the abnormalities of ECM caused by various physiological and pathological conditions [67]. The ECM composition, stiffness, and pH are influenced by disease, trauma, and aging, and changes in the ECM affect the response of cells to growth factors. Fracture healing provides an illustrative example, in which a hematoma rich in fibrin is formed immediately after the disruption of tissue and blood vessel integration [68]. The fibrin based hematoma is critical for bone healing, partly due to its ability to bind to multiple growth factors involved in bone regeneration [69,70]. In contrast, the absence of a fibrin clot during chronic wound healing severely interrupts the process of tissue regeneration and leads to formation of non-healing wounds [71]. The ECM abnormalities of non-healing wounds can

also include protease-inhibitor imbalance, high local pH and excessive scar formation which could affect growth factor activity locally [72]. For example, PDGF activation triggered by cell adhesion to fibronectin has been interrupted by high local pH at wound sites [73, 74]. Excess level of matrix metalloproteinases (MMPs) has caused loss of ECM, leading to the lack of adhesive sites for cell anchorage [40]. Cancer can also substantially change the ECM stiffness, pH value and composition, and influence the cellular response to growth factors [75]. For instance, Asthagiri et al. reported that matrix stiffing significantly sensitized cells to EGF and resulted in the loss of contact inhibition during proliferation [76].

3.2. pH

Microenvironmental pH has been considered an important factor influencing a variety of aspects of growth factor delivery, including release kinetics, and stability [77,78]. Most growth factor delivery studies are performed at near physiological pH, which is 7.35. However, the local pH at delivery sites in vivo can drastically change due to various physiological and pathological conditions [79–81]. For example, the acidic milieu created by osteoclasts can result in a local pH of 4.50 during bone remodeling [82]. Such pH changes could have a significant impact on both the carrier material and the growth factors to be delivered. Various reports have shown that the degradation rate of many carrier materials, including polyesters, calcium phosphates, and natural polymers was a function of solution pH [83–85]. Since carrier degradation is often directly correlated to GF release kinetics, the pH could substantially affect a GF release strategy. Proteins like GFs are often highly sensitive to pH variation due to their structural fragility. Exposure of proteins to extreme pH could be deleterious to their bioactivity, which could further affect the biological activity of GF delivery [86,87]. Here, we discuss the impact of pH on growth factor delivery from both natural and synthetic carriers. We also briefly discuss pH responsive drug delivery systems, as an example of a physiologically responsive “smart” drug delivery strategy.

3.2.1. Impact of pH on orthobiologics delivery in natural environments—

Although the pH of natural environments is usually maintained in the range of 7.35, many disease or injury conditions may substantially change this value. Inflammation of tissues is often associated with acidic pH caused by the accumulation of metabolic acid generated during inflammatory cell activation [80]. The low local pH observed in a fracture hematoma originates from similar sources, as inflammation is an early event after a fracture takes place [68]. Extremely low pH in natural microenvironments is also found near the milieu where osteoclasts are actively involved in bone remodeling. This acidic pH (~pH 4.5) is generated by secretion of hydrochloric acid (HCl) by osteoclasts [82]. The low pH environment created by osteoclasts may be of particular relevance to calcium phosphate, calcium sulfate, and calcium carbonate based growth factor delivery systems, as these minerals degrade faster in acidic pH [88]. Liu et al. identified that osteoclast-mediated degradation of calcium phosphate carriers as one of the major GF release mechanisms. Specifically, the release of BMP-2 from calcium phosphate coated implants was closely associated with local osteoclast activity [34,89]. On the other hand, pathological environments such as chronic wounds can cause pH increases up to 8.0, which affects the activity of MMPs [41]. In turn, the presence of active MMPs can degrade the growth factors delivered to the wounds and inhibit wound healing [90]. Local pH changes can also affect the growth factor binding to ECM

components. For example, Nugent et al. found that acidic pH could increase the binding of VEGF to endothelial cells and fibronectin [91]. Therefore, variation of pH in natural environments can affect the delivery of orthobiologics in multiple ways suggesting that design of delivery systems must fit the “microclimate” pH of the intended delivery site.

3.2.2. Impact of pH on drug delivery in synthetic microenvironment—Synthetic microenvironments within implants and drug carriers also affect growth factor release and bioactivity. pH changes in synthetic microenvironments are often caused by polymer degradation [92]. For instance, Schwendeman and colleagues have identified that an acidic microenvironment was one of the most important sources of irreversible inactivation of proteins encapsulated in polymeric carriers [93–97]. A study by Fu et al. showed that the minimum microenvironmental pH within poly(lactic-co-glycolic acid) (PLGA) microspheres could be as low as 1.50 [87]. Such strong acidity can cause protein unfolding which eventually led to protein aggregation. The low pH also promoted peptide bond hydrolysis, further disrupting bioactivity of the proteins [86,98]. Modulation of local pH within controlled release systems has become an attractive strategy to improve the stability of protein during delivery. For example, Zhu et al. successfully neutralized the acidity from polymer degradation byproducts by incorporating poorly soluble basic salts into the delivery devices. This method successfully prevented degradation of proteins such as BSA, bFGF and BMP-2 for up to one month [97]. In another approach, the use of bioresorbable polyphosphazenes eliminated the generation of acidic byproducts during degradation [99]. Interestingly, the acidic microenvironment could also be leveraged to stabilize proteins that are unstable at neutral pH. Camptothecin, an anticancer drug that undergoes rapid hydrolysis at pH = 7.40, was stabilized by encapsulating into PLGA microspheres due to the low pH generated during the degradation of the microspheres [100].

3.2.3. pH-responsive drug delivery systems—The variation of local pH in physiological environments has inspired the development of a series of environmental responsive drug delivery systems, which has been comprehensively summarized elsewhere [101–105]. Here we will focus on their application in the field of orthopedics. Akashi et al. developed a poly (γ -glutamic acid)-sulfonate matrix for pH-controlled release of bFGF in which bFGF release was successfully controlled by switching pH between 7.40 and acidic values [106]. Researchers have also designed stimulus responsive delivery systems which could respond to the pH of the wound sites and trigger release of therapeutic agents such as VEGF and EGF [91,104]. For example, Maiti et al. reported that the environmental pH within a healing wound initiated release of VEGF and bFGF encapsulated in a poly (NIPAAm-co-AAc) hydrogel without loss of bioactivity [107]. More recently, Farokhzad et al. developed a novel strategy leveraging the pH at the site of an infection to target antibiotic delivery to the bacteria cell wall. Vancomycin loaded in PLGA-PLH-PEG nanoparticles could rapidly bind to both Gram-positive and Gram-negative bacteria through the selective protonation of the imidazole groups of PLH at acidic pH [108]. Together, these results demonstrated that variation of pH can be successfully incorporated into the design of drug delivery system and provide stimuli-responsive release of therapeutics.

3.3. Growth factor carriers

3.3.1. Drug/carrier interaction—The interactions between cargo proteins and their carriers are major factors to control protein release. Interactions of GFs with the ECM and ECM-derived molecules have been reviewed in previous sections, so here we focus on synthetic growth factor carriers. Growth factors are typically incorporated into carriers through two main strategies: physical adsorption and covalent immobilization. Growth factors can physically adsorb to polymeric carrier surfaces via electrostatic interactions, such as hydrogen bonds between the functional groups on the protein and the carrier [85]. One study showed that insulin stability encapsulated in PLGA microspheres was partially determined by an acylation reaction during the attachment of lactic acid/glycolic acid to the amine groups of insulin [109]. The level of deamidation, acylation and proteolysis of a protein was also influenced after insulin binding to PLGA films [110]. Bone growth factors have also been covalently immobilized onto their carriers through selective chemical coupling [105,111]. This strong interaction can slow down GF degradation or internalization as well as prolong the release from a carrier. In one study, EGF covalently tethered on a solid substrate retained its biological activity while physically adsorbed EGF on the same substrate showed no activity [112]. However, the bioactivity of growth factor incorporated through this approach might be compromised due to the structural damage to the protein caused by inappropriate coupling site selections [33].

3.3.2. Carrier degradation—Carrier degradation has been regarded as a primary mechanism dictating drug release in various bioresorbable carriers. The growth factors incorporated into synthetic polymeric carriers have been released mainly through a combined diffusion/degradation mechanism influenced by hydrolysis, which is a process of cleavage of chemical bond by the presence of water [85]. For example, the release kinetics of BMP-2 from bioresorbable scaffolds was closely associated with the degradation and erosion of the scaffolds [113,114]. Zheng et al. also reported that the amount of released BMP-2 was proportional to the loss of polymer mass during release in vitro [115]. The degradation of polymer was classified into surface (or heterogeneous) erosion and bulk (or homogeneous) erosion [116], and these two different degradation mechanisms led to distinct growth factor release kinetics [117,118]. The surface erosion rate is determined by the surface area of the carrier, which may provide a simple route to control GF release kinetics [119]. However, most bioresorbable polymers such as PLA, PLGA and PCL undergo bulk erosion with distinct degradation kinetics. In addition, a series of polymer properties such as molecular weight [120,121], crystallinity [121], and hydrophobicity [122] can affect the degradation of the polymeric carriers and in turn, affect the release rate of the growth factors. Growth factor release also heavily relies on the fabrication parameters of the carrier, because they are also closely related to the carrier degradation rate. For example, the pore size of PLG scaffolds has considerably affected the release of proteins by changing the diffusion coefficient of proteins releasing from the scaffolds [92]. A similar effect of fabrication parameters on growth factors was also observed in other formats, including microspheres [123], hydrogels [124], nanoparticles [125] and nanofibers [126]. It is noteworthy that degradation byproducts are another important factor that can influence growth factor release. In an example noted above, the degradation byproduct of poly(α -hydroxyl esters) (e.g. PLGA, PLLA and PGA) pose a threat to growth factor, due to their

high acidity [93,97]. The low local pH has also catalyzed hydrolysis, which caused heterogeneous erosion inside the carrier and altered the designed growth factor release profiles [127].

Another important degradation mechanism is enzyme mediated carrier degradation which involves the use of enzyme-cleavable biopolymers. This concept has been elegantly demonstrated in studies by Hubbell et al. [128–130], in which a growth factor was fused with a factor XIIIa substrate sequence NQEQVSPL through its N-terminus and incorporated into a fibrin gel during coagulation. The release of growth factor was then controlled by the enzymatic degradation of the fibrin gel by invading cells resulting in a “cell-demanded” release mechanism coordinated with tissue growth at the desirable sites [128,130]. The technique has been successfully applied to regenerate blood vessels, bone, and neurons. A similar approach was applied to a synthetic MMP-sensitive hydrogel in which protein was liberated due to the proteolytic degradation during cell invasion into the hydrogels [131,132].

3.3.3. Minerals coatings as GF carriers—Calcium phosphate (CaP) mineral, as the major inorganic component of bone tissue, has been used for decades in orthopedics to achieve a rapid fixation and improved bone-implant bonding due to its excellent biocompatibility and osteoconductivity [133]. More recently, it has also been identified to be a versatile growth factor carrier, owing to its ability to bind to proteins via charge–charge interactions between $\text{Ca}^{2+}/\text{PO}_4^{3-}$ ions and protein side chains [134–136], and dissolve at controllable rates into non-toxic, physiological mineral ions. Through the last several years, we and others have developed a series of strategies to control the growth factor release by modulating the intrinsic properties of CaP minerals, including the dissolution/precipitation. Here we highlight the recent progress regarding these technologies and their application in orthopedics.

Mineral degradation (dissolution) is the primary mechanism for release of growth factors trapped/bound to mineral coated orthopedic implants [89], thus we have developed approaches to tailor the degradation of mineral coatings. Our approach involves forming various mineral coatings via a nucleation and growth process in a modified simulated body fluid (mSBF) (Fig. 3-A) [137]. Importantly, the characteristics of the mSBF can be systematically varied, and can dictate the properties of the mineral coatings, including morphology, composition, and dissolution. The systematic variation of these properties can be achieved without changing the nature of the degradation byproducts (principally Ca^{2+} , PO_4^{3-}). In addition, mineral nucleation and growth can be achieved on virtually any underlying material. Thus, nucleation/growth of mineral coatings provides an extraordinarily adaptable approach for GF delivery on a wide variety of medical devices. For instance, by changing the carbonate concentration in mSBFs, the nanoscale morphology, composition and dissolution rate of these coatings were precisely tailored. Higher carbonate concentration led to faster dissolution of mineral coating (Fig. 3-B) which could be attributed to the substitution of PO_4^{3-} by CO_3^{2-} in the lattice structure of hydroxyapatite (HA) and the associated change in the crystallinity of the mineral [137–141]. A similar approach was used to modulate the degradation properties of CaP materials such as β -

tricalcium phosphate (β -TCP) (Fig. 3-C) [138]. Importantly, release kinetics of growth factors in each of these studies was strongly dependent on the solubility of the mineral coatings (Fig. 3-D). Specifically, when the carbonate concentration was increased from low to high, the release of a VEGF-mimetic peptide was increased from 10% to 45% during a 30 day release study (Fig. 3-E) [140]. Similar results were also observed with other growth factors, including BMP-2, TGF- β and bFGF [138,142]. Furthermore, the released growth factors showed no significant loss of their bioactivity in multiple biological assays (Fig. 3-F) [138]. Controllable mineral coatings have been formed on various orthopedic implants including sutures, scaffolds, screws, plates, and microspheres, which combines the clinical advantages of these devices with the controllable growth factor delivery capability of the mineral coatings [137,140,143].

Another set of studies extended the control over coating degradation further by incorporation of fluoride [142]. Fluoride incorporation in the coating not only changed the nanostructure of coatings from plate-like to needle-like, but also significantly slowed down the dissolution and the release of BMP-2 (Fig. 4-A) [144]. By combining carbonate and fluoride incorporation into one coating system, we developed a multilayered microparticle platform for tunable delivery of multiple GFs. The release of BMP-2 and VEGF bound on different mineral coating layers was independently controlled by both the position and solubility of the coating. For example, one tailored microparticle formulation released up to 72% of VEGF but only 12% of BMP-2 within the same time frame (Fig. 4-B). Variation of the outer coating thickness was found to be another efficient way to modify BMP-2 release kinetics (Fig. 4-C) [142].

3.4. Cells present in microenvironments during bone growth factor release

3.4.1. Macrophages—Macrophages are differentiated cells from the mononuclear phagocyte system that play an important role in host response to foreign bodies [145]. Almost all biomaterial implants can cause a macrophage response, regardless of their composition, dimension, and shape. Once the implants are recognized by the body's immune system, macrophages conduct a series of actions to clear these foreign items via different cellular mechanisms, which could significantly affect the delivery of growth factor loaded on/into these implants. For example, micro/nano particles loaded with growth factors have often been cleared by macrophage via phagocytosis in a short time when these particles were injected to injury sites such as bone fractures [146]. The rapid removal of drug-releasing particles has been a significant obstacle for particulate controlled release systems [147]. To address this challenge, novel strategies such as PEGylation of the particles have been developed to mitigate phagocytosis via macrophages [148]. Recently, Zhang et al. remarkably increased the circulation lifetime of nanoparticles in vivo by disguising the particle surface with a layer of erythrocyte membrane. This biomimetic decoration of nanoparticles successfully "confused" macrophages and protected the particles from phagocytosis [149]. Macrophages are also actively involved in biodegradation of polymers, which can drastically change the release profiles of growth factors from these materials in vivo [150]. Macrophages can release reactive oxygen intermediates, enzymes and acid that serve as catalysts to accelerate implant degradation, which in turn may affect the release of growth factors loaded in these implants [151]. For instance, MMP-9 expression was found to

be elevated in adherent foreign body giant cells (FBGCs) which may influence the degradation rate of natural polymer based carriers such as collagens and fibrin [152]. Clover et al. reported host mediated degradation of collagen scaffolds was mainly executed by macrophages, and that crosslinking of the collagen scaffolds increased the resistance to macrophage-mediated degradation [153].

3.4.2. Platelets—Platelets circulating in blood can be rapidly activated by external stresses such as blood vessel rupture, and quickly stop bleeding by coagulation and elaboration of a blood clot at a wound site [154]. The resulting hematoma is mainly composed of a fibrin rich ECM, which plays two major roles during wound healing: 1) it provides a template for cell migration and 2) it serves as a provisional reservoir for binding of cytokines and growth factors [68]. Platelet-triggered hematoma formation may influence growth factor delivery strategies in a series of ways. First, the presentation of delivered growth factors to target cells is realized by recruitment of the target cells into the hematoma via a series of inflammatory cells [155]. On the other hand, the released growth factor is typically able to bind to ECM proteins (e.g. fibrin) in the hematoma, which can extend GF half-life and increase bioactivity [43]. For example, released bFGF has been shown to be retained on fibrin and subsequently liberated by plasmin during fibrin degradation [48]. Further, degranulating platelets can release multiple growth factors, including EGF, bFGF, PDGF and TGF- β . These factors can either act alone or synergistically cooperate with exogenous delivered growth factors to accelerate tissue repair and regeneration [156].

3.4.3. Osteoclasts—Osteoclasts are multinucleated cells differentiated from monocyte/macrophage precursor cells surrounding the bone surface. The major function of osteoclasts is to conduct bone resorption, which is a process of bone matrix degradation during bone remodeling [157,158]. Briefly, premature osteoclasts are activated by two key signals macrophage colony-stimulating factor (M-CSF) and receptor activator of NF κ B ligand (RANKL), and undergo polarization after adhering to bone surface. Mature osteoclasts generate a ruffled border that forms into a sealed compartment between the bone surface and the basal membrane of osteoclasts. Matrix degradation mediators such as hydrochloric acid and proteinases are then released into the so called “vacuole” and initiate bone resorption [158]. Osteoclasts not only play an important role in native bone remodeling, but also actively participates in implanted biomaterials degradation. The secretion of acid by osteoclasts can dissolve calcium phosphate mineral crystals, thereby influencing degradation of mineral-based biomaterials [159]. In particular, resorptive lacuna have been documented on HA, β -TCP, and biphasic calcium phosphate [160]. Importantly, osteoclast mediated resorption presents an important mechanism for growth factor liberation from mineral-based materials [89,161]. Wernike et al. showed that the presence of osteoclast-like cells sustained the long term release of protein when compared to passive diffusion, suggesting the prolonged release of protein was attributed to cell-mediated resorption [162]. Further, enzymes released by osteoclasts can degrade ECM-derived polymer, which could also influence growth factor release from polymer-based drug carriers. For example, cathepsin K, an important proteinase secreted by osteoclasts, has been shown to degrade type I and II collagens [163] as well as PEG hydrogel cross-linked by a cathepsin K degradable peptide sequence [164]. Notably, while the cell types discussed in this section do not encompass all

the cell types present during GF delivery, the studies we highlighted here provide evidence of common mechanisms by which cells can influence GFs and their carrier materials.

3.5. Biomechanics

Most tissues in the human body experience a variety of mechanical stimuli, such as compression in bone and cartilage, stretching in muscle, tension in tendon and shear force in blood vessels. The existence of a mechanically dynamic environment can profoundly influence various biological processes involving both resident cells and delivered growth factors during tissue development and metabolism [165]. For example, mechanical stimuli can be converted into biochemical signals and regulate cell behaviors [166]. In one illustrative example, Little et al. reported that cyclic stretch to smooth muscle cells stimulated bFGF release and promoted the growth of the cells [167]. In other examples, mechanical stimulation to fracture hematoma has been shown to enhance secretion of VEGF and improve angiogenesis during fracture healing [168]. In view of the importance of mechanical stimuli in the body, drug delivery systems responsive to these stimuli have been designed. Mooney et al. recapitulated aspects of the mechanically dynamic environment in vivo by providing mechanical stimulation to growth factor loaded alginate hydrogels. Deformation of the hydrogel led to up to a five times greater VEGF release rate when compared to static conditions. Importantly, the increase of VEGF release promoted blood vessel formation in both severe combined immunodeficiency (SCID) and non-obese diabetic (NOD) mice (Fig. 5-A–C) [169]. They also identified that greater interactions between VEGF and the hydrogel could affect drug release under mechanical stimuli, as the release of bound VEGF was more strongly influenced by a mechanical stimulus (Fig. 5-D–F) [170]. Wang et al. reported that release of BSA encapsulated in PLGA microspheres was accelerated under mechanical loading when the microspheres were embedded in polymeric scaffolds [171]. Mechanical stimulus parameters such as frequency, duration, and amplitude have also been used to modulate growth factor release [172]. While these studies demonstrate that intentional application of mechanical stimulation can influence GF release, it is important to recognize that the surrounding mechanics are likely to be a critical parameter in virtually all GF delivery systems in orthopedics. Collectively, these studies suggest that mechanical stimulus should be taken into consideration during growth factor delivery or leveraged as an important mechanism to design new drug delivery systems.

4. Impact of microenvironments on orthobiologics delivery

4.1. Loading and release of biologics

Localized, sustained release of orthobiologics can potentially offer several desirable features including; lower required dose, ability to achieve long term release, and avoidance of heterotopic or even systemic exposure and associated side effects. Depending on the strategy used for protein incorporation, and the surrounding microenvironment, one can control several aspects of soluble growth factor release. This section will describe methods for loading orthobiologics and the release of growth factors from these different approaches.

4.1.1. Adsorption—Perhaps, the simplest strategy for loading orthobiologics involves adsorption to a carrier matrix. Growth factors have been adsorbed to sponges, and also to

mineral surfaces. Absorbable collagen sponges are widely used as a delivery vehicle for growth factors including TGF- β , and bFGF, and they are the current clinical strategy for the release of rhBMP-2 and rhBMP-7 [173,174]. Collagen is an attractive biomaterial since it is the main non-mineral component of bone, has good biocompatibility, degradation into physiological end-products, and allows for cell infiltration. Loading of a growth factor into collagen sponges is achieved by soaking the sponge in the growth factor solution, which allows for control over the dosage by simply changing the growth factor concentration in the soaking solution. This approach can be used to load high dosages of growth factors, up to 12 mg [175]. Binding studies of rhBMP-2 to collagen sponges have demonstrated that changes in pH influence loading efficiency. At pH <4, binding of rhBMP-2 is negligible. As the pH increases from 4.5 to 6.5, more significant amounts can be loaded (up to 0.1–0.2 mg rhBMP-2 per mg collagen) and it has been reported that rhBMP2 can be loaded with over 90% efficiency [174]. However, there is typically some loss of the loaded growth factor due to mechanical manipulation of collagen sponges during implantation. BMP-2 loss might also be caused by leakage of BMP-2 due to bleeding and postoperative drains after implantation. Mok et al. studied the extravasation of rhBMP-2 after posterolateral spinal fusion and found a median 0.58% of implanted BMP-2 loss in the drains with 48 h of implantation [176], indicating the impact of these factors was in a manageable range.

In another approach, growth factors are commonly adsorbed to mineral surfaces. Metal implants, scaffolds, and polymeric devices have been coated with a mineral layer to improve the integration of the device with host tissue, and the coating can also serve as a carrier to load orthobiologics. Mineral coatings have been formed using methods such as plasma spraying, pulsed laser deposition, or electrophoretic deposition [177–179]. These methods allow for the formation of highly stable, stoichiometric hydroxyapatite. Dosages of proteins in the order of micrograms have been loaded into these coatings [180]. Another method to form mineral coatings has involved incubation of a device in a simulated body fluid solution that mimics the ionic constituents of blood plasma. The mineral formed using this method is highly porous on the nanoscale, and contains charged calcium and phosphate ions capable of efficiently binding acidic or basic growth factors via electrostatic interactions. This approach has been used to coat several devices, geometries including bone screws, sutures, microspheres, and various macroporous scaffolds [137,138,140,143,181–183]. Once the device is coated, growth factor incorporation is as simple as “dip coating” the device for several minutes in an aqueous buffer containing the growth factor of interest [183]. A broad range of growth factors and peptides have been bound to mineral coated devices using this approach including mineral-binding bone morphogenetic peptide (mBMP) [181,183,184], bFGF, rhVEGF [138,183]. This strategy offers numerous advantages, including binding of multiple proteins, control over dosage by simply changing the concentration of the growth factor solution or the dip coating time, and retention of biological activity of the bound orthobiologic [139,183]. The percentage of growth factor bound by adsorption varies since it depends on the growth factor-mineral affinity, the coating surface area, and the growth factor concentration in solution. It has been reported to range from over 90% for growth factors or peptides with high mineral binding affinity to 15% for growth factors with low mineral binding affinity [138,140,183]. This approach can be considered as a “modular

design” approach, in which a controllable biologics carrier is integrated into a structural device as a thin surface coating that does not compromise the device performance.

The release kinetics of growth factors adsorbed to a carrier are highly dependent on the carrier material. On one end of the spectrum, rhBMP2 release from collagen sponges has been characterized and shown to exhibit a burst release over the first 2 days after implantation (>60% rhBMP2 is released) resulting in substantial short term release [185]. In vivo retention of rhBMP-2 has been reported to be from 2.5–4 days [174,185]. On the other end of the spectrum, highly crystalline hydroxyapatite coated onto metal implants in many cases lacks macroporosity and does not degrade. This could, in principle, lead to bone formation on the surface that would encase the growth factor and prevent its availability. Previous work has demonstrated that 15–25% of TGF- β 2 loaded onto highly crystalline hydroxyapatite released in vitro within the first two days [180,186]. Release from mineral coatings is dynamic and is affected by many factors including crystallinity of the mineral [140], the affinity between the growth factor and the mineral [183], the pH, ionic composition of release medium [183], and the presence of proteins in the release medium. Mineral coatings formed after incubation in simulated body fluids can be resorbable and allow for sustained growth factor release. Suarez-Gonzalez et al. explored the differential affinity between growth factors and a coating material to achieve slower release kinetics with higher affinity, and faster release kinetics with lower affinity [183]. The release profile for rhVEGF and an engineered mBMP were characterized for periods of two months in two buffer systems; cell culture medium and simulated body fluid. Release kinetics of rhVEGF and mBMP were each slower in simulated body fluid than in cell culture medium. Simulated body fluid is supersaturated with calcium and phosphate ions, whereas cell culture medium has a lower concentration of the same ions. As the coating dissolves in SBF, the released protein may re-bind to existing or newly formed mineral or may become encapsulated within a growing mineral coating resulting in slower protein release. Re-precipitation of the mineral in cell culture medium is slower since the solution is not supersaturated with calcium and phosphate ions and therefore leads to faster release kinetics.

4.1.2. Co-precipitation—Proteins can also be loaded into materials such as mineral coatings by co-precipitation. The protein becomes incorporated into the growing mineral layer and is released gradually as the coating degrades. In a study by Liu et al., BSA was co precipitated with mineral on titanium-alloy implants [187]. BSA concentrations of 0.01–1000 μ g/ml were incorporated into the supersaturated solution used to form the coating. Other groups have used similar concentrations of BSA and used longer incubation times for mineral nucleation and growth [188]. This system allows for incorporation of the protein into the growing layer, however the amount of protein required could be high because materials are typically incubated for periods of days and even weeks to achieve a continuous mineral layer. Each time that the incubating solution is renewed, the protein must be renewed, resulting in a poor effective loading efficiency. Release of proteins incorporated into the growing mineral layer by co-precipitation can also serve as vehicles for long term-sustained release. In a study by Lee et al., BSA and lysozyme were released for periods of 30 days [139]. The presence of proteins during coating precipitation can also impact the properties of mineral coatings, and therefore the subsequent release profile. In a study by

Liu et al., coatings formed by incubation in simulated body fluids with and without BSA had different dissolution properties. Coatings formed in the presence of BSA degraded slower compared to those formed under the same conditions but without the protein content [187].

4.1.3. Encapsulation—Orthobiologics can also be encapsulated in various natural and synthetic polymeric matrices. The natural polymers include chitosan, alginate, dextran, and gelatin, and the synthetic polymers include PLGA, and PCL among others. Biodegradable polymer microspheres have been widely used as carriers for controlled release of growth factors. Fabrication of polymeric microspheres often relies on a water in oil emulsion or water-in-oil-in-water (W/O/W) double emulsion process with solvent extraction [189,190]. W/O/W involves dissolving the polymer in an organic solvent such as dichloromethane (DCM) and emulsifying the orthobiologic in that solution. The resulting solution is then re-emulsified in an aqueous phase. Following the double emulsion, the organic solvent is evaporated and the polymer precipitates with the orthobiologic encapsulated. Microspheres are then lyophilized for 24–48 h. Loading of proteins can be highly efficient reaching over 90% efficiency in some instances [191–193]. Milligram quantities of proteins have been loaded into polymeric microspheres [193]. Growth factors can also be incorporated in polymers used to coat metal surfaces [194]. In this case, incorporation of a growth factor is achieved by dissolving the polymer in an organic solvent followed by incorporation of the growth factor into the polymer solution [194,195]. Encapsulation of growth factors in a polymeric matrix in many instances compromises the integrity of the growth factor since it is exposed to organic solvents and polymer degradation byproducts which can result in growth factor denaturation, as discussed on previous sections [196]. Growth factor release from microspheres has drawn increasing interest and is accepted as a useful tool for controlled drug delivery due to their inherently small size, good drug loading efficiency, sustained delivery of orthobiologics, ability to release multiple growth factors, and ability to quickly respond to stimuli from the surrounding environment (e.g. temperature, pH, magnetic fields). Release kinetics from this carrier system are dependent on the properties of the polymer, as release typically involves drug diffusion through a polymer matrix that is degrading and eroding [197]. Microspheres typically exhibit burst release in the first few days (~4 days) followed by a more linear sustained release [143, 189]. A similar trend is observed when growth factors are released from polymer coatings — burst release followed by sustained release for days to weeks [143,189].

4.1.4. Affinity based binding—Natural ECM displays high affinity for many types of protein therefore is able to localize growth factor activity via the ECM binding domains on these growth factors [45,198]. This mechanism has inspired various non-covalent immobilization strategies of growth factors on orthopedic devices to achieve controllable delivery. GAGs are one of the most well-known ECM molecules used for bone growth factor immobilization owing to their high affinity to many growth factors via their negatively charged sulfate groups [199]. GAGs such as heparin and heparan sulfate have been widely exploited to load bone growth factors into different systems such as scaffolds, hydrogels and micro/nanoparticles [200]. Since their interactions with growth factors are non-covalent and reversible, this immobilization approach ensures the minimal influence on growth factor ternary structure and tends to preserve their bioactivity. A recent report by

Hettiaratchi et al. demonstrated that heparin microparticles retained large amount of bioactive BMP-2 and elicited biological response comparable to soluble BMP-2 treatment [201]. Other ECM molecules such as fibronectin, fibrin and hyaluronan have also shown their capability to localize growth factor and act as sustained release platform. For example, hyaluronic acid was successful used to retain BMP-2 in its scaffold and release the BMP-2 in a sustained manner [202].

Another emerging technique for affinity based growth factor immobilization is through layer-by-layer (LBL) route, in which a thin film is formed on a charged substrate by alternately dipping in complementary charged polymer solutions [203]. One of the most attractive advantages of using LBL assembly for bone growth factor delivery is its ability to incorporate large amount of proteins with a multilayer thin film. Up to 40 wt.% of drugs can be loaded onto the LBL thin film which is about 10 times higher than most drug encapsulation systems [204]. For example, Macdonald et al. demonstrated the extremely high loading capacity of BMP-2 on LBL coatings and showed its inductivity on bone tissue formation in vivo [205]. Additionally, the binding of growth factor to LBL films are conducted in aqueous solution at mild pH and ionic strength, which can effectively preserve the activity of proteins and lower the required dosage for therapeutic applications [206]. In a study by Crouzier et al., BMP-2 loaded on TCP/HAP ceramics with a polyelectrolyte multilayer coating showed more potent inductive effect in terms of alkaline phosphatase activity, indicating the polyelectrolyte multilayer could protect protein from denaturation [207].

4.1.5. Chemical immobilization—As previously mentioned, authors Kuhl and Griffith-Cima demonstrated the feasibility of covalent growth factor tethering while preserving bioactivity. Here the authors demonstrated an increased level EGF's mitogenic activity over adsorbed EGF when presented to rat hepatocytes. The authors argued that chemical immobilization via covalent tethering offered enhanced growth factor bioactivity via the presentation of precise concentrations and a reduction of cellular internalization and down regulation [112]. For materials used in orthopedic applications, advancements in surface chemistry have allowed for covalent tethering of growth factor orthobiologics to substrates such as titanium. Strategies for chemical immobilization on orthopedic implants often first involve functionalization of the material substrate with amines or thiols. Example functionalization techniques include the formation of a polydopamine coating on the material via dip coating, or the exposure of the material to allylic amides after plasma treatment. Attachment of the growth factor orthobiologics then proceeds through Michael Addition, imine formation, or amide formation after carbodiimide/N-hydroxysuccinimide activation of the surface amine. Variations of this strategy often employ covalent attachment of an intermediate material to the orthopedic implant surface, allowing for functionalization with a wider range of chemistries. This alternate approach often employs both natural and synthetic polymers as this intermediate, which also often confer additional surface qualities such as anti-bacterial or non-fouling properties. Examples of such polymers are the polysaccharide chitosan and PEG. Importantly, these strategies have been successful in vitro and in vivo animal models for growth factors of interest to orthopedic applications such as members of the BMP family [208,209] and VEGF [210].

Although these immobilization strategies have proved to be effective for certain specific tethering approaches, they are still dependent on the compatibility of the chemical immobilization with the growth factor variant of interest. Specifically, several of these non-selective covalent tethering strategies have resulted in reduced bioactivity of growth factors when multiple primary amines are present [33]. Chemistries that allow for tethering at selective sites on growth factors or that utilize selective electrostatic interactions are critical to improving upon chemical immobilization strategies for bioactivation of orthopedic implant surfaces. Since the publication by Kuhl and Griffith-Cima in 1996, investigators have developed several alternative strategies that avoid this limitation and have covalently tethered growth factors in ways that are more selective. Specifically, investigators have explored covalent tethering of ECM components and ECM-inspired ligands to bind growth factors to overcome this limitation [211]. Hu et al. demonstrated VEGF presented on a titanium substrate via a VEGF-binding heparin-catechol (HepC) tether. The authors demonstrated that the HepC-presented VEGF retained significantly higher activity over the direct covalent tethering of VEGF to hyaluronic acid-catechol (HAC). The authors attribute the difference in activity to the disruption of VEGF's tertiary structure by direct covalent-tethering to HAC. Importantly, the HepC-presented VEGF enhanced in vitro tubulogenesis and proliferation of endothelial cells and mineralization in osteoblasts/endothelial cell co-cultures over HAC-presented VEGF [211].

4.2. Impact of microenvironment on bone growth factor bioactivity during delivery

The efficacy of bone growth factors upon delivery is of paramount importance, as the success of their application in orthopedics and other fields is highly dependent on the physical integrity and biological activity of the delivered bone growth factors [198,199]. The impact of the microenvironment on bone growth factor efficacy is substantially influenced by the conformational structure of these growth factors, which is sensitive to external stress discussed in the previous sections [200]. During the course of incorporation and release, the bioactivity of growth factors is challenged by a series of microenvironmental factors, including hydrophobic interfaces, detergents used for protein encapsulation, elevated temperature and degradation byproducts of carrier materials [201]. In this section, several typical growth factor delivery systems are discussed to highlight the impact of the microenvironment on delivery efficacy.

Delivery of rhBMP-2 from collagen sponges has been successful in accelerating fracture repair, and the approach was introduced to the clinic in 2002 with the INFUSE® product. The use of INFUSE® in lumbar spine fusion has resulted in higher fusion rates, with results that are superior to lumbar fusion using autogenous bone grafts. A study by Burkus et al., compared the outcome of patients that had received the INFUSE product to patients that received iliac crest autografts. Patients treated with the INFUSE product had statistically superior outcomes with regards to operating time, blood loss, hospital stay, rate of secondary interventions, median time to return to work, and fusion rate [212]. Outcomes of the delivery of rhBMP2 from collagen sponges are summarized in [175], and serve as a demonstration that the biological activity of bone growth factors can be exploited using this carrier system. However, there is more exogenous BMP2 in a single dose of the INFUSE product than would be present in 1000 human bone defects as the physiological BMP-2 concentration is

around 100–200 pg/mL in human bone tissue [213]. At concentrations so much higher than physiological range it is unclear what percentage of released rhBMP2 is biologically active.

Release of orthobiologics from mineral coated devices also results in a high level of biological activity. Previous studies by our group have demonstrated the release of bioactive growth factors in vitro and in vivo from mineral coated devices [138,181,183,184]. Suarez-Gonzalez et al. released rhVEGF from β -TCP scaffolds in an ovine intramuscular model resulting in an increase in blood vessel ingrowth [183]. Lee et al. delivered bFGF from mineral-coated sutures in a chronic rotator cuff repair model in sheep, and the local release of bFGF improved the load at failure of the repaired tendon [137]. Lu et al., delivered mBMP from mineral-coated bioresorbable interference screws in a sheep tendon-bone healing model and demonstrated improved histologic scores of early tendon-bone healing [181]. Taken together, these studies and others suggest that the mechanism of growth factor-mineral binding and subsequent release maintains growth factor biological activity and serves as a simple and perhaps broadly applicable technique [138–140,143,184].

Growth factors released after encapsulation in synthetic polymers have been reported to stimulate a biological response, therefore suggesting the release of bioactive growth factors [214–217]. For example, Richardson et al., showed that rhVEGF and platelet derived growth factor released from PLG scaffolds stimulated angiogenesis [216]. TGF- β 1 released from a PLLA coating on titanium implants significantly improved biomechanical and histological aspects of fracture healing [194]. These examples show that poly(α -hydroxy ester) materials can release growth factors and influence tissue formation in vivo. This is perhaps surprising in view of studies that have reported concerns with bioactivity of growth factors released using this carrier system. Degradation of microspheres fabricated from poly(α -hydroxy-esters); the most frequently used synthetic polymers, result in acidic by products that can cause an inflammatory tissue response and denaturation/degradation of bioactive proteins [218,96]. Future studies may provide further insights into the stability of proteins encapsulated within, and released from, bioresorbable polymers.

5. Emerging directions

5.1. Relevance to other emerging molecular orthobiologics

Despite promise shown by strategies to improve bone growth factors' stability through chemical modification and biomaterial delivery strategies, challenges with narrow therapeutic indices, short half-lives, and narrow ranges of efficacious concentrations persist. Additionally, the recombinant DNA technology that has made many growth factor-based therapies more feasible does not always produce growth factors of equivalent bioactivity when compared to their naturally derived counterparts. This is particularly true for growth factors whose bioactivity is dependent on post-translational modifications. In light of these considerations, investigators have sought strategies for delivery of other classes of orthobiologics in addition to growth factors. Here we briefly describe other classes of orthobiologics used in delivery strategies.

5.1.1. Polynucleotides for gene delivery—In the early 1990s, investigators began exploring direct polynucleotide delivery for orthopedic applications as an alternative to bone

growth factor based therapies. In principle, polynucleotides in the form of plasmid DNA that encode growth factors can be delivered into target cells to induce local production of corresponding growth factor [219]. Compared to protein delivery, successful gene delivery can lead to sustained expression of physiological levels of growth factor, with authentic post-translational modification. This can potentially mitigate the need for supraphysiological dosages of recombinant growth factors, which are the major cause for the high cost and severe side effects observed in clinical studies [220]. In theory, in situ expression of bone growth factors would obviate many challenges associated with delivery of recombinant proteins. In addition, the growth factors commonly used as orthobiologics, such as BMP-2, VEGF, bFGF, and parathyroid hormone (PTH), could each be produced by cells through the delivery of their encoding genes [221–223].

Genes encoding bone growth factors must be delivered into target cells via vectors, which fall into the two broad classes of viral and non-viral vectors. Viral vectors are well known for their high efficiency of gene transfer to the cell [224]. The most commonly used viral vectors include adenovirus, retrovirus, lentivirus, and adeno-associated virus (AAV). Safety issues due to the potential for immune responses to viral gene delivery [225] and potential for insertional mutagenesis cause concerns that may limit broad clinical application [220]. However, measures can be taken to reduce safety concerns with viral vectors. Illustrating this concept, Musgrave et al. utilized a BMP-2 adenoviral vector to promote ectopic bone formation in both SCID and immunocompetent mice [226]. To extend on this concept, Okubo et al. also delivered a BMP-2 encoding gene and discovered that bone formation occurred, but immune-mediated clearance of transduced cells occurred before their beneficial effect could be realized in immunocompetent mice. They were able to circumvent this using transient immunosuppression [227]. Successful helper-virus strategies using vectors devoid of viral coding regions have shown potential for improving viral gene delivery safety [228], but viral transduction of pro-osteogenic genes in defect sites has yet to be realized in the clinic.

Investigators have also explored the use of non-viral polynucleotide vectors for gene delivery in orthopedic applications. For example, delivery of plasmid DNA encoding PTH via physical entrapment in a “gene activated matrix” resulted in sustained PTH expression for up to six weeks in a dog segmental bone defect. Remarkably, new bone on the scale of centimeters was successfully generated in the defect area [229]. Shea et al. reported that delivery of plasmid DNA encoding PDGF using a synthetic polymer matrix enhanced matrix deposition and vascularization in a rodent model [230]. Additionally, numerous non-viral vectors, such as liposomes, cationic polymers, calcium phosphates (nanoparticles and coatings) have been developed in an effort to achieve highly efficient non-viral gene delivery. However, the efficiency of non-viral vectors remains substantially lower than typical viral vectors [231]. However, substantial recent progress has been made to improve the efficiency of non-viral gene delivery by combining nanotechnology and mechanobiology, together with development of novel non-viral vectors [232–234]. With progress in both viral and non-viral gene delivery, the application of polynucleotides for gene delivery in orthopedic applications may soon become feasible in clinical settings.

5.1.2. Peptides—Peptides are another emergent therapeutic strategy in orthobiologics. Peptide-based orthobiologics are relatively short (<50 amino acids) sequences, often derived from intact growth factors and designed to mimic their biological functions [235]. These engineered peptides can mimic naturally occurring growth factors by binding to the appropriate receptor and promoting osteogenesis during defect repair [236,237]. Importantly, their lack of a complex tertiary structure addresses some of the aforementioned stability challenges that persist in protein delivery. For example, multiple forms of BMP-2-derived short peptides have demonstrated their capability to induce osteogenic differentiation both in vitro and in vivo [238]. In addition, the synthetic adaptability of peptides relative to proteins opens up new options for controlled delivery [239]. For example, Lee et al. engineered a VEGF mimicking peptide with a unique, high affinity HA binding sequence [236]. This peptide allowed for specific activation of VEGF-dependent endothelial cell proliferation and bound tightly to HA. A similar approach has now been used with BMP mimicking peptides and others. This form of modular peptide engineering has the potential for bioactivation of a range of orthopedic devices, as well as direct activation of native bone in the form of a defect site, autograft, or allograft.

5.1.3. Cytokines—Cytokines represent another category of orthobiologics that could be utilized as therapeutics for tissue healing such as bone fracture, ligament rupture and muscle injury. Cytokines are small nonstructural proteins secreted by inflammatory cells, such as degranulating platelets, macrophages, monocytes and lymphocytes during inflammation [240]. Important cytokines involved in tissue healing include IL-1, IL-4, IL-13 tumor necrosis factor- α (TNF- α), granulocyte colony-stimulating factor (GCSF) and macrophage colony-stimulating factor (MCSF) [241]. As host regulators of inflammation at the initial stage of tissue healing, cytokines play a critical regulatory role in the healing process by initiating related cascades, recruiting precursor cells and modulating immune responses [241]. For instance, IL-4 has been shown to activate connective tissue cells and stimulate ECM deposition [242]. Chamberlain et al. reported that treatment of a ligament wound with IL-4 significantly influenced ligament healing during the first week after injury [243]. Systemic administration has been the primary mode of cytokine delivery to date. However, cytokines cause considerable systemic toxicity, thus local delivery may be preferable in emerging studies to improve therapeutic performance [244,245]. Due to the similar physiochemical properties shared by growth factors and cytokines, the considerations for growth factor delivery, including the microenvironmental factors discussed throughout this review, are also applicable to the development of drug delivery systems for cytokines.

5.2. Future directions

The vast majority of drug release studies are performed in in vitro conditions designed to mimic some limited aspects of the in vivo environment. Classical settings for drug release use a near physiologic temperature of 37 °C, near physiologic pH of 7.40, and ion concentrations similar to those in blood plasma (SBF, e.g.) [246]. While these conditions can provide a convenient framework to compare results obtained from different studies, they fail to represent critical characteristics of the in vivo environment. For example, the pH of bone defects at initial stages is usually low due to inflammation, which is likely to change the release kinetics of growth factors from various delivery systems [88]. In addition, most

orthopedic implants directly contact blood and other complex biological fluids once implanted, and the release medium in vivo then becomes more complex due to the presence of serum proteins [247]. Studies from our group and others indicate that in vitro release media containing proteins can significantly influence growth factor release kinetics. For example, the presence of albumin concentrations that mimic serum amounts can significantly increase the VEGF release rate. The high complexity of growth factor release environments in vivo coupled with the influence of even simple parameters on growth factor release call for development of in vitro release environments that more closely mimic wound sites.

Another challenge for biologics delivery is maintaining bioactivity of these factors. While a substantial emphasis of growth factor release studies to date has been controlling pharmacokinetics, perhaps the more significant challenge is maintaining long term growth factor stability. Owing to their complex tertiary structure, growth factors can lose their function readily due to denaturation and associated aggregation. Denaturation can occur even more readily in response to environmental parameters such as changes in pH, temperature and solvent characteristics either during formulation or delivery [248]. Therefore, novel strategies that efficiently stabilize proteins within a carrier during these delivery systems could substantially improve the current performance of protein-based therapeutics, including bone growth factor delivery strategies. As the fundamental mechanisms causing protein denaturation have been gradually identified, corresponding protein stabilization strategies have been developed. For example, enhanced protein stability in sugar-glass nanoparticles indicates protein stabilization may be achieved by carefully controlling the microenvironment of the entrapped protein [249]. In another example, discovery of protein aggregation caused by microclimate pH changes in bioresorbable polymers have led to protein stabilization strategies that neutralize the local pH surrounding the protein during delivery [250]. These new trends in protein delivery may be translated into clinically applicable devices offering superior performance in the future.

Development of multi-component delivery systems allowing local, sustained release of multiple proteins also remains an active area of interest. While pro-osteogenic factors are of particular importance for orthopedics as they can actively promote bone formation, the presence of anti-inflammatory factors and antibiotics is also important for the success of orthopedic implants, particularly in polytrauma scenarios. For example, delivery of anti-inflammatory cytokines during the inflammatory phase of wound healing, followed by bone growth factor delivery might shorten the inflammation stage and accelerate the bone healing progress [251]. In addition, infection is still considered a primary reason for joint replacement failure, as 12% of patients receive revision replacement due to surgical or implant-related infection [252]. Thus, there is a strong need for sustained, local release of antibiotics through the process of bone healing. Delivery of multiple biologics with independent control of location, dose, and timing may be highly desirable in the context of musculoskeletal disease and trauma.

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Abbreviations

AAV	adeno-associated virus
BMPs	bone morphogenetic proteins
BSA	bovine serum albumin
β-TCP	β -tricalcium phosphate
CaP	calcium phosphate
DBM	demineralized bone matrix
DCM	dichloromethane
ECM	extracellular matrix
FBGCs	foreign body giant cells
FGFs	fibroblast growth factors
GAG	glycosaminoglycan
GF	growth factor
GFR	growth factor receptors
HA	hydroxyapatite
HAC	hyaluronic acid-catechol
HepC	heparin-catechol
IGF-1	insulin like growth factor 1
IL-1	interleukin-1
IL-4	interleukin-4
MMPs	matrix metalloproteinases
M-CSF	macrophage colony-stimulating factor
mBMP	mineral binding bone morphogenetic peptide
mSBF	modified simulated body fluid
PDGFs	platelet-derived growth factors
PG	proteoglycans
PLGA	poly (lactic-co-glycolic acid)
PEG	poly (ethylene glycol)
PLA	poly (lactic acid)

PCL	poly (ϵ -Caprolactone)
PTH	parathyroid hormone
RANKL	receptor activator of NF κ B ligand
RGD	Arginylglycylaspartic acid
TGF-β	transforming growth factor- β
TNF-α	tumor necrosis factor- α
VEGF	vascular endothelial growth factor
W/O/W	water-in-oil-in-water

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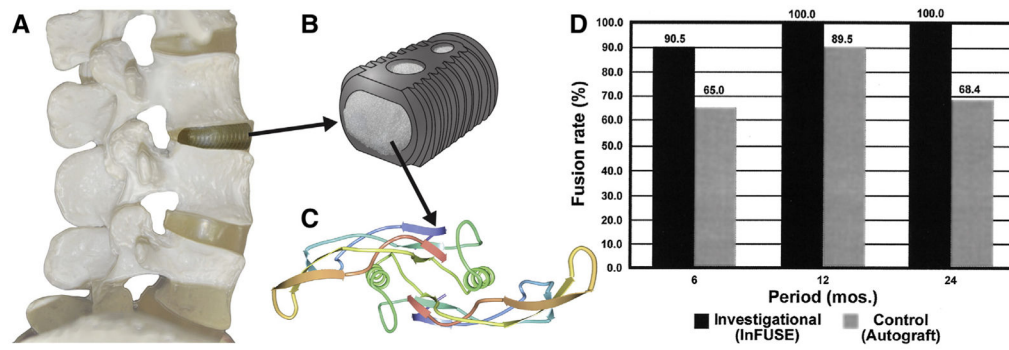


Fig. 1.

Application of InFUSE™ bone grafts: A) Implantation of a lumbar interbody fusion device for spinal fusion B) a lumbar interbody tapered fusion cage representation of the InFUSE™ bone graft from Medtronic C) Crystal structure of BMP-2 (modified from www.pdb.org) D) Comparison of postoperative fusion outcomes in the investigational group (InFUSE™ Bone Graft) and the control group (iliac crest autograft) [6]. Reproduced with permission.

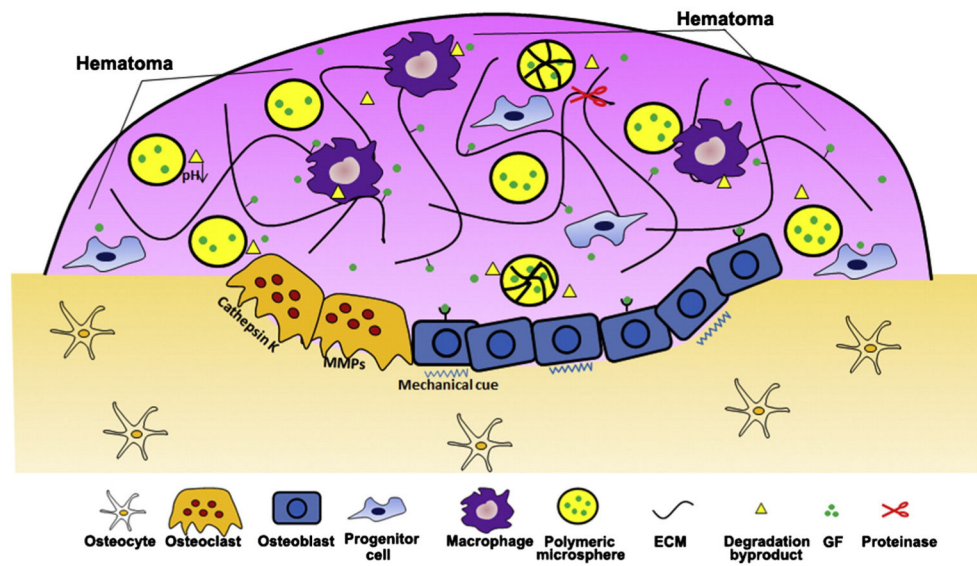


Fig. 2. Various aspects of dynamic microenvironment can influence the delivery of bone growth factor during bone healing. Osteoblasts and osteoclast directly participate in the catabolic and anabolic processes of bone formation. The carrier of the delivery system can directly control the release kinetics of the growth factors. pH at the bone defect sites can affect growth factor release by changing the degradation rate of the carrier. The presentation of growth factors to target cells is also modulated by ECM while ECM components is consistently remodeled by inflammatory and other types of cells via secretion of proteases such as MMPs. Besides, mechanical stimulus also play an importantly during the whole bone regeneration process.

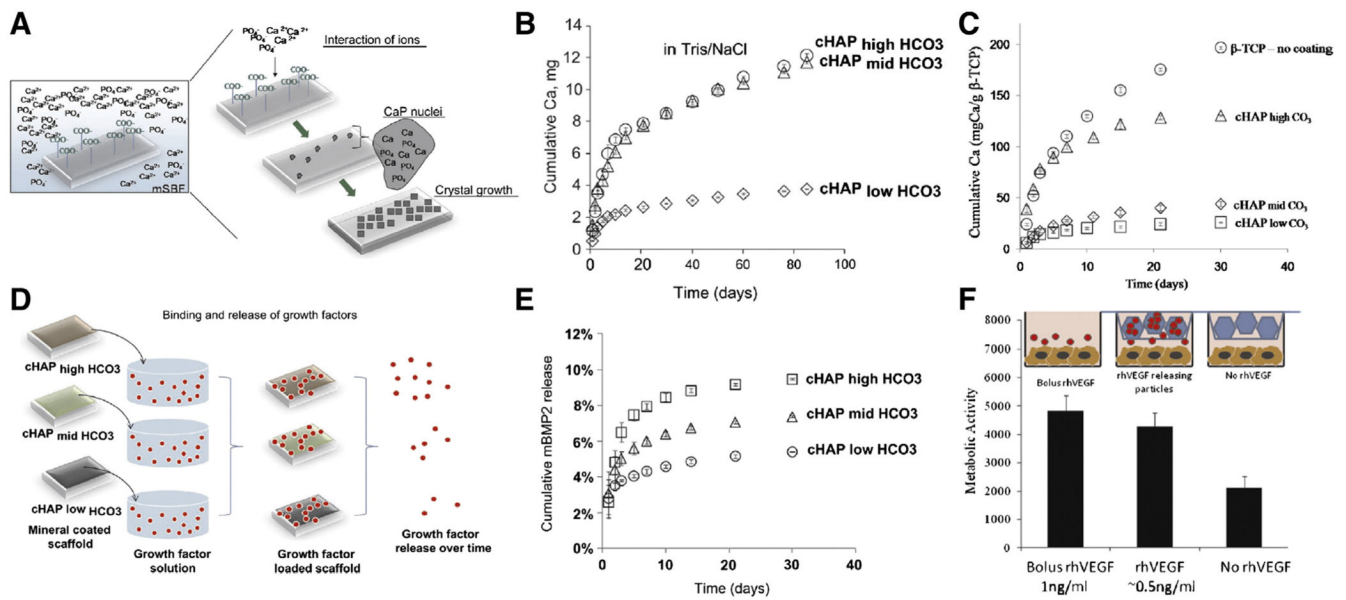


Fig. 3. Controlled release of growth factors via mineral coated based delivery systems. A) Schematic of mineral coating formation process using mSBF. B) Mineral coating stability was affected by the extent of carbonate substitution in mineral coating during coating formation. C) Formation of mineral coating on β-TCP surface helped slow down the dissolution of the materials. D) Schematic of release and binding mechanism of growth factors to mineral coating. With higher carbonate concentration, the amount of growth factor released is greater over time. E) The release kinetics of VEGF mimic was controlled by the dissolution rate of mineral coating. F) VEGF bound on mineral coating fully preserved its bioactivity [138,140]. Reproduced with permission.

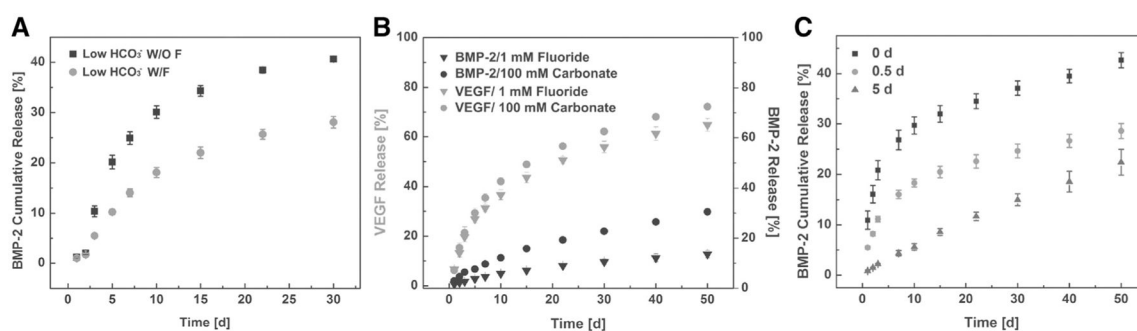


Fig. 4. Multiple GF release via multilayer mineral coating with tuned stability: A) Incorporation of fluoride into mineral coating decelerated the dissolution of mineral coating. B) Dual release of BMP-2/VEGF was achieved by using multilayered microparticles. BMP-2 was released in a sustained manner while VEGF was liberated from mineral coating relatively faster. C) Control over BMP-2 release by changing the coating thickness on the microparticles [142]. Reproduced with permission.

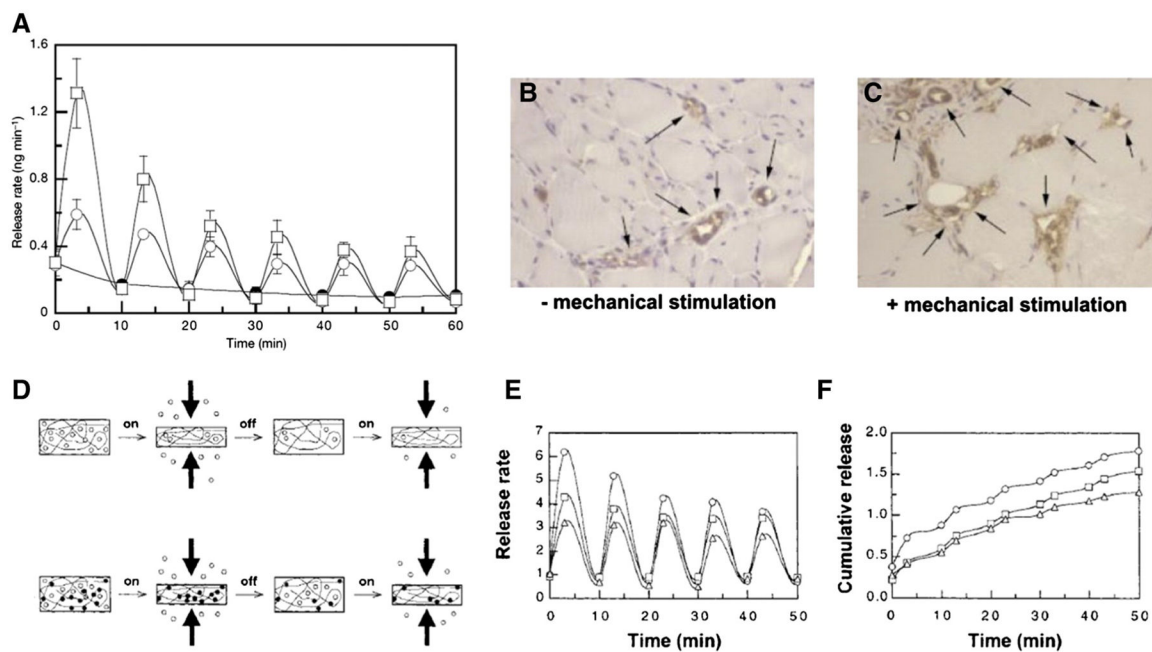


Fig. 5. Mechanical stimulation triggered growth factor release: A) Release rate of VEGF from alginate hydrogels under 10% (open circle) and 25% (open squares) strain amplitude, and with no compression (filled circles) as a control. In vivo response to VEGF-loaded hydrogels implanted into femoral artery ligation site: B) without mechanical stimulation, and C) with mechanical stimulation, D) Schematic of anticipated drug release from polymeric matrices under mechanical loading: open circle, free drug; filled circle, bound drug. In vitro release behaviors of trypan blue (triangle), methylene blue (square), and VEGF (circle) from alginate hydrogels under mechanical stimulus: E) in vitro release rate, and F) Cumulative release. Reproduced with permission [169,170].

Table 1

Summary of various dynamic factors influencing growth factor delivery in pathophysiological contexts.

Factor	Components	Influence on GFs delivery	Reference
ECMs	Proteoglycans, fibrin, collagen, fibronectin.	Directly binds to GFs via affinity interactions; augment GFs activity through synergistic effect with ECM; cooperatively work with integrins for activation signaling pathways	[33,45–47,57,58]
pH	Natural environment, engineering environment, pH responsive delivery systems	Change GFs release kinetics by changing carrier degradation; influence GFs bioactivity (protein aggregates)	[77,78,97]
GF carrier	Carrier materials, carrier degradation, enzyme-mediated degradation	Primary mechanism to control GF release rate; immobilize GF on carriers; cause damage to GFs due to the degradation byproducts	[85,105,111,116]
Surrounding cells	Macrophages, platelets, osteoclasts	Clearance of GF carriers via phagocytosis; formation of hematoma; participate in carrier degradation and resorption	[48,146,155,159,163]
Biomechanics	Mechanical stimuli to tissues and GF carrier systems	Mechanical loading increases GF release during healing; deformation of GF carrier by pressing causes GF release	[167,169,171]