



Biomaterials for the Delivery of Growth Factors and Other Therapeutic Agents in Tissue Engineering Approaches to Bone Regeneration

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Bone fracture followed by delayed or non-union typically requires bone graft intervention. Autologous bone grafts remain the clinical “gold standard”. Recently, synthetic bone grafts such as Medtronic’s Infuse Bone Graft have opened the possibility to pharmacological and tissue engineering strategies to bone repair following fracture. This clinically-available strategy uses an absorbable collagen sponge as a carrier material for recombinant human bone morphogenetic protein 2 (rhBMP-2) and a similar strategy has been employed by Stryker with BMP-7, also known as osteogenic protein-1 (OP-1). A key advantage to this approach is its “off-the-shelf” nature, but there are clear drawbacks to these products such as edema, inflammation, and ectopic bone growth. While there are clinical challenges associated with a lack of controlled release of rhBMP-2 and OP-1, these are among the first clinical examples to wed understanding of biological principles with biochemical production of proteins and pharmacological principles to promote tissue regeneration (known as regenerative pharmacology). After considering the clinical challenges with such synthetic bone grafts, this review considers the various biomaterial carriers under investigation to promote bone regeneration. This is followed by a survey of the literature where various pharmacological approaches and molecular targets are considered as future strategies to promote more rapid and mature bone regeneration. From the review, it should be clear that pharmacological understanding is a key aspect to developing these strategies.

Keywords: RNA interference, bone morphogenetic protein 2 (BMP-2), collagen, keratin, gelatin, fibrin, chitosan, regenerative medicine

CLINICAL SIGNIFICANCE AND CURRENT CLINICAL STRATEGIES

Bone tissue is a dynamic system which is continuously being remodeled on a day-to-day basis (Barrett, 2010). Because of this property, the normal healing process typically restores the biological and mechanical function of bone following fracture. Unfortunately, the native healing potential of bone is occasionally insufficient for underlying reasons such as smoking (Patel et al., 2013; Taormina et al., 2014), malnutrition (Alvear et al., 1986), congenital disease (Shah et al., 2012),

or large defects resulting from tumor resections (Qu et al., 2015). In addition, healthy individuals sometimes experience the inability for bone healing and a return to normal function due to large defects caused by trauma. Bone defects that surpass a size that can spontaneously heal during the lifetime of the individual are known as defects of critical size or critically-sized defects (Schmitz and Hollinger, 1986; Spicer et al., 2012), and are a specific type of non-union. These critically-sized defects frequently lead to secondary complications including morbidity and functional limitations in patients (Tseng et al., 2008). It is estimated that 10% of bone fractures in the United States result in impaired or incomplete healing known as delayed union and non-union, respectively (BMUS, 2014). Lack of osteogenic cells, signaling molecules, and osteoconductive matrix as well as inadequate vascularity all contribute to the inability of bone to heal in these situations (Harwood et al., 2010).

Clearly, bone defects leading to non-union substantially impair activities on a daily basis and impair quality of life. In order for a large defect or other non-union to be functionally restored, a surgical intervention and placement of a bone graft at the injury site is required to attempt to bridge the defect area. Bone grafts are currently the second most common type of tissue transplant in the United States with an estimated 500,000–1.5 million bone-grafting procedures performed yearly. As such, the market in the U.S. alone is ~\$1.6–\$2.5 billion (Cutter and Mehrara, 2006; Bishop and Einhorn, 2007). Thus, from both a quality of life and an economic perspective, bone grafts that lead to functional restoration following fracture are beneficial.

According to the American Academy of Orthopedic Surgeons the “ideal bone graft” should be “biocompatible, bioresorbable, osteoconductive, osteoinductive, structurally similar to bone, easy to use, and cost-effective” (Greenwald et al., 2001). Osteoconductive materials support bone healing through vascularization, architecture, chemical composition (e.g., calcium sulfate, calcium phosphate, calcium hydroxyapatite, etc.), and surface charge (Urist, 1980). Osteoinduction refers to the process that supports the migration and proliferation of mesenchymal stem cells as well as promoting differentiation of preosteocytes (Urist and Strates, 1971). When designing biomaterials for bone applications a number of factors (i.e., composition, porosity, mechanical properties) must be considered in order to recapitulate native bone. Structural and mechanical property differences between cortical and cancellous bone can be quite significant and must be considered when applying engineered bone substitutes to specific locations. For example, cortical porosity ranges from 5 to 10% while cancellous bone porosity ranges from 75 to 90%. The pore size of areas of native cortical and cancellous bone also differ dramatically (10–600 μm ; Polo-Corrales et al., 2014); however, it is suggested that the optimal range lies between 200 and 350 μm (Murphy et al., 2010; Guda et al., 2014). The scaffold pore size and shape has been shown to significantly modulate osteogenesis (Hulbert et al., 1970; Sanzana et al., 2014) while a heterogeneous distribution of pore size enhances osteogenic potential (Woodard et al., 2007; Di Luca et al., 2016). The mechanical properties of bone also vary widely over orders of magnitude (Polo-Corrales et al., 2014). Taken together, researchers must be vigilant

of their scaffold’s design and resulting application in the body.

The primary approaches to bone grafting include autografts, allografts, and (more recently) what are referred to as synthetic bone grafts. Below, we briefly discuss some of the drawbacks of autografts and allografts that have led to the push for “off-the-shelf” synthetic bone grafts and some of the ongoing needs in the development of these constructs. We then identify existing and promising strategies to improve bone regeneration via pharmacological approaches, and these strategies are summarized in **Figure 1**.

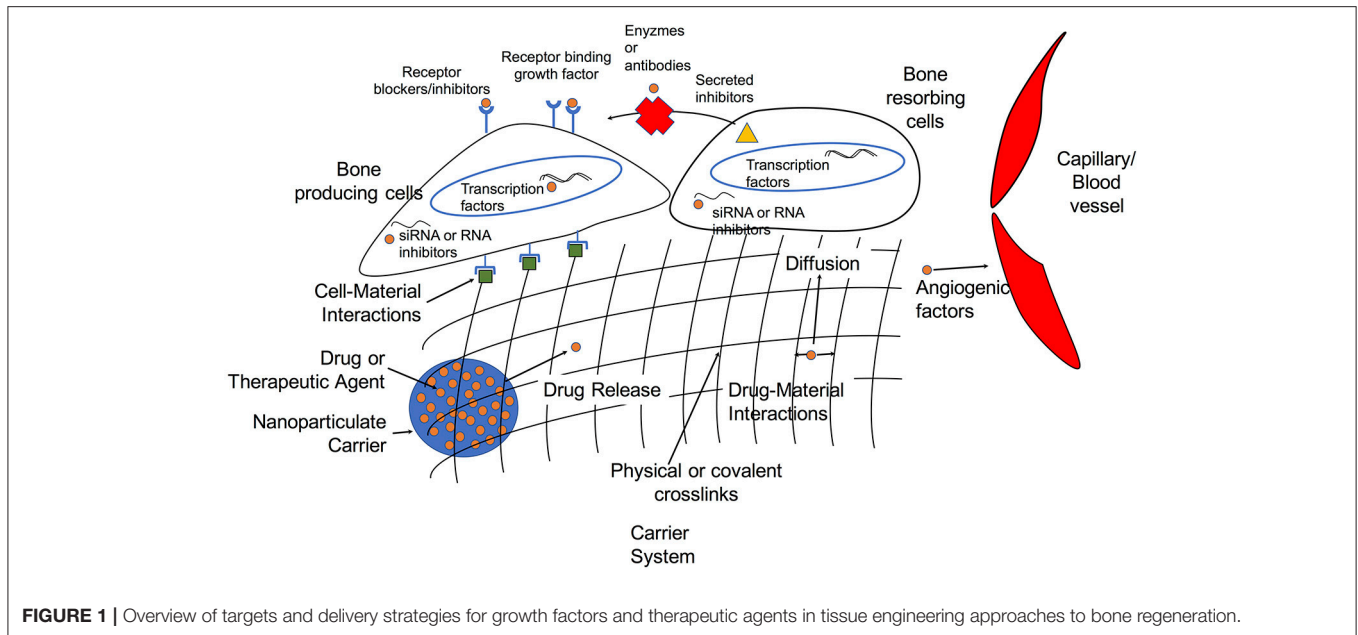
Autografts, Allografts, and Xenografts

Autologous bone grafts remain the clinical “gold standard” for bone defects because of their osteoconductive and osteoinductive properties. Autografts are most often harvested from the iliac crest or other bone (Greenwald et al., 2001). Even though autograft transplantation does not lead to immunogenic response, it has a limited supply, donor site morbidity, and up to 30% failure rate (Schoelles et al., 2005). One solution to overcoming limited supply and donor site morbidity of autologous bone grafts is to use allogeneic or xenogeneic bone sources.

Xenografts are primarily harvested from bovine origins and are osteoinductive/conductive. Research stemming from clinical trials has determined xenografts, primarily from bovine origins, to be unsuitable due to increased risk of infection, immunogenicity, and host rejection (Trice, 2009; Mehta et al., 2012).

Allografts harvested from cadaveric sources have the advantage of being osteoconductive and osteoinductive while being available through regional tissue banks in a variety of sizes and shapes. Again there can be limited supply, and allograft treatments are associated with risk of rejection and transmission of infectious disease (Grover et al., 2011). While tissue processing and sterilization through freezing or 25 kGy gamma irradiation virtually eliminates the possibility of disease transmission, irradiation causes a number of adverse effects on tissue properties such as weakening (Nguyen et al., 2007).

Demineralized bone matrix (DBM) products derived from allogeneic sources once comprised ~50% of the bone graft market (Cutter and Mehrara, 2006) but since the emergence of a number of FDA-approved synthetic bone grafts (see below), DBM now accounts for ~20% of the grafting market (Gruskin et al., 2012). The osteogenic potential of commercial DBM, however, is highly variable due differences in the amounts of osteogenic molecules (e.g., BMP-2 and BMP-7; Bae et al., 2010) likely due to variability between donors, sterilization techniques, and storage methods (Wang et al., 2007). Non-standardized production and lack of precise control conditions needed for storage to maintain bioactivity has resulted in inconsistent results with different products as well as product lot numbers (Wang et al., 2007; Gruskin et al., 2012). In essence, although the constituents are known, DBM is an undefined material. For this reason, the use of synthetic bone grafts as a tissue engineering approach has become an important consideration for clinical treatment of bone defects and other orthopedic applications.



Biomedical Materials as Bone Fillers

In the next section, the use of what are referred to as synthetic bone grafts are discussed. As used in the literature, “synthetic bone graft” is a bit of a misnomer. A synthetic bone graft need not be synthetic. More specifically, collagen is the most commonly used material component. Synthetic bone grafts also rely on the use of osteogenic molecules to promote bone growth.

Here, however, we briefly discuss some biomedical materials that have been used as bone grafts, and in this context they are often referred to as bone fillers. Common advantages to the use of such materials are that they, like synthetic bone grafts, are off-the-shelf products that can be manufactured in a repeatable manner and in sufficient quantities. These materials may or may not be osteoconductive and while they can promote bone tissue formation, they do not have the same degree of osteoinductive or osteogenic behavior as molecules such as bone morphogenetic protein 2 (BMP-2). A common feature, though, among these materials is they are traditional biomedical devices that do not achieve their primary function through chemical action (i.e., they are not drugs).

Ceramics

Examples of ceramic bone fillers include Bioglass, calcium phosphate, calcium sulfate, and hydroxyapatite. In general, an advantage of ceramic materials is their similar material and mechanical properties to the mineral composition of bone. Indeed, hydroxyapatite is the main inorganic constituent of bone, so its use as a biomedical material is logical.

More recently, ceramics have been used as composites with natural polymers in an effort to obtain the beneficial properties of each material. For example, Bioglass in conjunction with gelatin led to mechanical strength but with increased pore size (Aksakal and Demirel, 2017). Likewise, the use of Bioglass with a tricalcium phosphate/alkylene oxide copolymer or tricalcium

phosphate have been used in a large animal model as a moldable material for conformation to defect geometry to promote both intraosseous and intramuscular bone formation (Barbieri et al., 2017). Ceramic materials can also be used for the delivery of antibiotics to prevent infection (see further below on synthetic polymers) (Kanellakopoulou and Giamarellos-Bourboulis, 2000; Li and Chang, 2005).

Polymeric Materials

One of the most well-known bone filler materials is poly(methylmethacrylate) or pMMA, also known as bone cement. pMMA is a non-biodegradable material and can be considered a bone filler in the truest sense of the word. That is, a major drawback to the use of pMMA is its non-degradable nature, which is known to impede bone remodeling (Freeman et al., 1982; Jensen et al., 1991), likely through an unfavorable cellular microenvironment (Maloney et al., 1990). Like other polymers pMMA also suffers from poor mechanical properties (Saha and Pal, 1984). As noted above for some ceramic materials, one clinical use for synthetic polymers such as pMMA is for the delivery of antibiotics to prevent infection. A significant challenge for fractures requiring open reduction is bacterial infection (Seekamp et al., 2000). As such, the ability to deliver gentamicin (Klemm, 1993; Shi et al., 2006), vancomycin (Zelken et al., 2007; Li et al., 2010), and other antibiotics (Efstathopoulos et al., 2008; Shi et al., 2010) is often of considerable importance.

Biodegradable natural and synthetic polymers have been used as bone fillers, but their mechanical properties are not advantageous. Synthetic biodegradable polymers and natural polymers are widely used for synthetic bone grafts at the clinical and pre-clinical level, as discussed in the following section. However, a growing use for biodegradable polymers such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA), copolymers of PGA and PLA (PLGA), polycaprolactone (PCL) as well

as natural and non-degradable polymers is in 3-D printing technology (Taboas et al., 2003; Hollister et al., 2005, 2015). 3-D approaches allow for the presentation of well-defined pore sizes and organizational structures that can be optimized to promote favorable cellular response.

To date, bone formation obtained by bone fillers such as those described above simply do not achieve the levels of bone formation obtained from autografts. This unmet need has led to investigation of the use of the synthetic bone grafts that make use of some of the principles of tissue engineering such as knowledge of the growth factors responsible for bone formation.

Synthetic Bone Grafts: A Tissue Engineering Approach to Bone Regeneration

Tissue engineering can be considered to consist of the triad of biomaterial scaffolds, cells, and signals (either chemical/molecular or physical/mechanical; Zerhouni, 2005). To address the disadvantages of ceramic and polymer-based bone substitutes, tissue engineering techniques have been applied to develop a number of growth factor-based products. These approaches typically consist of a collagen sponge as the biomaterial and recruit endogenous cells to the defect site. These strategies also consist of molecular signals in the form of growth factors/morphogenetic proteins. While bone formation and regeneration is controlled by a cascade of molecules delivered at precise locations and times, only two commercially produced growth factors have been FDA approved for clinical use: bone morphogenetic protein-2 (BMP-2) and bone morphogenetic protein-7 (BMP-7), which is also known as osteogenic protein-1 (OP-1).

BMPs primarily act as cytokines mediating the differentiation of mesenchymal cells into cartilage and bone forming cells (Ebara and Nakayama, 2002). The functions of various BMP molecules have been described both in general (Wang et al., 2014) and in skeletal tissue (Rahman et al., 2015; Wu et al., 2016). In particular, BMP-2 and -7 (OP-1) have been identified as playing critical roles in bone formation and healing by their ability to induce osteoblast differentiation (Spector et al., 2001). The genetic sequence of BMP was first identified in 1988 which subsequently allowed for the commercial production of various BMPs through the use of recombinant gene technology (Wozney et al., 1988). This marked a notable increase in the use of BMPs in bone tissue engineering. BMP-2 and BMP-7 are highly osteoinductive growth factors and their adsorption onto osteoconductive carriers such as collagen is based on difficulties associated with the efficacy of solubilized rhBMP-2 such as a short half-life (Yamamoto et al., 2003) and up-regulation of receptor-binding antagonists (Ebara and Nakayama, 2002).

Given that collagen is the main organic component of bone, is osteoconductive, and can support mineralization and cell ingrowth it is reasonable that DBM (Niederwanger and Urist, 1996) or absorbable collagen sponges (ACS) (Nevins et al., 1996) were among the first carriers for BMP-2. Absorbable collagen sponges (ACS) and collagen particles were the first generation of tissue-engineered products on the market with FDA approval

(McKay et al., 2007). Initial clinical trials conducted with rhBMP carriers such as collagen for spinal fusion applications were not only successful but outperformed autologous bone grafts (Schimandle et al., 1995).

Like many first-generation products there have been a number of drawbacks to rhBMP-2 collagen carriers. Positioning of the ACS is often difficult and secondary displacement of the collagen sponge has been reported (Schmidmaier et al., 2007). Collagen is quickly degraded *in vivo*, causing voids to develop within the new bone matrix due to the inability of collagen to provide a structure which lasts long enough for cell migration (Friess et al., 1999). Perhaps the main issue associated with the ACS is an initial burst release of rhBMP-2 into the local environment, leading to heterotrophic ossification (Brown et al., 2011). Another side effect of this burst release is the activation of osteoclasts in the surrounding environment at high BMP-2 concentrations (Okamoto et al., 2006; Suliman et al., 2015), which may lead to bone resorption. Surrounding mesenchymal lineage progenitor cells in adjacent musculature also receive rhBMP-2 dosage, leading to differentiation into osteoblasts and subsequent mineral deposition in muscle tissue (Katagiri et al., 1994).

This burst release is a cause for concern since the current clinical modalities use 10- to 1,000-fold higher concentrations than that of native BMP-2 levels found in the body (Vaibhav et al., 2007). Therefore, novel methods of delivery and also alternative molecular targets continue to receive attention at the pre-clinical level. Below, we discuss several classes and types of alternative carrier systems being investigated. We then consider therapeutic strategies beyond the existing rhBMP-2 paradigm.

ALTERNATIVE BIOMATERIAL CARRIERS FOR MOLECULES PROMOTE BONE REGENERATION

Great effort has been placed on developing biomimetic scaffolds which provide a three-dimensional matrix for cell migration and proliferation. Although modern biomaterials lack the temporal and spatial complexity to fully mimic the native extracellular matrix (ECM) (Daley et al., 2008), they capture many of the essential elements. Through their mechanical, material, and chemical cues, biomaterials are able to influence a number of cellular functions (e.g., cell-matrix interactions) including proliferation, differentiation, apoptosis, and signaling (Daley et al., 2008). Ideally, these biomaterials should not only be non-toxic but also biodegradable so that the artificial matrix will degrade over time and give way to new tissue being formed at the site where the material was implanted. The degradation/resorption of the scaffold should be tailored to the rate of bone turnover and healing rates of the injured bone. For example, spinal fusion cases can require 9 months whereas craniofacial application are of a shorter (3–6 months) duration (Bose et al., 2012). For this reason, we focus here on engineered degradable polymers which are capable of degradation rates ranging from weeks to months.

In addition to their role in mimicking the structural aspects of the ECM, biomaterial carriers can also be used to promote

the controlled release of therapeutic agents. Bioactive molecules and therapeutics can be incorporated or physically entrapped within the polymer network. One important design parameter to regulate carrier degradation as well as rhBMP-2 release is to modify the mesh size of the polymer network through the crosslink density. When the overall crosslink density of a hydrogel network is increased, it decreases the molecular distance between crosslinks, resulting in a smaller hydrogel mesh size (Lowman and Peppas, 1999). This in turn lowers the diffusivity of the therapeutic agent, allowing tunable rates of release to be achieved by varying the crosslink density of a hydrogel (Saltzman, 2001). This is one approach used in many hydrogel systems since it is the simplest method for successful encapsulation of rhBMP-2. The use of electrospinning nanofibers in which the growth factor is embedded within the fibers employs a similar strategy (Li et al., 2006). A burst release is often observed (Huang and Brazel, 2001) followed by release of the therapeutic agent via some combination of diffusion and material degradation. In cases where there is minimal interaction between the therapeutic agent and the carrier, first order release is common (Saltzman, 2001).

While hydrophobic, van der Waals, and electrostatic interactions between the material and growth factor as well as entrapment in the polymer network are inherently present, additional molecules can be introduced into the biomaterial to retard the rate of release. Examples of these immobilization methods include affinity binding such as heparin-binding (Yang et al., 2010, 2012; Jeon et al., 2011; Wang et al., 2013), ionic interactions such as those provided by chondroitin sulfate (Wang Y. et al., 2010; Bae et al., 2012), cyclodextrins (Del Rosario et al., 2015), protease degradable tethers (Tokatlian et al., 2010), succinylation (Tsujiigiwa et al., 2006), alkylation (Tachibana et al., 2006; Han et al., 2015), and even covalent conjugation (Shen et al., 2009; Zhang et al., 2010).

In addition to direct modification of the material, rhBMP-2 can be encapsulated via micro, macro, and nanoparticles alone (White et al., 2013) or within these particles embedded within a scaffold (Park et al., 2009). In this way, rhBMP-2 release is regulated by the rate of particle degradation. When rhBMP-2 microspheres are entrapped in a hydrogel network they retain rhBMP-2 for significantly longer periods of time (compared to adsorption) resulting in prolonged bioactivity (Kempen et al., 2008). Synthetic polymers are commonly used for this purpose due to their predictable rates of degradation and release (Brown et al., 2011; Hernandez et al., 2012). Natural polymers such as chitosan (Niu et al., 2009; Hou et al., 2012; Cao et al., 2014), alginate (Abbah et al., 2013), and gelatin (Patel et al., 2008) are being increasingly used for rhBMP-2 microsphere encapsulation. In summary, examination of key parameters noted above (material network properties, material modification, and encapsulation) associated with rhBMP-2 release has allowed for the development of systems that approach zero-order release in some cases (Pillay et al., 2005).

Synthetic Materials for Bone Regeneration

Synthetic polymers have the advantage of being able to be manufactured in large quantities and customizable to show a wide range of material properties. A number of biodegradable

synthetic polymers are already FDA-approved for use in humans. Poly(a-hydroxy acids); poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and poly(lactic-co-glycolide) (PLGA) (Sokolosky-Papkov et al., 2007) have been used for decades in orthopedic applications (Puska et al., 2011; Razak et al., 2012). These polymers undergo bulk degradation by hydrolysis of which the rate of degradation can be tailored to the demands of the tissue. Their degradation products, lactic acid and glycolic acid, can be processed and excreted from the body. Unfortunately, these components can decrease the pH of the local environment (Suganuma and Alexander, 1993). Synthetic polymers have been reported to cause immunogenicity and toxicity due to chemical crosslinkers and polymerizers used in their production (Athanasidou et al., 1996; Williams et al., 2005). Many of these polymers, especially the FDA-approved polymers, are hydrophobic and lack cell-binding domains such as the arginine:glycine:aspartic acid (RGD) sequence, thus hindering cell attachment (Pierschbacher and Ruoslahti, 1984; Lieb et al., 2003).

Fortunately, RGD and other cell-binding domains can be incorporated into synthetic materials by incorporation of chemically labile groups in the polymer (Hersel et al., 2003). Synthetics can also be tuned for their rates of degradation (Ifkovits et al., 2009; Qiu et al., 2011), release of therapeutic agents (Ashley et al., 2013), and mechanical properties (Chiou et al., 1996; Martello et al., 2014). These materials can also be mixed with calcium phosphates (e.g., hydroxyapatite) (Pathi et al., 2010) or natural polymers (e.g., collagen) (Ochi et al., 2003; Niu et al., 2011) to enhance cell attachment and osteoconductivity. For synthetic polymers to achieve both osteoconductive and osteoinductive properties they must be a combination product composed of the synthetic polymer, calcium phosphate, a natural polymer or cell binding motif, and osteogenic growth factor (Niu et al., 2009; Lu et al., 2012).

Natural Materials for Bone Regeneration

Interest in natural polymers has been increasing due to a number of beneficial properties such as low cytotoxicity, favorable degradation byproducts, low immunogenicity, and similarity to the ECM. Living organisms synthesize a number of macromolecular components which a biological environment can recognize and degrade hydrolytically or metabolically (Mano et al., 2007). Polysaccharides (e.g., alginate, chitosan) and proteins (e.g., collagen, silk, fibrin, and keratin) are extracted from renewable plant (Plowman et al., 2010), algae (Percival, 1979), animal (Zhang et al., 2006; Plowman et al., 2010), or human (Mosesson and Sherry, 1966; Reichl, 2009) resources. Here, we briefly discuss several promising carrier systems for rhBMP-2 or other molecular therapeutics as discussed below.

Fibrinogen/Fibrin

Fibrin is a fibrous protein involved in hemostasis when soluble fibrinogen (340 kDa molecular weight) is polymerized in the presence of the enzyme thrombin activated in response to injury. Orthopedic surgeons have long appreciated the significantly positive effect blood clots (which include fibrin) have on bone regeneration. Even though fibrin is not a regular component of

the uninjured ECM, it serves as a temporary matrix for wounded tissue until remodeling can replace it with tissue-specific ECM (Clark et al., 1982). Not only does fibrin provide structure, but the material alone has been reported to induce osteogenic differentiation (Martino et al., 2009) and angiogenesis (Feng et al., 2013). Thrombin-mediated release of fibrinopeptides A and B (Profumo et al., 2003) attracts inflammatory cell migration to the site of injury (Senior et al., 1986) and induces cell proliferation (Sporn et al., 1995). Fibrin has numerous binding sites for not only cells, but for growth factors and other ECM components as well. Fibrin sequesters a multitude of growth factors which are essential for bone regeneration including FGF, VEGF, PDGF, IGF, and the TGF- β superfamily including BMP-2 (Martino and Hubbell, 2010). Thus, fibrin ultimately also provides a repository of signaling cues to direct cell behavior (Brown and Barker, 2014).

Fibrin is degraded (via fibrinolysis) into nontoxic components by the serine protease plasmin, which is initiated by the coagulation cascade. The rate of degradation depends on the fibrin fiber thickness and density; a fibrin network comprised of dense thin fibers degrades much more slowly than a network of thick loose fibers (Weisel, 2007). Fiber thickness has also been shown to play a large role in cell-specific signaling molecule expression (Shats et al., 1997). Fibrin material properties can be altered through polymerization by adjusting the concentrations of fibrinogen, thrombin, and Ca^{2+} /salt concentrations (Cox et al., 2004). In general, increasing fibrinogen concentration leads to increasingly dense plug formation. However, care must be taken not to make the hydrogel too dense or else it will inhibit cell migration (Karp et al., 2004). Decreasing thrombin concentration leads to increased material modulus, ultimate tensile strength, and increasing fibrin fiber diameters (Rowe et al., 2007). Sodium chloride concentration has an effect on hydrogel compressive modulus and fiber diameter, which in turn affects MSC differentiation and alkaline phosphatase expression (Davis et al., 2011). With all of these factors in mind, fibrin biomaterials have been used for decades for cell and biomolecule delivery (Rajangam and An, 2013).

Fibrin is FDA approved as a wound sealant, and clinically, fibrin sealants have been mixed with hydroxyapatite for craniofacial applications (Le Guehennec et al., 2004). Isoelectric points of fibrinogen (pH 5.5) and fibrin (pH 5.6) (Oka, 1981) would cause fibrin to have negative surface charge at the neutral pH, which would allow for rhBMP-2 “entrapment” and adsorption of nucleic acid complexes (Saul et al., 2007).

Keratin

Keratins are intermediate filament proteins and intermediate filament-associated proteins, which are widely found in nature and are best known as being the structural proteins referred to as hard or soft keratins. Wool and human hair are commonly used as sources for biomaterials due to their availability, renewable source, low toxicity, ease of sterilization, and ability to be hydrolytically degraded. Keratins can be processed into a number of biomaterials such as electrospun fibers, (Xing et al., 2010), scaffolds (Tachibana et al., 2002; Srinivasan et al., 2010), and films (Yamauchi et al., 2003). One salient feature of keratins is the high number of cysteine residues, which can form disulfide

bonds. This allows for control over the number of disulfide crosslinks within the material and thus the material properties and rates of degradation (Crewther et al., 1965). One approach is the chemical modification of cysteine residues via alkylation on keratin extracted by reductive means (known as kerateine) (Tachibana et al., 2006; Han et al., 2015). Alternatively, different chemical forms of the keratin resulting from oxidative (keratose) or reductive (kerateine) extraction can be mixed to tune the properties of the materials in a similar fashion to alkylation (Ham et al., 2015).

Keratin can be used as a traditional sponge (Tachibana et al., 2002; Katoh et al., 2004) or injectable gel (Aboushwareb et al., 2009; Saul et al., 2011; Kowalczewski et al., 2014) which allows for minimally invasive administration able to contour to any defect shape. A number of studies have already shown keratin's ability to achieve healing in injured bone as a carrier for hydroxyapatite (Tachibana et al., 2005; Dias et al., 2010a,b). Other studies have demonstrated healing in long bone (de Guzman et al., 2013) and mandible (Kowalczewski et al., 2014) models when keratin is used as a carrier for rhBMP-2.

Silk

Silks are proteins well-known for the high number of beta sheets in their secondary structure. Silk contains integrin binding motifs and is an appealing biomaterial for bone applications due to its very high tensile strength, osteoconductive nature (Meinel et al., 2005), and predictable rate of degradation (Altman et al., 2003). The two primary sources of silk are spider or silkworm (Arntzen and Ritter, 1994), and these can also be modified (e.g., by crosslinking tyrosine residues) to provide a more tunable carrier platform (Partlow et al., 2014). Silks can also be processed into a number of forms including films (Dutta et al., 2013), fibers (Mandal et al., 2012; Panda et al., 2015), hydrogels (McGill et al., 2017), scaffolds (Marolt et al., 2006; Zhu et al., 2014), and are compatible for fabrication of composites with ceramic (Jin et al., 2015) or synthetic polymers. Furthermore, these materials have been used as carriers for rhBMP-2 (Karageorgiou et al., 2006; Ma et al., 2016) and promoted bridging in a critically-sized rat femur defect model (Kirker-Head et al., 2007). Interestingly, domestication of silkworms (i.e., diet and environmental conditions) has resulted in higher silk yields compared to wild silkworms (Normile, 2009); however, it has resulted in alterations in silk properties (Holland et al., 2012; Fang et al., 2015). In particular, silk from silkworms fed non-mulberry diet have shown to be superior in applications for bone regeneration (Naskar et al., 2014; Sahu et al., 2015; Midha et al., 2016).

Alginate, Chitosan, and Other Polysaccharides

Each of the materials noted above (keratin, fibrin, silk) are protein-based materials. Polysaccharide-based materials have also been investigated as a potential carrier system. One drawback to many polysaccharides is that they do not bear the amino acid sequences typically associated with cell attachment via integrin binding domains. Cell attachment and osteoconductivity in polysaccharides are often enhanced by methods that include mixing with osteoconductive materials such as hydroxyapatite

(Danilchenko et al., 2011), chemical surface modifications (Luna et al., 2011), or incorporating cell adhesion proteins such as collagen (Lawson et al., 2004) or fibronectin (Kirchhof and Groth, 2008) or integrin binding sequences such as arginine-glycine-aspartic acid (RGD) (Ho et al., 2017). Although peripheral to this review, it is noteworthy that alginate has long been used for cell encapsulation techniques (e.g., for xenogeneic or allogeneic islet cell implantation for production of insulin for Type I diabetes; Lim and Sun, 1980). The ability to encapsulate cells and control matrix stiffness to mediate (stem) cell response makes such materials particularly interesting for cell-based approaches to bone repair (Darnell et al., 2017). A potential advantage of polysaccharide materials, when used alone or as part of a composite material, is their suitability for controlled release of small molecule drugs (Nafee et al., 2017), growth factors (Cao et al., 2017), and nucleic acids (Li et al., 2017) with or without cells.

MOLECULAR TARGETS TO PROMOTE BONE REGENERATION

Molecular Promoters of Bone Regeneration

TGF- β Superfamily and BMPs

In considering potential therapeutics to promote bone regeneration, it is informative to consider the molecular nature of the bone healing process. Molecular cues expressed during healing can be divided into three broad categories: (1) pro-inflammatory cytokines, (2) angiogenic factors, and (3) the transforming growth factor beta (TGF- β) superfamily and other growth factors (Ai-Aql et al., 2008).

Of the endogenous growth factors involved in bone healing, possibly none are as important as the transforming growth factor-beta (TGF- β) superfamily which acts upon a broad range of cells, influencing cellular activity, growth, differentiation, and extracellular matrix production. After initial blood clot formation, platelets release TGF- β to stimulate the proliferation of periosteal cells. Even though TGF- β plays a role in the production of extracellular proteins for callus formation, its osteoinductive potential is limited and does not have an effect on mineralization (Lind et al., 1993). TGF- β 's most important roles may lay in initiating the production of BMPs in osteoprogenitor cells while inhibiting osteoclast activation (Dimitriou et al., 2005).

The most important members of the TGF- β superfamily, in terms of bone regeneration, are likely the BMPs. BMPs are pleiotropic regulators of growth (Friedrichs et al., 2011), differentiation (Pera et al., 2004), and apoptosis (Hyzy et al., 2012) of a variety of cell types. One property of BMPs is their ability to be osteoinductive by themselves (especially BMP-2, -6, -7, and -9; Termaat et al., 2005), which makes them attractive therapeutically for tissue engineering products. They are strong promoters of differentiation of osteoprogenitor cells into osteoblasts. BMPs are expressed at various points along the phases of bone healing. BMP-2, -6, and -9 have been found to be the most potent inducers of pluripotent MSCs to differentiate into osteoblasts. Most other BMPs act more to

support the terminal differentiation and maturation of osteocytes (Cheng et al., 2003). As previously discussed, to-date only BMP-2 and BMP-7 have been approved for clinical use in the United States for bone substitutes. While BMP-2, in particular, has achieved clinical and commercial success, its side effects due to supraphysiological dosage suggest that other molecular targets might be used to reduce the dosages required to achieve bone healing.

One approach to this end is the use of peptide sequences or low molecular weight drugs sequences. Peptide sequences which consist of small components of BMP-2, can be used to promote bone healing while minimizing negative side effects (Saito et al., 2004; Li et al., 2011). The concept here is to identify the components responsible for promoting bone healing while minimizing negative side effects. Another approach is to use small molecules to facilitate the action of BMP-2 at lower doses. For example, the SVAK-12 compound interacts with the Smad binding site of Smurf-1 to prevent degradation of Smad, which play a role in the BMP/TGF- β signaling pathway (Kato et al., 2011). Other drugs such as simvastatin (Qi et al., 2013) and lovastatin (Yoshii et al., 2014) also work on the BMP/Smad pathway while bisphosphonates (Stadelmann et al., 2008), for example, work on alternative pathways. A particularly intriguing approach is the modification of known growth factors with so-called superaffinity domains, which allows these growth factors to achieve effects at a lower effective dose through better binding affinity to their carrier material or ECM proteins (Martino et al., 2014).

While each of the above approaches has advantages, there are several continuing challenges. One is that the potency of these molecules seems to be lower than BMP-2 with, for example, the P24 peptide having a dose three orders of magnitude greater than rhBMP-2 (Wu et al., 2008; Li et al., 2011), and thus requiring a larger overall mass to be delivered. Secondly, due to their smaller size, these molecules are susceptible to more rapid diffusion from their carriers, potentially shortening their activity.

Angiogenesis

An issue that has long been known but is yet to be fully solved for tissue engineering strategies is the need for vascularization of newly formed tissue (Nerem, 2006). In injured bone the initial hypoxic conditions during fracture healing stimulate angiogenesis, which is crucial for successful bone healing (Rowe et al., 1999). Platelet-derived growth factor (PDGF) is released from platelets entrapped within the hematoma in the early stages of bone healing and up-regulates vascular endothelial growth factor (VEGF) (Hankenson et al., 2011). The expression of VEGF (Deckers et al., 2002) and PDGF (Xie et al., 2014) by pre-osteogenic cells has been shown to be a crucial component in regulating the rate of neo-angiogenesis to correlate with the rate of bone formation (Gerstenfeld et al., 2003). In addition PDGF acts as a potent mitogen of inflammatory cells and mesenchymal stem cells (MSCs) (Dimitriou et al., 2005). Vascular ingrowth is also regulated by fibroblast growth factor (FGF) secreted by macrophages, mesenchymal stem cells, chondrocytes, and osteoblasts. FGF-1 and FGF-2, being the most commonly expressed growth factors in bone regeneration, partake by

increasing callus formation and osteoblast activity (Nakamura et al., 1998). Although angiogenic factors are crucial for effective bone healing, alone they are not osteoinductive. It might be expected that the combination of angio-genic factors in combination with BMP-2 might promote formation of more rapid healing or higher quality bone. However, combinatorial approaches have yielded minimal improvements (Patel et al., 2008), highlighting the need for highly tuned temporal control of delivery in such strategies (Young et al., 2009).

Cytokines and Other Growth Factors

Pro-inflammatory cytokines interleukin-1 (IL-1), interleukin6 (IL-6), and tumor necrosis factor alpha (TNF- α) are secreted within the first 24h of bone damage by macrophages which causes the initiation of the repair phase of bone regeneration. These cytokines initiate the downstream responses that, in-turn, initiate the induction of both angiogenic factors and growth factors. TNF- α activates osteoclast activity for the removal of bone debris and promotes recruitment of mesenchymal stem cells. The expression of these pro-inflammatory cytokines is highest during the first 24h of bone healing and the cytokines are subsequently expressed in smaller quantities during repair and remodeling phase (Kalfas, 2001). The ability to direct inflammatory response is therefore a potentially potent mediator of bone healing.

Insulin-like growth factors (IGF) play critical roles in skeletal development as well as fracture healing by promoting bone matrix formation such as collagen type 1 (Tsiridis et al., 2007). IGF-1 is the most potent in the IGF family and is localized in healing fractures (Andrew et al., 1993). IGF-1 stimulates chemotaxis and activity of osteoblasts, and has the greatest effect on bone formation when it is used in combination with TGF- β (Schmidmaier et al., 2003). One advantage of BMP-2 strategies is that this molecule is sufficient and necessary in the natural regeneration process, which has allowed it to be used alone to promote bone regeneration. Strategies that employ other and multiple growth factors may require improved understanding of the underlying biology and strategies to precisely time the delivery of these molecules. Although beyond the focus of this review, the advancement of small molecules and osteogenic drugs in bone regeneration can serve as alternatives to exogenous growth factors and cytokines (Han et al., 2013; Laurencin et al., 2014; Balmayor, 2015). A number of small molecules have been used alone (Papadimitriou et al., 2015) or in combination with growth factors such as rhBMP-2 (Cho et al., 2017) with success in bone regeneration. However, due to non-specific cellular uptake, adverse effects due to unwanted signaling cascades, and lack of efficient local sustained delivery (Brouwers et al., 2011; Laurencin et al., 2014) are limiting factors in the current progress of small-molecules.

Molecular Inhibitors of Bone Regeneration

Molecular control over fracture healing follows developmental osteogenesis very closely (Gilbert, 2000). Patterning and maintenance of tissue is overseen not only through molecular promoters, such as those discussed above, but is also by molecular inhibitors. Recent interest, as noted above for the SVAK-12 compound, has focused on bone regeneration

inhibitors in particular due to their mechanistic role in which negative feedback and crosstalk decrease the cellular exposure of the molecular promoters of bone regeneration. The effectiveness of these signaling regulators and how they can have significant negative impact on BMP efficacy can be appreciated when considering the therapeutic dose of BMPs in bone substitutes. One reason that BMP carriers are loaded with supraphysiological concentrations is likely related to the need to overcome the regulating factors of BMP inhibitors in order to achieve a therapeutic response. These inhibitors are present within the BMP signaling cascade at (1) intracellular locations, (2) as pseudo-receptors, and (3) in extracellular locations.

Intracellular inhibitors of BMP signaling include inhibitory SMADs which are dormant in the nucleus until BMP stimulation at which time they are released into the cytoplasm. After BMP binds to its receptor, SMADs inhibit the signal transduction by interacting with the BMP receptor. SMAD-6 specifically acts to inhibit BMP signaling while SMAD-7 targets the general TGF- β superfamily (Ishisaki et al., 1999). Another intracellular regulator is SMAD ubiquitin regulatory factor (SMURF), which controls intracellular BMP signal transduction by binding and degrading various positive signaling molecules or by degrading the BMP receptor (Murakami et al., 2003).

BMP and activin membrane bound inhibitor (BAMBI) is a pseudo-receptor which presents an extracellular domain similar to a BMP receptor domain; the difference is that BAMBI lacks the intracellular domain. Therefore, when BMPs bind to a pseudo-receptor it cannot form an active receptor complex in order to propagate the signal (Tsiridis et al., 2007).

Extracellular inhibitors, mainly produced by osteoblasts, are secreted proteins that act as BMP binding antagonists which prevent BMP from binding with its receptors. An increase in extracellular inhibitor expression is directly correlated with an increase in local BMP levels. The majority of the binding antagonists focus on BMPs with the strongest osteoinductive potential (BMP-2,-6, and -9; Termaat et al., 2005). Even though BMPs are mainly associated with being molecular promoters of bone regeneration, BMP-3 is an antagonist of osteogenic BMPs. Ironically, it is the most abundantly expressed BMP in adult bone (Tsiridis et al., 2007). The differential screening-selected gene aberrative in neuroblastoma (DAN) family proteins include gremlin and sclerostin. Gremlin binds and blocks BMP-2,-4, and -7. Gremlin is responsible for inhibiting osteoblast differentiation and reducing bone remodeling (Dimitriou et al., 2006). Sclerostin is produced by osteoclasts and directly competes with BMP-2,-4,-6, and -7 in binding to their receptors to inhibit osteoblast differentiation and bone remodeling. Sclerostin not only decreases MSC differentiation and osteoblast activity but also induces apoptosis in bone cells. Follistatin neutralizes BMP-2,-4,-15, and joins with high affinity to BMP-7 by forming a trimeric complex between itself, BMP, and the receptor (Abe et al., 2004). In embryogenesis, follistatin is known to inhibit all aspects of BMP activity (Iemura et al., 1998), but its role in adult bone healing is yet to be completely understood. Chordin binds BMP-2,-4, and -7 and blocks their ability to bind to BMP receptors and acts similarly to gremlin (Canalis et al., 2003). Noggin has the ability to bind to the greatest number of molecular

promoters of bone regeneration: BMP-2,-4,-5,-6, and -7 and prevents them from binding to BMP receptors. Noggin works in a complementary fashion with gremlin to develop a local zone which is devoid of BMP (Stafford et al., 2011).

Interfering RNA Approaches to Removing Inhibitors/Antagonists

Although each of the molecules discussed above play inhibitory or antagonistic roles in response to BMPs, they also play important roles in the homeostasis of healthy bone. As such, approaches to temporarily (not permanently) remove these molecules are important.

Small interfering RNA (siRNA) is a gene-silencing mechanism by which post-transcriptional gene silencing can occur (Cheema et al., 2007). Delivery of siRNA alone is not successful due to its susceptibility to degradation and overall negative charge, which prevents siRNA from passing through the cell membrane (Wang J. et al., 2010). To overcome these challenges and allow siRNA to be effective in the cytoplasm of targeted cells, siRNA can be packaged into viral or non-viral vectors.

Of particular interest in tissue engineering approaches is the ability to easily incorporate these constructs into biomaterial scaffolds. Krebs et al. were among the first to demonstrate the ability of a three dimensional hydrogel to achieve delivery of siRNA in a sustainable fashion (Krebs et al., 2009). Local delivery strategies now involve layer-by-layer (Hossfeld et al., 2013), nanoparticle embedding (Mittnacht et al., 2010), and direct incorporation into a cationic hydrogel (Ma et al., 2014).

In terms of promoting bone regeneration, focus has been on co-delivery of inductive molecules such as BMP-2 and siRNA targeting suppressive molecules. Targeting intracellular regulators of BMP-2, such as Smurf1 (Rodriguez-Evora et al., 2014), resulted in improved osteogenic activity of MSCs. "Pre-treatment" with noggin siRNA has also led to significantly increased expression of osteogenic markers in *in vitro*, and of bone regeneration *in vivo* (Wan et al., 2007). Substrate-mediated siRNA delivery for bone application has just begun to make an impact on the scientific community. The use of chitosan hydrogels as a reservoir for siRNA delivery has shown successful down regulation of osteoclast activity *in vitro* (Ma et al., 2014). Substrate-mediated delivery of noggin siRNA from a synthetic polymer has successfully enhanced osteogenic activity *in vitro* (Nguyen et al., 2014) and we recently achieved delivery of noggin

siRNA from the surface of fibrin hydrogel films (Kowalczewski and Saul, 2015).

CONCLUSIONS

In order to address the challenges and drawbacks of current augmentation strategies for critically-sized bone defects, tissue engineered bone substitutes have been designed to be both osteoconductive (collagen carrier) and osteoinductive (rhBMP-2). Like many first-generation products, there have been a number of drawbacks to rhBMP-2 collagen carriers such as edema and ectopic bone growth. In order to develop the next generation of bone substitutes it is important to understand the biological action and temporal expression during the healing cascade. Currently the focus has been placed on only incorporating molecules which promote bone regeneration (BMPs) without acknowledging the innate molecular controls achieved with inhibitory molecules (e.g., Noggin, Gremlin). In order to successfully decrease the therapeutic concentration of BMPs, novel carrier systems that maintain or enhance rhBMP-2 bioactivity must be designed and the negative feedback signaling caused by BMP antagonists must be addressed.

AUTHOR CONTRIBUTIONS

CK conceived of topics in the review, wrote initial draft of most sections of the manuscript, and conducted editing of the document; JS conceived of concept for the review for article, wrote initial draft of several sections of the manuscript, provided final revisions, and primary editing of the document.

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