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Stem cell- and scaffold-based tissue engineering approaches to osteochondral regenerative medicine

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Abstract

In osteochondral tissue engineering, cell recruitment, proliferation, differentiation, and patterning are critical for forming biologically and structurally viable constructs for repair of damaged or diseased tissue. However, since constructs prepared *ex vivo* lack the multitude of cues present in the *in vivo* microenvironment, cells often need to be supplied with external biological and physical stimuli to coax them towards targeted tissue functions. To determine which stimuli to present to cells, bioengineering strategies can benefit significantly from endogenous examples of skeletogenesis. As an example of developmental skeletogenesis, the developing limb bud serves as an excellent model system in which to study how an osteochondral structures form from undifferentiated precursor cells. Alongside skeletal formation during embryogenesis, bone also possesses innate regenerative capacity, displaying remarkable ability to heal after damage. Bone fracture healing shares many features with bone development, driving the hypothesis that the regenerative process generally recapitulates development. Similarities and differences between the two modes of bone formation may offer insight into the special requirements for healing damaged or diseased bone. Thus, endogenous fracture healing, as an example of regenerative skeletogenesis, may also inform bioengineering strategies. In this review, we summarize the key cellular events involving stem and progenitor cells in developmental and regenerative skeletogenesis, and discuss in parallel the corresponding cell- and scaffold-based strategies that tissue engineers employ to recapitulate these events *in vitro*.

Keywords

Tissue engineering; regenerative medicine; osteochondral; bone development; bone healing

1. Introduction

The field of regenerative medicine seeks to repair, replace, or regenerate tissues and organs damaged by injury or disease. Stem cells have emerged as a promising cell source to address these challenges. However, because the field of stem cells is fairly new there are many questions about how to best handle them for therapeutic applications. One major issue is the need to determine how much guidance or instruction stem cells require in order to regenerate tissues, and in what form these instructions should be provided. Many clues can be drawn from developmental and regenerative biology, where endogenous stem and progenitor cells are

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recruited to form new tissue in response to environmental stimuli. These stimuli can be provided through various means, including secreted or matrix-embedded signaling molecules, matrix chemistry and physical forces.

The extracellular matrix (ECM) is a particularly rich source of signals, acting as a structural support, a reservoir of growth factors, a transducer of mechanical signals, a source of spatial cues delivered via chemical epitopes, and many related features. Classical tissue engineering strategies aim to recreate this ECM environment to direct cell behavior on a scaffold of choice, with the eventual goal of implantation at the site of injury or disease to restore tissue function. Ideally, a microenvironment would be formed in which the ECM induces certain cell behavior, and cells would respond in turn by remodeling the substrate, establishing a dynamic feedback cycle that fashions the ECM according to the changing needs of the cell, allowing the cells and ECM to dictate the repair process.

Many combinations of cells and scaffolds have been utilized for tissue engineering; this review will focus on our particular approach culturing human mesenchymal stem cells on silk fibroin scaffolds as an example of a bioengineering strategy to regenerate connective tissues such as bone and cartilage. The aim of this review is to demonstrate how knowledge of endogenous cell biology can be applied to scaffold design to develop effective regenerative stem cell therapies.

The ability to tailor and control tissue formation *in vitro* suggests that tissue engineering can provide new options in the field of regenerative medicine. This impact is via the formation of clinically relevant pregrown human tissue replacements, as well as *ex vivo* human tissues serving as model systems. These *ex vivo* systems can be used to study human disease formation and therapeutic interventions, filling a niche between human cell screening and human clinical trials where currently animal models are used. Further, tissue engineering can provide a reciprocal benefit to the field of developmental biology and regeneration in general. Thus, while insight from development can inform and guide cell biology and tissue outcomes *in vitro* and *in vivo*, the availability of novel scaffold-cell systems with tight environmental control can provide new options to interrogate or control tissue formation processes both *in vitro* and *in vivo*. This would lead to new insight into how tissues regenerate, offering to impact approaches with which to promote tissue repair without scarring, approaches that are currently dominated by uncontrolled inflammatory wound healing pathways as opposed to true tissue regeneration pathways.

2.1. Endogenous skeletogenesis: progenitor and stem cell sources and cell recruitment

The early limb bud consists of undifferentiated mesenchymal cells that migrate into the limb field from the lateral plate and somatic mesoderm [1] (Figure 1A). Skeleton formation occurs through a process called endochondral ossification, in which the mesenchymal progenitor cells first aggregate into a cartilage template that is subsequently replaced by bone [2] (Figure 2). Fracture healing of long bones may also occur via a cartilage intermediate in endochondral ossification, but may also occur via direct bone formation in a process called intramembranous ossification [3,4]. Similar to development, regenerative healing requires skeletogenic mesenchymal stem cells (MSCs). Sources of these stem cells may include the periosteum, the membranous connective tissue surrounding bone; the surrounding soft tissues, such as muscle; the marrow spaces at the site of bone damage; granulation tissue; and the endosteum [4–14] (Figure 1B).

Several signaling molecules are involved in progenitor/stem cell recruitment and migration to the site of new bone formation. These molecules include transforming growth factor- β (TGF-

β), bone morphogenetic proteins (BMPs), and insulin-like growth factor-1 (IGF-1), which all act as stimuli for mesenchymal stem cell recruitment to the site of new bone growth [15–17]. Many of these molecules also promote mesenchymal cell proliferation and differentiation. Both the matrix and the cells at the site of repair may be sources of these molecules. For example, one of the initial responses to fracture injury is BMP release from the matrix as well as BMP secretion by primary mesenchymal cells recruited to the site of injury [18,19].

One major difference between initiation of bone formation in developmental skeletogenesis vs. in regenerative skeletogenesis is the significant role of inflammation in the healing process [20,21]. During skeletal repair, many important growth factors and cytokines that facilitate mesenchymal cell recruitment are secreted by inflammatory cells. For example, tumor necrosis factor α (TNF α), platelet-derived growth factor (PDGF), and interleukin-1 and -6 (IL-1, IL-6) are secreted by inflammatory cells and platelets, inducing mesenchymal cell migration and proliferation; chemotaxis of other inflammatory cells; and aggregation of platelets [4,19,22, 23].

2.2. Tissue engineering cell sources: adult mesenchymal stem cells

Since developmental and regenerative bone formation are both mediated by stem and progenitor cells, stem cells are a natural choice for osteochondral tissue engineering [24–26]. In addition, stem cells have become the main cell source of choice for tissue repair because they meet several major cell therapy requirements that differentiated primary cells do not meet [24–26]. For example, differentiated cells are often difficult to expand in culture and therefore yield insufficient numbers of cells for cell therapy. Furthermore, differentiated cells such as chondrocytes often lose their tissue-forming capacity *in vitro* and are therefore unable to support tissue repair or regeneration [27]. Stem cells, on the other hand, are defined by their self-renewal and differentiation capacity; they are able to proliferate in culture without losing their potential to form tissues.

Embryonic stem cells (ESCs) and adult mesenchymal stem cells (MSCs) are the main types of stem cells used for tissue engineering. ESCs have a broader differentiation spectrum because they can generate cell types from all three germ layers: endoderm, ectoderm, and mesoderm. However, many factors have limited their application to human cell therapy, including ethical concerns, immunological incompatibilities, potential for malignant tumor growth, heterogeneous differentiation, and an insufficient understanding of and control over ESC differentiation [25]. For these reasons, adult MSCs are currently the cell type of choice for therapeutic applications; these cells will be the focus of this review.

MSCs are characterized by several features. They were first obtained from whole bone marrow and separated from suspended hematopoietic stem cells by their ability to adhere to substrates and to form colony units [28–30]. MSCs are also often defined by their ability to differentiate into osteogenic, adipogenic, and chondrogenic lineages, making them an attractive cell source for osteochondral tissue engineering. Molecular characterization of MSCs, however, is difficult and controversial, as MSCs do not appear to uniquely express any molecule. Characteristic surface marker expression is somewhat inconsistent, but some groups look for positive expression of CD73, CD90, CD105, and absence of CD34, CD45 [25,31,32]. Because of a lack of unique identifying markers, it is difficult to study the activity of endogenous MSCs, especially in the context of their contributions to wound healing. Consequently, most studies of *in vivo* MSC activity examines the behavior of transplanted MSCs, which can be labeled *ex vivo*, then injected and monitored *in vivo* [32].

There are two main cell transplantation strategies: site-directed or systemic delivery of MSCs. Site-directed delivery of MSCs has shown that MSCs can engraft in host tissues, especially in models of injury in myocardium, spinal cord, and brain [33–37]. Systemic administration of

MSCs has further demonstrated the ability of MSCs to home to injured tissues, including brain, lung, and heart, although the degree of homing is less than with site-specific delivery [38–41].

The mechanisms underlying MSC recruitment from the circulatory system after injury are still unclear but are hypothesized to be similar to leukocyte trafficking across the blood vessel endothelium [32,42]. MSCs express a variety of chemokine receptors, adhesion molecules, and integrins that may be responsible for adhesion and rolling along blood vessel walls, including P-selectin and vascular cell adhesion molecule (VCAM) [32,42]. The ability of MSCs to migrate to sites of injury supports their use in tissue-engineered constructs, since it demonstrates that MSCs can sense and respond to factors and cytokines secreted in an injury environment. While MSC delivery appears promising for hematopoietic, myocardial, and neural repair, skeletal repair generally also requires a scaffold for structural and mechanical support at the injury site [31], while also serving the anchorage dependent function for the cells. Therefore, the majority of tissue engineering efforts aim to develop a suitable scaffold as a delivery vehicle for MSCs (Figure 1C).

3.1. Endogenous skeletogenesis: cell proliferation, differentiation, and interaction with the ECM

Developmental and regenerative bone formation occurs as a result of coordinated cell proliferation, differentiation, migration, and remodeling of the ECM (Figure 2). Prior to endochondral ossification, pre-chondrocytic mesenchymal cells that have been recruited into the bone-forming region secrete ECM largely composed of hyaluronan and collagen type I [26]. In the first major step of endochondral ossification, mesenchymal cells commit to the chondrogenic lineage and undergo condensation (aggregation) to form compact nodules [2]. Condensation involves changes in cell-cell and cell-matrix interactions, which are mediated by molecules including N-cadherin, fibronectin, syndecans, tenascins, thrombospondins, neural cell adhesion molecule, focal adhesion kinase and paxillin [26,43–46]. Condensation also is associated with a decrease in extracellular space, due to an increase in hyaluronidase activity and a denser distribution of collagens type I and III and fibronectin within the mesenchymal tissue [26,47–50]. Pre-chondrocytic cells then proliferate and differentiate to form a soft callus that provides mechanical support while acting as a template or scaffold for future hard callus formation [51]. Chondrocyte differentiation is characterized by synthesis of cartilage-supporting matrix, including collagens II, IX, and XI, and aggrecan and other proteoglycans [2,52]. Chondrocytes mature further and eventually undergo hypertrophy as they mineralize the ECM by depositing hydroxyapatite [2,53]. During hypertrophy, the proteinaceous composition of the ECM changes due to chondrocyte secretion of collagen type X and matrix metalloprotease 13 (MMP13) [2,53]. ECM degradation allows for vascular invasion; recruitment of chondroclasts, which remove apoptotic chondrocytes; and recruitment of new MSCs, which differentiate into osteoblasts that secrete bone matrix [2,54]. The soft cartilaginous callus is gradually replaced by a hard callus of woven bone. During this stage of primary bone formation, active osteogenesis produces bone matrix composed of proteinaceous and mineralized ECM. During the final stage of bone formation, referred to as secondary bone formation, the irregular and under-remodeled ECM of the hard callus is further remodeled into load-bearing cortical or trabecular bone [51].

In contrast to endochondral ossification, fracture healing by intramembranous ossification occurs when recruited MSCs from the underlying cortical bone and periosteum proliferate and differentiate directly into pre-osteoblasts and osteoblasts [4]. Interestingly, whether wound healing occurs via endochondral or intramembranous ossification depends upon the mechanical forces to which the injury is subjected. Endochondral ossification is enhanced by motion and mechanical stimulation and is inhibited by fixation [19,55]. Bending and shear

loading at the injury site thus favors chondrogenesis over osteogenesis as a mode of repair. Conversely, intramembranous ossification is favored when bone segments are stabilized during healing [20]. MSCs must therefore be sensitive to the mechanical environment provided by the ECM, in addition to the biochemical stimuli presented by the ECM.

3.2. MSC differentiation and scaffold considerations

In a scaffold-based approach to delivering MSCs to sites of osteochondral tissue defects, there are several design requirements to consider when choosing a biomaterial for the scaffold (Figure 3, Table 1). Knowledge of endogenous MSC activity in endogenous skeletogenesis, including the progression of cellular events and the sensitivity of cells to biochemical and mechanical stimuli, can inform many of these scaffold design choices. We will discuss these considerations mostly in the context of a particular biomaterial that has shown promise in supporting osteochondral growth *in vitro* and *in vivo*: silk fibroin from the silkworm *Bombyx mori*.

One basic requirement for a scaffold is that the material should support necessary cell activity leading to bone regrowth, including cell attachment, proliferation, and differentiation, as outlined in Section 3.1. Several studies have demonstrated the ability of bone marrow-derived mesenchymal cells to adhere, proliferate, and undergo osteogenic differentiation on two-dimensional silk fibroin films [56–58]. These films establish the suitability of silk fibroin as a stem cell-supporting biomaterial; however, due to their two-dimensional (2D) format, application of these films for wound healing is limited to use as coatings for other three-dimensional (3D) scaffolds to alter surface properties [59].

Because silk fibroin is a flexible material that can be processed in several different ways, it is not limited to 2D monolayer cell culture. Silk substrates can also be formed three-dimensional (3D) materials suitable for *in vivo* implantation into the site of bone or cartilage damage. For example, silk fibroin solution can undergo a sol-gel transition to form 3D hydrogels, which can be used as tissue culture substrates [60,61]. Hydrogels can also be further processed by lyophilization to generate porous sponges. Silk fibroin sponges and hydrogels have supported chondrocyte-based cartilage tissue engineering *in vitro* [62–64] and guided repair of critical-sized cancellous bone defects *in vivo* [65], respectively.

Another promising processing option is the formation of porous scaffolds from silk fibroin solutions by salt leaching, gas foaming, and freeze drying [66–68]. Scaffold topography and geometry play a critical role in tissue formation by dictating cell adhesion, proliferation, and distribution, as well as nutrient and oxygen availability. Thus, ideal scaffolds should be capable of forming various geometries for tissue-specific needs. The architecture and morphology of silk scaffolds can be controlled by processing options such as fibroin solution concentration, salt particle size, and solvent (aqueous or organic) [66]. Adjustment of these properties can result in favorable conditions for cartilage and bone engineering. For example, choice of solvent affects pore interconnectivity, surface topography and hydrophilicity, mechanical strength, and degradation rate [67] (Figure 4). Chondrogenesis of MSCs was supported on 3D porous aqueous-derived silk scaffolds, forming cartilage-like tissue whose spatial distribution of cells and ECM, chondrogenic gene expression, cell morphology, and zonal architecture resembled native tissue [69,70]. Chondrogenesis was also found to be improved on silk scaffolds compared to collagen scaffolds in terms of cell attachment, metabolic activity, proliferation, ECM deposition, and glycosaminoglycan (GAG) content [71,72]. Osteogenesis of MSCs resulting in bone-like trabeculae and mineralization was also improved on silk scaffolds compared to collagen scaffolds [73–75]. Pore size and porosity have also been shown to be important parameters for control over osteogenic and chondrogenic proliferation and differentiation. *In vitro*, small pore sizes favored osteoblast cell proliferation, while lower

porosity supported osteogenic differentiation, likely due to suppressed proliferation and increased cell aggregation [76–78]. *In vivo*, higher porosity allowed for cell recruitment and vascularization, leading to improved osteogenesis [77,79]. Interestingly, scaffold pore size and geometry was shown to dictate the mode of bone regeneration in an *in vivo* model of osteogenesis via BMP-2-loaded honey-comb-shaped hydroxyapatite (HA) scaffolds [80–82]. Small diameter ‘tunnels’ favored chondrogenesis followed by osteogenesis, while large diameter ‘tunnels’ favored direct bone formation [80–82]. Similarly, the geometry of HA scaffolds (honey-comb, porous particles, or porous blocks) determined the mode of bone formation [80]. Porous HA particles and blocks allowed for vascularization, providing sufficient oxygen and nutrient flow to permit direct bone formation, while honey-comb HA structures provided a low oxygen environment, stimulating initial chondrogenesis followed by osteogenesis [80]. These results suggest that scaffold architecture can be designed to favor one tissue type over another (e.g., cartilage vs. bone), as well as to favor a particular regeneration pathway (e.g., intramembranous vs. endochondral ossification). Given the responsiveness of cells to scaffold properties, silk scaffold processing was explored to determine whether scaffold features could be designed to achieve not only desired cell proliferation and differentiation, but also complex tissue architectures. The anatomical structure of newly formed bone was found to be pre-determined by initial silk scaffold geometry [83]. By changing pore interconnectivity within silk scaffolds, bone structures ranging from trabecular, plate-like bone to cortical-like bone networks were formed [83]. Building upon these results, silk scaffolds were engineered with two side-by-side domains of large and small diameter ranges of pore sizes to recreate the heterogeneous structure of bone, which can range from spongy, porous morphologies to compact morphologies [84]. The structure of the newly formed bone correlated with scaffold pore sizes, with the smaller pores supporting more trabeculae formation. Thus, scaffold properties such as porosity, pore size, and pore geometry can be tailored to dictate the mechanism of tissue regeneration and the structure of the resulting tissue, an important design consideration for large-scale tissue patterning.

An important consequence of altering the geometry of silk and other biomaterial scaffolds is that the mechanical properties of the scaffolds are altered. In an *in vivo* setting, such as healing of a bone defect, it is critical for the scaffold to provide mechanical support in addition to biological stimuli until the new tissue develops biomechanical properties that match the native tissue [31]. Silk is an attractive material for osteochondral tissue engineering because its mechanical properties are favorable for engineering load-bearing tissues: silk displays a higher elastic modulus and tensile strength compared to other natural biomaterials as well as synthetic biomaterials [85,86]. These mechanical properties of silk fibers translate to the 3D scaffold environment as well. The compressive strength and modulus of porous silk sponges are significantly higher than the corresponding properties of collagen, chitosan, hyaluronan, and polymeric porous sponges [60,68,86]. Thus, silk scaffolds are well-suited to address the mechanical challenges that contribute to the failure of natural materials like collagen, which are attractive materials because of their bioactivity and their presence in the ECM but which cannot support mechanical loading [87]. In addition to its role in reinforcing the injury site, the mechanical environment supplied by the scaffold may be important for controlling the mechanism of bone tissue regeneration. Endogenous examples of intramembranous and endochondral ossification show that mesenchymal cells at a fracture site respond to differences in mechanical stimulation (motion vs. fixation) by choosing one pathway over the other [19, 20]. Thus, it is possible that the differences in mechanical stability provided by porous scaffolds of different geometries may also favor one healing pathway over the other.

The biomechanical properties of an implanted scaffold may change as a function of time due to scaffold degradation and tissue ingrowth. Endogenous endochondral ossification involves several instances of ECM degradation and remodeling, including the transition from chondrogenesis to osteogenesis and the transition from irregular hard callus to cortical and/or

trabecular bone formation. Thus, to facilitate formation of mature bone in a tissue-engineered construct, scaffolds need to allow for control of degradation kinetics, as they would need to degrade in an appropriate timeframe to support new tissue growth and ECM deposition. Silk degradation has been characterized both *in vitro* and *in vivo* [88,89]. Silk scaffold degradation rates *in vivo* can be tailored by controlling several parameters, including processing solvent (aqueous vs. organic), silk solution concentration, and pore size [89]. Aqueous-processed scaffolds degrade within two to six months, while organic solvent-processed scaffolds persist for over one year when implanted in rats. Slower degradation is correlated to higher silk solution concentrations and smaller pore sizes, likely due to less tissue ingrowth. Importantly, these tunable properties allow the silk scaffold morphology to be designed to match the dynamic needs of the growing tissue.

4.1. Role of signaling molecules in endogenous skeletogenesis

Tailoring of the physical properties of biomaterials is necessary for developing an environment that promotes bone healing. Equally important are the biological signaling requirements of the healing tissue. The cellular events underlying *in vivo* skeletogenesis are regulated by an array of signaling factors, many of which have similar functions in both bone development and bone repair. Three major categories of signals have been identified: pro-inflammatory cytokines; growth and differentiation factors; and metalloproteinases and angiogenic factors [19,90,91]. Several of these factors will be highlighted below as examples of major similarities and differences between bone development and repair in terms of signaling factor activity and dynamics. Overall, the two modes of bone formation exhibit significant differences during initial stages, where inflammatory molecules play a role only in repair. The two modes then show increasing similarities during cartilage and bone growth: growth is mediated by similar signaling factors, sometimes operating under different temporal regimes. In the final stages of endochondral bone formation, development and repair show similar matrix- and angiogenesis-related activity.

Pro-inflammatory cytokine expression is a major feature of bone repair that distinguishes it from skeletal development. The inflammatory response observed early after injury plays a role in initiating the repair process. Elevated expression of cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF α) occurs within the first 24 hours after injury as well as during bone remodeling [4,19,23]. These cytokines are secreted by inflammatory cells and mesenchymal cells; their release stimulates chemotaxis of other inflammatory cells, ECM synthesis, angiogenesis, MSC recruitment, chondrocytic apoptosis, and osteoclast activity during endochondral bone growth [92].

Unlike inflammation-associated signals, many growth and differentiation factors regulate similar cell functions in both bone development and repair. Of the transforming growth factor-beta (TGF β) superfamily of proteins, bone morphogenetic proteins (BMPs) play diverse roles in skeletogenesis. BMP expression is regulated during skeletal development and plays a large role in osteochondral cell growth, differentiation, and apoptosis [17]. In the developing limb bud, the type of BMPs, their spatial distributions, and their temporal regulation are all critical parameters for patterning of the tissue structure [1,17,26]. BMP-2 and BMP-4 are expressed in the epithelium of the limb bud and act as signals for proliferation and differentiation of the underlying mesodermal progenitor cells [17]. BMP-2-induced differentiation is implicated in pattern formation along the anterior-posterior axis. Subsequent BMP-2, -4, -6, and -7 expression in the mesenchyme of the later bud regulates cartilage growth, differentiation, and apoptosis to form cartilaginous condensations during endochondral ossification [17,93]. Precise regulation of these morphogens allows for correct digit patterning: mesenchymal cells within the condensation are assigned a digital or interdigital fate for the formation of the

autopod [94]. BMP-6 contributes to cartilage hypertrophy, indicating a role in terminal chondrocyte differentiation [95,96].

While the same BMPs regulate fracture healing, they do so with different temporal dynamics. During endochondral ossification, BMP-2 expression is induced the earliest during mesenchymal cell recruitment and persists through chondrogenic and osteogenic differentiation to the stage of woven bone formation [19,97]. BMP-2 is hypothesized to trigger bone healing and induction of other morphogens [97]. In contrast, BMP-4 expression is more delayed and restricted in endochondral bone formation, reaching maximal expression during stages of active osteogenesis [17,97,98]. During intramembranous ossification, BMP-2/-4 expression is upregulated during early stages of repair, then downregulated during later stages in more differentiated cells [18]. In a rat model of bone fracture, BMP-7 is similarly upregulated during early stages of intramembranous and endochondral ossification, and subsequently downregulated in chondrocytes and in endochondral bone [17].

During the late stages of endochondral ossification, ECM degradation and blood vessel invasion are regulated similarly in both bone development and repair. Cartilage invasion by osteoclasts, osteogenic cells, and blood vessels requires the degradation of cartilage matrix elements such as collagen type II and aggrecan [19]. Matrix metalloproteinase 13 (MMP13), secreted by hypertrophic chondrocytes and newly recruited osteoblasts, acts together with MMP9, secreted by bone marrow-derived cells, osteoclasts, and endothelial cells, to degrade collagens and aggrecan to allow normal invasion of the ossification front [54]. Matrix degradation generates a permissive environment for vascular invasion, stimulated by vascular endothelial growth factor (VEGF). VEGF is secreted by chondrocytes and is upregulated during hypertrophy [54]. It acts upon vascular endothelial cells to promote angiogenesis and may also act upon osteoclasts to stimulate bone resorption.

4.2. Delivery of signaling molecules within tissue-engineered scaffolds

From the large pool of biochemical factors known to stimulate developmental and regenerative bone formation, tissue engineers can select the most promising target molecules for incorporation into scaffolds to stimulate tissue regeneration. In addition to the strategic choice of signaling molecules, scaffold design is an important component of efficient delivery of biochemical stimuli. There are several strategies for incorporating biological stimuli in a bioengineered scaffold in order to enhance tissue functionality when cultured *in vitro* and, more importantly, when implanted *in vivo*. Many of these approaches have been taken with silk fibroin materials to improve osteochondral tissue formation, taking into account the growth factors, cytokines, and other factors that are known to play a critical role in endogenous skeletogenesis. These strategies include coupling of silk to various bioactive molecules and encapsulation of molecules for controlled delivery [61,86]. In addition to materials modification, biomolecule delivery via gene therapy in MSCs has drawn recent attention as an efficient means of sustained biological stimulation [99].

The scaffold can serve not only as a substrate of bound signaling factors, but also as a reservoir that releases these factors in soluble form as a function of protein desorption and diffusion, matrix degradation, and other variables [3]. Silk fibroin films and scaffolds have been modified to present bioactive molecules to cells, thus functionalizing the silk materials for improved osteogenic and chondrogenic differentiation. BMP-2 is one of the most common signaling factors delivered to tissue-engineered bone. BMP-2 has been delivered via silk substrates in several ways. Loading of BMP-2 into silk fibroin scaffolds by physical adsorption resulted in significant release of BMP-2 in the first week of a 4-week *in vitro* osteogenesis study, and was sufficient to elevate osteogenic activity and mineralization compared to unloaded scaffolds [100]. When these tissue-engineered scaffolds were subsequently implanted into mouse cranial

defects, bone healing was significantly improved, with evidence of new mature bone formation and integration with the host tissue [100]. BMP-2 was also successfully incorporated into the fabrication process of silk electrospun scaffolds [101]. The high porosity of these scaffolds allowed sufficient BMP-2 delivery to stimulate to upregulate osteogenesis of hMSCs. Nanolayered silk coatings are currently being explored as a method to tailor the biomaterial surface to control protein release kinetics [59].

Chemical coupling of bioactive molecules to the tissue-engineered scaffold is another delivery option. Coupling of the cell adhesion peptide RGD to silk fibroin films and fibers improved the attachment and proliferation of MSCs [57,102]. RGD-coupled silk films and scaffolds also improved osteogenesis, stimulating increased osteogenic gene expression, calcification of the matrix, and formation of bone-like trabeculae compared to unmodified silk materials [75]. Parathyroid hormone (PTH), which stimulates proliferation and differentiation of osteoprogenitor cells in callus formation *in vivo*, was also found to stimulate proliferation of osteoblasts cultured on PTH-coupled silk [57]. Functionalization of silk films by covalent conjugation of BMP-2 enhanced osteogenic differentiation of MSCs compared to soluble delivery of BMP-2 [56]. The differences in bioactivity of immobilized vs. soluble growth factors is interesting when considering the main sources of signaling molecules in the endogenous bone environment: secretion from cells (soluble) vs. matrix component (immobilized). Presentation of growth factors in a scaffold may therefore need to take into account the way the growth factor is presented to cells *in vivo*.

For *in vivo* repair of skeletal defects, several limitations of direct protein incorporation into scaffolds have led to the use of gene therapy to deliver growth factors in a tissue-engineered scaffold. Because proteins have fast half-lives in the body and because scaffolds can only serve as finite reservoirs of proteins, direct loading of growth factors into scaffolds may not provide an adequate supply of growth factors for support of long-term bone repair [99,103]. Furthermore, recombinant proteins usually lack post-translational modifications compared to proteins synthesized *in vivo*, and may therefore be less biologically active [99]. In one common gene therapy approach to growth factor delivery, cells are transfected with DNA encoding a growth factor and subsequently express the desired protein. Transfected cells are then seeded into a scaffold and implanted at the injury site, where they continuously express and secrete the protein. The implanted cells can therefore not only participate in tissue repair directly, but also produce the therapeutic factors that stimulate endogenous cells to participate in the repair of the tissue defect [103]. Most gene therapy applications in bone engineering have focused on BMP transfection, and some recent efforts have explored the synergistic effects of delivery of multiple genes, including combinations of BMP-2, BMP-4, BMP-7, and VEGF [103].

5. Conclusions

The fields of developmental biology and tissue engineering both seek to understand and control the cues needed to stimulate cells to construct or reconstruct tissues and organs. To undertake such a problem, both approaches must integrate knowledge of progenitor and stem cell behavior; the role of the ECM or scaffold; and the role of signaling molecules. Drawing from developmental biology's knowledge of the cellular participants in tissue formation, tissue engineers have identified adult mesenchymal stem cells as promising cell sources that possess the necessary plasticity to perform similar cell functions *in vitro* and *in vivo*. In addition, developmental biology has uncovered a host of ECM-bound and soluble inductive factors that regulate developmental patterning. Tissue engineers can use this knowledge to guide their choice of biomolecules to incorporate in their systems, whether as a scaffold biomaterial or as a released factor. At the same time, because tissue engineering is a bottom-up approach to regenerative medicine, scaffold-based tissue formation raises up several important considerations that may not be emphasized in developmental biology. These include the

physical microenvironment of the regenerated tissue, which is largely dictated by the materials properties of the scaffold and which may regulate a wide range of parameters, including cell proliferation, cell differentiation, mode of healing, scaffold persistence *in vivo*, and release or presentation of delivered growth factors. By the mutual efforts of these two fields, progress may be made toward discovering the appropriate balance between biochemical and physical cues for tissue formation and toward fine-tuning the spatiotemporal delivery of these cues for large-scale tissue patterning. Such knowledge is critical for engineering complex tissues *in vitro*. Functional engineered tissues have great potential to advance regenerative medicine efforts both by addressing the current clinical need for tissue replacements and by providing platforms to investigate treatment strategies for stimulating tissue regeneration.

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Abbreviations

BMP	bone morphogenetic protein
ECM	extracellular matrix
ESC	embryonic stem cell
GAG	glycosaminoglycan
HA	hydroxyapatite
IGF	insulin-like growth factor
IL	interleukin
MMP	matrix metalloproteinase
MSC	mesenchymal stem cell
PDGF	platelet-derived growth factor
PTH	parathyroid hormone
TGF-β	transforming growth factor- β
TNFα	tumor necrosis factor α

VEGF

vascular endothelial growth factor

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Figure 1.

Progenitor and stem cell sources for bone development, bone healing, and bone tissue engineering

(A) During limb bud development, mesenchymal progenitor cells from the lateral plate mesoderm (LPM) migrate into the presumptive limb bud (LB) region and initiate endochondral ossification under the apical ectodermal ridge (AER).

(B) During bone fracture healing, mesenchymal cells migrate into the wound from the periosteum (P), the surrounding soft tissues such as muscle (M), the bone marrow (BM), and the neighboring cortical bone (CB).

(C) Many bone tissue engineering approaches utilize human mesenchymal stem cells, which are commonly derived from bone marrow aspirates taken from the iliac crest (IC) of the pelvic bone. These cells are seeded onto a porous scaffold (PS) of choice and cultured under osteogenic stimulatory conditions.

Figure 2.**Matrix changes during endochondral ossification**

Tissue engineers invest much effort into biomaterial design to provide cells with the appropriate growth-inducing microenvironment. As an *in vivo* example of the critical role of the extracellular matrix (ECM) during tissue development, Figure 2 illustrates ECM changes during endochondral ossification. The ECM evolves in each stage of the ossification process: it is remodeled by the cells while also providing physical and biochemical stimuli to the cells.

Figure 3.

Biologically-informed design specifications for biomaterials in tissue engineering. Scaffold properties such as material biocompatibility, geometry, porosity, mechanical strength, degradation rate, and incorporation of signaling molecules can be optimized to address various physiological requirements of an engineered tissue.

Figure 4.

Silk fibroin-based porous scaffolds

Silk fibroin can be processed into porous sponges in organic (A) or aqueous (B) solvents.

Scaffolds were prepared by salt leaching using 500–600 μm -sized porogens. Aqueous-based silk fibroin sponges have rougher pore surfaces and demonstrate better cell attachment, greater mechanical strength, and faster degradation rates compared to organic solvent-based sponges. Scale bar = 500 μm .

Modulation of scaffold properties for osteochondral tissue engineering

Table 1

Scaffold property	Rationale for modulation	Method of control	Biomaterials for which scaffold property has been investigated for osteochondral TE	Examples of osteochondral TE outcomes, especially as related to silk	References
Porosity	affects cell recruitment and attachment; vascularization	choice of porogen; size of porogen; concentration of starting solution; processing method (saltpolylactide-co-glycolide leaching, gas foaming, freeze-drying, electrospinning); crosslinking; polymer molecular weight	(PET), poly(L-lactide-co-D,L-lactide) (PLDL), polylactic acid (PLA), polyglycolic acid (PGA), saltpolylactide-co-glycolide, collagen, hyaluronic acid, hydroxyapatite (HA), β -tri-calcium phosphate (TCP), silk fibroin, and combinations thereof	<i>In vitro</i> , higher porosity increased proliferation and oxygen/nutrient transport; lower porosity supported cell aggregation and osteogenic differentiation. <i>In vivo</i> , higher porosity supported tissue ingrowth and bone formation. Silk: Porous silk scaffolds supported osteogenic and chondrogenic differentiation of MSCs <i>in vitro</i> and bone healing <i>in vivo</i> .	[70,72,73,75,77]
Pore size	affects cell infiltration, migration, proliferation, distribution; ECM deposition and distribution; nutrient and oxygen exchange	size of porogen; concentration of starting solution; molecular weight of biomaterial	same as for porosity	Optimal pore radius for bone ingrowth is thought to be 50–150 μ m. Smaller pores favored chondrogenesis followed by osteogenesis; larger pores favored direct osteogenesis. Silk scaffolds: Smaller pore sizes favored osteoblast cell proliferation <i>in vitro</i> .	[77,84,104]
Pore interconnectivity	determines geometry of resulting tissue	controlled porogen melting; gas foaming	silk fibroin, HA, TCP, various synthetic polymers and copolymers, polymer-ceramic composites	Pore interconnection sizes of 50 and 100 μ m are recommended for bone ingrowth. Silk scaffolds: Bone architecture (trabecular vs. cortical) was pre-determined by pore interconnectivity.	[83,104–106]
Degradation	allows for deposition of native matrix by growing tissue; provides structural support until native matrix is deposited; nontoxic byproducts	material properties; concentration of starting solution; processing solvent; scaffold pore size; crosslinking; materials ratios (for composites)	many natural and synthetic polymers, including collagen, silk, PLGA, polycaprolactone (PCL)	Silk degradation shows some advantages over other natural and synthetic materials. E.g., silk degradation is slower than collagen degradation, which is too fast to maintain structural tissue support. Slower silk degradation was achieved with higher silk solution concentrations and smaller pore sizes.	[61,75,83,86,89,107]
Mechanical strength	match mechanical properties of native tissue; retain 3D structure and space for growing tissue	form of scaffold (hydrogel, sponge, mats, fibers); porosity; crosslinking	many natural and synthetic polymers, bioactive glasses, ceramic materials	Few porous biodegradable polymers have sufficient mechanical strength to match the mechanical properties of bone. Silk fibroin's elastic modulus and ultimate tensile strength, however, are appropriate for trabecular and cortical bone.	[108–111]
Incorporation of biochemical signaling	provides stimuli for cell adhesion, proliferation, differentiation, vascularization, etc.	method of incorporation (adsorption, encapsulation, covalent crosslinking, delivery via transfected cells)	PLGA, CaP, TCP, chitosan, HA, collagen, silk, and others	Factors such as RGD, FGF-2, BMP-2, BMP-4, BMP-7, TGF β 1, TGF β 2, TGF β 3, VEGF, PDGF, IGF have been used in osteochondral tissue engineering.	[87,103,111,112]