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Tissue Engineered Bone Mimetics to Study Bone Disorders *Ex Vivo*: Role of Bioinspired Materials

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Abstract

Recent advances in materials development and tissue engineering has resulted in a substantial number of bioinspired materials that recapitulate cardinal features of bone extracellular matrix (ECM) such as dynamic inorganic and organic environment(s), hierarchical organization, and topographical features. Bone mimicking materials, as defined by its self-explanatory term, are developed based on the current understandings of the natural bone ECM during development, remodeling, and fracture repair. Compared to conventional plastic cultures, biomaterials that resemble some aspects of the native environment could elicit a more natural molecular and cellular response relevant to the bone tissue. Although current bioinspired materials are mainly developed to assist tissue repair or engineer bone tissues, such materials could nevertheless be applied to model various skeletal diseases *in vitro*. This review summarizes the use of bioinspired materials for bone tissue engineering, and their potential to model diseases of bone development and remodeling *ex vivo*. We largely focus on biomaterials, designed to re-create different aspects of the chemical and physical cues of native bone ECM. Employing these bone-inspired materials and tissue engineered bone surrogates to study bone diseases has tremendous potential and will provide a closer portrayal of disease progression and maintenance, both at the cellular and tissue level. We also briefly touch upon the application of patient-derived stem cells and introduce emerging technologies such as organ-on-chip in disease modeling. Faithful recapitulation of disease pathologies will not only offer novel insights into diseases, but also lead to enabling technologies for drug discovery and new approaches for cell-based therapies.

1. Introduction

Animal models have greatly advanced our understanding of the origin and progression of many human diseases and tissue repair. The most widely used animal models are in mice (i.e. transgenic mice) that re-create many aspects of disease pathologies and have been a

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vital tool for basic understandings and discovery of new therapeutic targets and approaches. However, only a few of these models replicate all the underlying features mostly due to species differences in genetic background, and often fall short in predicting the pathophysiology of many human diseases [1]. On the other hand, employing human cells along with the necessary microenvironment (i.e., niche) could be an alternative approach; however the commonly used two-dimensional (2-D) cultures often do not replicate the complex three-dimensional (3-D) environment as well as the dynamic physicochemical cues of the microenvironment [2]. Improved *ex vivo* systems that facilitate modeling of human disease by providing key attributes of the native environment may overcome the drawbacks of both the animal studies and *in vitro* 2D cultures [3, 4]. To this end, efforts have been made to develop *ex vivo* cultures that are 3-D, with tissue-specific physicochemical cues including, but not limited to mechanical properties, chemical components, growth factor signaling, oxygen tension, and fluid flow [5–9].

"Bioinspired" materials are materials engineered to mimic the structure, properties, and function of naturally occurring extracellular matrices (ECM). Natural biological materials such as bone ECMs are hierarchically organized displaying nanoscale to macroscopic features and combine chemical and physical properties that integrate and perform specific functions at individual size scales. The multifaceted effects of these distinct properties of the ECM are manifested at the cellular, tissue, and organ level. This is important for skeletal tissues that are known to be functionally hierarchical, highly organized, and undergo coordinated spatiotemporal events during development and repair [10]. Over the years, a substantial number of biomaterials—decellularized bone tissues, ECM proteins and polysaccharides, and synthetic materials—have been developed as bone grafts or scaffolds for engineering bone tissues, which elicit the necessary cellular response that contribute to bone regeneration [11–14]. While decellularized bone ECM provides various physicochemical cues relevant to bone tissues, synthetic or hybrid biomaterials are modular, which means individual elements of the biomaterial can be manipulated independently to enable defined experimental conditions, relatively easy to manufacture, and reproducible.

Particular attention has been given to designing biomaterials that mimic different physicochemical attributes of bone ECM during bone developmental processes or homeostasis [15]. These bioinspired materials are fabricated by incorporating key factors that are important for bone formation and function. They can be categorized into their functionalized components based on *inorganic* substances in the form of minerals (e.g. calcium phosphate; CaP) or *organic* elements (i.e. collagenous and non-collagenous) [15], or matrix metalloproteinases (MMP)-sensitive materials [16]. While there are a substantial number of biomaterials used to engineer bone tissues to study bone metastasis [17] and bone repair [18], less attention has been given to their potential to study bone disorders. When combined with appropriate cells and/or under the right culture conditions (e.g. biochemical factors), these same materials could potentially be applied to develop *ex vivo* models recapitulating various attributes of bone diseases. Different cell populations such as bone marrow-derived MSCs (BM-MSCs) and pluripotent stem cells (embryonic stem cells, ESCs and induced pluripotent stem cells, iPSCs) have been used to engineer bone tissues *ex vivo* due to their ability to differentiate into osteoblasts [19–21]. Given the importance of BM-MSCs in bone repair and iPSCs in disease modeling, respectively, we will focus on studies

involving these two cell populations. Based on our current understandings of bone ECM and bone biology in development and disease, we will outline various potential strategies available for disease modeling involving bioinspired materials, diseased cells, and induction protocols. *In vitro* platforms with increasing complexity, such as bone-on-a-chip and bioengineered systems, which integrate mechanical stimuli will also be discussed.

2. ECM and growth factors during bone development and remodeling

During development, bone is formed by either intramembranous (IM) or endochondral (EC) ossification [10]. Flat bones are formed by IM ossification in which neural crest-derived mesenchymal cells proliferate and condense into compact nodules and differentiate into osteoblasts. The osteoblasts secrete a collagen-proteoglycan matrix (osteoid), which mineralizes to form calcified tissue. On the other hand, long bones are formed by EC ossification. Longitudinal growth of skeletal bones is characterized by EC ossification with ordered zones of proliferating and differentiating chondrocytes in the growth plate. These processes are characterized by distinct ECM composition and properties. The ECM in the growth plate is composed of type II, IX, X, and XI collagen, large proteoglycans (aggrecan) that contain glycosaminoglycans (GAGs; chondroitin sulfate), hyaluronic acid (HyA), and other molecular components such as matrilins, cartilage oligomeric matrix protein, and MMPs [22, 23]. The proteoglycans interacts noncovalently via hyaluronan-binding motifs with HyA, an important nonproteoglycan polysaccharide [24]. The most abundant collagen in the growth plate cartilage is type II collagen [22], a homotrimer composed of three identical α1(II) collagen chains encoded by the *COL2A1* gene [25]. Hypertrophic chondrocytes in the growth plate secrete collagen type X and also induces expression of the collagenase matrix metalloproteinase-13 (MMP13), which is a prerequisite for invasion of blood vessels, osteoclasts, and osteogenic cells to form ossification and maturation of bone [23].

Bone is a highly dynamic tissue constantly undergoing remodeling throughout the lifetime of an individual [26]. The function and homeostasis of bone tissues are maintained by highly coordinated activities of cells residing within the bone organ, which include osteoblasts, osteocytes, and osteoclasts; 90% of cells in mature bone tissue are osteocytes [27–29]. Once bone tissue is formed, osteoblasts get trapped within the mineralized ECM and transform into osteocytes. In addition to osteoblasts and osteocytes, bone tissue also contains multinucleated osteoclasts, which are formed by the fusion of mononuclear progenitors of macrophages. The osteoclasts degrade the bone matrix by creating an acidic microenvironment that liberates Ca^{2+} and PO_4^{3-} ions during bone remodeling [30]. Bone remodeling is a highly coordinated process involving communication between multiple cell types present in the bone tissue. Osteoblasts can affect osteoclast formation, differentiation, and apoptosis through OPG/RANKL/RANK, RANKL/LGR4/RANK, Ephrin2/ephB4, and Fas/FasL pathways. Conversely, osteoclasts influence bone formation by osteoblasts via the d2 isoform of the vacuolar (H+) ATPase (v-ATPase) V0 domain (Atp6v0d2), complement component 3a, semaphorin 4D, microRNAs, and growth factors, released from the resorbed bone matrix, such as TGF-β and IGF-1 [31]. Each of the processes involves a complex spatiotemporal progression at the molecular, cellular and tissue level where the cells, soluble factors, and ECM have defined roles. In addition to the bone tissue specific cell types, other

cells that are involved in bone development and remodeling, include endothelial cells of the vasculature, peripheral neurons, and immune cells of the hematopoietic system [32–36].

Collagenous and non-collagenous proteins make up the organic components in mature bone while minerals make up the inorganic components. Type I collagen, a triple-helical molecule containing two identical α1(I) chains and a α2(I) chain, is the major collagenous protein in bone [37]. Collagen α chains are modified by several post-translational modifications including hydroxylation of lysyl or prolyl residues, glycosylation of hydroxylysine with galactose and glucose residues, and formation of intra- and intermolecular covalent crosslinks [38]. Collagen molecules are ~1.5 nm in width that co-assemble laterally to form a microfibril. Multiple microfibrils further assemble to form a fibril of ~50-300 nm in width. [39, 40]. In the periodic 67 nm cross-striated pattern of the collagen fibril, the less dense 40 nm-long gap zone has been implicated as the place where apatite crystals nucleate to form an amorphous phase, and subsequently grow and spread through the fibrils, leading to arrays of HA nanocrystals embedded within and oriented along the collagen fibrils, forming intrafibrillar mineralization [41–45]. Following intrafibrillar mineralization, HA crystals also grow on the surface of the collagen fibrils, leading to extrafibrillar mineralization [46].

In addition to type I collagen, trace amounts of type III, V, and VI may be present during certain stages of bone formation and may regulate collagen fibril diameter [47–49]. On the other hand, non-collagenous proteins comprise 10-15% of the total protein content in bone. These include various families of proteins encompassing serum-derived proteins (e.g. albumin), matricellular proteins (e.g. thrombospondins), small integrin-binding ligand-linked glycoprotein (SIBLING; e.g. bone sialoprotein), carboxylated proteins (e.g. osteocalcin), small leucine rich proteoglycans (SLRP; e.g. biglycan), and MMPs [50, 51]. Among the MMPs, MMP-2, MMP-9, MMP-13, and MMP-14 have a major role in modulating bone remodeling [52]. A series of growth factors are also involved in bone development and fracture repair[53, 54], including Bone Morphogenic Protein (BMP) [55], Vascular Endothelial Growth Factor (VEGF) [56], Notch [57, 58], Wnt [59], Sonic hedgehog [60], Indian hedgehog [61], Transforming Growth Factor β (TGFβ), Fibroblast Growth Factor (FGF) [62], Platelet-derived Growth Factor (PDGF) [63], and Insulin-like Growth Factor (IGF) [64]. Amongst the many growth factors, BMPs are the most important growth factors for bone formation during embryogenesis and in postnatal tissues.

Bone is a unique tissue in which one of the main ECM component is inorganic mineral and hence structurally bone ECM can be characterized as a composite. The apatite-like mineral phase of the bone ECM is around 60-70% dry weight depending upon the site and stage of development [37]. In addition to calcium and phosphate, the mineral phase of the apatite contains various substituents such as carbonate, magnesium, and acid phosphate [51]. There is a substantial heterogeneity with respect to crystallinity and the mineral content of bone tissue. The initially nucleated crystals are amorphous CaP (ACP) [65–67] that matures to crystalline HA minerals [41, 43, 68]. Thus, with time, bone mineral develops from a poorly crystalline apatite with high HPO_4^{2-} content to a mineral with more organized structure of higher crystallinity, more carbonate substitutions, and lower acid phosphate content [69–71].

3. Cells sources to engineer bone tissues *ex vivo*

Various stem cell populations such as pluripotent and multipotent stem cells have been extensively used to engineer bone tissues due to their ability to differentiate into bone forming cells, osteoblasts [19, 20, 72]. Amongst them multipotent BM-MSCs have been extensively studied towards engineering bone tissues. BM-MSCs from patients with skeletal pathologies have also been used to determine the cellular changes associated with various bone diseases [73, 74]. The ability to derive induced pluripotent stem cells (iPSCs) from somatic cells has opened up immense opportunities to model developmental and hereditary genetic disorders *in vitro* [75]. Stem cells are suitable for disease modeling because they 1) allow relatively high cell number for experimental manipulation, 2) recapitulates disease progression during commitment, differentiation, and maturation, and 3) can be isogenic and allows differentiation into multiple lineages to study the contribution of cell-matrix and cell-cell interactions. Patient-derived cells from bone diseases could model to illuminate the dynamic epigenetic, transcriptional/post-transcriptional, and translational/post-translational processes during different stages of disease progression. Disease modeling of monogenic diseases can be readily achieved by using iPSCs [76, 77] because they are cell autonomous and usually have defined readouts reflecting the pathophysiology of the affected cells. In contrast, developing *in vitro* models of most diseases [78, 79], require complex systems, such as multiple cell populations that can organize into rudimentary organ subunits and tissue specific extracellular cues. Recent efforts in the area of organoids and organ-on-chip systems have allowed the modeling of various human diseases with relevant pathophysiology.

3.1. iPSCs derived from patients with skeletal disorders

A number of iPSC lines are established from patients with rare genetic disorders. Osteogenesis imperfecta (OI) is caused by mutations in the *COL1A1* or *COL1A2* genes. To understand the cardinal features of the diseases *ex vivo*, iPSCs have been derived from patients with OI and the corresponding gene-corrected cells [80]. In OI, the folding process of collagen into a triple helical structure is disrupted resulting in an abnormal collagen protein structures. The most common type of mutations observed in OI are single base changes that lead to the replacement of one Gly in the $(Gly\text{-}Xaa\text{-}Yaa)_n$ repeating sequence of the triple helix by another amino acid residue. Such changes are postulated to affect $\alpha 2\beta 1$-integrin binding [81], which could have significant impact in cell-matrix interactions. Another disease with bone abnormalities is craniometaphyseal dysplasia (CMD), which is a rare disorder characterized by progressive hyperostosis of craniofacial bones and widened metaphyses of long bones. Patients with CMD suffer from deafness, blindness, facial paralysis and severe headache due to hyperostosis and compression of the brain. Mutations of the autosomal dominant form have been identified in the progressive ankylosis gene (ANKH), a pyrophosphate (PPi) transporter that channels intracellular PPi into extracellular matrix, and in Connexin 43 (Cx43) for a recessive form [82, 83]. Extracellular PPi acts as a potent inhibitor of mineralization, thus influencing the ECM properties. Furthermore, Cx43 is a main component of gap junctions in osteoblasts, osteocytes, osteoclasts. Cx43 are required for osteoblast and osteoclast differentiation and function [84], and the compromised Cx43 could significantly limit the cell-cell communication needed for osteoclastogenesis

and bone remodeling. Studies showed that hiPSCs with ANKH mutations formed fewer osteoclasts, expressed lower levels of osteoclast specific genes, and resorbed less bone [85]. Another disease with iPSCs generated from patients is Marfan syndrome (MFS), a life-threatening, autosomal dominant disease with mutations identified in Fibrillin-1 (FBN1) microfibrils and elastic fibers [86]. FBN1 sequesters BMP and TGF-β in the ECM and its aberrant changes during MFS disease affects their bioavailability, leading to premature depletion of MSCs and osteoprogenitor cells and enhanced bone resorption [87–89]. Skeletal abnormalities include long limbs and digits, deformities of vertebrae and anterior chest, and increased height. hiPSCs generated from MFS patients were found to faithfully represent pathologic skeletogenesis associated with MFS and impaired osteogenic differentiation due to activation of TGF-β signaling and its crosstalk with BMP signaling [90, 91].

Similarly, several iPSCs from patients with developmental disorders related to EC ossification have been derived. Individuals with a lethal form of metatropic dysplasia have aberrant EC ossification and cortical bone morphology, caused by a dominant mutation in the calcium channel gene TRPV4. TRPV4 act as a transducer of mechanical loading to regulate cartilage ECM biosynthesis [92]. Another pathology related to compromised EC ossification is Thanatophoric dysplasia type 1 and achondroplasia caused by gain-of-function mutations in the FGFR3 gene. Accumulation of FGFR3 protein suppresses the differentiation and proliferation of chondrocytes [93], and patients manifest symptoms such as femoral bowing, short limb stature, and skeletal dysplasia. Expression profiling in FGFR3-related chondrodysplasias revealed about 8% of the modulated genes are related to extracellular matrix (ECM) structure and dynamics, including those coding the basement membrane or ECM structural components, aggrecan turnover, glycosaminoglycan (GAG) and proteoglycan biosynthesis, diversification and sulfation. Genes involved in cell-cell interaction or adhesion, including CD44, NCAM1, integrins, cadherins and protocadherins are also affected [94]. Fibrodysplasia ossificans progressive (FOP) is another rare, debilitating genetic disease caused by constitutive activation of the ACVR1 gene [95]. Mutations in the ACVR1 gene lead to overgrowth of bone and cartilage and fusion of joints. Concurrent with disease manifestation, FOP-derived iPSCs exhibited enhanced mineralization and chondrogenesis *in vitro* [96].

iPSCs have also been generated from patients with rheumatoid arthritis [97] and osteoarthritis [97, 98]. Although bone loss is a secondary symptom as a consequence of immune-related dysfunction, integration of hiPSC-derived organoids with diseased immune cells and an arthritic environment (biochemical and biomechanical) could lead to disease models *in vitro*. Bioreactors involving mechanical loading and organ-on-chip platforms could be used to establish multifaceted complex environments pertaining to the diseases to study secondary conditions and the influence of immune environment on disease maintenance and progression.

3.2. MSCs isolated from bone diseases

Given the inherent potential of MSCs to contribute to adipose, cartilage and bone tissues [99–103], they have been extensively studied towards their application to regenerate skeletal

tissues and their function during skeletal diseases [74, 99, 104–108]. Cell studies comparing the differentiation potential of MSCs derived from osteoporotic patients with that of normal MSCs have shown dysregulated osteogenic and adipogenic processes, involving increased adipocyte formation, along with reduced differentiation potential into osteogenic cells [73]. Osteoporotic MSCs demonstrate decreased TGF-β production, as well as decreased capacity to synthesize and maintain a type I collagen-rich ECM. Furthermore, these cells had diminished alkaline phosphatase activity and less calcium deposition, and reduced ability to form mature bone cells [104]. Dysregulated cell-cell interactions between osteoblasts, osteocytes and osteoclasts regulating the RANK/RANKL, osteoprotegerin, Wnt/sclerostin pathways contribute to disease progression [109]. Autoimmune diseases such as rheumatoid arthritis (RA) and its management using glucocorticoids, increase the risk for bone loss [106]. MSCs isolated from patients with RA display impaired inhibition of Th17 cells. Th17 cells secrete IL-17, an important cytokine that induces osteoclast differentiation and bone resorption [74, 107]. These studies demonstrate the potential of MSCs derived from patients to study diseases of bone loss by re-creating tissue specific environments *ex vivo*.

4. Bioinspired materials for bone disease modeling

Substantial knowledge of skeletal development, fracture healing, and diseases have identified various key ECM molecules/properties and growth factors that contribute to bone formation, as well as maintenance of tissue homeostasis [10]. These understandings have enabled design of biomaterials with chemical and physical cues as well as growth factor immobilization to direct cellular functions relevant to bone tissue formation. To our knowledge, there are no studies directly employing bioinspired materials to study bone disorders in development and remodeling. Most bioinspired materials developed so far are used as grafts for bone repair or as scaffolds (i.e., artificial ECM) to engineer bone tissue surrogates [17, 18, 110–113]. However, these approaches can be easily extended to create *ex vivo* models of bone disorders in development and remodeling. Bioinspired materials can contribute to bone disease modeling either as an (i) active component, or (ii) passive component. In the case of an active component, the bioinspired materials can be used to model and study the role of ECM regulation in bone tissue functions and how perturbations in these interactions could contribute to bone diseases. In the latter case, the bioinspired materials can be used as a scaffold to create functional bone tissues from diseased cells. In this section we discuss the role of bioinspired materials in bone tissue engineering and their potential contributions to disease modeling.

4.1 Bioinspired materials to mimic biochemical cues

4.1.1 CaP and collagen-based materials—CaP-based biomaterials are the most commonly used scaffolds for bone tissue engineering. CaP-based materials are either osteoinductive, osteoconductive, or a combination of both. A comprehensive list of CaP-based biomaterials for bone tissue repair is summarized elsewhere [18]. CaP-biomaterials such as ceramics have been used for bone and dental restorations [114]. Composite materials containing both organic phase and inorganic CaP minerals have been developed by physical dispersion of CaP particles [115–121] or through biomineralization [122, 123]. Biomineralization is a naturally occurring process that supports mineralization of living

systems. Biomineralization requires functional groups of the organic matrices to nucleate CaP minerals and support their growth. A number of factors such as structure, hydrophobicity and functional groups of the organic template has been shown to play a key role in promoting biomineralization [41, 124–128]. Similarly, the crystalline nature of CaP minerals can be controlled through different experimental parameters such as the pH, temperature, and reactants [114].

Another widely used material for bone tissue formation is collagen, individually or as hybrids with calcium phosphate minerals. Several physical forms of collagens were developed over the years, including injectable hydrogels, membranes, or sponges with a number of materials as commercialized products. Synthetic materials that combine type I collagen with HA or other forms of CaP, have also been developed [129–131]. Some examples include, injectable collagen hydrogels in the form of composites such as collagen/HA/chitosan or collagen/HA/alginate [132, 133]. In addition to this review, we suggest literature summarizing collagen materials for bone tissue engineering elsewhere [134]. Besides collagen, collagen-derived peptides are also heavily used in bone tissue engineering. For example, collagen-derived peptides Ala-Gly-Glu-Ala (DGEA) that engage with $\alpha 2\beta 1$ integrin was used to improve the biofunctionality of alginate hydrogels. MSCs cultured with the help of these hydrogels exhibited increased levels of osteocalcin expression and mineral deposition [135]. In fact, many of the collagen-based products are commercialized for bone tissue repair [136–138]. These include collagen sponges such as Collagraft™ that combines HA/TCP with bovine collagen. INFUSE® Bone Graft is a recombinant human bone morphogenetic protein-2 (rhBMP-2) applied to an absorbable collagen sponge carrier for lumbar spine fusion, tibial fracture repair, and maxillofacial and dental bone grafting. OssiMend™ Bone Graft Matrix combines porous mineral with collagen for use in orthopaedic and spinal surgery.

4.1.2. CaP/Collagen-based biomaterials for disease modeling—Osteoinductive biomaterials such as CaP-based materials can induce osteogenic differentiation of stem cells *in vitro* and also support neo-bone tissue formation through recruitment of endogenous cells [20, 101, 139, 140] without the addition of exogenous osteoinductive factors. Thus, these materials offers a tool to engineer *ex vivo* bone tissue surrogates either through the use of cells derived from patients or healthy individuals. For example, patient-derived diseased stem cells can be *in vitro* cultured in 3-D osteoinductive materials (e.g. TCP, BCP) to undergo material-induced osteogenic commitment and mineralization to study IM ossification (Figure 1A). Addition of biochemical factors and immune cells could further be used to replicate diseases that arise from systemic biochemical or cellular factors such as hyperparathyroidism, treatment-induced bone loss, or autoimmune diseases [141–143]. Moving forward, the cell-laden constructs could be implanted *in vivo* in immune-compromised animals to undergo vascularization and host cell infiltration (e.g. hematopoietic progenitors, osteoprogenitors) [144–146] and establishment of bone remodeling. In addition, disease models can also be established with osteoconductive materials (e.g. HA, collagen) with diseased cells, where the latter often requires addition of exogenous osteoinductive molecules such as chemicals or BMPs, for osteogenesis and mineralization (Figure 1B). Such tissue-engineered platforms could be used to decouple the

contribution of ECM, cells, and microenvironmental cues on disease progression and maintenance.

4.1.3 Bioinspired materials to study disorders involving endochondral ossification—During skeletal development and repair, EC ossification remains a key biological process with the involvement of cartilage formation. Development through EC ossification and fracture healing processes are spatiotemporally dynamic and do not completely resemble the homeostatic native bone ECM [147, 148]. This lack of resemblance suggests that materials design aimed at mimicking the composition of mature bone ECM (mainly CaP minerals and collagens) may be insufficient to faithfully recreate the process of EC ossification. Biomaterial designs that leverage the temporal ECM changes during the developmental process have the potential to recapitulate EC ossification. Specifically, materials that facilitate the initial inflammatory or cartilaginous tissue formation in the early reparative stage of fracture healing may be used. In order to achieve EC ossification, the materials should support the underlying biological features including hypertrophic chondrocyte growth and eventual bone tissue formation. Most of the traditional tissue engineering steps involving stem cells include: 1) priming with chondrogenic supplements for a cartilage template, 2) hypertrophic conditions with thyroxine, β-glycerophosphate, dexamethasone, ascorbic acid, and IL-1β to generate hypertrophic cartilage, and finally 3) implanting subcutaneously into immune-deficient mice for calcification. This strategy, referred to as “developmental engineering”, is envisioned to be more adequate for bone regeneration by following the highly regulated spatiotemporal stages of EC ossification. Although a number of different biomaterials are used for chondrogenic differentiation [149], we refer only to those used specifically for studies related to EC ossification. Some materials have been found to better facilitate the process over others. For example, increased EC ossification foci were observed in human demineralized bone (DBM) implants suspended in a HyA carrier compared with DBM implants suspended in a glycerol solution [150]. *In vitro* priming of MSCs within collagen/HyA scaffolds supported significantly more cartilage tissue formation and VEGF production *in vitro*, and led to more bone formation within critical-sized bone defects compared to collagen/hydroxyapatite materials [151]. HyA is known to be a major component of cartilage ECM [152] and, therefore, could play a regulatory role in both chondrogenesis and EC ossification [23]. Alginate, chitosan, and fibrin hydrogels were used for chondrogenesis and hypertrophy of MSCs *in vitro* and EC ossification *in vivo*. Both alginate and fibrin constructs facilitated vascularization, endochondral bone formation, as well as the development of a bone marrow *in vivo*. Alginate constructs accumulated significantly more mineral and supported greater bone formation especially in the central regions of the constructs [153]. To shorten the *in vitro* chondrogenic priming period, MSC micropellets were mixed with fibrin that demonstrated comparable bone formation to standard pellets implanted *in vivo* [154]. It is interesting to note that although fibrin participates during early stages of bone repair, it was determined as nonessential for fracture repair [155]. Other studies have recapitulated EC ossification by mixing MSCs with ECM components such as collagen-GAG [156, 157], type I collagen mesh/IL-1β [158, 159], hyaluronan-gelatin [160], gelatin methacrylate embedded with cartilage-derived matrix particles [161], collagen/HyA acid [151], collagen/HA [151], and HA/TCP composites [162]. Using an alternative strategy, endochondral bone formation by

scaffold-free constructs was developed that comprised of human BM-MSCs embedded with bioactive microparticles for controlled delivery of TGF-β1 and BMP-2. The microparticles were formulated to release TGF-β1 to induce cartilage formation followed by BMP-2 in a sustained manner to promote remodeling into bone. Microparticle-incorporated constructs with both TGF-β1 and BMP-2 promoted the most bone formation and bone bridging compared to those with no growth factor or single growth factor [163].

In order to model skeletal diseases due to aberrant endochondral ossification, biochemical cues are required, while materials serve as scaffolds that provide the necessary 3-D environment and ECM-based physicochemical cues to support chondrogenesis, followed by hypertrophy and bone tissue formation (Figure 2). Upon culture, the embedded cells secrete their own ECM as a function of time. The intermediate tissue-engineered hypertrophic cartilage can also be implanted into immune-deficient animals to promote vascularization and eventual bone formation (Figure 2). While *in vivo* implantation forms ossification, however, to avoid xenogenic effects, more efforts should be emphasized to establish methods that enable abundant *in vitro* calcification similar to the levels achieved *in vivo*. Such an *in vitro* approach could limit the artifacts, if any, involved with *in vivo* transplantation of human cells into mice recipients. A recent study demonstrated that large 3-D MSC ball-like constructs cultured under a hypoxia condition formed region-specific chondrogenic differentiation and mineralization within the cartilage tissue *in vitro* [164], suggesting that all stages of EC ossification could be recapitulated *in vitro*.

4.1.4 CaP materials to study bone mineral environment—The mineral environment of the bone tissue has been touted to pay a key role in maintaining tissue homeostasis and repair. Indeed, bone mineral density (BMD) serves as a marker for bone health. The mineral environment of native tissue is very dynamic due to tissue remodeling. Such a dynamic environment can be mimicked *ex vivo*, in the absence of osteocytes and osteoclasts, through the crystalline nature of the CaP minerals. The dissolution of CaP minerals to Ca^{2+} and PO_4^{3-} ions often results in their supersaturation, leading to subsequent precipitation of Ca^{2+} and PO_4^{3-} ions onto CaP minerals [114, 165]. Such processes, termed as dynamic dissolution-precipitation, govern the local levels of extracellular Ca^{2+} and PO_4^{3-} ions and has been shown to regulate osteogenic commitment of stem cells [101, 102, 166]. A study by Germaini et al. compared the functions of carbonated HA (CHA) with that of HA and results suggest that carbonated HA has distinctive effects on osteoblasts and pre-osteoclasts compared to HA materials [167]. Studies have demonstrated TCP undergoes higher dissolution rates compared to HA [165, 168], which correlates with their ability to promote osteogenic differentiation of MSCs [169] and bone regeneration [168]. However, excessive dissolution of CaP moieties from ACP surfaces adversely affect osteogenic differentiation of MSCs compared to crystalline CaP surfaces [170, 171]. This is further supported by a recent study which reported the osteostimulatory effect of exogenous Ca^{2+} and PO_4^{3-} supplements on MSCs observed within a stringent concentration range [172]. Together the findings suggest that only those mineralized materials that maintain a tightly regulated and optimal extracellular concentration of extracellular Ca^{2+} and PO_4^{3-} can support osteogenic differentiation of stem cells.

Several pathways have been postulated regarding the contribution of CaP-materials assisted osteogenic differentiation and bone regeneration through extracellular Ca^{2+} and PO_4^3 ions [102, 169, 173, 174]. Ca^{2+} ions have been shown to promote osteogenic differentiation of MSCs through L-type voltage-gated calcium channels (L-VGCC) [173, 174]. BMP2 signaling is regulated by calcium phosphate ions. For example, BMP2 expression increases when cells are cultured in presence of TCP, calcium phosphate crystals, and Ca^{2+} [175–177]. The dissolution- precipitation of CaP minerals could facilitate various biological signaling through the adsorption/sequestration of growth factors and their subsequent release, including BMP2 [178–180]. Our studies using biomineralized materials and BM-MSCs have shown that phosphate ions released from the materials, due to dissociation of CaP minerals, promote osteogenic differentiation of progenitor cells via SLC20a1-ATP-adenosine signaling pathway (Figure 3) [102, 181]. In addition to promoting the osteogenic differentiation, cells on these materials undergo limited adipogenic differentiation [181]. This CaP-material mediated inhibition of adipogenic differentiation could be reversed through adenosine A2B receptor signaling intervention [181]. Emerging studies implicate the importance of purinergic P1 and P2 family of adenosine and adenosine triphosphate (ATP) receptors in bone and cartilage functions [182–184]. Reflecting the findings from biomaterial studies, mouse models with knockout of adenosine A2B receptor showed low bone mass, delayed fracture repair with MSCs displaying diminished osteogenic differentiation [185] and bone formation [186]. That the *in vivo* findings mirror the *in vitro* findings using biomineralized materials suggest the potential of bioinspired materials as an active component to study bone disorders and the underlying mechanisms *ex vivo*. Findings from materials study could be used to investigate whether alterations to SLC20a1-ATP-adenosine signaling occurs in diseased patient cells. Furthermore, disorders of mineral metabolism including hyperphosphatemia/hypophosphatemia and abnormal calcium regulation can potentially be studied using such materials. Osteoinductive CaP-based biomaterials could thus be used as an *in vitro* tool to explore and unravel the molecular mechanism through which the mineral environment of bone ECM maintains bone tissue regeneration and homeostasis (Figure 4A). CaP-based materials could also be prime tools to study diseases of bone loss or excess bone formation associated with abnormal bone remodeling. In native bone tissue, *in situ* bone resorption by osteoclasts creates local increases in the extracellular calcium [187] (and phosphate), which could be important for osteoblast formation during remodeling.

4.2 Bioinspired materials to mimic biophysical cues

Besides biochemical cues, collagen-based biomaterials also offer an ECM-like nanofibrous topography and facilitate ECM degradation by endogenous enzymes that promote tissue remodeling. The mechanical property of the biomaterials determines cell behaviors in spreading, polarization and differentiation by modulating mechanotransduction. The influence of mechanical properties of the biomaterial on osteogenic differentiation of stem cells and progenitor cells have been extensively studied [188–194]. Cell behaviors such as spreading, polarization, proliferation, and differentiation are determined by integrins sensing the mechanical cues of ECM to modulate mechanotransduction [195–199]. Integrin associated complexes connect ECM proteins through integrin transmembrane receptors with the intracellular actin cytoskeleton. Hydrogels are attractive tools for studying

mechanobiology given that their mechanical properties can be easily tuned to achieve the targeted values and properties such as linear elasticity. Most hydrogels are non-adhesive and to improve the cell-matrix interaction they are often coated with ECM proteins or functionalized with peptide units such as integrin-binding ligands [195–198]. The ability of MSCs to differentiate into osteoblasts is increased on relatively more rigid materials [200]. hMSC differentiation is also impacted by stress stiffening in nonlinear elastic materials [201]. This phenomenon was found to affect through a previously unknown stress-stiffening-mediated mechanotransduction pathway involving microtubule-associated protein DCAMKL1. Unlike most hydrogels that are used *ex vivo*, natural ECMs such as collagens are viscoelastic. Using viscoelastic hydrogels with dynamic mechanical cues, recent studies have shown materials with relatively faster stress-relaxation increased cell spreading, proliferation, osteogenic differentiation of MSCs, and bone formation [202, 203]. In diseases such as OI, absence of α2(I) chains in the collagen fibrils correlate with reduced stiffness, as well as a reduced failure strength of collagen fibrils [204]. Materials mimicking or allowing control of the dynamic physical properties of ECM proteins [205] can thus be applied to unravel unknown mechanotransducers that are potentially relevant to *in vivo* disease settings (Figure 4B).

4.3 Cell-responsive materials to mimic ECM degradation

A number of synthetic materials has been employed towards bone tissue engineering [21, 170]. While such synthetic materials offer control over many material properties, they lack degradation which is important in bone tissue formation and remodeling. MMPs are highly involved in bone development, remodeling, and disease [52, 206]. By leveraging this native biological process, linear oligopeptide substrates of MMPs were crosslinked with poly(ethylene glycol) (PEG) macromers functionalized with integrin-binding domains to create 3-D scaffolds for bone tissue regeneration [16]. When physically bound with BMP-2, the MMP-degradable hydrogel promoted cell infiltration and enhanced bone regeneration in a critical rat cranium defect. Furthermore, MMP-sensitive hyaluronic acid (HyA)-based hydrogel was used for BMP-2 delivery where maleimide-modified HyA macromers were crosslinked with difunctional MMP-sensitive peptides to permit protease-mediated hydrogel degradation and BMP-2 release [207]. Degradation of MMP-responsive materials can be used to study the effect of MMP on dynamic self-regulation of cells. For example, secretion of MMP by cells degrade the surrounding ECM, which could affect cell-matrix interaction by regulating signaling related to cell spreading, *de novo* ECM secretion, growth factor release; all of which influence osteogenesis and bone formation. Diseased cells involved in bone development can be cultured in MMP-responsive materials to explore MMP function and related targets, in diseases previously unknown to involve MMPs. Similarly, cell-responsive disulfide-modified PEG hydrogels that undergo tunable degradation in response to extracellular molecules with thiol groups [208] can be applied to study the effect of matrix degradation on ECM dynamics (Figure 4B).

4.4 Bioinspired materials with immobilized growth factors to explore coupling of biochemical cues and mechanotransduction

In native tissue, growth factors are bound to the ECM, as both proteins and GAGs have growth factor binding sites [209]. Growth factors can also be sequestered by the ECM

through electrostatic interactions [210]. Towards this, many growth factors or their derivatives have been covalently tethered or sequestered through physical interactions to collagen or CaP based materials [211–216]. BMPs are the most frequently used growth factor for bone tissue repair owing to its osteoinductive properties. To anchor BMP molecules onto collagen-based scaffolds, The CBDs have been incorporated into recombinant BMP2 [217, 218], BMP3 [219], and BMP4 [220, 221]. When coupled with collagen carriers, these CBD-BMP fusion proteins were found to stimulate greater bone formation compared to materials soaked with recombinant BMPs. In contrast to collagen-based scaffolds, CaPs offer minimal functional groups for covalent bonding. Thus, efforts have been focused on engineering BMP-mimetic peptides with a CaP-binding domain to improve their anchoring to CaP carriers. In one such instance, a modular peptide containing a BMP2 mimetic sequence was fused with an HA-binding domain of a γ-carboxyglutamate-rich motif of osteocalcin [222]. HA biomaterials functionalized with this modular peptide promoted osteogenic differentiation of MSCs *in vitro*, and enhanced bone repair *in vivo* [223, 224]. To circumvent the undesirable off-target effects of BMP2 in the surrounding tissue, peptide mimics of BMP2 were covalently bound to alginate hydrogels to enable local presentation and to control stem cell differentiation [225].

Angiogenesis is key to bone formation and is influenced by angiogenic factor VEGF [226]. New blood vessels bring oxygen and nutrients, as well as inflammatory and bone precursor cells to reach the regenerating tissue. Hence, several materials encoded with VEGF were used for bone tissue formation and repair [227–229]. To improve the coupling of recombinant VEGF to CaP carriers, a co-precipitation method for VEGF deposition onto BCP ceramics was developed [230]. Compared with superficially adsorbed VEGF, co-precipitated BCP/VEGF exhibited reduced VEGF burst release, and showed enhanced vascularization and osteointegration when implanted into mouse cranial defects [230]. Apart from these activities, a peptide termed “QK” that mimics the region in the VEGF binding interface to VEGF receptor, was synthesized with an osteocalcin-derived HA-binding domain. This peptide-coated HA microparticle was found to stimulate endothelial cell proliferation and migration [231]. When implanted intramuscularly into sheep, the HA-binding QK peptide that was coated onto β-TCP disks, through immersion, stimulated significantly higher blood vessel ingrowth [232]. A synthetic bone graft involving β-TCP matrix combined recombinant PDGF has been approved by the FDA as a dental product (GEM21S®). Similar to BMPs, PDGF molecules has been engineered with CBD domains to enhance anchoring to collagen-based scaffolds [215]. In another study, heparin was first crosslinked onto collagen-rich demineralized bone matrix (DBM), and recombinant PDGF was attached to the heparin-DBM via the heparin binding domains of PDGF [233]. Furthermore, by modeling natural growth factor-ECM interactions, collagen has been covalently affixed with heparin, followed by the noncovalent binding of growth factors that naturally bear heparin-binding domains [234, 235].

When combined with substrates of varying mechanical property, the direct interaction between GF receptors and integrins on mechanotranduction and cell behavior, and possibly disease, can be studied. It has been shown that integrin α1β1 and bone morphogenetic protein receptor (BMPR) IA formed a complex and co-localized in several cell types [236] and the coupling of physical and biochemical signaling was investigated on how growth

factors were presented to cells [237]. For example, murine C2C12 cells on stiff surfaces responded to both matrix bound BMP-2 and soluble BMP-2 in the culture media while that on softer matrices responded only to matrix bound BMP-2. Matrix bound BMP-2 was sufficient to induce β3 integrin–dependent C2C12 cell spreading by overriding the low mechanical rigidity of the biomaterial (i.e., soft matrices). This phenomenon is due to convergence of BMP receptor and β3 integrin signaling that control both focal adhesion dynamics and Smad signaling to cell migration and fate commitment [238]. However, the growth factor induced osteogenic differentiation of the C2C12 cells required a minimal threshold stiffness (3.5 kPa) below which the presence of bound BMP-2 mimetic-peptide had no effect [239]. These results imply that compared to physical properties of biomaterials, chemical attributes such as those presented by growth factors are more robust on affecting cell function. Similarly, studies have shown increased osteogenic differentiation of stem cells with higher adhesion peptide density [240], and osteogenic differentiation of stem cells on soft matrices when cultured in osteogenic medium [199]. Furthermore, studies investigating matrix rigidity often employ primed cells (cells exposed to pro-differentiating medium) [241]. Together these findings suggest that chemical cues are active regulators of cell processes whereas physical cues support the effects of chemical cues in a secondary role. Furthermore, a distinct mechanism of matrix-bound GF presentation that is different to soluble cues occurs, possibly coupled through the direct interaction between integrins and growth factor receptors that feel the substrate stiffness. During aging and disease, the mechanical property of bone ECM is drastically affected [242, 243], which could impact GF binding and affect the coupled integrin-GF receptor signaling. Such materials could be used to identify unexplored signaling targets for intervention (Figure 4C).

5. Bioinspired materials in complex *ex vivo* systems to study bone function

Biomechanical cues are important to the function of native bone tissue. Employing systems that incorporate the relevant biomechanical cues could be applied to materials to study disease *ex vivo*. Various forms of mechanical stimuli such as shear stress and compressive loading are present in bone [244, 245], all of which are possible to be applied *in vitro* to 3-D cultures [246, 247]. In the latest advancement, organ-on-chip platforms that attempt to mimic tissue-specific structure and function are promising tools to improve *in vitro* disease modeling and enable validation of drug efficacy [9]. To induce pathophysiology, various forms of mechanical or biochemical stress could be provided using inflammatory cytokines and cells, bacterial or viral challenge, and mechanical stretch and strain. Integrating mechanical loading with organ-on-chips could lead to better mimics of bone modeling. This section introduces some systems that can be integrated to disease models for higher complexity with potential to further improve recapitulation of disease in the body.

5.1 Ex vivo systems providing mechanical stimuli

Bone is constantly influenced by mechanical loading. The strain magnitude during walking was estimated to be approximately -430 με (compression strain), but up to 850 με (tensile strain) when running [248]. During daily functional activities, large strains (>1000 με) occur only a few times a day, while small strains (<10 με) occur thousands of times a day [245]. Under experimental settings, increased osteogenic differentiation was observed when tensile

forces were applied to MSCs seeded in collagen-GAG scaffolds cyclically (1 Hz) at 5% strain magnitude [246], and collagen coated silicone scaffolds at 2.5% at 0.17 Hz [249]. A 3-D *in vitro* co-culture system was also adopted to investigate the effect of loading on osteocyte-osteoblast interactions [5]. Osteocyte MLO-Y4 cells were embedded in type I collagen gels with MC3T3-E1 or MG63 cells layered on top. Cyclic compression of 10 Hz at 2.5 N to induce 4000-4500 με within gels increased prostaglandin E2 (PGE2) in MLO-Y4 cells, and increased type I pro-collagen synthesis in the co-culture. This co-culture model facilitates the elucidation of osteocyte-osteoblast communication during bone formation as a result of mechanical loading. In addition to mechanical loading, fluid shear stress had been postulated to occur in the lacunar-canalicular pores [244] and bone marrow [250]. The effect of fluid flow-induced shear stress on osteoprogenitor cells in 3-D scaffolds has been extensively studied. In general, unidirectional flow promoted early proliferation and osteogenic differentiation [247, 251], and intermittent pulsatile and oscillatory flow also increased cell response compared to continuous flow [252, 253].

5.2 Organ-on-a-chip systems

The development of technological platforms that generate three-dimensional (3D) organoids recapitulating the structural and biological features of native tissues has led to promising *in vitro* systems for disease modeling. Current research in this area has focused on the development of organ- and tissue-on-chip platforms ranging from traditional monolayer cultures to multicellular three-dimensional organoids. Organ-on-a-chip, which contain engineered microtissues that capture the physiological complexity of the native tissues within a continuous perfusion device, can be developed in a reproducible and cost-effective manner. These microphysiological systems also have the potential to supplement preclinical animal studies during drug discovery and development in order to improve the translatability of the drugs to the clinic. In fact, functional organs-on-chips simulating the lungs, gut, bone, heart, skeletal muscle, tumor, motor neurons, blood-brain barrier, and immune function have been developed [9, 254–260]. During bone development and remodeling, the vasculature, peripheral neurons, and hematopoietic system are highly involved in its physiological and pathological states. It would be ideal if a combination of some or all of the relevant cell types can be integrated into an *ex vivo* platform to model various etiologies of bone disorders. To date, only the vasculature has been shown to be integrated with bone [261–263]. A number of microphysiological systems consisting of the vasculature have been developed that are applied to studies in cancer metastasis, tumor angiogenesis, endothelial dysfunction, and organ regeneration using reconstituted natural ECM materials such as type I collagen [264–272], a mixture of thymosinbeta4/chitosan/collagen [273], and fibrin [262]. Below we discuss the advancements in the area of bone-on-chip systems. Though the focus of these studies were not bone disease modeling, when used appropriate cells and culture conditions these systems can be easily extended to study bone diseases.

5.2.1 Bone-on-a-chip platform—A triculture bone perivascular (BoPV) niche-on-a-chip system was developed to investigate the progression and drug resistance of breast cancer cells colonizing the bone. Human endothelial and bone marrow-derived mesenchymal stem cells (MSCs) were cultured in a native decellularized bone matrix measuring 6 mm height × 3 mm length × 1 mm thickness and exposed to interstitial flow and oxygen gradients. Breast

cancer cells were perfused into the engineered bone and the cells were found to colonize within the tissue. Furthermore, disseminated breast cancer cells exposed to flow exhibited a slow proliferative state and increased drug resistance in the BoPV niche-on-a-chip model [261]. In another bone-on-a-chip model, multiple cell populations comprising of primary hMSCs, hMSC-derived osteoblasts, and HUVECs were encapsulated in fibrin gels to create a bone-mimicking environment of osteoblasts with perfused microvasculature and mural cells. Employing this system, the authors have validated the extravasation of cancer cells, which showed that the human breast cancer cells were able to be perfused into the microvasculature and extravasate. These studies demonstrate the efficacy of such systems as a drug screening platform and a potential tool to investigate specific molecular pathways involved in cancer progression [262].

5.2.2 Bone marrow-on-a-chip platform—To establish the cellular diversity and complex functions of living bone marrow *in vitro*, bone marrow-on-a-chip was developed with a functional hematopoietic niche [263, 274] (Figure 5). This was achieved by filling type I collagen gel containing bone-inducing (i.e., osteoinductive) demineralized bone powder and BMP2 or BMP4 in a microfabricated poly(dimethylsiloxane) (PDMS) device with a central cylindrical cavity measuring 1 mm in height and 4 mm in diameter, and implanting subcutaneously in the back of mouse. The implanted device was excised once the bone marrow was formed, and inserted into a microfluidic system for *in vitro* culture. The engineered bone marrow (eBM) retained hematopoietic stem and progenitor cells with *in vivo*-like ratios for at least 1 week *ex vivo*. In addition, the eBM modeled organ-level marrow toxicity responses, and protective effects of radiation countermeasure drugs that were not possible with conventional bone marrow culture methods.

6. Summary and future outlook

Tremendous advances in bone biology, materials science, stem cells, and tissue engineering have led to *in vitro* tools to improve the potential of translating discoveries from the bench to bedside. In contrast to the frequent use of bioinspired materials as bone grafts for bone tissue repair and as scaffolds for bone tissue engineering, such materials are less commonly used as tools to study congenital disease of development and disorders of bone remodeling. These material-based tools and tissue engineered bone surrogates are valuable for acquiring novel insights to disease progression that are not able to be studied on 2-D tissue culture surfaces, and enables validation of drugs for therapeutic targets. Needless to say, the integration of bioinspired material, cells, and soluble factors are minimal elements required to model disease *in vitro* and *in vivo*. The value of bioinspired materials is their ability to test and dissect the role of individual factors through controllable manufacturing. However, technical limitations to biomaterials-based approaches described in this review exist and can be improved. For example, in addition to inducing cell differentiation, a material that facilitates cells to undergo tissue morphogenesis that forms macroscopic lamellar structures of native bone would be desirable. When stem cell-laden biomaterials are used to study developmental process such as EC ossification or remodeling, the final bone formation steps often require implantation into animals. Although this aids bone formation, the participation of host xenogenic, immune-compromised environment could introduce deviations and may

not recapitulate the development process entirely. In addition to establishing and perfecting the components necessary for generating models of tissues, analyses of these tissue surrogates to determine how adequate they mimic the disease initiation, progression, and maintenance comparable to actual human disease are also crucial. Advancements in biomaterial synthesis, microfabrication, and imaging can lead to *in vitro* platforms with increased complexity by providing controlled microenvironmental stimuli, such as geometry, mechanical force, pH, temperature, and oxygen tension. Yet, it is important to be aware that increasing complexity does not necessarily emulate the physiological state, and it is necessary to ensure that the stimuli applied to cells *ex vivo*, are representative of what they are "felt" in the body. Rapid developments in organ-on-chip systems affirm that bone-on-a-chip can soon be integrated with other tissues/organ-chips as a multi-organ-on-chip system to study the systemic effects of bone on other organs, and vice versa, during drug treatments and disease. This will tremendously improve our ability to acquire accurate new insights into disease progression, and validation of therapeutic treatments for humans that are not possible in current cultures or animal models.

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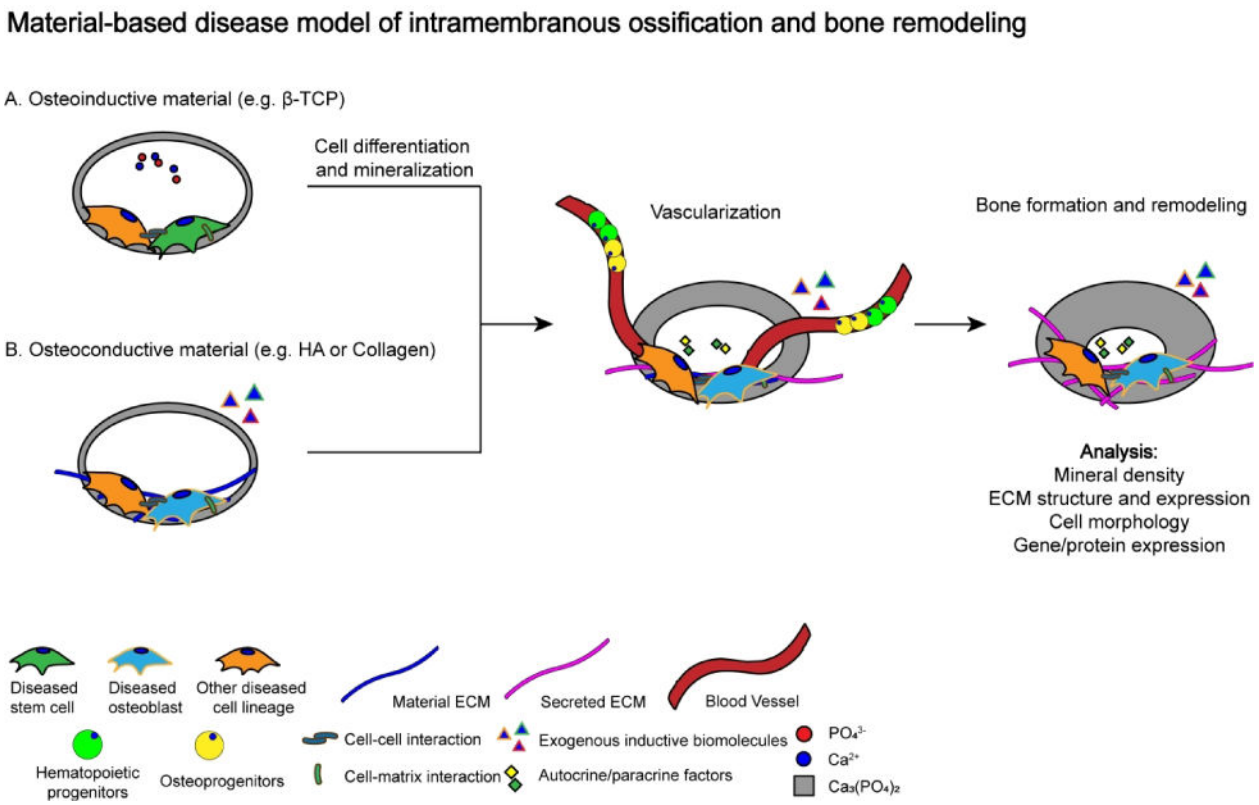

Figure 1.

Proposed disease model of material-based intramembranous ossification and bone remodeling. (A) Diseased bone formation using osteoinductive material (i.e. β-TCP, BCP). (B) Diseased bone formation using osteoconductive material (i.e. HA, collagen). Diseased stem cell: iPSC, ESC, BMMSC. Other diseased cell lineage: pre-osteoclast, immune cells, endothelial cell etc.

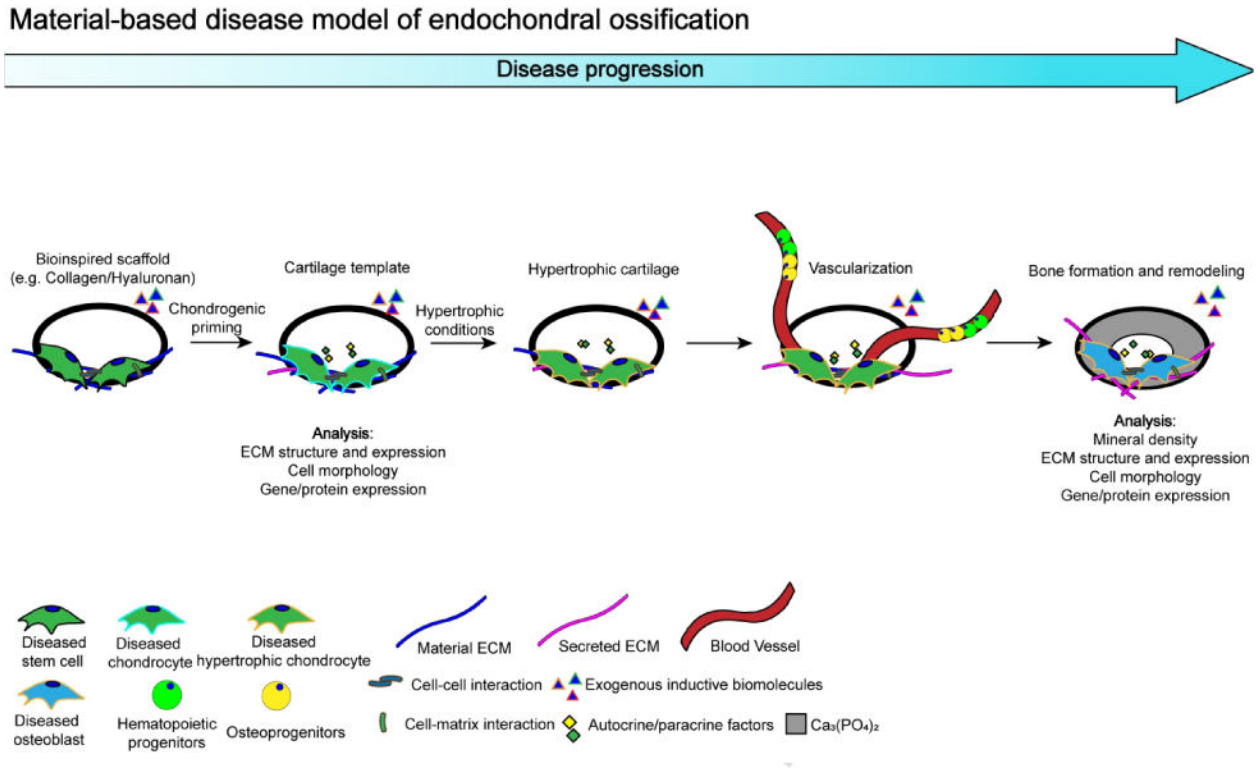

Figure 2.

Proposed disease model of material-based endochondral ossification. Diseased bone formation through endochondral ossification using bioinspired scaffold (i.e. collagen, hyaluronan, fibrin). Diseased stem cell: iPSC, ESC, BMMSC. Other diseased cell lineage: pre-osteoclast, immune cells, endothelial cell etc. Diseased cell: osteoblast.

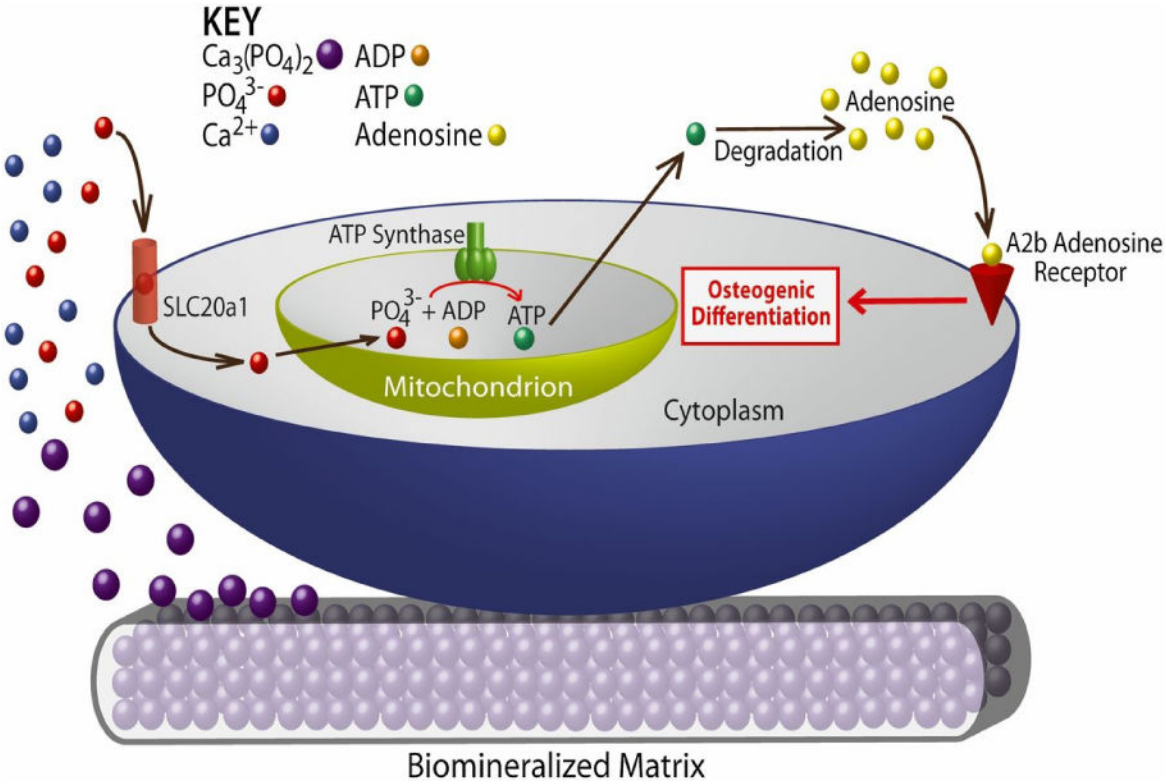

Figure 3.

Schematic model of mineralized matrix-induced osteogenic differentiation through inorganic phosphate (adapted from Ref. [102]).

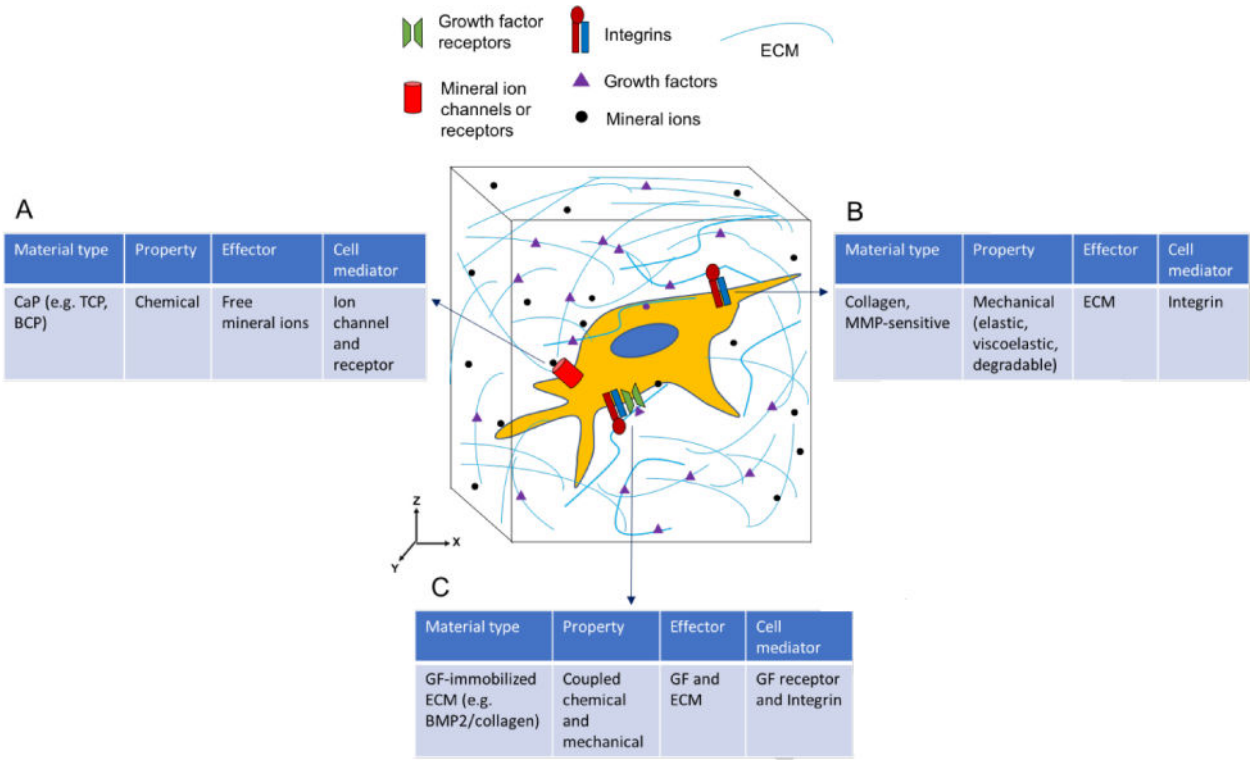

Figure 4.

Types of materials to explore molecular targets involved during osteogenesis. (A) Osteoinductive calcium phosphate materials release mineral ions to bind ion channels or receptors. (B) Mechanical properties of ECM modulate integrin and mechanotransduction. (C) Immobilized growth factor (GF) on ECM couples chemical and mechanical signaling through direct interaction between GF receptors and integrins.

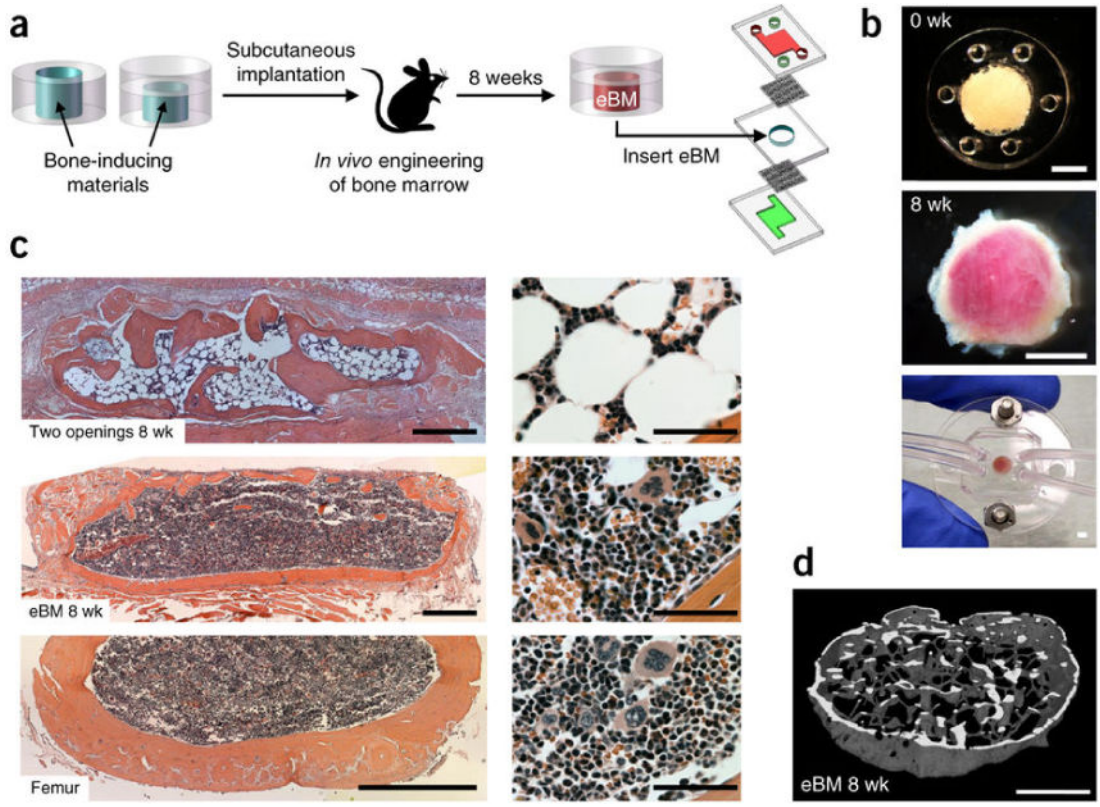

Figure 5.

Bone marrow-on-a-chip. (A) Workflow to generate a bone marrow-on-a-chip system. (B) Top, PDMS device containing bone-inducing materials before implantation. Center, white cylindrical bone with pink marrow within eBM 8 weeks after implantation. Bottom, *in vitro* bone marrow (BM) chip microdevice. (C) H&E-stained sections of the eBM formed in the PDMS device with two openings (top), or one lower opening (center) at 8 weeks following implantation, bone BM in a normal adult mouse femur (bottom). (D) 3-D reconstruction of micro-CT data from eBM 8 weeks after implantation (from Ref. [263] with permission).