Introduction

Bone defects resulting from trauma, congenital defects, cancer, infection, and arthritis have become a global healthcare problem. The reconstruction of extensive bone defects continues to be a great challenge medically and socioeconomically. Currently available approaches for bone defect reconstruction, such as the use of autografts, allografts, xenografts, and synthetic grafts, all have specific indications and limitations. Thus, extensive research has been performed to identify substitute approaches for the reconstruction of bone defects. BTE, which aims to regenerate new biological bone tissue with structures, mechanical properties, and functions similar to those of naturally occurring bone tissue, has become a valid approach for bone defect reconstruction.

The classic BTE paradigm frequently relies on several key elements: (1) an osteoconductive scaffold that closely mimics the natural bone extracellular matrix (ECM) niche; (2) osteogenic cells capable of initiating bone formation at the graft site or, at least, creating an osteoinductive microenvironment to induce resident cells to do so; (3) osteoinductive molecules to trigger implanted or resident cells to form...
bone tissue; and (4) sufficient vascularization to meet the needs of the growing tissue for a supply of nutrients and the clearance of waste. In the past three decades, BTE has traditionally focused on mimicking an intramembranous ossification (IMO) pathway, by which mesenchymal stem cells (MSCs) are induced to undergo osteogenic differentiation with subsequent formation of a bone-like matrix. This approach has achieved major progress in terms of bioreactor design, scaffold engineering, and long-term tissue construct maintenance, but there have also been encountered setbacks and delays in clinical translation. An emerging paradigm of developmental BTE, which advocates engineering cartilage constructs by replicating certain aspects of ECO for bone defect reconstruction, has been gradually adopted by researchers to address the limitations of traditional BTE strategies. However, the basis of ECO strategies is not widely understood. Furthermore, several key design criteria regarding the choice of cell sources, biomaterials, and endochondral priming protocols remain to be elucidated. Thus, this review introduces the concept of developmental bone engineering, explores the routes of endochondral bone engineering, and summarizes the current experimental data on large bone defect reconstruction via ECO-based strategies. Within this framework, emphasis is placed on the superiority of ECO-based strategies, and future trends in the clinical translation of these strategies are discussed.

**Engineering endochondral bone: A developmental engineering strategy**

**Embryonic bone development and fracture healing**

All bone is formed through two mechanisms: IMO and ECO. Both mechanisms begin with MSC migration to sites of future bone. Here, MSCs form condensations of high cellular density that outline the shape and size of the future bone. Within these condensations, the MSCs either differentiate into osteoblasts to directly form flat bone (intramembranous bone formation, Figure 1(a)), or differentiate into chondrocytes and form a cartilaginous template that is responsible for long bone formation (Figure 1(b)) as well as bone fracture healing (Figure 1(c)) (endochondral bone formation).

The replacement of cartilage with mineralized bone in endochondral bone is a complex process. Osteogenesis begins when proliferating chondrocytes within the template enter a non-proliferating, hypertrophic state. These hypertrophic chondrocytes secrete osteogenic and angiogenic factors, such as vascular growth factor (VEGF) and alkaline phosphatase (ALP). Concurrently, the tissue is invaded by vasculature, which delivers MSCs, osteoclasts, endothelial cells, and hematopoietic cells to the diaphysis, thereby forming a primary ossification center (POC).

Traditionally, hypertrophic chondrocytes undergoing programmed cell death are believed to be removed by osteoclasts from the template. MSCs then differentiate into osteoblasts to produce bone matrix. However, recent works have highlighted an alternative fate of hypertrophic chondrocytes, consisting of transdifferentiation into osteoblasts and osteocytes in the final stages of endochondral bone formation. Additional vascularization near the ends of the bone will establish one or more secondary ossification centers (SOCs), which contribute to the growth of the bony epiphyses and articular cartilage (Figure 1(b)).

Bone fracture healing differs from natural bone development. In general, bone fracture healing consists of three overlapping phases: the inflammatory, reparative, and remodeling phases. ECO and IMO occur concurrently during the reparative phase. When a bone fracture occurs, the inflammatory phase begins immediately. The damaged vasculature and bone marrow create a hypoxic microenvironment that recruits MSCs, fibroblasts, and endothelial cells to the fracture site. The reparative phase consists of two subphases: the soft callus and the hard callus phases. In the soft callus phase, the recruited MSCs start differentiating in two different ways according to their microenvironment. IMO primarily occurs along the periosteal surface of the bone adjacent to the fracture site as MSCs differentiate into osteoblasts that lay down woven bone. However, ECO occurs predominantly at the center of the fracture site, where MSCs differentiate into chondrocytes. These chondrocytes then form a cartilaginous soft callus, and the synthesized cartilage ECM mineralizes through chondrocyte apoptosis. In the hard callus phase, osteoblasts migrate to where blood vessels invade the calcified cartilage to produce a hard callus. Finally, after the fracture has been filled with new woven bone, osteoclastic activity occurs at the outer surface to initiate periosteal callus resorption and the remodeling phase (Figure 1(c)).

**Endochondral bone engineering: A promising solution for large bone defect reconstruction**

Inspired by the process of bone development and fracture healing, current BTE strategies strive to generate bone substitutes by emulating the body’s biochemical and physical environment. Traditional BTE strategies mimic the embryological process of IMO, by which MSCs are induced to undergo osteogenic differentiation with the subsequent formation of a bone-like matrix in vitro. This strategy has clear potential and has made major progress. A major drawback such strategies for engineering constructs is the limited size of the construct. In vitro osteogenic induction results in extensive matrix deposition on the surface of the construct, which hampers nutrient delivery and makes it difficult to scale up the size. Furthermore, extensive bone matrix on the surface hinders the invasion of blood vessels upon construct implantation. Thus, such traditional BTE approaches often fail due to
poor perfusion, leading to avascular necrosis and core degradation. Consequently, attention has shifted toward an alternative route of “developmental engineering,” which strives to stimulate in vivo developmental processes and imitates natural factors governing cell differentiation and matrix production.

In contrast to IMO-based approaches, approaches based on developmental engineering involve engineering cartilaginous constructs by replicating certain aspects of ECO for bone defect reconstruction. Briefly, MSCs are induced to differentiate into chondrocytes in vitro to form a hypertrophic cartilage construct, which contains essential “biological instructions” to initiate the ECO process after implantation, and the defect is subsequently repaired by endochondral bone formation (Figure 2). This strategy offers a solution to overcome problems associated with poor vascularization after implantation: (1) chondrocytes that are born in an avascular environment can intrinsically resist...
hypoxia, while MSCs and osteoblasts are less tolerant to a hypoxic environment. (2) Hypertrophic chondrocytes can induce neovascularization and ossification through the release of VEGF, vesicles containing hydroxyapatite, and bone morphogenic proteins (BMPs); and (3) endochondrally primed grafts allow for faster host integration and bone formation after implantation in vivo. Therefore, ECO-based strategies have brighter prospects and interest in ECO has gradually increased in recent years.

**Engineering strategies for the in vitro recapitulation of ECO**

Developmental engineering strategies for recapitulating endochondral bone formation typically involve two steps: (1) engineering a cartilaginous intermediate in vitro and (2) implanting the cartilaginous template in the defect site to induce bone regeneration. As these are tissue engineering approaches, some fundamental factors related to cell sources, bioscaffolds, biochemical factors, and priming protocols have been studied extensively to mimic natural bone development.

**Cell sources**

Cell from an ideal cell source for endochondral bone engineering must have the capacity to undergo hypertrophic chondrocyte differentiation and synthesize hypertrophic cartilage-specific ECM. Cells derived from numerous sources, including bone marrow, adipose tissue, embryonic tissue, and the nasal septum, have been successfully applied in endochondral bone engineering.

**Adult MSCs.** Bone marrow-derived mesenchymal stem cells (BMSCs) are currently the most frequently used cells in endochondral bone engineering. These cells are favored for their potential to differentiate into chondrocytes and subsequently develop a hypertrophic phenotype in vitro. Generally, the chondrogenic differentiation of BMSCs in vitro shows similarities to the process of ECO: the cells progressively produce collagen type II (COL II) and COL X, when a phosphate donor is added to the culture medium, mineralization can occur in the engineered constructs; and when the chondrogenically primed constructs are implanted in vivo, bone formation, mineralized matrix deposition, and blood vessel ingrowth can be observed. Furthermore, BMSCs have shown greater chondrogenic potential and 15-fold greater COL X expression than adipose-derived stem cells (ASCs) under the same culture conditions. In donor-matched comparison studies, BMSCs have shown a significantly higher chondrogenic capacity than ASCs after 21 days of culture in chondrogenic differentiation medium (CHM). Cartilaginous constructs engineered from BMSCs follow a process similar to that of ECO, with greater COL X expression and mineralization than constructs engineered from joint tissue-derived stem cells under the same conditions. The use of allogeneic MSCs, which have shown the ability to elicit endochondral bone regeneration in critical-sized femoral defects in immunocompetent rats, represents an interesting alternative to overcome these limitations. However, further research on achieving robust bone formation with allogeneic MSCs is needed. Although BMSCs have shown excellent performance in endochondral bone engineering, limitations of BMSCs, including donor variability, invasive harvesting protocols, and difficulty expanding cells in vitro, have also been reported.

ASCs are a heterogeneous population of cells that are harvested from subcutaneous adipose tissue and exhibit several distinct translational advantages over MSCs from other tissues. ASCs have multilineage potential comparable to that of BMSCs, but they are easily accessible...
in abundant quantities by a minimally invasive procedure and have a higher proliferative capacity. ASCs have shown the ability to recapitulate ECO in vitro and in vivo. ASCs, either cultured as micromass pellets or spheroids or seeded onto collagen sponges in CHM, can mature into hypertrophic cartilage tissue. Upon subcutaneous implantation, these hypertrophic cartilage constructs are able to undergo an ECO process and develop into bone containing bone marrow elements. However, the clinical translation of this approach may still be hampered by the complex ASC processing protocol and long-term in vitro expansion procedures, which impair their multilineage differentiation potential. Therefore, our team innovatively fractionated human liposapirates into small adipose tissue particles and used them as numerous ASC niches and native scaffolds for hypertrophic cartilage engineering. The resulting constructs develop a hypertrophic cartilage phenotype and even show higher efficacy in endochondral bone formation than ASC-seeded collagen sponges (Figure 3). These studies provide a clinically translatable approach for bone defect repair.

Figure 3. Engineering hypertrophic cartilaginous tissue directly from human adipose tissue: (a) Human adipose tissue was harvested during liposuction surgery; the human adipose tissue was positive for COL IV but negative for fibronectin, COL II, and COL X. After 3 weeks of culture in proliferation medium, the adipose tissue was positive for COL IV and fibronectin but negative for COL II and COL X, indicating that proliferative culture results in more stromal cells in the adipose tissue. (b) Then, the cultured adipose tissue was subjected to endochondral priming. After 4 weeks of chondrogenic priming, the engineered constructs showed a cartilaginous phenotype, which was characterized by positive safranin O staining for GAG, weakly positive staining for COL I and COL X, and strongly positive staining for COL II. The chondrogenically primed constructs were cultured in HYM for 2 weeks, which resulted in strong positive staining for COL X. (c) The endochondrally primed constructs were subcutaneously implanted into nude mice for 12 weeks. MicroCT scanning of the retrieved constructs showed a bony shell around bone trabeculae inside. Bone tissue formation and morphological evidence of bone marrow in the retrieved constructs were identified histologically by hematoxylin and eosin (H&E) staining and osteocalcin (OCN) staining. Intensive bone resorption by osteoclasts, characterized as tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells, was observed in the inner margins of the bone marrow-like cavity surrounded by newly formed bone tissue.
Another clinically relevant cell type for recapitulating ECO is periosteum-derived cells (PDCs), which are found in the inner cambium of the periosteum and play a prominent role during fracture healing. In the context of BTE, PDCs exhibit strong MSC-like multipotency characteristics at the single-cell level but greater clonogenicity, differentiation potential, and bone regeneration capacity. Furthermore, PDCs acquire the capacity for endochondral bone formation in response to injury, which has made them an attractive cell type for endochondral bone engineering. Callus organoids engineered by the chondrogenic priming of human PDC (hPDC) microspheroids in vitro can form bone microorganoids in an ectopic environment and heal murine critical-sized long bone defects. In another study, the preconditioning of hPDC microaggregates in vitro resulted in the formation of intermediate cartilage tissue, which could ectopically develop into bone tissue via ECO and facilitate bone defect healing.

Embryonic stem cells (ESCs). ESCs possess the potential to differentiate into any cell type and have been shown to achieve bone formation via ECO. The chondrogenic induction of mouse ESCs in vitro results the deposition of cartilage matrix on ceramic scaffolds, which in turn demonstrate robust endochondral bone formation upon implantation in subcutaneous sites or in critical-sized cranial defects. The chondrogenesis of differentiated ESCs is typically characterized by five overlapping stages, which are similar to the stages of the embryonic developmental processes of ECO: (1) condensation of differentiated ESCs; (2) differentiation and fibril scaffold formation; (3) ECM deposition and cartilage formation; (4) hypertrophy and degradation of cartilage; and (5) bone replacement with membranous calcified tissues.

However, concerns regarding ethical objections, immune rejection, and teratoma formation continue to impede the clinical implementation of ESCs. To produce functional bone tissue without these risks, MSCs derived from ESCs (ESC-MSCs), which possess the lineage-specific differentiation potential of MSCs but enhanced proliferative and immunosuppressive capabilities, have also shown the capacity to recapitulate the ECO process and repair bone defects semiautomatically without preimplantation differentiation into osteo- or chondroprogenitors. Furthermore, embryonic limb-derived progenitor cells can also form bone via the ECO pathway in an ectopic environment, which has been harnessed to bridge parietal bone defects in a mouse model.

Chondrocytes. Chondrocytes, which constitute mature and functional cartilage, are a logical cell type for endochondral bone engineering. As mentioned before, hypertrophic chondrocytes within the fracture callus stimulate osteogenesis and vasculogenesis during bone fracture healing, and hypertrophic chondrocytes isolated from fracture calluses, even from callus tissue directly, have demonstrated the capacity to promote bone regeneration. These inherent biological features suggest that the use of hypertrophic chondrocytes is a logical therapeutic strategy for bone regeneration.

Articular cartilage is normally a permanent tissue that resists hypertrophy, vascularization, and ossification. Under physiological conditions, articular chondrocytes remain in a resting state and refrain from proliferation or terminal differentiation. However, under the conditions of osteoarthritis, articular chondrocytes enter ECO-like cascades of proliferation and phenotypical dysregulation, becoming hypertrophic and abnormally expressing genes such as COL X, matrix metalloproteinase (MMP)-13, and ALP. Therefore, by leveraging these inherent biological characteristics, human osteoarthritic articular chondrocytes have been used to engineer endochondral constructs that subsequently undergo ECO after implantation either subcutaneously or orthotopically. Interestingly, ectopic bone formation has also been observed in healthy articular chondrocyte-engineered constructs, indicating the bone-forming potential of healthy articular chondrocytes. Indeed, healthy articular cartilage lesions often undergo progressive degeneration toward osteoarthritis under pathological conditions, and continuous efforts have been directed to optimize conditions for redirecting articular chondrocytes toward hypertrophy. A recent study has demonstrated that upon transforming growth factor (TGF)-β1 administration during ex vivo expansion, human articular chondrocytes are redirected toward a hypertrophic phenotype, which is an undesirable effect for cell culture but could be useful in endochondral bone engineering. Similarly, with the help of BMP-2, primary porcine articular chondrocytes can produce hypertrophic cartilage matrix on poly(ε-caprolactone) (PCL) scaffolds in vitro and even result in bone and bone marrow formation upon implantation. Furthermore, ECO with marrow cavity formation can be observed in the long term after the ectopic implantation of primary human articular chondrocytes isolated from the epiphyseal cartilage of infants in nude mice, even without in vitro expansion, induction, and scaffold seeding. Although promising results have been reported, the clinical translation of articular chondrocyte-engineered constructs is still limited by phenotype stability, donor site morbidity, and in vitro expansion.

Chondrocytes derived from other tissues have also shown potential as a cell source for endochondral bone engineering. Nasal septal cartilage derives from the same multipotent embryological segment that gives rise to the majority of maxillofacial bones and has been considered an autologous cell source for cartilage and BTE. The hypertrophic induction of adult human nasal chondrocyte-based micromass pellets in vitro results in the formation of mineralized cartilaginous tissue rich in COL X, but the tissue remains avascular and reverts to stable hyaline cartilage upon subcutaneous implantation in nude mice. In contrast, chondrogenically primed rat nasal
chondrocyte-seeded constructs are not only rich in COL X but also show angiogenesis, mineralization, and bone formation upon subcutaneous and orthotopic implantation. These results demonstrate the plasticity of nasal chondrocytes in engineering endochondral bone. In addition, chondrocyte-like progenitors possessing a transient phenotype in vitro have been effectively induced toward endochondral bone formation in vivo.

**Biomaterials**

Generally, an ideal scaffold for endochondral bone engineering should have mechanical characteristics that resemble those of hypertrophic ECM, possess appropriate cues to enhance cellular attachment and subsequent differentiation, and provide an architecture for allowing cellular adhesion, matrix production, and vessel ingrowth in vivo. Typically, four types of biomaterials, natural polymers, synthetic polymers, bioceramics, and decellularized/devitalized ECM (dECM), are used in endochondral bone engineering.

**Natural polymers.** Natural polymers, such as hyaluronic acid (HyA), collagen, gelatin, and fibrin, are widely used in endochondral bone engineering. Collagen and HyA are both key components of the native cartilage matrix. Highly porous collagen-based scaffolds have been extensively used to support the in vitro growth and differentiation of MSCs toward both osteogenic and chondrogenic lineages due to their biocompatible and biodegradable nature. Porous collagen sponges were first used to generate cartilage in vitro and have recently also been used to generate endochondral bone. HyA is an important physiological component of the cartilaginous ECM that provides a favorable environment for endochondral bone engineering and has achieved promising results. HyA-based scaffolds have been shown to support cell migration and differentiation. MSCs seeded into HyA hydrogel have shown greater COL II and chondroitin sulfate production but less COL I deposition than other materials. Therefore, composite scaffolds developed using collagen and HyA have shown greater hypertrophic cartilage formation in vitro and endochondral bone formation in vivo but a high HyA concentration in such composite scaffolds may result in a low proportion of cells with a hypertrophic phenotype in the engineered constructs.

Gelatin is a naturally derived protein obtained by collagen hydrolysis. Due to its biocompatibility, biodegradability, and ability to form hydrogels, gelatin plays a significant role in 3D cell culture models and has been used alone and as a basic material for improving endochondral bone regeneration. Porous gelatin sponge scaffolds have been shown to support chondrogenesis in vitro and calvarial healing via ECO. Gelatin-methacrylamide (GelMA) hydrogels produced by the chemical modification of gelatin retain some properties of collagen and gelatin, such as cell adhesion domains, thermosensitivity, and biodegradability. Thus, this material can be used as an embedding material and in the fabrication of bioprintable scaffolds for endochondral bone engineering. For example, GelMA hydrogels have been loaded with MSCs and chondroinductive particles, and the resulting composite constructs stimulated endochondral bone formation in a subcutaneous rat model. Additionally, MSC-loaded GelMA hydrogels are used to print constructs with an interconnected microchannel network which are subsequently used for engineering cartilaginous constructs. The 3D-printed microchannels within the cartilage template can promote osteoclast/immune cell invasion, hydrogel degradation, and vascularization during endochondral bone formation.

Fibrin is a biopolymer of the monomer fibrinogen that plays critical roles in blood clotting, cell-matrix interactions, inflammation, and wound healing. Fibrin hydrogels facilitate MSC-mediated vascularization, endochondral bone formation, and bone marrow development. Fibrin hydrogels reduce the standard chondrogenic priming duration from 28 days to 7 days but yield comparable endochondral bone formation. Fibrin supports cell attachment, condensation, and proliferation but is mechanically weak and degrades rapidly. Hyaluronan is mechanically stronger and degrades much more slowly than fibrin. Therefore, a novel hybrid system composed of 70% hyaluronan and 30% fibrin has been developed to closely mimic the ECM microenvironment. This combination supports cell microaggregation and differentiation and demonstrates the healthy development of chondrogenic and hypertrophic stages with abundant stage-specific ECM components. Similarly, fibrin glue combined with MSCs and tricalcium phosphate (β-TCP) particles can enhance heterotopic endochondral bone formation.

Other naturally derived polymers, such as alginate, chitosan, and agarose, have also been used as biocompatible hydrogel materials in endochondral bone engineering. Alginate hydrogels have been used to engineer endochondral bone tissue in subcutaneous spaces or bone defects. Furthermore, similar to GelMA hydrogels, alginate hydrogels are also used as beads for cell encapsulation or as bioinks for 3D printing for endochondral bone engineering. Chitosan is structurally similar to various glycosaminoglycans (GAGs) and has been shown to support hypertrophic cartilage matrix deposition and endochondral bone formation. However, compared with alginate and fibrin hydrogels, chitosan hydrogels are resistant to vascularization and bone remodeling. Agarose is a polysaccharide that has been used to encapsulate MSCs, MSC pellets, or other materials in endochondral bone engineering. By leveraging the good mechanical and thermoreversible properties of agarose hydrogels, agarose hydrogels containing an array of microchannel structures have also been shown to support vascularization and conversion to endochondral bone.
Synthetic polymers. Natural polymers are sometimes limited by their poor mechanical strength and rapid degradation. Synthetic polymers including poly(lactic acid) (PLA), polylactide-co-glycolide (PLGA), poly(lactic-co-glycolic acid) (PLGA), PCL, and their copolymers have been extensively utilized for endochondral bone engineering due to their good chondrogenic conduction, biocompatibility, slow degradation, and easily altered mechanical properties.

Synthetic polymers have demonstrated good chondrogenic conduction both in vitro and in vivo. PLGA scaffolds loaded with chondrogenically predifferentiated rat BMSCs have been shown to heal large bone defects in rats by mimicking ECO. Similarly, nasal chondrocyte-seeded PGA scaffolds can promote endochondral bone formation in rat cranial defects. The biochemical cues provided by synthetic polymer scaffolds control cellular proliferation and differentiation. PLGA/PLLA copolymer scaffolds can induce human ESC differentiation via an ECO pathway, while hydroxyapatite (HAp)-based PLGA/PLLA composite scaffolds result in bone formation via an IMO pathway. However, cell adhesion and proliferation are limited due to the hydrophobicity of these synthetic polymer scaffolds.

Synthetic polymers have better mechanical properties and flexibility than natural polymers. Porous PLGA scaffolds have been added to hyaluronan-fibrin hydrogels to enhance their mechanical strength and fabricate a load-bearing scaffold system. In this polymer-gel hybrid scaffold, the gel phase provides a microenvironment to guide the endochondral process, while the polymer phase is expected to provide mechanical strength to the overall polymer-gel structure. PCL possesses an appropriately high bulk stiffness to facilitate MSC differentiation toward skeletal lineages and has been selected as a scaffold material to support chondrogenesis and hypertrophic mineralization. In addition, bioprinted PCL microfiber networks have been used to reinforce the mechanical strength of engineered cartilaginous templates, which support the development of a vascularized bone organ containing trabecular-like bone and a supporting hematopoietic marrow structure.

Synthetic polymer scaffolds with nanofibers similar in size to natural cartilage matrix fibers facilitate tissue regeneration. 3D PLGA/PCL scaffolds fabricated by electrospinning have been demonstrated to support the chondrogenic differentiation of rat BMSCs in vitro and endochondral bone formation in vivo. Furthermore, the pore size and architecture of nanofibrous polymer scaffolds affect the conversion of cartilage to bone tissue. Under the same in vitro priming and in vivo implantation conditions, nanofibrous PLLA scaffolds with large pores (425–600 μm) have been shown to support endochondral bone formation by allowing blood vessel ingrowth, whereas scaffolds with very small pores (60–125 μm) have been shown to allow cartilage formation but inhibit ECO. In another study, an electrospun PCL 3D nanofibrous scaffold with interconnected and hierarchically structured pores morphologically similar to natural ECM could lead to high cell viability. More importantly, it could promote the BMP-2-induced chondrogenic differentiation of mouse BMSCs in vitro and act as a favorable synthetic ECM for endochondral bone regeneration in vivo.

Bioceramics. Known for their good biocompatibility, bone bioactivity, and osteoconductivity, bioceramics, such as HAp, β-TCP, and biphasic calcium phosphate (BCP), have been utilized as scaffolds for endochondral bone engineering. However, due to their high brittleness, bioceramics are typically combined with various natural or synthetic polymers to create highly porous biocomposite materials with improved mechanical properties.

HAp is one of the main inorganic components of the natural bone matrix. HAp-based scaffolds have been used extensively to promote bone regeneration because of their good biocompatibility and high osteoconductivity. Recently, nano-HAp particles, when used to coat titanium scaffolds, have been shown to support the chondrogenic differentiation of MSCs and enhance endochondral bone regeneration in mandibular defects in rats. Furthermore, when incorporated into biodegradable polymers, such as collagen and poly(vinyl alcohol), HAp particles enhance the mechanical properties of scaffolds, resulting in mechanical strength very similar to that of bone or cartilage. Moreover, the osteoinductivity of HAp can affect the chondrogenesis, hypertrophy, and ECO of MSCs. In a bioprintable HyA-based hydrogel system, a small number of HAp particles has been shown to promote both the chondrogenic and the hypertrophic differentiation of ASCs, whereas larger numbers of HAp particles promote hypertrophic conversion and early osteogenic differentiation of ASCs. In another study, CaP-coated HAp mineral particles have been shown to promote the gene expression of chondrogenic markers and enhance the hypertrophic phenotype of ESC aggregates in vitro in a dose-dependent manner.

β-TCP has been shown to promote cartilage regeneration and biomineralization because of its biodegradability, biocompatibility, and bioactivity. β-TCP is often combined with other materials to enhance the chondrogenic and endochondral potential of scaffolds. It has been shown that the addition of β-TCP particles to a 3D biomimetic hydrogel scaffold not only induces abundant expression of the chondrogenic markers COL II and aggrecan but also, most notably, results in overexpression of the hypertrophic marker COL X and the osteogenic marker ALP.

BCP consists of two CaP phases, namely, a stable HAp phase and a soluble β-TCP phase, in distinct proportions. These two materials are proportionally combined to obtain
a suitable balance between the predictable biodegradability offered by β-TCP and the improved resiliency provided by HAp. The chemical and structural cues provided by porous HAp/TCP scaffolds have been shown to support progressive lamellar-like bone formation and mature bone marrow development.125 Our previous study showed that porous HAp/β-TCP granules support endochondral bone formation at ectopic sites by enhancing mineralization.56 Furthermore, porous HAp/TCP scaffolds are more suitable for implantation in loadbearing areas than other scaffolds, including those made of polyurethane foam, electrospun PLGA/PCL fibers, and COL I gel, due to their excellent mechanical properties.114

dECM. dECM not only retains the original 3D morphological architecture of the tissue but also retains a complex mixture of proteins and macromolecules that facilitate the proliferation and differentiation of endogenous or exogenous cells.126 Importantly, the structural and functional proteins of the ECM are highly conserved across species, which allows xenogeneic dECM to be implanted in recipients of different species without an immune reaction.127,128 dECM derived from natural bone and cartilage tissues has been used as scaffolds for endochondral bone engineering. Recent works have shown that endochondral constructs can be engineered by culturing ASC-seeded decellularized bone matrix (dBM) sequentially in CHM and then hypertrophic medium (HYM). These engineered constructs have been shown to enhance bone deposition, bone remodeling, and bone marrow formation in critical-sized femoral defects in rats.59 In addition, decellularized cartilage matrix (dCM), which consists predominantly of COL II but lacks GAGs and cells, is a promising scaffold material for endochondral bone engineering.129 The biological integrity of dCM can stimulate endochondral bone regeneration by enhancing the chondrogenesis of MSCs, PDCs, or chondrocytes in vitro and eliciting a regenerative response upon implantation in vivo.96,128 but has no significant effect on construct mineralization96 and a lower potential for endochondral bone formation.130 Therefore, the use of dCM for endochondral bone engineering remains controversial, and studies have demonstrated that dCM promotes the chondrogenic differentiation but inhibits the hypertrophic differentiation of MSCs in vitro and subsequent ECO in vivo.131–133 It has long been realized that viable hypertrophic cartilage will form bone in vivo; decellularized hypertrophic cartilage matrix (dHCM), which is manufactured from native epiphyseal plates or fractured callus tissue, can also trigger the natural ECO process upon implantation.134 A major drawback of these types of naturally derived dECMs is the limited donor source, which hinders their clinical application. Recent works have sought to engineer off-the-shelf decellularized/devitalized tissue engineered-cartilaginous matrix (dTECM) for endochondral bone engineering, which is expected to induce regenerative processes not only through specific “organomorphic” structures but also through the physiological presentation of different cocktails of regulatory molecules in a suitable environment.135,136 Bourgine et al. engineered a novel hypertrophic cartilage construct using immortalized human MSCs, in which decellularization was achieved by the induction of apoptosis with efficient ECM preservation. The resulting dHCM could efficiently remodel to form endochondral bone tissue of host origin, including a mature vasculature and a hematopoietic compartment.137–139 Furthermore, dTECM engineered from various cell sources, such as ATDC5 cells,105 adipose-derived stromal vascular fraction (SVF) cells,140 and BMSCs,141,142 has shown the ability to be activated by MSCs to initiate ECO and induce new bone formation upon implantation without long-term endochondral priming in vitro. These promising results offer an alternative solution to overcome the drawbacks of naturally derived dECM and the extensive production of dECM using allogenic MSCs.

Optimal endochondral priming medium and duration

Chondrogenesis is the first stage of the ECO process, involving with differentiation and maturation of chondrocytes. Different endochondral priming protocols have been used to replicate the initial stages of ECO by driving progenitors toward a hypertrophic phenotype.

Endochondral priming medium. Endochondral priming largely relies on the chondrogenic differentiation stage, as chondrocytes possess specific metabolic features and secrete diverse biochemical cues when they are in different developmental stages. Generally, the engineering of a cartilage template from MSCs in vitro is necessary to recapitulate the process of ECO because chondrogenically differentiated MSCs tend to simultaneously acquire a certain degree of hypertrophic properties upon persistent exposure to CHM.143,144 However, chondrogenic induction alone usually results in “early hypertrophic” tissues with low and localized COL X expression,51,87 which is specifically produced by hypertrophic chondrocytes.145 Due to the critical roles of hypertrophic chondrocytes during ECO, an additional in vitro hypertrophic priming step is often applied to the chondrogenically primed constructs to elicit the most extensive ECO.146,147 The differentiation of MSCs in vitro into mature and hypertrophic chondrocytes requires a precise combination of growth and differentiation factors. However, standard medium formulations for chondrogenic differentiation and hypertrophic induction are lacking. CHM is typically defined as Dulbecco’s modified Eagle’s medium (DGMEM) supplemented with 100 U/mL penicillin/streptomycin, 100 μg/mL sodium pyruvate, 40 μg/mL L-proline, 50 μg/mL L-ascorbic acid 2-phosphate, 4.7 μg/mL linoleic acid, 1.5 mg/mL bovine serum albumin (BSA), 1× insulin-transferrin-selenium (ITS)
premix, and 100 nM dexamethasone, along with various growth factors. Serum is sometimes used, but serum-free media are more common because serum-free cultures closely mimic the serum-free physiological environment in which chondrocytes reside. Growth factors play a dominant role in the differentiation of MSCs toward chondrogenic phenotypes in vitro; these growth factors include TGF-βs and BMPs, which play fundamental roles in early-stage MSC differentiation and chondrogenic phenotype maintenance. Some of these factors are also involved in terminal differentiation.

TGF-β superfamily members including TGF-β1, TGF-β2, and TGF-β3 are the core components of most chondrogenic differentiation protocols. In vitro, TGF-βs induce chondrogenesis, proliferation, and matrix deposition in MSCs, with eventual progression toward hypertrophy. TGF-β1 and TGF-β3 initiate MSC condensation during the early stage of endochondral bone development. TGF-β3 has stronger effects than TGF-β1 or TGF-β2 on the chondrogenic differentiation of MSCs. Furthermore, BMPs, which are morphogens in the TGF-β superfamily, have been shown to regulate chondrogenesis and osteogenesis during skeletal development. BMP-2, BMP-4, and BMP-6 are the most commonly used BMPs for chondrogenic differentiation. BMP-2 has been reported to induce chondrogenic differentiation by upregulating the levels of COL II and aggrecan in various types of stem cells in vitro and to promote chondrocyte hypertrophy by increasing COL X and ALP expression as well as upregulating of Indian hedgehog expression. BMP-4 plays a fundamental role in early-stage MSC chondrogenic differentiation and chondrogenic phenotype maintenance and is also involved in regulating the terminal differentiation of chondrocytes. BMP-6 participates in mediating chondrocyte hypertrophy. The treatment of chondrocytes with BMP-6 has been widely shown to stimulate COL X gene expression.

Various combinations of TGF-βs and BMPs have shown a synergistic ability to enhance chondrogenic differentiation and hypertrophy and have been widely used in endochondral bone engineering. For example, a combination of TGF-β1 and BMP-2 has been shown to increase the GAG and collagen content and initiate robust endochondral lineage commitment. The dual delivery of TGF-β1 and BMP-2 within BMSC aggregates has been shown to result in enhanced chondrogenesis and an enhanced osteogenic phenotype, as well as a greater degree of mineralization and COL X expression. Additionally, compared to TGF-β1 alone, combinations of TGF-β1 and BMP-6 can enhance the chondrogenic potential of BMSCs and ASCs. Among numerous growth factor combinations, TGF-β3 in conjunction with BMP-6 appears to be the most effective for chondrogenic induction and ECO in ASCs because TGF-β3 alone is a potent inducer of chondrogenic differentiation, whereas BMP-6 acts synergically with TGF-β3 by inducing the expression of TGF-β receptor I, which is usually not expressed by ASCs.

Additional culture in HYM has been applied to promote chondrocyte hypertrophy and ossification in vitro. HYM is typically defined as CHM without growth factors, with a reduced dexamethasone concentration (1–10 nM), and with β-glycerophosphate (β-GP, 10 mM) and thyroxine (1–50 nM) or triiodothyronine (T3, 1 nM). The reduced dexamethasone concentration can induce Runx2 upregulation, followed by COL I upregulation. β-GP can act as a source of phosphate for HAp. In addition to these supplements in HYM, other molecules or growth factors have been added to improve the efficiency of hypertrophic induction. Thyroid hormone and T3 have been shown to induce morphological and hypertrophic marker expression without inducing proliferation. Furthermore, inflammatory cytokines, such as interleukin-1β, have been used to induce inflammation to improve hypertrophic cartilaginous construct remodeling into bone tissue without hampering mineralization.

**Endochondral priming duration.** Endochondral priming protocols typically aim to reach a stage with a certain degree of hypertrophy before implantation. For chondrogenic priming, the in vitro induction duration varies from 1 to 5 weeks for different cells, media, and culture systems but usually lasts for 3 to 4 weeks. For clinical translation, prolonged in vitro culture is associated with high treatment costs and a large regulatory burden, which are not ideal. Fine coordination between the progression of chondrogenesis and endochondral transformation needs to be achieved by choosing the optimal duration of HYM. It has been shown that the priming of BMSC pellets in vitro for 3 weeks in CHM followed by 4 weeks in HYM optimized GAG production and mineralization, resulting in a construct with mineralization throughout the core. However, a longer chondrogenic priming duration resulted in a significant increase in the homogeneous deposition of cartilage matrix, whereas the bone volume was not affected by the priming duration. Two weeks of chondrogenic priming in vitro is sufficient to generate a substantial amount of vascularized endochondral bone in vivo.

**Bone defect reconstruction via the endochondral route: current state of the art**

Cartilaginous constructs engineered via endochondral priming tend to undergo hypertrophy and generate endochondral bone tissue at ectopic sites, which opens the possibility of using endochondrally primed constructs for bone defect repair. Such an ectopic model is useful for investigating vascular invasion and mineralization but does not offer an ideal environment for assessing the efficacy of bone defect reconstruction. In an orthotopic environment, bone regeneration starts with a low-grade inflammatory...
phase, low oxygen tension, and continuous biomechanical stimuli, which are all known to affect bone regeneration. To analyze the current state of bone defect reconstruction using ECO-based strategies, we searched the PubMed databases for articles published between January 1, 2000, and March 1, 2021, using the search terms “endochondral ossification,” “bone defect reconstruction,” “bone regeneration,” and “bone tissue engineering.” Here, we include 25 publications related to bone defect reconstruction in animal model. The included studies must meet all the above criteria at the same time. The excluded criteria were: (1) not an original article; (2) full text was not available; (3) not English language; (4) duplicate publications. Reports meet any of the above criteria were excluded.

Figure 4. Flow diagram for study selection.
The inclusion criteria were as follows: (1) the constructs were engineered via chondrogenic and/or hypertrophic priming in vitro; (2) bone defect reconstruction in animal model. The included studies must meet all the above criteria at the same time. The excluded criteria were: (1) not an original article; (2) full text was not available; (3) not English language; (4) duplicate publications. Reports meet any of the above criteria were excluded.

Additional hypertrophic priming promotes the efficiency of endochondral bone formation

Although chondrogenically primed constructs have shown a certain degree of hypertrophy and can recapitulate ECO upon implantation in vivo, insufficient construct ossification and vascularization have also been observed in several studies. Additional hypertrophic priming of chondrogenically primed constructs not only maintains the chondrogenic features but also promotes the abundant expression of hypertrophic markers and ultimately results in abundant vascularization and mature bone matrix formation. Similar results have also been observed in adipose tissue-derived SVF-based cartilaginous constructs. These results from ectopic bone formation models suggest that the hypertrophic priming of cartilaginous grafts provides a valuable solution for enhancing endochondral bone regeneration and accelerating vascularization. Unfortunately, to date, no studies have compared the efficiency of bone formation between cartilage constructs engineered via chondrogenic priming alone and those engineered via a combination of chondrogenic and hypertrophic priming in orthotopic bone formation models.

Chondrogenically primed cartilaginous grafts act as logical templates for bone repair at orthotopic sites

As shown in Table 1, a wide range of distinct approaches have been adopted to engineer cartilaginous grafts by chondrogenic priming. In 2006, a cartilaginous construct engineered via the chondrogenic priming of autologous BMSC-seeded biodegradable scaffolds for 3 weeks effectively prevented carpal collapse in a New Zealand white rabbit model. This is the first report demonstrating that tissue-engineered cartilaginous grafts could recapitulate the ECO process and support bone formation at orthotopic sites. Inspired by this study, attempts have been made to engineer cartilaginous grafts in vitro for critical-sized bone defect reconstruction and have yielded promising results. Tissue-engineered cartilaginous grafts have been observed to mature and form bone tissue in critical-sized calvarial defect models, although they are not the most logical model for endochondral bone formation because craniofacial bones form through IMO. For example, compared with sham implants, cartilaginous constructs engineered via the chondrogenic priming of mouse ESC-seeded ceramic scaffolds could transform into bone tissue in the inner circle of the constructs in 8-mm rat cranial defects. Moreover, cartilaginous grafts engineered via the chondrogenic priming of other cell sources, such as nasal chondrocytes and PDCs, have shown promising results in critical-sized bone defect reconstruction. To further translate the use of chondrogenically primed cartilaginous constructs for therapeutic applications, cartilaginous grafts engineered by the chondrogenic priming of BMSC-seeded PLGA scaffolds have been used to heal both critical-sized (5-mm) and massive (15-mm) full-thickness femoral defects in rats. After 8 weeks, the mean biomechanical strength of femora with 15-mm implants reached 75% of that of the normal rat femur, while there was no significant difference in the strength of femora with 5-mm implants. Collectively, this evidence suggests that engineering of cartilaginous grafts via chondrogenic priming alone is a viable, underexplored strategy for critical-sized bone defect reconstruction.
Table 1. Cartilaginous constructs engineered by chondrogenic priming alone for bone defect reconstruction.

| Reference                  | Cell source                | Biomaterial       | In vitro priming condition                                           | Bone defect model                           | Highlighted results                                                                                                                                                                                                 |
|----------------------------|----------------------------|-------------------|-----------------------------------------------------------------------|---------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| limori et al. (2021)       | hiPSC line                 | /                 | Scaffoldless suspension culture; 10, 12, or 17 weeks in CHM containing TGF-β, BMP-2, and GDF-5 | 3.5-mm femoral defect in SCID mice          | The hiPSC-derived cartilage produced new bone via reminiscent of SOC-ECO process in the defects. Less time for chondrogenic differentiation of hiPSCs resulting in faster bone formation.                                         |
| Longoni et al. (2020)      | hBMSCs; rat BMSCs          | COL I gel         | Spheroid culture; 4 weeks in CHM containing TGF-β and BMP-2          | 6-mm femoral defect in Brown Norway rats    | The amount of endochondral bone formation was proportional to the degree of host-donor relatedness. No full bridging of the defect was observed in the hBMSCs group, whereas 2/8 and 7/7 bridges formed in allogeneic and syngeneic group, respectively. |
| Nilsson Hall et al. (2020) | hPDCs                      | /                 | Microspheroid culture; 4 weeks in chemically defined CHM containing BMP-2, TGF-β, GDF-5, BMP-6, and FGF-2 | 4-mm tibial defect in NMRI nu/nu mice       | Engineered callus organoids spontaneously bioassembled in vitro into large, engineered tissues able to heal murine critical-sized long bone defects via ECO.                                                                 |
| Freeman et al. (2020)      | hBMSCs; hUVECs             | PCL scaffold      | Endochondral priming: 3 weeks in CHM containing TGF-β; Osteogenic priming: 3 weeks in osteogenic medium | 4-mm calvarial defect in immunocompromised mice | The addition of hUVECs alone or a coculture of hUVECs and hBMSCs did not benefit for either the vascularization or mineralization potential of the scaffolds. Endochondral priming alone was sufficient to induce vascularization and subsequent mineralization. |
| Wang et al. (2018)         | Rat BMSCs                  | HAp-coated porous Ti₆Al₄V scaffolds | 4 weeks in CHM                                                      | 5-mm full-thickness circular mandibular defect in SD rats | The HAp-coated Ti₆Al₄V scaffolds improved the chondrogenic differentiation of BMSCs in vitro and increased new bone formation via ECO in vivo. 3D-printed hypertrophic cartilage grafts with microchannels promoted osteoclast/immune cell invasion, hydrogel degradation, and vascularization following implantation. |
| Daly et al. (2018)         | Rat BMSCs                  | GelMA hydrogel with 3D printed microchannels | 4 weeks in CHM containing TGF-β and BMP-2 | 5-mm femoral defect in Fischer rats | Serum-free preconditioning in CDM enhanced BMP-2-induced osteochondrogenic differentiation of PDCs. Combined in vitro priming by BMP-2 treatment and aggregation led to endochondral bone formation and critical-size bone defect healing in vivo. |
| Bolander et al. (2017)     | hPDCs                      | COL I gel         | Cell aggregate culture; 6 days of preconditioning in serum-free CDM or growth medium followed by 6 days of stimulation by BMP-2, BMP-4, BMP-6, BMP-7, BMP-9, and GDF-5 in CDM | 4-mm tibial defect in NMRI nu/nu mice | Constructs derived from nasal chondrocytes had the capacity to express features of hypertrophic chondrocytes. Nasal chondrocytes can be used to engineer hypertrophic cartilage and repair bone defects. |
| Bardsley et al. (2017)     | Rat nasal chondrocytes     | PGA scaffold      | Constructs were cultured in basic medium containing insulin and ascorbic acid for 5 weeks | 4-mm full-thickness calvarial defect in Wistar rats |                                                                                       |

(Continued)
| Reference | Cell source | Biomaterial | In vitro priming condition | Bone defect model | Highlighted results |
|-----------|-------------|-------------|----------------------------|-------------------|---------------------|
| van der Stok et al. (2014) | hBMSCs | / | Pellet culture; undifferentiated pellets: 3 days in CHM containing TGF-β1; Chondrogenically differentiated pellets: 3 weeks in CHM containing TGF-β1 | 6-mm femoral defect in RUN rats | Chondrogenically differentiated pellets resulted in significantly more bone and vascularization in critical bone defects through ECO than undifferentiated pellets. |
| Harada et al. (2014) | Rat BMSCs | PLGA scaffold | 3 weeks in CHM containing TGF-β1 and BMP-2 | 5-mm or 15-mm femoral defect in Fischer rats | The large 15-mm implants reached 75% of the strength of the normal rat femur, while there was no significant difference in the strength of the 5-mm implants. |
| Mikael et al. (2014) | hBMSCs | Donut-shaped Heals scaffold disc | Pellet culture, 16 days in CHM containing TGF-β1 | 3.5-mm calvarial defect in NSG mice | Precartilage template formed in vitro induced mineralized tissue formation via a cartilage-mediated process. |
| Bahney et al. (2014) | hBMSCs; hACs | PEGDA scaffolds | Pellet culture, 3 weeks in CHM containing TGF-β1; Scaffold culture, 6 weeks in CHM containing TGF-β1 | 2-mm segmental tibial defect in Nude mice | Cartilage grafts from fracture callus produced well-vascularized and integrated bone regeneration via ECO in bone defects. |
| Jukes et al. (2008) | Mouse ESC line IB10 | Ceramic scaffolds | 3 weeks in serum-free CHM containing TGF-β1 | 8-mm calvarial defect in immunodeficient rats | Significantly more bone ingrowth was observed in the inner circle of the tissue-engineered cartilaginous constructs. |
| Huang et al. (2006) | Rabbit BMSCs | Composite sponge of 70% esterified hyaluronan and 30% gelatin | 3 weeks in serum-free CHM containing TGF-β1 | Lunate excision in adult New Zealand white rabbits | Cartilaginous implants formed abundant bone tissue and blood vessels through ECO. |

BMSCs, bone marrow-derived mesenchymal stem cells; CDM, chemically defined medium; CHM, chondrogenic medium; ESCs, embryonic stem cells; GDF-5, growth/differentiation factor 5; GelMA, gelatin-methacrylamide; hACs, human articular chondrocytes; HAp, hydroxyapatite; hiPSCs, human induced pluripotent stem cells; hPDCs, human periosteum-derived cells; hUVECs, human umbilical vein endothelial cells; PCL, poly(ε-caprolactone); PEGDA, poly(ethylene glycol) diacrylate; PGA, polyglycolic acid; PLGA, poly(lactic-co-glycolic acid). CHM is typically defined as DMEM supplemented with 100 U/mL penicillin/streptomycin, 100 μg/mL sodium pyruvate, 40 μg/mL L-proline, 50 μg/mL L-ascorbic acid 2-phosphate, 4.7 μg/mL linoleic acid, 1.5 mg/mL BSA, 1× ITS, 100 nM dexamethasone, and 10 ng/mL human TGF-β1 or TGF-β3.
| Reference                  | Cell source                  | Biomaterial                                                                 | In vitro priming condition                        | Bone defect model             | Highlighted results                                                                 |
|---------------------------|------------------------------|------------------------------------------------------------------------------|----------------------------------------------------|-------------------------------|--------------------------------------------------------------------------------------|
| Mikael et al. (2020)      | hBMSCs                       | Hybrid matrix scaffolds composed of PLGA microspheres and HyA-fibrin hydrogel | 2 weeks in CHM containing TGF-β3 followed by 2 weeks in HYM | 3.5-mm calvarial defect in NSG mice | Hybrid matrix recruited host cells, leading to new bone formation and remodeling through ECO. |
| Zhang et al. (2020)       | Mouse gingiva-derived iPSCs  | /                                                                            | 3D rotary suspension culture pellet culture; 2 weeks in CHM containing TGF-β3 and BMP-4 followed by 2 weeks in HYM | 5-mm circular calvarial defect in SD rats | The hypertrophic cartilage pellets derived from iPSCs were capable of vascularized bone regeneration via ECO in the bone defects. |
| Li et al. (2019)          | Mouse BMSCs                  | Ceria nanoparticle modified cancellous bones                                | 2 weeks in CHM containing TGF-β3 followed by 2 weeks in HYM | 3-mm femoral defect in FVB/N mice | Ceria nanoparticles significantly promoted ECO-based bone regeneration by ensuring sufficient hypertrophic differentiation via DHX15 activation. |
| Petersen et al. (2018)    | hBMSCs                       | Collagen scaffold with a channel-like pore architecture                      | 3 weeks in CHM containing TGF-β3 followed by 2 weeks in HYM | 5-mm femoral defect in SD rats | Channel-like macroporous architecture had the potential to induce the ECO process for bone healing. |
| Matsiko et al. (2018)     | MSCs                         | Collagen-HyA scaffolds                                                      | 3 weeks in CHM followed by 2 weeks in HYM           | 5-mm femoral defect in Fischer rats | Collagen-based scaffolds acted as suitable templates for the development of ECO constructs capable of supporting early-stage bone repair. |
| Bai et al. (2018)         | Murine BMSCs                 | /                                                                            | Pellet culture; 2 weeks in CHM containing TGF-β3 and mangiferin followed by 2 weeks in HYM | 2-mm femoral defect in BALB/c mice | Mangiferin promoted the chondrogenic and hypertrophic differentiation of BMSCs in vitro and enhanced ECO-based bone repair in vivo. |
| Dang et al. (2017)        | hBMSCs                       | Gelatin microparticles loaded with TGF-β3; mineral-coated HAp microparticles loaded with BMP-2 | Microparticle-loaded hBMSC sheets; 2 weeks in serum-free CHM followed by 3 weeks in serum-free osteogenic medium | 5-mm circular calvarial defect in athymic rats | Constructs containing microparticles loaded with TGF-β3, and BMP-2 promoted the greatest degree of healing with bony bridging via ECO. |
| Bernhard et al. (2017)    | hASCs                        | Decellularized bovine trabecular bone matrix                                | Hypertrophic chondrocyte graft: 2 weeks in CHM containing TGF-β3 and BMP-6 followed by 3 weeks in HYM; osteoblast graft: 5 weeks in osteogenic medium | 5-mm femoral defect in RUN nude rats | Hypertrophic chondrocyte grafts enhanced bone regeneration by recapitulating ECO in critical-sized orthotopic long bone defects. |
| Thompson et al. (2016)    | Rat BMSCs                    | Collagen-HyA scaffolds; ECO constructs: 3 weeks in CHM containing TGF-β3 followed by 2 weeks in HYM; collagen-HA scaffolds | 7-mm circular calvarial defect in Fischer rats | Collagen-HyA hypertrophic constructs supported the greatest new bone formation within the defects. | ECO-based constructs yielded more new bone formation within the defects than IMO-based constructs, which may be associated with VEGF secretion in the ECO-based constructs. |
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Reference Cell source Biomaterial In vitro priming condition Bone defect model Highlighted results

Bahney et al. (2016) Human OA chondrocytes Pellet culture; 1 week in CHM containing TGF-β1 and BMP-4 followed by 3 weeks in CHM without growth factors 3-mm tibial defect in immunocompromised mice Endochondrally primed cartilage grafts generated from passaged OA chondrocytes underwent ECO, variably remodelled into woven bone, and integrated with host bone at 15/16 junctions. Cartilage grafts formed from primary OA chondrocytes without endochondral priming did not undergo ECO in vivo. Chondrogenically primed BMSC-alginate constructs acted as templates to treat critical-sized defects within bones formed through either IMO or ECO.

Cunniffe et al. (2015) Rat BMSCs Alginate hydrogels 4 weeks in CHM containing TGF-β3 followed by 3 weeks in HYM 5-mm femoral defect and 7-mm circular calvarial defect in Fischer rats Chondrogenically primed BMSC-alginate constructs acted as templates to treat critical-sized defects within bones formed through either IMO or ECO.

Table 2. (Continued) However, several studies have revealed the superiority of hypertrophically primed constructs over traditional IMO-based constructs in bone defect reconstruction. Thompson et al. compared the orthotropic bone formation efficiency of constructs engineered via chondrogenic and hypertrophic priming with that of constructs engineered by osteogenic priming. Because hypertrophic chondrocytes can secrete osteogenic and angiogenic signals, the endochondral constructs showed better results than the osteogenic constructs in terms of bone regeneration, vascularization, and remodeling.90 Similar results have been observed in a rat model of critical-sized femoral defects repaired by hASC-based grafts. The hypertrophic grafts engineered by 3 weeks of chondrogenic priming followed by 2 weeks of hypertrophic priming substantially enhanced bone regeneration associated with extensive bone remodeling and hematopoietic marrow formation. Furthermore, the hypertrophic cartilaginous grafts resulted in significantly greater bone volume in the defect space than the osteoblast grafts and acellular scaffolds.35 However, the additional in vitro hypertrophic priming step prolongs the endochondral bone engineering period. A delicate balance between chondrogenic differentiation and hypertrophic induction should be further investigated to improve the efficiency of ECO-based strategies in the future.

Future perspectives
To date, numerous studies have shown promising results in bone defect reconstruction using endochondrally primed constructs in animal models. However, the translation of these ECO-based strategies from the bench to the bedside is still ongoing and will face many challenges.

Integration of “top-down” tissue engineering and developmental engineering approaches provides a new solution for repairing large bone defects

Generally, tissue-engineered grafts for bone defect reconstruction should ensure osteogenesis, angiogenesis, and survivability after implantation. To date, successes in large bone defect reconstruction using ECO-based strategies have been achieved in small animal models, but no studies have verified the practicability of such a strategies in a large animal model, which is closer to the actual clinical situation. For repairing large bone defects in large animal models or under clinical conditions, scaled-up endochondral constructs are needed to fit the defects, which poses a new challenge.

Classic approaches for recapitulating ECO adopt a “top-down” strategy that relies on seeding progenitor cells onto scaffolds and then guiding the ECO process with growth factors. Such strategies are limited in the fabrication of large tissue constructs in vitro. Conversely, an
emerging “bottom-up” strategy for engineering large endochondral constructs is the scaffold-free culture technique, which aims to precondition cells to form modular tissue units represented by spheroid culture techniques, including cell pellets, cell sheets, and cell aggregates. These high-density cell cultures provide a more homogeneous 3D culture format than other cultures to allow cell-cell interactions that are similar to the precartilage condensation process during embryonic bone development. Importantly, stem cell condensation has been shown to enhance chondrogenic differentiation. By integrating the principles of “bottom-up” tissue engineering and “developmental engineering” approaches, endochondrally primed spheroids derived from MSCs or ESCs can spontaneously fuse with each other and recapitulate ECO events, making them ideal building blocks for engineering large-scale bone grafts. This integrated approach has several advantages that may support its clinical translation: (1) the possibility of scaling up tissue-engineered bone grafts to a clinically relevant size; (2) the ability to create endochondral bone tissues with high cellular densities without scaffolds; and (3) the potential to model an endochondral bone graft with a complex geometric shape. The engineering of endochondral constructs via cell aggregates and pellets has been reported in the studies regarding critical-sized bone defect reconstruction and large bone graft prefabrication.

**dTECM: An off-the-shelf material capable of recapitulating ECO**

A major obstacle to the clinical translation of ECO-based strategies is the long-term in vitro endochondral priming period. Furthermore, other issues, such as cost-effectiveness, engineering process complexity, the need for two surgical procedures, and tissue engineering-associated regulatory hurdles, also need to be overcome. These limitations have driven the development of dTECM as an off-the-shelf and immune-compatible alternative to living grafts with the capability of recapitulating the ECO process for bone defect reconstruction. The dTECMs could be used to directly attract endogenous MSCs toward the scaffold by leveraging bioactive cues embedded within the dTECM or activated by living cells prior to implantation, with the assumption that the dTECM is capable of directing these cells to differentiate into hypertrophic chondrocytes. To date, various chemical, enzymatic, and physical procedures have been developed to eliminate the cellular components of tissue-engineered cartilaginous tissue while minimally disrupting the ECM. Cunniffe et al. created porous scaffolds by freeze-drying hypertrophic cartilage constructs engineered from allogeneic BMSCs. The resulting scaffolds retained their proangiogenic ability and capacity to direct host-mediated orthotopic bone regeneration in critical-sized femoral defects. Bourgine et al. developed a decellularization methodology that induces the apoptosis of cells within tissue-engineered hypertrophic cartilage. Compared to standard production and freeze/thaw treatment, the resulting dHCM showed superior ECM preservation, leading to enhanced bone formation upon implantation. Therefore, developing a reproducible and cost-effective technique to manufacture a large amount of human tissue-derived dTECM may have good potential for clinical translation. Overall, the “off-the-shelf” availability and immune-compatible properties of dTECM may determine the extent of its clinical use.

**Conclusion**

Comprehensively, these initial results demonstrated that ECO-based strategies can be considered highly promising approaches for large bone defect reconstruction. Although limited success has been observed in clinical cases, these strategies have shown tremendously promising results in critical-sized bone defect reconstruction in animal models and have provided new insights into the fabrication of large, vascularized bone grafts. Nevertheless, research in this field is ongoing, and extensive research is undoubtedly needed to further improve bone output, scale up constructs, and enhance graft vascularization in the future.

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