Materials Today Bio 3 (2019) 100009

Contents lists available at ScienceDirect

Materials Today Bio

journal homepage: www.journals.elsevier.com/materials-today-bio

# Biomaterial-based endochondral bone regeneration: a shift from traditional tissue engineering paradigms to developmentally inspired strategies

E.J. Sheehy [a,b,d], D.J. Kelly [a,b,c,d], F.J. O'Brien [a,b,c,d,*]

[a] Tissue Engineering Research Group (TERG), Department of Anatomy and Regenerative Medicine, Royal College of Surgeons in Ireland, Dublin, Ireland
[b] Trinity Centre for Biomedical Engineering, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland
[c] Department of Mechanical and Manufacturing Engineering, School of Engineering, Trinity College Dublin, Dublin, Ireland
[d] Advanced Materials and Bioengineering Research Centre (AMBER), Royal College of Surgeons in Ireland and Trinity College Dublin, Dublin, Ireland

ARTICLE INFO

*Keywords:*
scaffold
mesenchymal stem cell
endochondral ossification
hypertrophy
biochemical factors
oxygen tension

ABSTRACT

There is an urgent, clinical need for an alternative to the use of autologous grafts for the ever increasing number of bone grafting procedures performed annually. Herein, we describe a developmentally inspired approach to bone tissue engineering, which focuses on leveraging biomaterials as platforms for recapitulating the process of endochondral ossification. To begin, we describe the traditional biomaterial-based approaches to tissue engineering that have been investigated as methods to promote in vivo bone regeneration, including the use of three-dimensional biomimetic scaffolds, the delivery of growth factors and recombinant proteins, and the in vitro engineering of mineralized bone-like tissue. Thereafter, we suggest that some of the hurdles encountered by these traditional tissue engineering approaches may be circumvented by modulating the endochondral route to bone repair and, to that end, we assess various biomaterials that can be used in combination with cells and signaling factors to engineer hypertrophic cartilaginous grafts capable of promoting endochondral bone formation. Finally, we examine the emerging trends in biomaterial-based approaches to endochondral bone regeneration, such as the engineering of anatomically shaped templates for bone and osteochondral tissue engineering, the fabrication of mechanically reinforced constructs using emerging three-dimensional bioprinting techniques, and the generation of gene-activated scaffolds, which may accelerate the field towards its ultimate goal of clinically successful bone organ regeneration.

## Introduction

Bone is a dense, connective tissue that functions to produce blood cells, store minerals, facilitate locomotion, and support and protect the vital organs of the body. As a highly dynamic material, bone possesses an intrinsic capacity for regeneration and constantly undergoes a remodeling process involving the resorption of old bone and deposition of new bone. However, clinical situations exist, whereby bone regeneration is required in large quantities, such as for reconstruction of large bone defects caused by trauma, infection, tumor resection, and skeletal abnormalities, or in cases where the regenerative process is compromised, such as in avascular necrosis, atrophic non-unions, and osteoporosis [1]. As a result, bone is the second most implanted tissue in the body after blood [2].

The gold standard treatment to promote regeneration of large bone defects is an autologous bone graft. This involves harvesting a section of a patient's own bone, usually from the anterior or posterior iliac crest of the pelvis, and then implanting the harvested bone back into the defect site. While this is a well-established approach, there are a number of drawbacks associated with the therapy; harvesting of the bone, for example, requires an additional surgical procedure, which can result in donor site morbidity, and there is a limitation on the quantity of bone available for harvest. Such limitations can be addressed through the use of an allogeneic bone graft, which can be obtained from human cadavers or living donors. However, as allogeneic bone grafts are devitalized prior to implantation, they may not possess the same osteoinductive potential as autologous bone grafts and complications can also arise due to immunogenicity and potential infection transmission [3]. Therefore, there is an urgent need for alternatives to autologous and allogeneic grafts in order to promote the regeneration of bone.

Tissue engineering has emerged as a multidisciplinary field which utilizes materials science, as well as aspects of cell biology and

* Corresponding author. Tissue Engineering Research Group (TERG), Department of Anatomy, Royal College of Surgeons in Ireland, Dublin, Ireland.
*E-mail address:* fjobrien@rcsi.ie (F.J. O'Brien).

https://doi.org/10.1016/j.mtbio.2019.100009
Received 18 April 2019; Received in revised form 23 May 2019; Accepted 24 May 2019
Available online 31 May 2019

engineering, in order to repair or regenerate damaged tissues through a typical combination of three-dimensional (3D) biomaterial scaffolds, cells, and signaling molecules [4,5]. Within this tissue engineering triad, the biomaterial scaffold is a key component which can be leveraged to deliver bioactive cues and determine the fate of cells. To date, bone tissue engineering applications have generally focussed on the direct osteogenic differentiation of mesenchymal stem cells (MSCs) [6] seeded on 3D scaffolds in a process resembling intramembranous ossification [7]. However, for large defects, this approach has been hampered by the lack of a functional vascular supply upon implantation, required for the necessary delivery of oxygen and nutrients into the scaffold to ensure cell survival [8]. Furthermore, in vitro osteogenic priming of an engineered tissue can inhibit vascularization of the graft by sealing up the pores of a scaffold with calcified matrix, resulting in core degradation [9]. These issues have, to a large extent, hindered the translation of intramembranous bone tissue engineering strategies into the clinic.

Recently, there has been a shift away from utilizing biomaterials in traditional tissue engineering paradigms towards strategies that leverage biomaterials in order to recapitulate embryonic processes, an approach which has been termed 'developmental engineering' [10]. The long bones of the body develop not by intramembranous but by endochondral ossification, where MSCs undergo chondrogenic differentiation into chondrocytes forming an initial cartilaginous template, followed by hypertrophic differentiation resulting in the release of pro-angiogenic factors facilitating vascularization and remodeling of the cartilaginous template into bone [11]. It follows that the endochondral approach to bone tissue engineering may circumvent the hurdles associated with the traditional intramembranous approach as chondrocytes normally reside in an avascular tissue and, as a result, are designed to function in a low oxygen environment, similar to what they would encounter upon implantation into a poorly vascularized defect [12]. Furthermore, bone marrow–derived MSCs cultured chondrogenically in vitro have an inherent tendency to become hypertrophic, the next step in the endochondral ossification pathway and a critical factor in promoting the conversion of avascular tissue to vascularized tissue [13]. However, a number of key design criteria surrounding the choice of biomaterial scaffolds, cells, and signaling factors required to engineer hypertrophic cartilaginous grafts in vitro capable of regenerating damaged or diseased bones in vivo remain to be elucidated, see Fig. 1.

This review will first evaluate the biomaterial-based approaches traditionally taken to promote bone regeneration through intramembranous ossification. Thereafter, the regulatory mechanisms that govern bone development during endochondral skeletogenesis and fracture repair will be described and the various cells and signaling factors which can be harnessed to promote hypertrophy of engineered cartilaginous grafts will be examined. Furthermore, the 3D scaffolds that can be leveraged to upscale tissue-engineered hypertrophic cartilaginous grafts suitable for promoting bone regeneration through endochondral ossification will be investigated. Finally, future scientific trends in biomaterial science which may accelerate the endochondral approach towards implementation in a clinical setting will be discussed and evaluated.

## Traditional biomaterial-based approaches for bone tissue engineering applications

### *Implantation of acellular scaffolds*

The use of 3D porous biomaterial-based scaffolds, which once implanted could direct the migration, proliferation, and differentiation of host cells, has shown some success as alternatives to autologous bone grafting for regenerative applications, albeit generally for small defects only. Typically, three types of biomaterials are used to fabricate such scaffolds: ceramics, natural polymers, and synthetic polymers. Hydroxyapatite (HA) is the major mineral constituent of bone and has garnered much attention in the field of bone tissue engineering. Early work investigated the capacity of dense and porous HA ceramics to promote ectopic ossification following implantation in dogs [14,15]. These studies found evidence of new bone deposition via intramembranous ossification in the porous ceramics only, highlighting the importance of a porous network in order to provide the appropriate milieu for osteogenesis. Additional studies have demonstrated that the osteogenic capacity of ceramics, when implanted intramuscularly in goats and sheep, can be enhanced through the combination of HA and β-tricalcium phosphate (β-TCP) in a biphasic calcium phosphate ceramic [16,17]. The brittle nature of ceramic materials, however, and a difficulty with tailoring their degradation characteristics, may limit their use in bone tissue engineering applications [18].

Issues surrounding the biodegradability of ceramic scaffolds for tissue engineering applications can be addressed by combining ceramics with natural derived polymers in a single scaffold. To that end, biomimetic bone scaffolds consisting of collagen and HA have been fabricated which show a synergistic effect whereby the ductile properties of collagen enhance the fracture toughness of HA [19]. Furthermore, in addition to improving the mechanical properties of a collagen-based scaffold, the incorporation of a ceramic compound also enhances permeability by better maintaining the interconnected pore structure of the scaffold [20]. Such collagen/HA scaffolds, which mimic the natural composition of bone tissue, have shown the capacity to recruit host cells and promote bone formation following

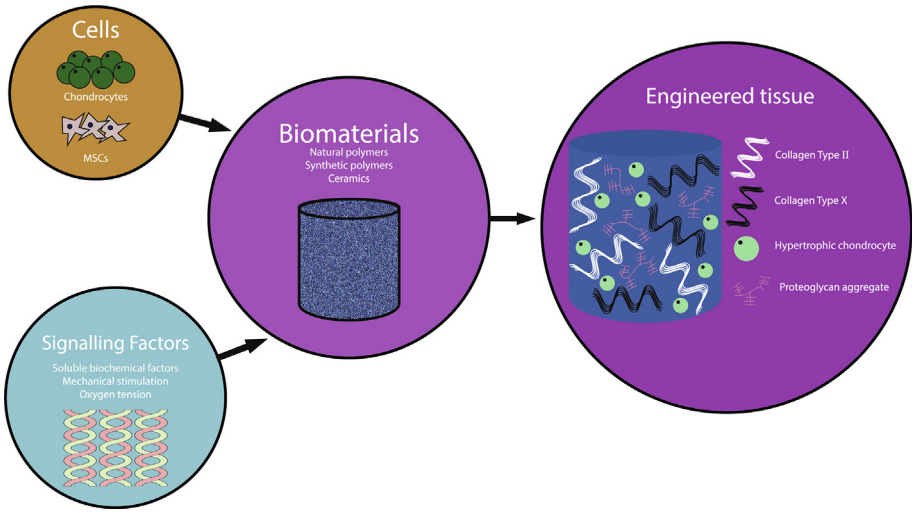


**Fig. 1.** Schematic illustrating combinations of biomaterials, cells, and signaling factors for the tissue engineering of hypertrophic cartilaginous grafts in vitro. Signaling factors such as soluble biochemical factors, mechanical stimuli, and oxygen tension can be harnessed to direct chondrogenesis and subsequent hypertrophy of cells such as chondrocytes and MSCs. Biomaterial scaffolds fabricated from natural polymers, synthetic polymers, and/or ceramics facilitate the upscaling of tissue-engineered grafts to clinically relevant sizes and can be leveraged to further guide cells down the endochondral pathway. The resultant engineered tissue should contain hypertrophic chondrocytes, to promote vascularization upon implantation, as well as the key extracellular matrix components of cartilage and hypertrophic cartilage, such as proteoglycans and collagens types II and X.

implantation into bone defects [21]. A comprehensive assessment of such porous scaffolds for bone tissue engineering applications is beyond the scope of this manuscript, and therefore, the reader is referred to the following review articles, which describe the topic in more detail [19,22,23].

*Scaffold-mediated delivery of growth factors and recombinant proteins*

In an effort to enhance their regenerative capacity, scaffolds have been functionalized with growth factors and recombinant proteins capable of promoting the deposition of extracellular matrix by host cells [24]. In this context, members of the transforming growth factor (TGF) superfamily such as TGF-β3 and bone morphogenetic protein-2 (BMP-2) have been explored for tissue engineering applications [25]. For example, poly(L-lactide-CO-D, L-lactide) scaffolds loaded with TGF-β3 and BMP-2 and implanted into a rat segmental defect model demonstrated enhanced bone regeneration compared to empty defects and showed a trend towards enhancing bone formation compared to scaffolds not containing TGF-β3 and BMP-2 [26]. Furthermore, polycaprolactone (PCL)–tricalcium phosphate scaffolds loaded with platelet-rich plasma (PRP) enhanced vascularization and bone bridging in the same rat defect model, compared to scaffolds without PRP [27]. However, the mechanical properties of the tissues generated in these studies remained an order of magnitude lower than those of age-matched intact femurs, suggesting perhaps that the slow resorption rate of the materials was negatively impacting mechanical restoration of the tissue.

Although growth factors and recombinant proteins have been shown to be powerful promoters of regeneration, a number of adverse effects have been reported following their use. The INFUSE™ bone graft from Medtronic (a collagen sponge soak-loaded with human recombinant BMP-2), for example, has been associated with complications including osteolysis, ectopic bone formation, infection, and cancer risk [28–32]. These issues, which are suggested to be the result of the uncontrolled release of supraphysiological levels of BMP-2 from the sponge [33], have led to increased interest in technologies that aim to control or reduce growth factor and recombinant protein delivery while retaining regenerative capacity [34–36]. For example, alginate hydrogels covalently coupled with the arginine–glycine–aspartic acid peptide to facilitate cell adhesion and loaded with BMP-2, have been shown to promote the regeneration of bone with mechanical properties similar to native bone [37]. In a follow-up study, this hybrid system also demonstrated enhanced bone defect repair when compared to the clinical standard sponge [34]. The binding between peptides and growth factors in these functionalized hydrogels has also been shown to enhance the survival of transplanted cells [38]. Other studies have utilized alginate and poly(lactic-co-glycolic acid) (PLGA) microparticles as a vehicle for the sustained delivery of BMP-2 and vascular endothelial growth factor (VEGF) demonstrating that, when delivered in this controlled manner, much lower levels of BMP-2 can be leveraged while still facilitating bone regeneration [35,39]. Recent work has also sought to recapitulate the role native ECM plays in growth factor sequestration by immobilizing growth factors within 3D scaffolds through covalent binding, thereby utilizing the scaffold as a reservoir for signaling proteins and for the more persistent presentation of proteins to cells [40,41]. Additionally, affinity-binding scaffolds can be fabricated so as to capture circulating growth factors through the incorporation of molecules such as heparin or by utilizing polysaccharides with an affinity for heparin-binding proteins [42,43]. A more extensive description of scaffold-mediated delivery of bioactive molecules for bone tissue engineering applications may be found in these referenced documents [44,45].

*Osteogenic priming of cell-seeded scaffolds*

Although combinations of scaffolds and growth factors may be harnessed successfully to regenerate certain bone defects, an alternative approach is the in vitro engineering of functional bone tissue. This approach requires the identification of a cell source capable of promoting the deposition of bone-specific extracellular matrix onto biomaterial scaffolds. Osteoblasts isolated from bone biopsies have been investigated as a potential source, but the relatively low numbers of cells obtained upon in vitro isolation and expansion are seen as a limitation [18]. Conversely, MSCs, which can be harvested with relative ease from tissues such as bone marrow, have the capacity to undergo numerous population doublings in vitro and can be directed to differentiate down a number of tissue-specific lineages [6]. In this regard, biomaterial characteristics such as substrate stiffness [46] and surface topography [47] can be utilized to guide MSCs down an osteogenic lineage. Furthermore, the application of biophysical stimuli such as fluid flow has been shown to enhance osteogenesis of MSCs in both monolayer [48] and 3D culture [49]. The in vitro osteogenic differentiation of MSCs is typically achieved by stimulation with dexamethasone, ascorbic acid, and β-glycerophosphate (β-GP), whereby dexamethasone induces and enhances expression of the osteogenic transcription factor Runx2, ascorbic acid facilitates osteogenic differentiation by increasing the secretion of collagen type I and β-GP acts as a source of phosphate for HA [50]. When applied to MSCs in monolayer or 3D culture, this cocktail of factors results in osteogenic differentiation within 21 days in a process resembling intramembranous ossification [51,52].

In order to investigate the capacity of in vitro expanded MSCs to promote bone regeneration in vivo, MSCs have been seeded onto HA scaffolds and implanted into critically sized long bone defects in sheep [53]. This study found accelerated bone repair and more homogenous bone distribution with the cell-seeded scaffold compared to the cell-free scaffold. Further studies have demonstrated the capacity of osteogenically induced MSCs loaded onto porous β-TCP and coralline scaffolds to repair mandibular bone defects in dogs [54,55]. The first clinical case study involving the use of cell-based constructs for the treatment of long bone defects involved 3 patients implanted with macroporous HA scaffolds seeded with in vitro expanded autologous MSCs [56]. A follow-up report on this case noted good osteointegration with no further complications 6–7 years after surgery but also highlighted limitations in the study, such as the lack of an acellular scaffold control group and lack of new bone quantification [57].

A major hurdle associated with in vitro bone tissue engineering is the lack of a functional vascular supply. When a cell-based construct is implanted, an insufficient vascular supply may result in the cells not receiving nutrients (such as oxygen, glucose, and amino acids) nor being capable of eliminating waste products from their local environment, thus threatening cell and construct viability [8]. An additional limitation of the intramembranous ossification approach to bone tissue engineering is that the in vitro culture of a construct can lead to extensive matrix mineralization, sealing the pores of the scaffold and further restricting in vivo vascularization. For example, studies have demonstrated superior bone healing with the implantation of an acellular collagen/calcium phosphate scaffold in a rat cranial defect compared to an MSC-cultured scaffold [9], in which the extensive in vitro engineered matrix inhibited in vivo remodeling, preventing host cell ingrowth and ultimately resulting in core degradation at the center of the implanted construct, see Fig. 2. These limitations associated with the intramembranous approach have led to a search for alternative strategies for bone tissue engineering applications.

## Endochondral bone development and fracture repair

Embryonic bone develops via two processes. The flat bones of the skull, for example, develop by intramembranous ossification, where MSCs form condensations and differentiate directly into bone-forming osteoblasts which lay down matrix. The long bones of the body, however, develop by endochondral ossification, which involves the remodeling of an intermediate cartilaginous template into bone [58–60]. During the endochondral development process, condensations of MSCs initiated by the expression of the transcription factor SOX9 differentiate

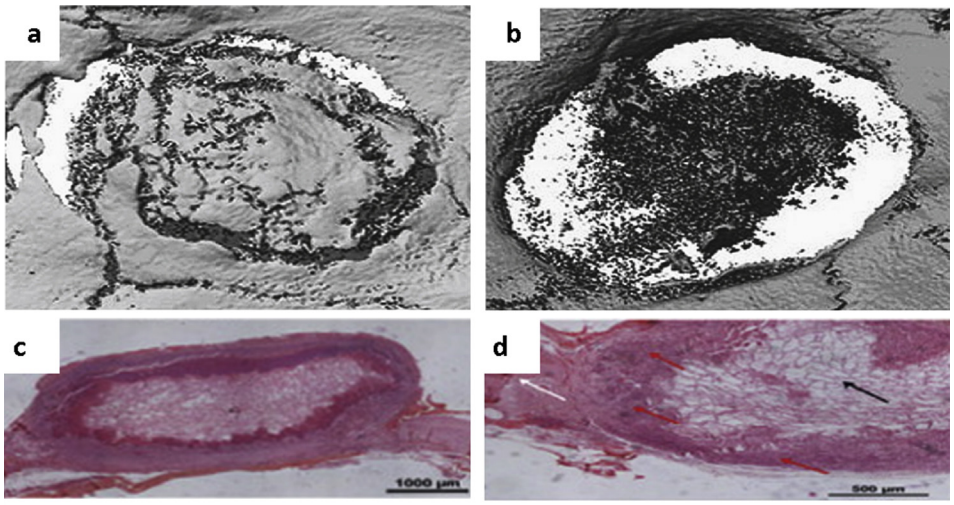


**Fig. 2.** Assessment of repair of rat cranial bone defects by osteogenically primed tissue-engineered constructs compared to cell-free scaffolds. Tissue-engineered constructs were formed by seeding collagen/calcium phosphate scaffolds with bone marrow–derived MSCs and culturing the constructs in an osteogenic medium for 4 weeks prior to implantation. (a) Micro–computed tomography (μCT) image of the cell-free collagen/calcium phosphate scaffold showing good levels of healing 4 weeks after implantation in comparison to (b) μCT the tissue-engineered construct which showed limited levels of repair. (c) Low magnification image of the tissue-engineered construct (stained with hematoxylin and eosin) showing the formation of a dense capsule around the periphery of the construct which at higher magnification (d) shows the original host bone adjacent to the defect site (white arrow) in comparison to the dense layer of tissue around the periphery of the implanted construct (red arrow) which has resulted the formation (black arrow) of a necrotic area in the center of the implanted construct. Modified with permission from Ref. [9].

into chondrocytes and form a template of cartilaginous tissue which enlarges as chondrocytes proliferate and lay down a matrix rich in sulfated glycosaminoglycan and collagen type II. Thereafter, chondrocytes in the center of the cartilaginous template undergo hypertrophy, whereby cells increase in volume and change their collagen production from type II to type X. Cycles of chondrocyte hypertrophy and proliferation then continue in accordance with the Indian hedgehog (Ihh)–parathyroid hormone-related protein (PTHrP) feedback loop [61,62], whereby Ihh expressed by central hypertrophic chondrocytes results in the synthesis of PTHrP by chondrocytes at the ends of the bone, which promotes proliferation and delays hypertrophy of adjacent cells [63]. Hypertrophic chondrocytes attract blood vessels and promote mineralization through production of factors such as VEGF, placental growth factor (PGF), matrix metalloproteinase-13, and BMP [64], and the resultant cartilaginous template acts as a scaffold for osteoclasts and osteoblasts to invade and deposit bone.

At the ends of the bone, secondary ossification centers form through cycles of chondrocyte hypertrophy and vascularization. A key feature in the formation of the secondary ossification center is the presence of cartilage canals, which facilitate angiogenesis by acting as conduits for blood vessel migration into the epiphysis [65–68]. Cartilage canals also act to eliminate waste and nourish chondrocytes beyond the reach of diffusive nutrients and provide the cartilaginous template with progenitor cells of the osteogenic lineage, which contribute to ossification within the epiphysis [66,69]. In the final stages of the endochondral process, chondrocyte proliferation continues causing lengthening of the bone and hematopoietic stem cells establish the site for hematopoietic marrow. At this point, the primary and secondary ossification centers fuse together, and the only cartilage left is the permanent articular cartilage at each end of the bone.

Similar to embryonic bone development, fracture repair also occurs by two mechanisms. If there is no movement between the fracture surfaces, the bone heals via remodeling directed by osteoclasts that remove trenches of bone followed by osteoblasts that lay down new bone in a process resembling intramembranous ossification [70]. In situations where movement occurs between two fracture surfaces, initiation of wound-healing pathways results in a soft callus forming around the fracture surfaces. SOX9 expression within the fracture site promotes the production of cartilage extracellular matrix, and the subsequent hypertrophy of chondrocytes in the soft callus results in the expression of collagen type X and the release of a cascade of pro-angiogenic and pro-osteogenic factors (VEGF, PGF and BMP) similar to those observed during the embryonic process of endochondral ossification [71].

There is some debate as to the role hypertrophic chondrocytes play in the final stages of endochondral bone formation. Traditionally, it was accepted that terminally differentiated hypertrophic chondrocytes underwent programmed cell death, facilitating chondrocyte deletion from the epiphyseal growth plate [72–74] and allowing for the development of the marrow cavity. However, more recent work has highlighted an alternative fate for hypertrophic chondrocytes, whereby these cells possess the capacity to transdifferentiate into osteoblasts and osteocytes [64,75–77], and this transformation has been shown to be regulated by blood vessel infiltration which activates the expression of pluripotency genes in the chondrocytes [78,79]. Recent advances in the field of bone tissue engineering towards the goal of functional bone organ regeneration have sought to leverage the capacity of chondrogenically differentiated cells to, firstly, survive the initial hypoxic environment that cell-based grafts are subjected to upon implantation and, secondly, promote vascularization and colonization of the graft with bone-forming cells resulting in the restoration of the tissue, see Fig. 3.

## Modulating the phenotype of cells for endochondral bone tissue engineering applications

### *Cells possessing the endochondral phenotype*

Cell sources for endochondral bone tissue engineering applications must first have the capacity to undergo chondrogenic differentiation and lay down a cartilaginous matrix. Thereafter, the cells must be capable of undergoing hypertrophic differentiation, releasing the pro-angiogenic factors required to facilitate vascularization and bone formation. Early work in the field attempted bone defect repair with tissue-engineered cartilage by implanting articular chondrocyte-seeded polymer scaffolds into rat cranial defects, although the neotissue formed in these engineered constructs was reported as stable cartilage resistant to vascularization and ossification [80]. A subsequent study comparing the capacity of tissues engineered using periosteal cells or chondrocytes derived from articular cartilage to promote bone defect repair demonstrated that while chondrocyte-derived tissues generated a stable cartilage resistant to ossification, periosteal cells generated a tissue which appeared to undergo morphogenesis from cartilage into bone, reminiscent of the process of endochondral ossification [81].

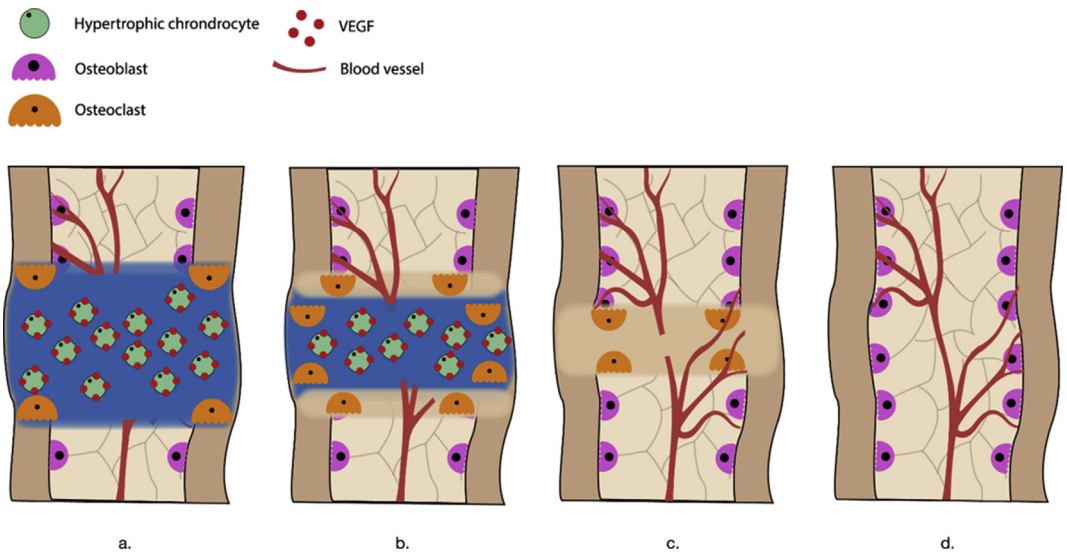


**Fig. 3.** Schematic illustration of hypothesized biomaterial-based endochondral bone regeneration in vivo. (a) Tissue-engineered hypertrophic cartilaginous grafts are implanted into the bone defect site. The ability of chondrogenic cells to survive in avascular environments maintains the viability of the engineered tissue during this initial hypoxic phase. (b) VEGF released by hypertrophic chondrocytes within the engineered tissue promotes the invasion of blood vessels. The cartilaginous matrix begins to degrade and osteoclasts and osteoblast begin to remodel the engineered tissue into bone. (c) Blood vessels, osteoclasts, and osteoblasts encroach further into the defect site promoting remodeling of any remaining cartilaginous matrix into bone. (d) The vascular network and bone marrow cavity is fully restored, and the bone is healed. VEGF, vascular endothelial growth factor.

The phenotype of chondrocytes isolated for subsequent endochondral bone tissue engineering applications is an important consideration. For example, when cephalic and caudal chondrocytes were isolated from the upper and lower sternum of chick embryos, respectively, cephalic chondrocytes demonstrated the potential to undergo hypertrophy in vitro and endochondral ossification in vivo, whereas caudal chondrocytes maintained their stable chondrogenic phenotype [82,83]. Although chondrocytes from healthy articular cartilage are phenotypically stable, in diseased states such as osteoarthritis, articular chondrocytes become hypertrophic and have been shown to generate bone through endochondral ossification when implanted as scaffold-free cartilaginous constructs in nude mice [84]. Cartilage from nasal septum has also recently been investigated as a source of hypertrophic chondrocytes and hypertrophic cartilaginous grafts engineered using this cell source were found to promote endochondral bone regeneration in a rat cranial defect [85]. Interestingly, chondrocytes derived from articular cartilage cotransplanted with osteoblasts were shown to organize into a structure that morphologically resembled the growth plate and grew in mass recapitulating aspects of long bone development when delivered within an alginate carrier, indicating an underlying mechanism of cross-talk between chondrocytes and osteoblast, which may be playing a role in growth plate development [86].

One limitation associated with the use of chondrocytes for tissue engineering applications is their potential loss in phenotype upon expansion in vitro [87,88]. As mentioned previously, stem cells have the capacity to undergo numerous population doublings in vitro, and embryonic stem cell–based tissue engineering of bone through endochondral ossification has been analyzed by seeding murine embryonic stem cells onto ceramic scaffolds, culturing the constructs chondrogenically for 21 days, and then implanting the constructs subcutaneously into immunodeficient mice [89]. This study also found that a cartilaginous template, and not just chondrogenically differentiated cells, was required for bone formation. The ethical issues surrounding the use of embryonic stem cells for tissue engineering applications can be addressed through the use of adult MSCs. MSCs first emerged as an attractive cell source for endochondral bone tissue engineering applications due, in part, to their inherent tendency to undergo hypertrophy when chondrogenically primed. Indeed, a number of studies which initially explored the use of MSCs as a potential cell source for articular cartilage tissue engineering applications actually provided the motivation for future work investigating their capacity to facilitate endochondral ossification [90,91]. For example, chondrogenically primed MSC pellets were found to follow an aberrant differentiation pathway with an initial induction of type X collagen preceding type II collagen production, followed by an upregulation in alkaline phosphatase activity in vitro which correlated with in vivo calcification and vascularization of MSC pellets which still contained proteoglycan and collagen type II [91]. Interestingly, some studies have shown that the chondrogenic phenotypes of MSCs following implantation are dependent on the tissues from which the cells are isolated, with bone marrow–derived MSCs demonstrating the capacity to proceed down the endochondral route following chondrogenic induction, whereas synovium and infrapatellar fat pad–derived MSCs demonstrated a tendency toward fibrous dedifferentiation [92,93]. Indeed, when human bone marrow–derived MSCs were cultured to undergo chondrogenesis and hypertrophy in vitro, subsequent implantation into nude mice resulted in a number of the features typical of endochondral skeletogenesis being recapitulated, including the formation of a bony collar surrounding trabecular bone and bone marrow foci [94], and furthermore, chondrogenically primed human bone marrow–derived MSC pellets were shown to promote significantly more bone formation compared to undifferentiated pellets when implanted into a rat femoral defect [95]. A recent study also demonstrated recapitulation of endochondral bone using human adipose tissue–derived stem cells, a finding which offers a viable alternative to the bone marrow–derived MSCs for endochondral bone tissue engineering applications [96].

### *Signaling mechanisms for regulating hypertrophy*

The phenotype of cells for endochondral applications can be regulated via biochemical cues delivered through soluble biochemical factors or mechanical stimulation, see Table 1. Early work in the field demonstrated that a chondro-osseous rudiment could be generated by culturing bone marrow–derived MSC pellets in a chondrogenic medium containing TGF-β for an initial three-week period before switching to a β-GP–loaded medium to induce mineralization [97]. In vitro hypertrophic differentiation of chondrogenically primed MSCs was stimulated through the removal of TGF-β, the reduction of dexamethasone (from 100 nM to 1 nM), and the addition of triiodothyronine, with or without the addition

**Table 1**
Cells and signaling factors for regulating hypertrophy and endochondral ossification of engineered cartilaginous constructs.

| Reference | Cell source | Biomaterial | In vitro culture conditions | Observations |
|---|---|---|---|---|
| Oliveira et al. [82,83] | Chondrocytes derived from the sterna of chick embryos. | Chitosan sponge | 10 days in the presence of newborn serum with an additional 10 days in the presence of 100 nM retinoic acid. | Cephalic chondrocyte seeded sponges underwent hypertrophy in the presence of retinoic acid and endochondral ossification in vivo, whereas caudal chondrocytes seeded sponges formed stable cartilaginous tissues. |
| Bardsley et al. [85] | Chondrocytes derived from the nasal septum of rats | PGA scaffold | 42 days in the presence of 10 ng/mL fibroblast growth factor-2 | Cell-seeded constructs showed evidence of hypertrophy (increase cell size and collagen type X expression) in vitro and remodeling into bone in vivo. |
| Scotti et al. [94] | MSCs derived from humans | Biomaterial-free construct | 21 days in chondrogenic medium, with an additional 14 days in the hypertrophic medium. | In vitro, constructs stained positively for collagen type II, collagen type X, and safranin-O in central regions with positively staining for collagen type I, bone sialoprotein, and alizarin red in peripheral regions. Constructs remodeled into trabecular-like bone following implantation. |
| Mumme et al. [99] | MSCs derived from humans | Collagen type I mesh | 21 days in chondrogenic medium supplemented with 50 pg/mL IL-β1 with an additional 14 days in hypertrophic medium supplemented 50 pg/mL IL-β1. | Histomorphometric analysis demonstrated more rapid degradation of cartilaginous tissue, accompanied by an increase in bone marrow, in implanted constructs treated with 50 pg/mL IL-β1 during the in vitro culture period. |
| Bian et al. [101] | MSCs derived from humans | Hyaluronic acid hydrogel | Compressive loading (10% peak strain at a frequency of 1 Hz; 4 h/day, 5 days/week) for 14 days in chondrogenic medium with an additional 15 days loading in hypertrophic medium | Dynamic compressive loading enhanced GAG and collagen deposition and suppressed hypertrophic differentiation and calcification. |
| Carroll et al. [104] | MSCs and infrapatellar fat pad stem cells derived from pigs | Agarose hydrogel | Hydrostatic pressure (10 MPa amplitude at a frequency of 1 Hz; 4 h/day, 5 days/week) for 21 days in chondrogenic medium with an additional 14 days loading in hypertrophic medium. | Dynamic hydrostatic pressure enhanced the functional development of cartilaginous tissues, suppressed calcification in MSCs, and promoted a more stable chondrogenic phenotype in infrapatellar fat pad–derived stem cells. |
| Sheehy et al. [112] | MSCs derived from pigs | Agarose hydrogel and biomaterial-free pellet | Up to 42 days in chondrogenic medium at 5% $pO_2$. | Maintenance in a low oxygen environment (5% $pO_2$) enhanced collagen type II production and suppressed collage type I production, collagen type X production, and calcification of constructs. |
| Leijten et al. [114] | MSCs derived from humans | Biomaterial-free construct | 35 days in chondrogenic medium at 2.5% $pO_2$. | Constructs maintained at 2.5% $pO_2$ in vitro were metabolically programmed to remain chondrogenically stable upon implantation, whereas constructs maintained at 20 %$pO_2$ underwent endochondral ossification. |
| Osinga et al. [96] | Adipose tissue stem cells derived from humans | Collagen type I mesh | 28 days in chondrogenic medium with an additional 14 days hypertrophic medium supplemented with 50 pg/mL IL-β1. | When implanted, engineered cartilaginous tissues underwent cartilage remodeling and developed bone ossicles including bone marrow elements. |

MSCs, mesenchymal stem cells; PGA, polyglycolic acid; GAG, glycosaminoglycan.
Chondrogenic medium 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 bovine serum albumin, 1 x insulin–transferrin–selenium, 100 nM dexamethasone, and 10 ng/mL human TGF-β3. Hypertrophic medium is typically defined as chondrogenic medium with the removal of 10 ng/mL human TGF-β3, a reduction in dexamethasone (1–10 nM), the addition of thyroxine (1–50 nM) or 1 nM triiodothyronine, and the addition of 10 mM β-GP.

of β-GP [98]. The culture of constructs in such hypertrophic media has been shown to enhance endochondral bone formation following implantation [94]. Furthermore, inflammatory cytokines such as interleukin 1-β, which play a key role during fracture healing, can be leveraged to direct more efficient remodeling of engineered hypertrophic cartilaginous constructs into bone [99].

Mechanical stimulation is also known to be a potent regulator of cell phenotype. Studies have demonstrated that mechanical loading such as dynamic compression can inhibit hypertrophy of engineered cartilaginous constructs in vitro [100,101] with further work finding that this inhibition only occurs under certain strain rates [102]. Studies have also demonstrated that hydrostatic pressure can stabilize the chondrogenic phenotype of MSCs and infrapatellar fat pad–derived stem cells [103,104]. However, recent work has shown that the application of fluid shear stress [105] and cyclic tension [106] can direct chondrogenically primed MSCs down a hypertrophic pathway in vitro and that, when applied in vivo, mechanical loading can facilitate bone regeneration and vascularization of constructs undergoing endochondral ossification [107].

Another factor which has been shown to play a key role in modulating hypertrophy is the local oxygen environment. The process of endochondral ossification is characterized by a gradient increase in oxygen, with initial MSC condensation and chondrogenic differentiation occurring at a low oxygen tension and subsequent hypertrophy, vascularization, and mineralization occurring at elevated levels of oxygen tension [108]. To that end, oxygen has been suggested as having a regulatory effect on the osteogenic and chondrogenic differentiation of MSCs, and the effects of oxygen on the hypertrophic differentiation of MSCs have also been examined. Adipose tissue–derived MSCs differentiated in a low oxygen environment have been shown to favor a chondrogenic phenotype, whereas cells differentiated at 20% $pO_2$ exhibited a hypertrophic phenotype [109]. Alkaline phosphatase activity was demonstrated to be inhibited by low oxygen conditioning during chondrogenic differentiation of the embryonic precursor cell line C3H10T1/2 [110], and reactive oxygen species, which can be generated when cells are placed in a hyperoxygenated state, have also been shown to induce hypertrophy in chondrocytes [111]. Furthermore, work examining the role of oxygen tension has demonstrated that hypoxia inhibits hypertrophy of bone marrow–derived MSCs in aggregate cultures as well as in pellets and hydrogels [112,113]. A key paper has also recently demonstrated that in vitro metabolic programming of MSCs by oxygen tension can direct the subsequent formation of either stable or hypertrophic cartilage tissue in vivo [114]. When leveraging oxygen tension in this manner, however, the size of the implanted tissue-engineered graft and its gradient effect on oxygen availability throughout the graft should also be considered. Indeed, recent work has demonstrated that, beyond a critical size, the activation of hypoxia signaling through the inhibition of the oxygen sensor prolyl hydroxylase domain–containing protein 2 can promote endochondral bone formation in tissue-engineered grafts by enhancing

chondrogenesis and matrix synthesis in central regions of implanted tissue [115]. The modulation of oxygen tension, either through environmental conditions in vitro or through the onset of vascularization in vivo [78], may, therefore, be harnessed as a mechanism for determining the ultimate fate of chondrogenic cells.

## Biomaterials for endochondral bone tissue engineering applications

### *Naturally derived scaffolds and hydrogels*

Naturally derived materials possess certain biological advantages for tissue engineering applications, and a number of studies investigating the use of tissue-engineered hypertrophic cartilage as grafts for promoting endochondral ossification have utilized naturally derived polymers such as hyaluronic acid, gelatin, and collagen, as scaffolds, see Table 2. These natural polymers can be processed using manufacturing techniques such as lyophilization to generate highly porous scaffolds, the porosity of which can be regulated by varying the final freeze-drying temperature of the process [116]. The capacity of such naturally derived polymers to promote endochondral bone regeneration was demonstrated in a seminal study when hyaluronic acid/gelatin sponge scaffolds, seeded with bone marrow–derived MSCs and cultured chondrogenically, were implanted into the excised lunate space of rabbits [117]. After a 12-week in vivo period, the engineered cartilage had developed into osteochondral tissue consisting of a peripheral cartilaginous layer surrounding a region of endochondral bone containing marrow space, which was mechanically functional for the duration of the experiment. Another key study investigating the engineering of a bone organ through endochondral ossification seeded human MSCs onto type I collagen meshes, cultured the constructs to undergo chondrogenesis and subsequent hypertrophy, and demonstrated that an outer cortical-like bone was formed by host cells laying down bone over a premineralized area and the inner trabecular-like bone developed with donor cells forming bone over a cartilaginous template [118]. Furthermore, the bone marrow generated in the upscaled tissues reconstituted multilineage long-term hematopoiesis in lethally irradiated mice, thus demonstrating the functionality of the engineered organ. It should be noted, however, that the central region of the in vitro engineered cartilage was devoid of cells and matrix, signifying the challenges in scaling up engineered tissues to clinically relevant sizes and highlighting the importance of utilizing a suitable scaffold material when doing so.

**Table 2**
Biomaterials for in vivo endochondral ossification of chondrogenically primed MSC-seeded constructs.

| Reference | Biomaterial | Animal model | Observations |
|---|---|---|---|
| Huang et al. [117] | Hyaluronic acid/gelatin sponge | Excised lunate regions in rabbits | Seminal study demonstrating endochondral bone regeneration after 12 weeks in a load-bearing environment. |
| Thompson et al. [120] | Collagen/ hyaluronic acid scaffold | Cranial defects in rats | Chondrogenically primed collagen-based scaffolds demonstrated enhanced cranial bone defect repair when compared to osteogenically primed scaffolds which may have been due to increased release of VEGF. |
| Matsiko et al. [121] | Collagen/ hyaluronic acid scaffold | Femoral defects in rats | Collagen-based scaffolds demonstrated enhanced bone healing at the early time point of 4 weeks. |
| Sheehy et al. [129] | Alginate, fibrin, and chitosan hydrogels | Subcutaneous implantation in mice | Alginate and fibrin hydrogels supported the progression from engineered hypertrophic cartilage into bone, whereas chitosan hydrogels were resistant to vascularization and bone remodeling. |
| Daly et al. [155] | Alginate bioink reinforced with PCL fibers | Subcutaneous implantation in mice | An alginate bioink incorporating Arg–Gly–Asp adhesion peptides printed in the shape of a vertebral body supported the development of an endochondral bone organ containing marrow tissue. |
| Harada et al. [142] | PLGA scaffold | Femoral defects in rats | Chondrogenically primed constructs promoted regeneration of femoral defects three times the critical size. |
| Janicki et al. [130] | β-TCP/fibrin scaffold | Subcutaneous implantation in mice | Enhanced endochondral bone formation by constructs with β-TCP particle size $< 0.7$ mm. Endochondral bone formed by chondrogenically primed constructs was of donor origin whereas marrow was of host origin. |

MSC, mesenchymal stem cell; VEGF, vascular endothelial growth factor; PCL, polycaprolactone; PLGA, poly(lactic-*co*-glycolic acid); B-TCP, β-tricalcium phosphate.

As collagen-based scaffolds support the differentiation of MSCs down both osteogenic and chondrogenic lineages [52], they are useful models to compare the capacity of MSCs to promote bone formation through either intramembranous or endochondral ossification. When primed osteogenically in vitro, subcutaneous implantation of MSC-seeded collagen-based scaffolds into nude mice showed increased mineralization but poor cell viability with no vascularization, whereas chondrogenically primed constructs did not mineralize but had good cell viability with evidence of vascularization [119]. Furthermore, upon implantation into rat cranial defects, chondrogenically primed MSC-seeded collagen-based scaffolds were found to undergo a greater degree of bone formation when compared to osteogenically primed scaffolds, with the authors speculating that higher VEGF production by the chondrogenically primed constructs in vitro translated to greater vascularization in vivo [120]. The efficacy of these constructs have also recently been evaluated in a rat femoral defect model, with MSC-seeded collagen/hyaluronic acid scaffolds, cultured in vitro to undergo chondrogenic and hypertrophic differentiation, being found to support early-stage bone healing at 4 weeks [121].

The pore size and pore architecture of scaffolds are key considerations which can be harnessed to guide biological processes towards regeneration. For example, studies have demonstrated that a smaller pore size facilitates cellular attachment by increasing surface area and offering a higher ligand density for cells to bind to [122], whereas larger pore sizes promote greater migration of cells into central regions of the scaffold [123]. Furthermore, directional freeze-drying can be utilized to generate aligned porous architectures, which have been shown to direct processes such as axonal growth for nerve repair applications [124]. This approach of fabricating longitudinally aligned pores within a collagen scaffold has recently been used to promote endochondral healing of bone defects [125]. Interestingly, endochondral bone regeneration in this case was achieved not by promoting hypertrophy of transplanted cells but by utilizing the aligned pores of the scaffold to preferentially recruit host progenitor cells from the bone marrow while preventing the in-growth of blood vessels from surrounding tissues. The resultant hypoxic environment within the scaffold provided a pro-chondrogenic environment, and as blood vessels followed the migrational route initially taken by the progenitor cells, chondrogenically differentiated cells within the scaffold were stimulated to undergo hypertrophy resulting in the recruitment of osteoblasts and osteoclasts and eventual remodeling of the scaffold into

endochondral bone. This important work demonstrates that a cell-free scaffold-based approach to endochondral ossification can be executed by utilizing the pore architecture of the scaffold to guide host cells down the endochondral route.

As an alternative to porous scaffolds, hydrogels may be a powerful tool in the engineering of hypertrophic cartilaginous grafts for endochondral bone tissue engineering applications as their high water content mimics that of native cartilaginous extracellular matrix. Furthermore, the architecture of a hydrogel can also be modified so as to contain channels or conduits facilitating infiltration of the graft with host endothelial and osteogenic cells upon implantation resulting in enhanced mineralization and vascularization in both ectopic [126] and orthotopic [127] sites. A key factor in selecting a hydrogel for an endochondral application would appear to be the degradation rate of the hydrogel as slow degrading hydrogels have been shown to impede endochondral ossification when compared to fast degrading hydrogels modified through gamma irradiation [128]. When the capacities of MSC-encapsulated alginate, chitosan, and fibrin hydrogels to promote endochondral bone formation in vivo following chondrogenic induction in vitro were examined, alginate constructs facilitated the greatest degree of remodeling and also promoted the greatest amount of endochondral bone formation [129], with the different processing and cross-linking regimes of the hydrogels possibly playing a role in determining their rate of degradation. Although fibrin constructs generated bone in lower quantities, the material still facilitated transition of the MSC-encapsulated construct down the endochondral route to ossification with associated infiltration of host vasculature, a finding which has been confirmed in other studies [130,131].

Naturally derived scaffolds for endochondral bone tissue engineering applications can also be fabricated by decellularizing cartilaginous tissues using physical, chemical, or enzymatic treatments leaving behind an extracellular matrix containing functional proteins that can be used as a scaffold for generating endochondral bone [132,133]. Although such decellularized cartilaginous particles have been shown to enhance chondrogenesis of MSCs encapsulated within gelatin methacrylamide hydrogels [134], they may be more attractive from a commercial translational perspective if utilized as devitalized grafts capable of recruiting host cells and promoting endochondral ossification and thereby providing 'off-the-shelf' therapies for bone repair [94,135]. This approach has recently been utilized to fabricate spatially complex constructs for osteochondral defect repair [136]. Recent work has also sought to investigate the capacity of non-cartilaginous extracellular matrix–derived scaffolds to promote endochondral ossification, by utilizing fractioned adipose tissue obtained by liposuction to generate scaffolds through in vitro culture [137].

As an interesting approach to tissue engineering, a 3D construct is utilizing a biomaterial as an interim scaffolding system. Recent work developed a living scaffold-free hyaline cartilage graft by culturing chondrocytes in an alginate hydrogel containing cavities for micro tissue nodule formation [138]. Following cartilaginous extracellular matrix formation, the alginate was removed by treatment with sodium citrate, with the 3D network anchored by neotissue formation within the original cavities remaining intact. This living cartilage graft was evaluated in vivo as a template for bone formation, where osteogenesis was reported both with and without additional seeding with MSCs [139].

### *Synthetically derived polymer scaffolds and bioceramics*

Although naturally derived polymers are often favored for tissue engineering applications due to their biological characteristics, they are sometimes limited by their relatively low mechanical properties. In contrast, synthetically derived polymer scaffolds provide superior mechanical properties. Furthermore, synthetically derived fibrous polymer scaffolds with fiber diameters similar to those of native extracellular matrix can be processed using fabrication techniques such as electrospinning, whereas the fluoroalcohols used during these processes have been shown to denature natural polymers like collagen [140]. PLGA/PCL scaffolds fabricated through electrospinning were shown to support chondrogenic differentiation of MSCs in vitro and endochondral ossification when implanted subcutaneously in nude mice [141]. In another study, MSC-seeded PLGA scaffolds were shown to promote endochondral bone regeneration of a very large rat femoral bone defect when first cultured chondrogenically in vitro [142] with similar results being obtained when nasal chondrocytes cultured chondrogenically on a polyglycolic acid (PGA) scaffold were implanted into a rat cranial defect [85].

Some bioceramics have also been leveraged as scaffolds for generating bone through the endochondral route [89]. HA scaffolds were used, for example, to demonstrate enhanced vascularization and host endothelial cell recruitment by implanted cells progressing down the endochondral route as compared to cells progressing down the intramembranous route [143]. Furthermore, human adipose tissue–derived stem cells, differentiated into hypertrophic chondrocytes on decellularized bone scaffolds, were shown to enhance bridging of the femoral defects when compared to acellular scaffolds and scaffolds seeded with osteogenically primed stem cells, which may have been due to the presence of proregenerative M2 macrophages within the hypertrophic grafts [144]. The endochondral capacity of such scaffolds has also been shown to be dependent on the type of bioceramic used. β-TCP scaffolds seeded with MSCs, for example, were found to generate greater endochondral bone formation in vivo when compared to MSC-seeded HA/TCP scaffolds [130]. Interestingly, HA particles have been shown to promote chondrogenic differentiation of MSCs when used as coatings on titanium scaffolds leading to greater bone formation when implanted into rat mandibles [145].

## Emerging trends in biomaterial-based approaches to endochondral bone regeneration

The field of endochondral bone tissue engineering has shown significant promise and is advancing towards ambitious strategies that aim to regenerate entire joints and bones. One approach, which could potentially be leveraged to generate osteochondral grafts suitable for the treatment of degenerative joint diseases such as osteoarthritis, is the fabrication of bilayered chondrogenically primed constructs in vitro capable of spatially regulating endochondral ossification in vivo [146]. This strategy has also recently been investigated by the authors as a means to engineer an anatomically shaped bone, in the form of the distal phalanx of the finger, complete with an articular cartilaginous surface, which may provide a framework for next-generation technologies aimed at regenerating entire bones [147], see Fig. 4. If this approach is to be viable, however, a key element will be the utilization of a cell source for the articular surface which is not only capable of generating a tissue resistant to endochondral ossification but also suitable for upscaling to clinically relevant sizes. In this context, the chondrocytes used to form the articular surfaces in the aforementioned studies are not ideal, as an age-related loss in the chondrogenic phenotype has been associated with the use of chondrocytes [148], and furthermore, the in vitro expansion of chondrocytes can result in fibrous dedifferentiation of the phenotype [88]. However, some studies have shown that stable cartilaginous tissues can be engineered using smaller quantities of chondrocytes if applied in coculture models with MSCs [149,150], whereby trophic factors released by MSCs drive subsequent proliferation of chondrocytes [151,152]. Indeed, the efficacy of a chondrocyte/MSC coculture approach to engineering scaled-up articular cartilage has recently been demonstrated following subcutaneous implantation in a nude mouse model [153], and future work should investigate whether the strategy could be successful if trialed within a load-bearing region such as the joints of the lower limbs.

Although some work has suggested that engineered cartilaginous constructs can remain mechanically functional in load-bearing bone sites of small animals [117], mechanical reinforcement of cartilaginous grafts may be required to support the transmission of forces human joints and bones are subjected to in everyday life, which can often be multiple times

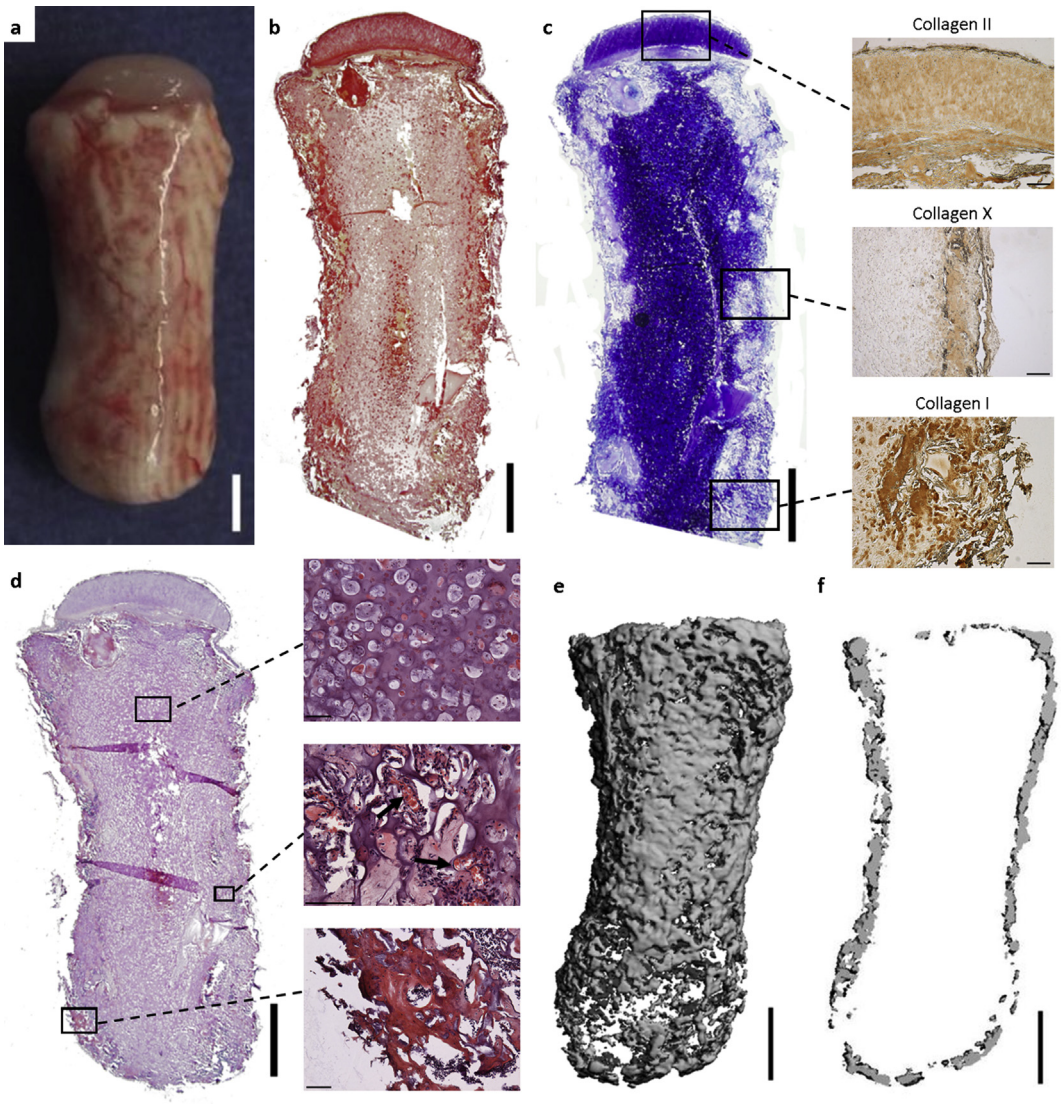


**Fig. 4.** Anatomically shaped phalanx constructs, consisting of an osseous component comprising an MSC-encapsulated alginate hydrogel and a chondral component comprising self-assembled chondrocytes, generated through spatial regulation of endochondral ossification. (a) Macroscopic image demonstrating a vascularized osseous component and an integrated chondral component which was not vascularized. (b) Picrosirius red staining for collagen. (c) Aldehyde fuchsin/alcian blue staining for sulfated glycosaminoglycan. Insets show collagen type II (top), collagen type X (center), and collagen type I (bottom staining). (d) H&E staining. Arrows indicate blood vessel structures. (e) Micro–computed tomography image of the whole construct. (f) Micro–computed tomography image of the center section of construct. Main image scale bars are 2 mm. Inset scale bars in (c) are 250 μm. Inset scale bars in (d) are 100 μm. Reproduced with permission from Ref. [147]. MSC, mesenchymal stem cell.

one's body weight. The rapid growth in additive manufacturing technologies may provide a solution. For example, 3D bioprinting has emerged as a biofabrication technique which allows for precise control over the mechanical properties and spatial distribution of cells and biomolecules within an engineered construct [154]. The rapid growth in new applications of this technology may play a key role in future developments within the field of endochondral bone tissue engineering, as it may allow for the fabrication of anatomically shaped constructs reinforced with polymer meshes containing cell-laden or growth factor–laden hydrogels designed to target the endochondral pathway as a route to regeneration [155]. Three-dimensional bioprinting may also allow for the generation of multizonal grafts containing pro-osteogenic and pro--chondrogenic compounds in adjacent layers as a means to fabricate osteochondral grafts [156]. Critical in the expansion of this technology for endochondral ossification therapies will be the development of novel printable hydrogels, or 'bio-inks', which must not only be capable of facilitating the conversion of hypertrophic cartilaginous tissue into vascularized bone but should also have a tailored viscosity so as to make the material printable. To that end, increased investigation into the interface between the material science and biological properties of different bioinks should accelerate their use in the field.

In intramembranous bone tissue engineering applications, biomaterial scaffold systems capable of delivering therapeutic agents such as growth factors and recombinant proteins have been investigated extensively [26,27,35,39]. However, the strategy of directing host cells down a route of hypertrophic chondrogenesis, by implanting drug-loaded matrices, has only recently been explored in endochondral bone tissue engineering applications. In this context, a gelatin-based system for the delivery of human recombinant proteins involved in the endochondral ossification cascade has been developed [157]. Temporally regulated dual growth factor delivery of TGF-β1 and BMP-2 designed to promote initial chondrogenesis and subsequent osteogenesis has also been examined and evaluated in vitro [158] and in vivo [159]. Furthermore, the immobilization of growth factors within scaffolds can be leveraged to direct

spatially organized endochondral ossification [160]. The time at which mechanical loading is applied has been shown to influence the degree of endochondral bone formation by drug-loaded matrices, with a delayed loading onset of 4 weeks after implantation being shown to enhance bone formation and stimulate vascular remodeling [161]. These drug delivery approaches may reduce the need for an in vitro chondrogenic culture period while maintaining the beneficial effects of generating a tissue in vivo that is programmed to withstand the initial hypoxic environment and is also capable of promoting the development of vascularized bone. With drug-loaded matrices, however, protein-induced expression within host cells is transient, and therefore, the release profile from such devices often involves initial supraphysiological levels of drug elution which diminish rapidly. This limitation could potentially be addressed through the use of gene-activated scaffolds, which aim to transfect host cells to express a desired transgene resulting in stable and prolonged expression of the protein, an approach which, if harnessed successfully, could have enormous therapeutic benefits. Indeed, recent studies have utilized genes such as BMP-2, TGF-β3, and VEGF as regulators of the phenotype of chondrogenic cells progressing down the endochondral pathway [162–165]. However, some safety concerns exist regarding the use of viral vectors in gene-activated scaffolds, which can be leveraged to deliver therapeutic genes to the site of interest [166]. Reports have shown retrovirus and adenovirus vectors to be the cause of leukemia-like disease [167,168] and fatality [169], respectively, in patients participating in clinical trials involving gene therapy. This has led to increased research into non-viral delivery vectors for gene therapy applications, which offer a safer alternative to the viral vector, although questions remain surrounding their efficacy for gene delivery [170]. In this context, a recent study has found the endochondral phenotype of MSCs in vitro to be dependent on the choice of non-viral vector [171]. Furthermore, chitosan [172] and nano-HA [173] have recently been showed to be effective non-viral delivery vectors for genes involved in promoting angiogenesis and osteogenesis in vivo and could potentially also be explored as mechanisms to promote hypertrophic chondrogenesis and endochondral ossification. Indeed, the field of endochondral bone tissue engineering has only very recently been opened up to the technology of gene delivery, and it is envisaged that investigations into the delivery of genes involved in the endochondral cascade, complexed to non-viral vectors, will become of particular interest to the field in upcoming years.

To conclude, knowledge of the mechanisms governing endochondral bone regeneration through tissue engineering is expanding within the scientific community, and in order to capitalize fully on the potential of this approach, further advances in the use of biomaterials, gene editing, and next-generation biomolecules [174] as means of controlling cell responses, as well as an enhanced understanding of developmental and stem cell biology, will be required. And although significant challenges remain in these areas, the endochondral route to regeneration, which was inspired by recapitulating the developmental process that drives bone formation during embryogenesis, will undoubtedly continue to be explored and investigated as a means to treat bone and joint-based ailments that require surgical intervention.

## Declaration of interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

Fergal O'Brien holds stock and is an inventor on two patents currently being commercialized by SurgaColl Technologies. Integra LifeSciences and Johnson & Johnson fund ongoing research projects in his lab.

## Acknowledgements

Supported by the Enterprise Ireland Commercialisation Fund Programme (CF20144051) and the European Union BlueHuman Intereg Atlantic Area Project (EAPA_151/2016).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mtbio.2019.100009.

## References

[1] R. Dimitriou, E. Jones, D. McGonagle, P.V. Giannoudis, Bone regeneration: current concepts and future directions, BMC Med. 9 (2011) 66.
[2] H. Shegarfi, O. Reikeras, Review article: bone transplantation and immune response, J. Orthop. Surg. 17 (2) (2009) 206–211.
[3] D. Donati, C. Di Bella, M. Col angeli, G. Bianchi, M. Mercuri, The use of massive bone allografts in bone tumour surgery of the limb, Curr. Orthop. 19 (5) (2005) 393–399.
[4] C.J. Koh, A. Atala, Tissue engineering, stem cells, and cloning: opportunities for regenerative medicine, J. Am. Soc. Nephrol. 15 (5) (2004) 1113–1125.
[5] R. Langer, Tissue engineering, Mol. Ther. 1 (1) (2000) 12–15.
[6] A.I. Caplan, Mesenchymal stem cells, J. Orthop. Res. 9 (5) (1991) 641–650.
[7] G.J. Meijer, J.D. De Bruijn, R. Koole, C.A. Van Blitterswijk, Cell-based bone tissue engineering, PLoS Med. 4 (2) (2007) 0260–0264.
[8] M.I. Santos, R.L. Reis, Vascularization in bone tissue engineering: physiology, current strategies, major hurdles and future challenges, Macromol. Biosci. 10 (1) (2010) 12–27.
[9] F.G. Lyons, A.A. Al-Munajjed, S.M. Kieran, M.E. Toner, C.M. Murphy, G.P. Duffy, et al., The healing of bony defects by cell-free collagen-based scaffolds compared to stem cell-seeded tissue engineered constructs, Biomaterials 31 (35) (2010) 9232–9243.
[10] P. Lenas, M. Moos, F.P. Luyten, Developmental engineering: a new paradigm for the design and manufacturing of cell-based products. Part I: from three-dimensional cell growth to biomimetics of in Vivo development, Tissue Eng. B Rev. 15 (4) (2009) 381–394.
[11] H.M. Kronenberg, Developmental regulation of the growth plate, Nature 423 (6937) (2003) 332–336.
[12] D. Gawlitta, E. Farrell, J. Malda, L.B. Creemers, J. Alblas, W.J.A. Dhert, Modulating endochondral ossification of multipotent stromal cells for bone regeneration, Tissue Eng. B Rev. 16 (4) (2010) 385–395.
[13] E. Farrell, S.K. Both, K.I. Odörfer, W. Koevoet, N. Kops, F.J. O'Brien, et al., In-vivo generation of bone via endochondral ossification by in-vitro chondrogenic priming of adult human and rat mesenchymal stem cells, BMC Muscoskelet. Disord. 12 (2011).
[14] H. Yamasaki, H. Sakai, Osteogenic response to porous hydroxyapatite ceramics under the skin of dogs, Biomaterials 13 (5) (1992) 308–312.
[15] C. Klein, K. de Groot, W. Chen, Y. Li, X. Zhang, Osseous substance formation induced in porous calcium phosphate ceramics in soft tissues, Biomaterials 15 (1) (1994) 31–34.
[16] H. Yuan, M. Van Den Doel, S. Li, C.A. Van Blitterswijk, K. De Groot, J.D. De Bruijn, A comparison of the osteoinductive potential of two calcium phosphate ceramics implanted intramuscularly in goats, J. Mater. Sci. Mater. Med. 13 (12) (2002) 1271–1275.
[17] D. Le Nihouannen, G. Daculsi, A. Saffarzadeh, O. Gauthier, S. Delplace, P. Pilet, et al., Ectopic bone formation by microporous calcium phosphate ceramic particles in sheep muscles, Bone 36 (6) (2005) 1086–1093.
[18] A.J. Salgado, O.P. Coutinho, R.L. Reis, Bone tissue engineering: state of the art and future trends, Macromol. Biosci. 4 (8) (2004) 743–765.
[19] D.A. Wahl, J.T. Czernuszka, Collagen-hydroxyapatite composites for hard tissue repair, Eur. Cells Mater. 11 (2006) 43–56.
[20] J.P. Gleeson, N.A. Plunkett, F.J. O'Brien, Addition of hydroxyapatite improves stiffness, interconnectivity and osteogenic potential of a highly porous collagen-based scaffold for bone tissue regeneration, Eur. Cells Mater. 20 (2010) 218–230.
[21] F.G. Lyons, J.P. Gleeson, S. Partap, K. Coghlan, F.J. O'Brien, Novel microhydroxyapatite particles in a collagen scaffold: a bioactive bone void filler? Clin. Orthop. Relat. Res. 472 (4) (2014) 1318–1328.
[22] V. Karageorgiou, D. Kaplan, Porosity of 3D biomaterial scaffolds and osteogenesis, Biomaterials 26 (27) (2005) 5474–5491.
[23] M.M. Stevens, Biomaterials for bone tissue engineering, Mater. Today 11 (5) (2008) 18–25.
[24] R.E. Guldberg, M.E. Oest, K. Dupont, A. Peister, E. Deutsch, Y. Kolambkar, et al., Biologic augmentation of polymer scaffolds for bone repair, J. Musculoskelet. Neuronal Interact. 7 (4) (2007) 333–334.
[25] J. Groppe, C.S. Hinck, P. Samavarchi-Tehrani, C. Zubieta, J.P. Schuermann, A.B. Taylor, et al., Cooperative assembly of TGF-β superfamily signaling complexes is mediated by two disparate mechanisms and distinct modes of receptor binding, Mol. Cell 29 (2) (2008) 157–168.
[26] M.E. Oest, K.M. Dupont, H.J. Kong, D.J. Mooney, R.E. Guldberg, Quantitative assessment of scaffold and growth factor-mediated repair of critically sized bone defects, J. Orthop. Res. 25 (7) (2007) 941–950.
[27] B. Rai, M.E. Oest, K.M. Dupont, K.H. Ho, S.H. Teoh, R.E. Guldberg, Combination of platelet-rich plasma with polycaprolactone-tricalcium phosphate scaffolds for segmental bone defect repair, J. Biomed. Mater. Res. A 81 (4) (2007) 888–899.
[28] E.J. Carragee, G. Chu, R. Rohatgi, E.L. Hurwitz, B.K. Weiner, S.T. Yoon, et al., Cancer risk after use of recombinant bone morphogenetic protein-2 for spinal arthrodesis, J. Bone Joint Surg. Am. Vol. 95 (17) (2013) 1537–1545.

[29] J.A. Rihn, R. Patel, J. Makda, J. Hong, D.G. Anderson, A.R. Vaccaro, et al., Complications associated with single-level transforaminal lumbar interbody fusion, Spine J. 9 (8) (2009) 623–629.
[30] C.A. Tannoury, H.S. An, Complications with the use of bone morphogenetic protein 2 (BMP-2) in spine surgery, Spine J. 14 (3) (2014) 552–559.
[31] J. Vavken, A. Mameghani, P. Vavken, S. Schaeren, Complications and cancer rates in spine fusion with recombinant human bone morphogenetic protein-2 (rhBMP-2), Eur. Spine J. 25 (12) (2016) 3979–3989.
[32] E.J. Woo, Recombinant human bone morphogenetic protein-2: adverse events reported to the Manufacturer and User Facility Device Experience database, Spine J. 12 (10) (2012) 894–899.
[33] N.E. Epstein, Pros, cons, and costs of INFUSE in spinal surgery, Surg. Neurol. Int. 2 (2011) 10.
[34] Y.M. Kolambkar, J.D. Boerckel, K.M. Dupont, M. Bajin, N. Huebsch, D.J. Mooney, et al., Spatiotemporal delivery of bone morphogenetic protein enhances functional repair of segmental bone defects, Bone 49 (3) (2011) 485–492.
[35] E. Quinlan, E.M. Thompson, A. Matsiko, F.J. O'Brien, A. López-Noriega, Long-term controlled delivery of rhBMP-2 from collagen–hydroxyapatite scaffolds for superior bone tissue regeneration, J. Control. Release 207 (2015) 112–119.
[36] E. Quinlan, A. Lopez-Noriega, E. Thompson, H.M. Kelly, S.A. Cryan, F.J. O'Brien, Development of collagen-hydroxyapatite scaffolds incorporating PLGA and alginate microparticles for the controlled delivery of rhBMP-2 for bone tissue engineering, J. Control. Release : Off. J. Contr. Release Soc. 198 (2015) 71–79.
[37] Y.M. Kolambkar, K.M. Dupont, J.D. Boerckel, N. Huebsch, D.J. Mooney, D.W. Hutmacher, et al., An alginate-based hybrid system for growth factor delivery in the functional repair of large bone defects, Biomaterials 32 (1) (2011) 65–74.
[38] A.K. Jha, K.M. Tharp, J. Ye, J.L. Santiago-Ortiz, W.M. Jackson, A. Stahl, et al., Enhanced survival and engraftment of transplanted stem cells using growth factor sequestering hydrogels, Biomaterials 47 (2015) 1–12.
[39] E. Quinlan, A. Lopez-Noriega, E.M. Thompson, A. Hibbitts, S.A. Cryan, F.J. O'Brien, Controlled release of vascular endothelial growth factor from spray-dried alginate microparticles in collagen-hydroxyapatite scaffolds for promoting vascularization and bone repair, J. Tissue Eng. Regenerat. Med. 11 (4) (2017) 1097–1109.
[40] J.E. Saik, D.J. Gould, E.M. Watkins, M.E. Dickinson, J.L. West, Covalently immobilized platelet-derived growth factor-BB promotes angiogenesis in biomimetic poly(ethylene glycol) hydrogels, Acta Biomater. 7 (1) (2011) 133–143.
[41] S. Metzger, P.S. Lienemann, C. Ghayor, W. Weber, I. Martin, F.E. Weber, et al., Modular poly(ethylene glycol) matrices for the controlled 3d-localized osteogenic differentiation of mesenchymal stem cells, Adv. Healthc. Mater. 4 (4) (2015) 550–558.
[42] T. Re'em, Y. Kaminer-Israeli, E. Ruvinov, S. Cohen, Chondrogenesis of hMSC in affinity-bound TGF-beta scaffolds, Biomaterials 33 (3) (2012) 751–761.
[43] I. Freeman, A. Kedem, S. Cohen, The effect of sulfation of alginate hydrogels on the specific binding and controlled release of heparin-binding proteins, Biomaterials 29 (22) (2008) 3260–3268.
[44] P.C. Bessa, M. Casal, R.L. Ruis, Bone morphogenetic proteins in tissue engineering: the road from laboratory to clinic, part II (BMP delivery), J. Tissue Eng. Regenerat. Med. 2 (2–3) (2008) 81–96.
[45] S.-H. Lee, H. Shin, Matrices and scaffolds for delivery of bioactive molecules in bone and cartilage tissue engineering, Adv. Drug Deliv. Rev. 59 (4) (2007) 339–359.
[46] A.J. Engler, S. Sen, H.L. Sweeney, D.E. Discher, Matrix elasticity directs stem cell lineage specification, Cell 126 (2006).
[47] J. Fiedler, B. Özdemir, J. Bartholomä, A. Plettl, R.E. Brenner, P. Ziemann, The effect of substrate surface nanotopography on the behavior of multipotnent mesenchymal stromal cells and osteoblasts, Biomaterials 34 (35) (2013) 8851–8859.
[48] Y.J. Li, N.N. Batra, L. You, S.C. Meier, I.A. Coe, C.E. Yellowley, et al., Oscillatory fluid flow affects human marrow stromal cell proliferation and differentiation, J. Orthop. Res. 22 (6) (2004) 1283–1289.
[49] G.N. Bancroft, V.I. Sikavitsas, J. van den Dolder, T.L. Sheffield, C.G. Ambrose, J.A. Jansen, et al., Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner, Proc. Natl. Acad. Sci. Unit. States Am. 99 (20) (2002) 12600–12605.
[50] F. Langenbach, J. Handschel, Effects of dexamethasone, ascorbic acid and β-glycerophosphate on the osteogenic differentiation of stem cells in vitro, Stem Cell Res. Ther. 4 (5) (2013) 117.
[51] D.P. Lennon, J.M. Edmison, A.I. Caplan, Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivo osteochondrogenesis, J. Cell. Physiol. 187 (3) (2001) 345–355.
[52] E. Farrell, F.J. O'Brien, P. Doyle, J. Fischer, I. Yannas, B.A. Harley, et al., A collagen-glycosaminoglycan scaffold supports adult rat mesenchymal stem cell differentiation along osteogenic and chondrogenic routes, Tissue Eng. 12 (3) (2006) 459–468.
[53] E. Kon, A. Muraglia, A. Corsi, P. Bianco, M. Marcacci, I. Martin, et al., Autologous bone marrow stromal cells loaded onto porous hydroxyapatite ceramic accelerate bone repair in critical-size defects of sheep long bones, J. Biomed. Mater. Res. 49 (3) (2000) 328–337.
[54] J. Yuan, L. Cui, W.J. Zhang, W. Liu, Y. Cao, Repair of canine mandibular bone defects with bone marrow stromal cells and porous β-tricalcium phosphate, Biomaterials 28 (6) (2007) 1005–1013.
[55] J. Yuan, W.J. Zhang, G. Liu, M. Wei, Z.L. Qi, W. Liu, et al., Repair of canine mandibular bone defects with bone marrow stromal cells and coral, Tissue Eng. 16 (4) (2010) 1385–1394.
[56] R. Quarto, M. Mastrogiacomo, R. Cancedda, S.M. Kutepov, V. Mukhachev, A. Lavroukov, et al., Repair of large bone defects with the use of autologous bone marrow stromal cells, N. Engl. J. Med. 344 (5) (2001) 385–386.
[57] M. Marcacci, E. Kon, V. Moukhachev, A. Lavroukov, S. Kutepov, R. Quarto, et al., Stem cells associated with macroporous bioceramics for long bone repair: 6- to 7-year outcome of a pilot clinical study, Tissue Eng. 13 (5) (2007) 947–955.
[58] S.H. Ralston, Bone structure and metabolism, Medicine 37 (9) (2013) 469–474.
[59] E.J. Mackie, Y.A. Ahmed, L. Tatarczuch, K.S. Chen, M. Mirams, Endochondral ossification: how cartilage is converted into bone in the developing skeleton, Int. J. Biochem. Cell Biol. 40 (1) (2008) 46–62.
[60] E.M. Thompson, A. Matsiko, E. Farrell, D.J. Kelly, F.J. O'Brien, Recapitulating endochondral ossification: a promising route to in vivo bone regeneration, J. Tissue Eng. Regenerat. Med. 9 (8) (2015) 889–902.
[61] S.J. Karp, E. Schipani, B. St-Jacques, J. Hunzelman, H. Kronenberg, A.P. McMahon, Indian hedgehog coordinates endochondral bone growth and morphogenesis via parathyroid hormone related-protein-dependent and -independent pathways, Development 127 (3) (2000) 543–548.
[62] B. St-Jacques, M. Hammerschmidt, A.P. McMahon, Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation, Genes Dev. 13 (16) (1999) 2072–2086.
[63] H.M. Kronenberg, PTHrP and skeletal development, Ann. N. Y. Acad. Sci. 1068 (2006) 1–13.
[64] C.S. Bahney, D.P. Hu, T. Miclau 3rd, R.S. Marcucio, The multifaceted role of the vasculature in endochondral fracture repair, Front. Endocrinol. 6 (2015) 4.
[65] T.M. Ganey, J.A. Ogden, J. Sasse, P.J. Neame, D.R. Hilbelink, Basement membrane composition of cartilage canals during development and ossification of the epiphysis, Anat. Rec. 241 (3) (1995) 425–437.
[66] B. Ytrehus, C.S. Carlson, N. Lundeheim, L. Mathisen, F.P. Reinholt, J. Teige, et al., Vascularisation and osteochondrosis of the epiphyseal growth cartilage of the distal femur in pigs - development with age, growth rate, weight and joint shape, Bone 34 (3) (2004) 454–465.
[67] M.J.F. Blumer, S. Longato, H. Fritsch, Cartilage canals in the chicken embryo are involved in the process of endochondral bone formation within the epiphyseal growth plate, Anat. Rec. Part A Discoveries in Molecular, Cellular, and Evolutionary Biology 279 (1) (2004) 692–700.
[68] M.J.F. Blumer, C. Schwarzer, M.T. Pérez, K.Z. Konakci, H. Fritsch, Identification and location of bone-forming cells within cartilage canals on their course into the secondary ossification centre, J. Anat. 208 (6) (2006) 695–707.
[69] M.J.F. Blumer, S. Longato, H. Fritsch, Structure, formation and role of cartilage canals in the developing bone, Ann. Anat. 190 (4) (2008) 305–315.
[70] N. Little, B. Rogers, M. Flannery, Bone formation, remodelling and healing, Surgery – Oxford Int. Ed. 29 (4) (2011) 141–145.
[71] A. Schindeler, M.M. McDonald, P. Bokko, D.G. Little, Bone remodeling during fracture repair: the cellular picture, Semin. Cell Dev. Biol. 19 (5) (2008) 459–466.
[72] M. SI, S. AC, F. Theresa, S. Vickram, Fate of the hypertrophic chondrocyte: microenvironmental perspectives on apoptosis and survival in the epiphyseal growth plate, Birth Defects Res. Part C Embryo Today - Rev. 75 (4) (2005) 330–339.
[73] Y.A. Ahmed, L. Tatarczuch, C.N. Pagel, H.M.S. Davies, M. Mirams, E.J. Mackie, Physiological death of hypertrophic chondrocytes, Osteoarthritis Cartilage 15 (5) (2007) 575–586.
[74] H.I. Roach, T. Aigner, J.B. Kouri, Chondroptosis: a variant of apoptotic cell death in chondrocytes? Apoptosis 9 (3) (2004) 265–277.
[75] L. Yang, K.Y. Tsang, H.C. Tang, D. Chan, K.S.E. Cheah, Hypertrophic chondrocytes can become osteoblasts and osteocytes in endochondral bone formation, Proc. Natl. Acad. Sci. Unit. States Am. 111 (33) (2014) 12097–12102.
[76] X. Zhou, K. von der Mark, S. Henry, W. Norton, H. Adams, B. de Crombrugghe, Chondrocytes transdifferentiate into osteoblasts in endochondral bone during development, postnatal growth and fracture healing in mice, PLoS Genet. 10 (12) (2014) e1004820.
[77] J. Park, M. Gebhardt, S. Golovchenko, F. Perez-Branguli, T. Hattori, C. Hartmann, et al., Dual pathways to endochondral osteoblasts: a novel chondrocyte-derived osteoprogenitor cell identified in hypertrophic cartilage, Biology Open 4 (5) (2015) 608–621.
[78] D.P. Hu, F. Ferro, F. Yang, A.J. Taylor, W. Chang, T. Miclau, et al., Cartilage to bone transformation during fracture healing is coordinated by the invading vasculature and induction of the core pluripotency genes, Development 144 (2) (2017) 221–234.
[79] C.S. Bahney, D.P. Hu, A.J. Taylor, F. Ferro, H.M. Britz, B. Hallgrimsson, et al., Stem cell-derived endochondral cartilage stimulates bone healing by tissue transformation, J. Bone Miner. Res. : Off. J. Am. Soc. Bone Miner. Res. 29 (5) (2014) 1269–1282.
[80] W.S. Kim, C.A. Vacanti, J. Upton, J.P. Vacanti, Bone defect repair with tissue-engineered cartilage, Plast. Reconstr. Surg. 94 (5) (1994) 580–584.
[81] C.A. Vacanti, W. Kim, J. Upton, D. Mooney, J.P. Vacanti, The efficacy of periosteal cells compared to chondrocytes in the tissue engineered repair of bone defects, Tissue Eng. 1 (3) (1995) 301–308.
[82] S.M. Oliveira, I.F. Amaral, M.A. Barbosa, C.C. Teixeira, Engineering endochondral bone: in vitro studies, Tissue Eng. 15 (3) (2009) 625–634.
[83] S.M. Oliveira, D.Q. Mijares, G. Turner, I.F. Amaral, M.A. Barbosa, C.C. Teixeira, Engineering endochondral bone: in vivo studies, Tissue Eng. 15 (3) (2009) 635–643.

[84] C.S. Bahney, L. Jacobs, R. Tamai, D. Hu, T.F. Luan, M. Wang, et al., Promoting endochondral bone repair using human osteoarthritic articular chondrocytes, Tissue Eng. 22 (5–6) (2016) 427–435.

[85] K. Bardsley, A. Kwarciak, C. Freeman, I. Brook, P. Hatton, A. Crawford, Repair of bone defects in vivo using tissue engineered hypertrophic cartilage grafts produced from nasal chondrocytes, Biomaterials 112 (2017) 313–323.

[86] E. Alsberg, K.W. Anderson, A. Albeiruti, J.A. Rowley, D.J. Mooney, Engineering growing tissues, Proc. Natl. Acad. Sci. U. S. A. 99 (19) (2002) 12025–12030.

[87] A. Barbero, S. Grogan, Scha?fer D, Heberer M, Mainil-Varlet P, Martin I. Age related changes in human articular chondrocyte yield, proliferation and post-expansion chondrogenic capacity, Osteoarthritis Cartilage 12 (6) (2004) 476–484.

[88] J. Diaz-Romero, J.P. Gaillard, S.P. Grogan, D. Nesic, T. Trub, P. Mainil-Varlet, Immunophenotypic analysis of human articular chondrocytes: changes in surface markers associated with cell expansion in monolayer culture, J. Cell. Physiol. 202 (3) (2005) 731–742.

[89] J.M. Jukes, S.K. Both, A. Leusink, L.M.T. Sterk, C.A. Van Blitterswijk, J. De Boer, Endochondral bone tissue engineering using embryonic stem cells, Proc. Natl. Acad. Sci. U. S. A. 105 (19) (2008) 6840–6845.

[90] C. De Bari, F. Dell'Accio, F.P. Luyten, Failure of in vitro-differentiated mesenchymal stem cells from the synovial membrane to form ectopic stable cartilage in vivo, Arthritis Rheum. 50 (1) (2004) 142–150.

[91] K. Pelttari, A. Winter, E. Steck, K. Goetzke, T. Hennig, B.G. Ochs, et al., Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice, Arthritis Rheum. 54 (10) (2006) 3254–3266.

[92] A. Dickhut, K. Pelttari, P. Janicki, W. Wagner, V. Eckstein, M. Egermann, et al., Calcification or dedifferentiation: requirement to lock mesenchymal stem cells in a desired differentiation stage, J. Cell. Physiol. 219 (1) (2009) 219–226.

[93] T. Vinardell, E.J. Sheehy, C.T. Buckley, D.J. Kelly, A comparison of the functionality and in vivo phenotypic stability of cartilaginous tissues engineered from different stem cells sources, Tissue Eng. 18 (11–12) (2012) 1161–1170.

[94] C. Scotti, B. Tonnarelli, A. Papadimitropoulos, A. Scherberich, S. Schaeren, A. Schauerte, et al., Recapitulation of endochondral bone formation using human adult mesenchymal stem cells as a paradigm for developmental engineering, Proc. Natl. Acad. Sci. U. S. A. 107 (16) (2010) 7251–7256.

[95] J. van der Stok, M.K. Koolen, H. Jahr, N. Kops, J.H. Waarsing, H. Weinans, et al., Chondrogenically differentiated mesenchymal stromal cell pellets stimulate endochondral bone regeneration in critical-sized bone defects, Eur. Cells Mater. 27 (2014) 137–148, discussion 48.

[96] R. Osinga, N. Di Maggio, A. Todorov, N. Allafi, A. Barbero, F. Laurent, et al., Generation of a bone organ by human adipose-derived stromal cells through endochondral ossification, Stem Cells Transl Med 5 (8) (2016) 1090–1097.

[97] A. Muraglia, A. Corsi, M. Riminucci, M. Mastrogiacomo, R. Cancedda, P. Bianco, et al., Formation of a chondro-osseous rudiment in micromass cultures of human bone-marrow stromal cells, J. Cell Sci. 116 (Pt 14) (2003) 2949–2955.

[98] M.B. Mueller, R.S. Tuan, Functional characterization of hypertrophy in chondrogenesis of human mesenchymal stem cells, Arthritis Rheum. 58 (5) (2008) 1377–1388.

[99] M. Mumme, C. Scotti, A. Papadimitropoulos, A. Todorov, W. Hoffmann, C. Bocelli-Tyndall, et al., Interleukin-1β modulates endochondral ossification by human adult bone marrow stromal cells, Eur. Cells Mater. 24 (2012) 224–236.

[100] S.D. Thorpe, C.T. Buckley, A.J. Steward, D.J. Kelly, European Society of Biomechanics S.M. Perren Award 2012: the external mechanical environment can override the influence of local substrate in determining stem cell fate, J. Biomech. 45 (15) (2012) 2483–2492.

[101] L. Bian, D.Y. Zhai, E.C. Zhang, R.L. Mauck, J.A. Burdick, Dynamic compressive loading enhances cartilage matrix synthesis and distribution and suppresses hypertrophy in hMSC-laden hyaluronic acid hydrogels, Tissue Eng. 18 (7–8) (2012) 715.

[102] E.A. Aisenbrey, S.J. Bryant, Mechanical loading inhibits hypertrophy in chondrogenically differentiating hMSCs within a biomimetic hydrogel, J. Mater. Chem. B, Mater. Biol. Med. 4 (20) (2016) 3562–3574.

[103] T. Vinardell, R.A. Rolfe, C.T. Buckley, E.G. Meyer, M. Ahearne, P. Murphy, et al., Hydrostatic pressure acts to stabilise a chondrogenic phenotype in porcine joint tissue derived stem cells, Eur. Cells Mater. 23 (2012) 121–132, discussion 33-4.

[104] S.F. Carroll, C.T. Buckley, D.J. Kelly, Cyclic hydrostatic pressure promotes a stable cartilage phenotype and enhances the functional development of cartilaginous grafts engineered using multipotent stromal cells isolated from bone marrow and infrapatellar fat pad, J. Biomech. 47 (9) (2014) 2115–2121.

[105] S.M. Goldman, G.A. Barabino, Hydrodynamic loading in concomitance with exogenous cytokine stimulation modulates differentiation of bovine mesenchymal stem cells towards osteochondral lineages, BMC Biotechnol. 16 (1) (2016) 10.

[106] S.F. Carroll, C.T. Buckley, D.J. Kelly, Cyclic tensile strain can play a role in directing both intramembranous and endochondral ossification of mesenchymal stem cells, Front. Bioeng. Biotechnol. 5 (NOV) (2017).

[107] A.M. McDermott, S. Herberg, D.E. Mason, J.M. Collins, H.B. Pearson, J.H. Dawahare, et al., Recapitulating bone development through engineered mesenchymal condensations and mechanical cues for tissue regeneration, Science Translational Medicine 11 (495) (2019).

[108] T. Ma, W.L. Grayson, M. Fröhlich, G. Vunjak-Novakovic, Hypoxia and stem cell-based engineering of mesenchymal tissues, Biotechnol. Prog. 25 (1) (2009) 32–42.

[109] M.C. Ronzière, E. Perrier, F. Mallein-Gerin, A.M. Freyria, Chondrogenic potential of bone marrow- and adipose tissue-derived adult human mesenchymal stem cells, Bio Med. Mater. Eng. 20 (3–4) (2010) 145–158.

[110] M. Hirao, N. Tamai, N. Tsumaki, H. Yoshikawa, A. Myoui, Oxygen tension regulates chondrocyte differentiation and function during endochondral ossification, J. Biol. Chem. 281 (41) (2006) 31079–31092.

[111] K. Morita, T. Miyamoto, N. Fujita, Y. Kubota, K. Ito, K. Takubo, et al., Reactive oxygen species induce chondrocyte hypertrophy in endochondral ossification, J. Exp. Med. 204 (7) (2007) 1613–1623.

[112] E.J. Sheehy, C.T. Buckley, D.J. Kelly, Oxygen tension regulates the osteogenic, chondrogenic and endochondral phenotype of bone marrow derived mesenchymal stem cells, Biochem. Biophys. Res. Commun. 417 (1) (2012) 305–310.

[113] D. Gawlitta, M.H.P. Van Rijen, E.J.M. Schrijver, J. Alblas, W.J.A. Dhert, Hypoxia impedes hypertrophic chondrogenesis of human multipotent stromal cells, Tissue Eng. 18 (19–20) (2012) 1957–1966.

[114] J. Leijten, N. Georgi, L. Moreira Teixeira, C.A. van Blitterswijk, J.N. Post, M. Karperien, Metabolic programming of mesenchymal stromal cells by oxygen tension directs chondrogenic cell fate, Proc. Natl. Acad. Sci. U. S. A. 111 (38) (2014) 13954–13959.

[115] P.-J. Stiers, S. Stegen, N. van Gastel, R. Van Looveren, S. Torrekens, G. Carmeliet, Inhibition of the oxygen sensor PHD2 enhances tissue-engineered endochondral bone formation, J. Bone Miner. Res. 34 (2) (2019) 333–348.

[116] F.J. O'Brien, B.A. Harley, I.V. Yannas, L. Gibson, Influence of freezing rate on pore structure in freeze-dried collagen-GAG scaffolds, Biomaterials 25 (6) (2004) 1077–1086.

[117] J.I. Huang, M.M. Durbhakula, P. Angele, B. Johnstone, J.U. Yoo, Lunate arthroplasty with autologous mesenchymal stem cells in a rabbit model, J. Bone Jt. Surg. 88 (4) (2006) 744–752.

[118] C. Scotti, E. Piccinini, H. Takizawa, A. Todorov, P. Bourgine, A. Papadimitropoulos, et al., Engineering of a functional bone organ through endochondral ossification, Proc. Natl. Acad. Sci. U. S. A. 110 (10) (2013) 3997–4002.

[119] E. Farrell, O.P. Van Der Jagt, W. Koevoet, N. Kops, C.J. Van Manen, C.A. Hellingman, et al., Chondrogenic priming of human bone marrow stromal cells: a better route to bone repair? Tissue Eng. C Methods 15 (2) (2009) 285–295.

[120] E.M. Thompson, A. Matsiko, D.J. Kelly, J.P. Gleeson, F.J. O'Brien, An endochondral ossification-based approach to bone repair: chondrogenically primed mesenchymal stem cell-laden scaffolds support greater repair of critical-sized cranial defects than osteogenically stimulated constructs in vivo, Tissue Eng. 22 (5–6) (2016) 556–567.

[121] M. Amos, M. TE, L.G. Cai, M. CG, V. Tatiana, P. GJ, et al., An endochondral ossification approach to early stage bone repair: use of tissue-engineered hypertrophic cartilage constructs as primordial templates for weight-bearing bone repair, J. Tissue Eng. Regenerat. Med. 12 (4) (2018) e2147–e2150.

[122] F.J. O'Brien, B.A. Harley, I.V. Yannas, L.J. Gibson, The effect of pore size on cell adhesion in collagen-GAG scaffolds, Biomaterials 26 (4) (2005) 433–441.

[123] C.M. Murphy, M.G. Haugh, F.J. O'Brien, The effect of mean pore size on cell attachment, proliferation and migration in collagen-glycosaminoglycan scaffolds for bone tissue engineering, Biomaterials 31 (3) (2010) 461–466.

[124] A.J. Ryan, W.A. Lackington, A.J. Hibbitts, A. Matheson, T. Alekseeva, A. Stejskalova, et al., A physicochemically optimized and neuroconductive biphasic nerve guidance conduit for peripheral nerve repair, Adv. Healthc. Mater. 6 (24) (2017) 1700954.

[125] A. Petersen, A. Princ, G. Korus, A. Ellinghaus, H. Leemhuis, A. Herrera, et al., A biomaterial with a channel-like pore architecture induces endochondral healing of bone defects, Nat. Commun. 9 (1) (2018) 4430.

[126] E.J. Sheehy, T. Vinardell, M.E. Toner, C.T. Buckley, D.J. Kelly, Altering the architecture of tissue engineered hypertrophic cartilaginous grafts facilitates vascularisation and accelerates mineralisation, PLoS One 9 (3) (2014).

[127] A.C. Daly, P. Pitacco, J. Nulty, G.M. Cunniffe, D.J. Kelly, 3D printed microchannel networks to direct vascularisation during endochondral bone repair, Biomaterials 162 (2018) 34–46.

[128] C.A. Simmons, E. Alsberg, S. Hsiong, W.J. Kim, D.J. Mooney, Dual growth factor delivery and controlled scaffold degradation enhance in vivo bone formation by transplanted bone marrow stromal cells, Bone 35 (2) (2004) 562–569.

[129] E.J. Sheehy, T. Mesallati, T. Vinardell, D.J. Kelly, Engineering cartilage or endochondral bone: a comparison of different naturally derived hydrogels, Acta Biomater. 13 (2015) 245–253.

[130] P. Janicki, P. Kasten, K. Kleinschmidt, R. Luginbuehl, W. Richter, Chondrogenic pre-induction of human mesenchymal stem cells on β-TCP: enhanced bone quality by endochondral heterotopic bone formation, Acta Biomater. 6 (8) (2010) 3292–3301.

[131] C.A. Knuth, J. Witte-Bouma, Y. Ridwan, E.B. Wolvius, E. Farrell, Mesenchymal stem cell-mediated endochondral ossification utilising micropellets and brief chondrogenic priming, Eur. Cells Mater. 34 (2017) 142–161.

[132] D. Gawlitta, K.E. Benders, J. Visser, A.S. van der Sar, D.H. Kempen, L.F. Theyse, et al., Decellularized cartilage-derived matrix as substrate for endochondral bone regeneration, Tissue Eng. 21 (3–4) (2015) 694–703.

[133] B. Quang Le, C. Van Blitterswijk, J. De Boer, An approach to in vitro manufacturing of hypertrophic cartilage matrix for bone repair, Bioengineering 4 (2) (2017).

[134] J. Visser, D. Gawlitta, K.E. Benders, S.M. Toma, B. Pouran, P.R. van Weeren, et al., Endochondral bone formation in gelatin methacrylamide hydrogel with embedded cartilage-derived matrix particles, Biomaterials 37 (2015) 174–182.

[135] G.M. Cunniffe, T. Vinardell, J.M. Murphy, E.M. Thompson, A. Matsiko, F.J. O'Brien, et al., Porous decellularized tissue engineered hypertrophic cartilage as a scaffold for large bone defect healing, Acta Biomater. 23 (2015) 82–90.

[136] G.M. Cunniffe, P.J. Diaz-Payno, E.J. Sheehy, S.E. Critchley, H.V. Almeida, P. Pitacco, et al., Tissue-specific extracellular matrix scaffolds for the regeneration of spatially complex musculoskeletal tissues, Biomaterials 188 (2019) 63–73.
[137] J. Guerrero, S. Pigeot, J. Müller, D.J. Schaefer, I. Martin, A. Scherberich, Fractionated human adipose tissue as a native biomaterial for the generation of a bone organ by endochondral ossification, Acta Biomater. 77 (2018) 142–154.
[138] K. Su, T.T. Lau, W. Leong, Y. Gong, D.-A. Wang, Creating a living hyaline cartilage graft free from non-cartilaginous constituents: an intermediate role of a biomaterial scaffold, Adv. Funct. Mater. 22 (5) (2012) 972–978.
[139] T.T. Lau, L.Q.P. Lee, B.N. Vo, K. Su, D.A. Wang, Inducing ossification in an engineered 3D scaffold-free living cartilage template, Biomaterials 33 (33) (2012) 8406–8417.
[140] D.I. Zeugolis, S.T. Khew, E.S. Yew, A.K. Ekaputra, Y.W. Tong, L.Y. Yung, et al., Electro-spinning of pure collagen nano-fibres – just an expensive way to make gelatin? Biomaterials 29 (15) (2008) 2293–2305.
[141] W. Yang, F. Yang, Y. Wang, S.K. Both, J.A. Jansen, In vivo bone generation via the endochondral pathway on three-dimensional electrospun fibers, Acta Biomater. 9 (1) (2013) 4505–4512.
[142] N. Harada, Y. Watanabe, K. Sato, S. Abe, K. Yamanaka, Y. Sakai, et al., Bone regeneration in a massive rat femur defect through endochondral ossification achieved with chondrogenically differentiated MSCs in a degradable scaffold, Biomaterials 35 (27) (2014) 7800–7810.
[143] F. Tortelli, R. Tasso, F. Loiacono, R. Cancedda, The development of tissue-engineered bone of different origin through endochondral and intramembranous ossification following the implantation of mesenchymal stem cells and osteoblasts in a murine model, Biomaterials 31 (2) (2010) 242–249.
[144] J. Bernhard, J. Ferguson, B. Rieder, P. Heimel, T. Nau, S. Tangl, et al., Tissue-engineered hypertrophic chondrocyte grafts enhanced long bone repair, Biomaterials 139 (2017) 202–212.
[145] Y. Wang, X. Cai, J. Huang, Y. Zhou, T. Jiang, Y. Wang, Bone regeneration in critically sized rat mandible defects through the endochondral pathway using hydroxyapatite-coated 3D-printed Ti6Al4V scaffolds, RSC Adv. 8 (55) (2018) 31745–31754.
[146] E.J. Sheehy, T. Vinardell, C.T. Buckley, D.J. Kelly, Engineering osteochondral constructs through spatial regulation of endochondral ossification, Acta Biomater. 9 (3) (2013) 5484–5492.
[147] E.J. Sheehy, T. Mesallati, L. Kelly, T. Vinardell, C.T. Buckley, D.J. Kelly, Tissue engineering whole bones through endochondral ossification: regenerating the distal phalanx, BioResearch Open Access 4 (1) (2015) 229–241.
[148] A. Barbero, S. Grogan, D. Schäfer, M. Heberer, P. Mainil-Varlet, I. Martin, Age related changes in human articular chondrocyte yield, proliferation and post-expansion chondrogenic capacity, Osteoarthritis Cartilage 12 (6) (2004) 476–484.
[149] M.E. Cooke, A.A. Allon, T. Cheng, A.C. Kuo, H.T. Kim, T.P. Vail, et al., Structured three-dimensional co-culture of mesenchymal stem cells with chondrocytes promotes chondrogenic differentiation without hypertrophy, Osteoarthritis Cartilage 19 (10) (2011) 1210–1218.
[150] L. Bian, D.Y. Zhai, R.L. Mauck, J.A. Burdick, Coculture of human mesenchymal stem cells and articular chondrocytes reduces hypertrophy and enhances functional properties of engineered cartilage, Tissue Eng. 17 (7–8) (2011) 1137–1145.
[151] L. Wu, J.C.H. Leijten, N. Georgi, J.N. Post, C.A. Van Blitterswijk, M. Karperien, Trophic effects of mesenchymal stem cells increase chondrocyte proliferation and matrix formation, Tissue Eng. 17 (9–10) (2011) 1425–1436.
[152] C. Acharya, A. Adesida, P. Zajac, M. Mumme, J. Riesle, I. Martin, et al., Enhanced chondrocyte proliferation and mesenchymal stromal cells chondrogenesis in coculture pellets mediate improved cartilage formation, J. Cell. Physiol. 227 (1) (2012) 88–97.
[153] T. Mesallati, E.J. Sheehy, T. Vinardell, C.T. Buckley, D.J. Kelly, Tissue engineering scaled-up, anatomically shaped osteochondral constructs for joint resurfacing, Eur. Cells Mater. 30 (2015) 163–185, discussion 85-6.
[154] V. Mironov, T. Trusk, V. Kasyanov, S. Little, R. Swaja, R. Markwald, Biofabrication: a 21st century manufacturing paradigm, Biofabrication 1 (2) (2009).
[155] A.C. Daly, G.M. Cunniffe, B.N. Sathy, O. Jeon, E. Alsberg, D.J. Kelly, 3D bioprinting of developmentally inspired templates for whole bone organ engineering, Adv. Healthc. Mater. 5 (18) (2016) 2353–2362.
[156] Y. Wang, S. Wu, M.A. Kuss, P.N. Streubel, B. Duan, Effects of hydroxyapatite and hypoxia on chondrogenesis and hypertrophy in 3D bioprinted ADMSC laden constructs, ACS Biomater. Sci. Eng. 3 (5) (2017) 826–835.
[157] L.A.J. Ahrens, D. Vonwil, J. Christensen, V.P. Shastri, Gelatin device for the delivery of growth factors involved in endochondral ossification, PLoS One 12 (4) (2017) e0175095.
[158] P.N. Dang, N. Dwivedi, L.M. Phillips, X. Yu, S. Herberg, C. Bowerman, et al., Controlled dual growth factor delivery from microparticles incorporated within human bone marrow-derived mesenchymal stem cell aggregates for enhanced bone tissue engineering via endochondral ossification, Stem Cells Transl. Med. 5 (2) (2016) 206–217.
[159] P.N. Dang, S. Herberg, D. Varghai, H. Riazi, D. Varghai, A. McMillan, et al., Endochondral ossification in critical-sized bone defects via readily implantable scaffold-free stem cell constructs, Stem Cells Transl. Med. 6 (7) (2017) 1644–1659.
[160] C. Stüdle, Q. Vallmajó-Martín, A. Haumer, J. Guerrero, M. Centola, A. Mehrkens, et al., Spatially confined induction of endochondral ossification by functionalized hydrogels for ectopic engineering of osteochondral tissues, Biomaterials 171 (2018) 219–229.
[161] J.D. Boerckel, B.A. Uhrig, N.J. Willett, N. Huebsch, R.E. Guldberg, Mechanical regulation of vascular growth and tissue regeneration in vivo, Proc. Natl. Acad. Sci. U. S. A. 108 (37) (2011) E674–E680.
[162] N. Zhou, Q. Li, X. Lin, N. Hu, J.Y. Liao, L.B. Lin, et al., BMP2 induces chondrogenic differentiation, osteogenic differentiation and endochondral ossification in stem cells, Cell Tissue Res. 366 (1) (2016) 101–111.
[163] C.Y. Lin, Y.H. Chang, K.C. Li, C.H. Lu, L.Y. Sung, C.L. Yeh, et al., The use of ASCs engineered to express BMP2 or TGF-beta3 within scaffold constructs to promote calvarial bone repair, Biomaterials 34 (37) (2013) 9401–9412.
[164] T. Gonzalez-Fernandez, E.G. Tierney, G.M. Cunniffe, F.J. O'Brien, D.J. Kelly, Gene delivery of TGF-beta3 and BMP2 in an MSC-laden alginate hydrogel for articular cartilage and endochondral bone tissue engineering, Tissue Eng. 22 (9–10) (2016) 776–787.
[165] A. Marsano, C.M. Medeiros da Cunha, S. Ghanaati, S. Gueven, M. Centola, R. Tsaryk, et al., Spontaneous in vivo chondrogenesis of bone marrow-derived mesenchymal progenitor cells by blocking vascular endothelial growth factor signaling, Stem Cells Transl. Med. 5 (12) (2016) 1730–1738.
[166] C.E. Thomas, A. Ehrhardt, M.A. Kay, Progress and problems with the use of viral vectors for gene therapy, Nat. Rev. Genet. 4 (5) (2003) 346–358.
[167] S. Hacein-Bey-Abina, C. von Kalle, M. Schmidt, F. Le Deist, N. Wulffraat, E. McIntyre, et al., A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency, N. Engl. J. Med. 348 (3) (2003) 255–256.
[168] E. Check, A tragic setback, 420, 2002, p. 116.
[169] Assessment of adenoviral vector safety and toxicity: report of the national institutes of health recombinant DNA advisory committee, Hum. Gene Ther. 13 (1) (2002) 3–13.
[170] M.S. Al-Dosari, X. Gao, Nonviral gene delivery: principle, limitations, and recent progress, AAPS J. 11 (4) (2009) 671.
[171] T. Gonzalez-Fernandez, B.N. Sathy, C. Hobbs, G.M. Cunniffe, H.O. McCarthy, N.J. Dunne, et al., Mesenchymal stem cell fate following non-viral gene transfection strongly depends on the choice of delivery vector, Acta Biomater. 55 (2017) 226–238.
[172] R.M. Raftery, I. Mencía Castaño, G. Chen, B. Cavanagh, B. Quinn, C.M. Curtin, et al., Translating the role of osteogenic-angiogenic coupling in bone formation: highly efficient chitosan-pDNA activated scaffolds can accelerate bone regeneration in critical-sized bone defects, Biomaterials 149 (Suppl. C) (2017) 116–127.
[173] C.M. Curtin, E.G. Tierney, K. McSorley, S.-A. Cryan, G.P. Duffy, F.J. O'Brien, Combinatorial gene therapy accelerates bone regeneration: non-viral dual delivery of VEGF and BMP2 in a collagen-nanohydroxyapatite scaffold, Adv. Healthc. Mater. 4 (2) (2015) 223–227.
[174] R.J. McCoy, A. Widaa, K.M. Watters, M. Wuerstle, R.L. Stallings, G.P. Duffy, et al., Orchestrating osteogenic differentiation of mesenchymal stem cells–identification of placental growth factor as a mechanosensitive gene with a pro-osteogenic role, Stem Cell. 31 (11) (2013) 2420–2431.