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Corresponding Author	Family Name	**Gadjanski**
	Particle	
	Given Name	**Ivana**
	Suffix	
	Division	
	Organization/University	Research fellow at BioSense Institute, University of Novi Sad
	Address	Novi Sad, Serbia
	Division	
	Organization/University	Assistant professor at Belgrade Metropolitan University
	Address	Belgrade, Serbia

Abstract

In order to engineer biomimetic osteochondral (OC) construct, it is necessary to address both the cartilage and bone phase of the construct, as well as the interface between them, in effect mimicking the developmental processes when generating hierarchical scaffolds that show gradual changes of physical and mechanical properties, ideally complemented with the biochemical gradients. There are several components whose characteristics need to be taken into account in such biomimetic approach, including cells, scaffolds, bioreactors as well as various developmental processes such as mesenchymal condensation and vascularization, that need to be stimulated through the use of growth factors, mechanical stimulation, purinergic signaling, low oxygen conditioning, and immunomodulation. This chapter gives overview of these biomimetic OC system components, including the OC interface, as well as various methods of fabrication utilized in OC biomimetic tissue engineering (TE) of gradient scaffolds. Special attention is given to addressing the issue of achieving clinical size, anatomically shaped constructs. Besides such neotissue engineering for potential clinical use, other applications of biomimetic OC TE including formation of the OC tissues to be used as high-fidelity disease/healing models and as in vitro models for drug toxicity/efficacy evaluation are covered.

Highlights

Biomimetic OC TE uses “smart” scaffolds able to locally regulate cell phenotypes and dual-flow bioreactors for two sets of conditions for cartilage/bone

Protocols for hierarchical OC grafts engineering should entail mesenchymal condensation for cartilage and vascular component for bone

Immunomodulation, low oxygen tension, purinergic signaling, time dependence of stimuli application are important aspects to consider in biomimetic OC TE

Keywords (separated by " - ")	Hierarchical scaffold - Bioreactor - Osteochondral - Cartilage, Bone

Chapter 7
Mimetic Hierarchical Approaches for Osteochondral Tissue Engineering

Ivana Gadjanski

Abstract In order to engineer biomimetic osteochondral (OC) construct, it is necessary to address both the cartilage and bone phase of the construct, as well as the interface between them, in effect mimicking the developmental processes when generating hierarchical scaffolds that show gradual changes of physical and mechanical properties, ideally complemented with the biochemical gradients. There are several components whose characteristics need to be taken into account in such biomimetic approach**,** including cells, scaffolds, bioreactors as well as various developmental processes such as mesenchymal condensation and vascularization, that need to be stimulated through the use of growth factors, mechanical stimulation, purinergic signaling, low oxygen conditioning, and immunomodulation. This chapter gives overview of these biomimetic OC system components, including the OC interface, as well as various methods of fabrication utilized in OC biomimetic tissue engineering (TE) of gradient scaffolds. Special attention is given to addressing the issue of achieving clinical size, anatomically shaped constructs. Besides such neotissue engineering for potential clinical use, other applications of biomimetic OC TE including formation of the OC tissues to be used as high-fidelity disease/healing models and as in vitro models for drug toxicity/efficacy evaluation are covered.

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- Biomimetic OC TE uses "smart" scaffolds able to locally regulate cell phenotypes and dual-flow bioreactors for two sets of conditions for cartilage/bone
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I. Gadjanski (✉)
Research fellow at BioSense Institute, University of Novi Sad, Novi Sad, Serbia

Assistant professor at Belgrade Metropolitan University, Belgrade, Serbia

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7.1 Introduction

In an osteochondral defect, the osteochondral (OC) unit is disturbed. Native, healthy OC unit is organized in a stratified, hierarchical way, with avascular/aneural cartilaginous zonal layer composed of chondrocytes embedded in the organic extracellular matrix (ECM), situated above the osseous, i.e., bone, part. Bone component comprises subchondral trabecular (cancellous) bone, highly vascular, enervated, with three different cell types (osteoblasts, osteocytes and osteoclasts) in the ECM composed of organic matrix and inorganic hydroxyapatite crystals. Osteochondral tissues are closely connected through the OC interface and function as one unit due to various mechanisms formed during development by the process of endochondral ossification.

In order to attempt any kind of reconstructing such a complex stratified structure comprised of vastly different components, a multifaceted approach needs to be implemented, which addresses both tissues as well as the connections between them, in effect mimicking the developmental processes.

Such approach is termed **biomimetic osteochondral tissue engineering (OC TE)** which aims to recapitulate in vitro the main elements of the in vivo development, i.e., of the endochondral ossification. In practice, this means fabrication of the stratified hierarchical constructs that should, ideally, achieve the structure of the native OC unit. This aim is proving to be very difficult, due to the complexity of the OC unit both from the developmental and structural aspects, particularly when the goal is to engineer living, clinically sized, physiologically stiff neotissue grafts, customized to the patient and to the defect requiring treatment.

Besides such neotissue engineering for potential clinical use, other applications of biomimetic OC TE include formation of the OC tissues to be used as high fidelity disease/healing models and as in vitro models for drug toxicity/efficacy evaluation [1, 2].

There are several components to take into account in biomimetic OC TE:

(a) **Cells**: type/source, differentiation protocols
   - Cell-free techniques

(b) **Scaffolds**: biomaterials, architecture/design and microstructure, fabrication methods
   - Scaffold-less techniques

(c) **Bioreactors:** design, parameters

(d) **Other components:**
   - Growth factors
   - Mechanical stimulation

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- Purinergic signaling
- Low oxygen conditioning
- Immunomodulation
- OC interface engineering methods

Majority of these components are the same whether the goal is to engineer neotissue for potential clinical use or a model system for drug evaluation or disease modeling. However, for the former—the formation of large, clinically sized OC grafts composed of the neotissue, an additional aspect is preferred: **anatomical shape**. To this aim, the use of additive manufacturing and custom-tailored bioreactors is of particular importance. Conceptually, there are several ways these components can be implemented to achieve **stratified hierarchical structural organization in the engineered OC construct**, [3–6]:

1. Scaffold-free cartilage layer and scaffold for the bone layer
2. A different scaffold for each layer, including the OC interface: biphasic, triphasic, multilayered (particularly when mimicking the zonal structure of the cartilage layer)
3. A single heterogenous scaffold for the whole OC construct = scaffolds with morphological/physical gradients
4. A single homogenous scaffold for the whole OC construct = scaffolds with biochemical gradients

However, up to now, there was no defined scaffold structure and biomaterial that was able to meet all the necessary requirements for the formation of a native-like OC-tissue [1] which is why **the current state-of-the-art approach in OC TE is to use multilayered hybrid scaffolds, with biochemical, structural and mechanical gradients**.

The OC constructs can be either cell-free or loaded with the primary OC cells (chondrocytes/osteoprogenitors) or with cells with both chondrogenic and osteogenic capacity, i.e., stem and stromal cells.

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7.1.1 Cells as Biomimetic System Component

For the small OC lesions, a cell-free approach might be implemented, where only scaffold and the growth factors are used to initiate localized repair and endogenous cell recruitment [7]. However, for the larger, unconfined OC defects, with lesions in the wound bed, the use of cells as part of the OC graft is necessary [3].

There are two main types of cells used in cellular therapy of osteochondral defects: **primary cells** (chondrocytes and osteoblasts-like, i.e., osteoprogenitor cells), preferably autologous and **stem/stromal cells**, autologous or allogeneic, isolated from various tissues. In this chapter, only the tissue engineering methods are covered, while different methods of cellular therapy of OC defects such as ACI (autologous chondrocyte implantation), MACI (Matrix-induced autologous chondrocyte implantation), and mosaicplasty are covered in detail elsewhere [6].

106 #### 7.1.1.1 Chondrocytes

107 Even though these are the native cells of the cartilage, there are several drawbacks to
108 the use of mature chondrocytes in OC TE: (1) harvesting is not very efficient due to
109 following factors: i) very low number of chondrocytes in the native cartilage tissue—
110 only 5% of total cartilage volume ~ 1 million cells/cm^3 [8]; (2) aggressive enzymatic
111 procedure with collagenase needs to be performed to decompose the collagen from
112 the extracellular matrix (ECM), which can also harm the cells (3) phenotype instabil-
113 ity of chondrocytes in 2D (monolayer) cell culture [9] that is usually used in order to
114 achieve high cell numbers: chondrocytes in the monolayer undergo dedifferentia-
115 tion, stop expressing the chondrogenic markers (e.g., collagen II and aggrecan) and
116 lose their distinctive spherical shape while attaining fibroblast-like morphology [10].
117 Various methods have been utilized in order to achieve maximum harvest yield
118 with optimal cell viability while preserving the chondrocyte phenotype [11–13].
119 Majority of these are implemented on animal chondrocytes that are usually used as
120 control cells in the experiments with engineered constructs.
121 In spite of described drawbacks, chondrocyte-based cartilage tissue engineering
122 remains a useful source of information, particularly when performed in combination
123 with (human) **mesenchymal stromal cells (MSCs)**-based engineering methods [14].
124 Important to mention is that physiologic mechanical properties can be achieved
125 when engineering cartilage from primary chondrocytes, while the highest compres-
126 sive moduli reported for cartilage engineered from human MSCs (without enabling
127 mesenchymal condensation—*see below*) was only ~ 50% of the normal values [15].
128 The use of human chondrocytes in OC TE is still largely prevented by additional
129 challenges: donor-site morbidity and low ECM production in culture.
130 These challenges can be potentially overcome through the use of **human mesen-**
131 **chymal stromal cells (MSCs)** that possess a number of characteristics advantageous
132 to the OC TE: (i) can be isolated from various sources with very low donor-site mor-
133 bidity (e.g., from the adipose tissue); (ii) can maintain multipotency even after multiple
134 passages and (iii) can be induced to both chondrogenesis and osteogenesis [16–18].
135 In addition, MSCs represent a very natural choice of cells for OC TE, since they
136 originate from mesenchymal connective tissues of mesodermal nature that, in the
137 course of development, give rise to all osteochondral components.
138 Here it is important to note the common confusion regarding the name of this
139 type of cells. Minimal classification criteria for “mesenchymal stem cells” were
140 established by the International Society for Cell Therapy (ISCT): A) plastic adher-
141 ence B) osteogenic, chondrogenic, and adipogenic differentiation in the bulk culture
142 (not on a single-cell clone) C) cell surface expression of CD73, CD90, and CD105
143 concurrent with absent expression of CD11b or CD14, CD45, CD34, CD79a, or
144 CD19, and human leukocyte antigen (HLA)-DR [19].
145 The problem is that only a minority of the cells in the bulk culture (less than half
146 of total cell number) that fulfill these criteria (A-C) also exhibit: (1) high prolifera-
147 tive capacity (colony-forming ability—CFU-F) [20] and (2) multipotency (when
148 appropriately tested on the basis of a single cell clone) [21].
149 This is why it is not accurate to use the term “mesenchymal stem cells” for the
150 bulk cell population, which is exactly what happens in majority of the tissue engi-

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neering studies: the term is non-critically extended to all fibroblast-like cells obtained after one or more culture passages starting from primary bone marrow (and later adipose tissue, cord blood, umbilical cord) mononuclear cells [21].

The only way to detect multipotent stem cells in the bulk population is to assay their colony-forming capacity (CFU-F) according to the initial Friedenstein's functional definition [18, 22]. Only the cells that are able to give rise in vitro to fibroblast colonies (i.e., possess CFU-F ability) can be called stem cells, provided they also exhibit another property: multipotency. If the cells of one single colony are capable of giving rise to at least three cell types (adipocytes, osteoblasts and chondrocytes) then the initial cell that gave rise to the colony was multipotent.

Interestingly, when individual clones were analyzed for their proliferative and differentiation capacities, data showed that only ~34% of CFU-F cells exhibit trilineage potential, ~60% osteogenic and chondrogenic, while 6% can differentiate into only one line (these are termed "committed progenitors") [21, 23].

Different methods were used to select for the "real mesenchymal stem cells", by concentrating the CFU-F in some phenotypically defined populations, but they only allowed enriching of the "real MSC" population to a limited extent.

In conclusion, in order to term cells as multipotent mesenchymal stem cells they need to fulfill two basic conditions: be able to form clonogenic colonies (CFU-F) and differentiate into osteogenic, chondrogenic, and adipogenic lineages. If the CFU capacity has not been evaluated, the most accurate is to term the cells as **Mesenchymal Stromal Cells.**

As mentioned, majority of the tissue engineering studies performed up to now did not pay attention to these aspects. This renders most of the results difficult to transfer to a clinical setting because the conclusions drawn from such studies do not reflect the behavior of the "real stem cells". In fact, the use of such heterogenous populations of mesenchymal cells, without preselection for CFU-F, led to various results such as generation of fibrocartilage and hypertrophic chondrocytes [24] and even non-articular cartilage formation within the defect, after implantation [25, 26].

Based on the above, the abbreviation MSCs in this chapter refers to the **mesenchymal stromal cells**. It is worth noting that there are initiatives (spearheaded by Dr. Arnold Caplan) to change the name of exogenously supplied MSCs (in clinical setting) to Medicinal Signaling Cells to more accurately reflect the fact that these cells home in on sites of injury or disease and secrete bioactive factors that are immunomodulatory and trophic (regenerative). These cells do not differentiate into neotissue, but stimulate via various biofactors the patient's own site-specific and tissue-specific resident stem cells and progenitors that construct the new tissue [27].

Concerning the use of MSCs for engineering the osseous component of the OC construct, the osteogenic capacity has been confirmed for MSCs derived from various sources, where the most used are bone marrow-MSCs (BMSCs) and adipose-derived stromal cells (ASCs). The other MSC types are covered in detail in an excellent review by Vonk et al. [18].

- BMSCs, isolated from bone marrow stroma are the most studied source for bone regeneration. One of the challenges associated with BMSCs use is high inter-patient variability in cell numbers within specific bone marrow aspirate (0.001–

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0.01% of the nucleated marrow cells) [28] which makes it necessary to expand them in culture to reach clinically relevant numbers for therapeutic purposes [29]. As described above, during expansion, one needs to keep in mind the heterogeneity of the cell population.

- ASCs came to use more recently, but are becoming a solution-of-choice due to the high cell numbers present in lipoaspirates harvested through liposuction techniques [30], that are less invasive than bone marrow aspiration. On average, several liters of lipoaspirate with a relatively high frequency of ASCs (1–5% of isolated nucleated cells) can be obtained [28]. In fact, the stromal vascular fraction of adipose tissue contains more MSCs compared with bone marrow (as measured in a colony-forming unit fibroblast (CFU-F) assay) [18]. Isolation protocols involve density gradient centrifugation of collagenase-digested tissue (lipoaspirate or minced adipose) followed by selection and culture of adherent cell populations. Various studies report successful cultivation of bone-like tissue using scaffolds seeded with ASCs [31, 32].

However, it should be stated that the transplantation of MSCs into bone defects primarily enhances bone repair via immunomodulatory effects, as opposed to their direct differentiation into bone-forming cells [28].

7.1.1.2 Osteoblast-Like Cells

Cells with osteoprogenitor characteristics can be harvested from adult bone tissue and periosteum, via preparation of explant cultures from dissected tissues, or enzymatic release of progenitor cells from endosteal and periosteal layers [28, 33, 34]. Osteogeneicity of these cells is confirmed when cultured on porous scaffolds yielding bone-like tissue [35, 36]. Importantly, these cells were also confirmed to have mesenchymal multipotency, demonstrated by single-cell lineage analysis [37].

7.1.1.3 Pluripotent Stem Cells

Pluripotent stem cells show unlimited self-renewal and can differentiate into all three germ layers (ectoderm, endoderm and mesoderm). The fact they can differentiate into mesodermal derivatives is of most importance for OC engineering, because of the **mesenchymal condensation** phenomenon—*see below*.

Human Embryonic Stem Cells (hESC)

hESCs have been used in a number of studies for inducing osteogenic and chondrogenic differentiation: through embryoid bodies (EBs) [38]; by coculture/conditioned culture with fully differentiated chondrocytes [39], MSCs [40], ESC-derived MSCs [41]; or by directed differentiation to chondrogenic and osteogenic cells [42, 43].

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It is important to note that, in their directed differentiation protocol, Oldershaw et al. demonstrated that hESCs progress through primitive streak or mesendoderm to mesoderm, before differentiating into a chondrocytic cell aggregates [43], confirming the importance of recapitulating the stage of mesenchymal condensation—*explained in detail below*.

Human Induced Pluripotent Stem Cells (hiPSCs)

When findings by Yamanaka, Takahashi and Gurdon enabled obtaining autologous pluripotent cells from somatic cells (fibroblasts, keratinocytes, blood cells) of a patient, these naturally seemed like a go-to solution for clinical use.

However, now, more than 10 years after publication of the key papers by Yamanaka and Takahashi, our knowledge on human induced pluripotent stem cells is still not sufficient to allow for a straightforward clinical application of hiPSCs [44]. One of the biggest challenges, raising real safety concerns, is the genomic instability of hiPSCs, which became obvious particularly with the advance of high-throughput technologies such as next-generation sequencing [45].

The application of hiPSCs in OC engineering is also somewhat limited by the current protocols for chondrogenic differentiation that are complicated and inefficient primarily due to the need for intermediate embryoid body (EB) formation, required to generate endodermal, ectodermal, and mesodermal cell lineages [1].

Recently, Nejadnik et al. reported a new, straightforward approach for chondrogenic differentiation of hiPSCs, which avoids embryoid body formation and instead is driving hiPSCs directly into mesenchymal stromal cells (MSC) and chondrocytes. hiPSC-MSC-derived chondrocytes showed significantly increased expression of chondrogenic genes compared to hiPSC-MSCs. Following transplantation of hiPSC-MSC and hiPSC-MSC-derived chondrocytes into osteochondral defects of arthritic joints of athymic rats, MRI studies showed engraftment, and histological correlations showed the production of hyaline cartilage matrix [46].

Suchorska et al. compared four methods to generate chondrocyte-like cells from hiPSCs: (1) monolayer culture with addition of defined mesodermal and chondrogenic growth factors (GFs) (DIRECT protocol), (2) EBs differentiated in chondrogenic medium with TGF-β3 cells (TGF-β3 protocol), (3) EBs differentiated in chondrogenic medium conditioned with human chondrocytes (HC-402-05a cell line) (COND protocol) and (4) EBs differentiated in chondrogenic medium conditioned with human chondrocytes and supplemented with TGF-β3 (TGF-β3 + COND protocol). Two fastest and most cost-effective methods were the monolayer culture with GFs (DIRECT) and the medium conditioned with human chondrocytes (COND) [47]. De Peppo et al. engineered functional bone substitutes by culturing hiPSC-derived mesenchymal progenitors on osteoconductive scaffolds in perfusion bioreactors, and confirmed their phenotype stability in a subcutaneous implantation model [48].

Along these lines, Wu and colleagues state in their recent review that efficient in vitro differentiation of hiPSCs into downstream cells, such as mesenchymal stem/stromal cells (MSCs), osteoblasts or osteocyte-like cells is necessary to limit unde-

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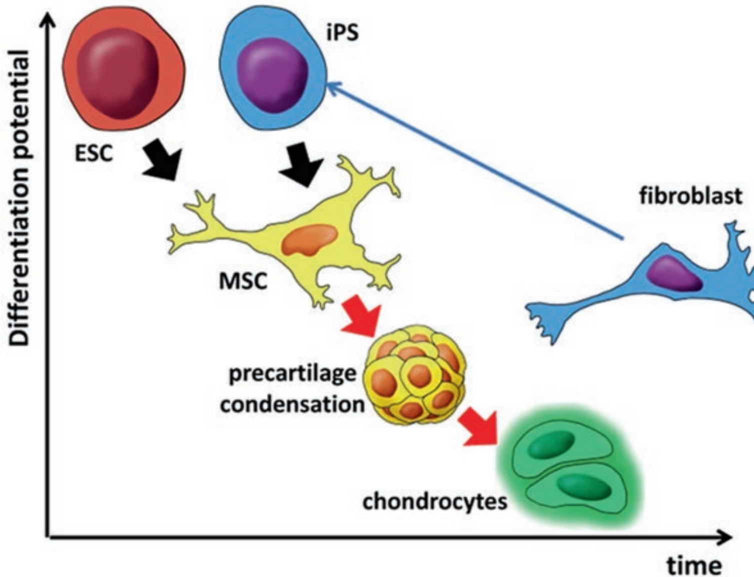

Fig. 7.1 Importance of mesenchymal (precartilage) condensation in chondrogenesis

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272 sired tumorigenesis associated with the pluripotency of hiPSCs [49]. They also give
273 good comparisons of the current techniques utilized to confer the induction of hiP-
274 SCs into the osteogenic lineage, an evaluation of osteogenic potentials of cells
275 derived from each technique and cells derived from different somatic origins and
276 comparisons of hiPSC-derived MSCs and BMSCs [49].

277 Mesenchymal Condensation: Necessary Requirement for Chondrogenesis

278 From the recent studies using both hESC and hiPSCs, a conclusion emerged that in
279 order to achieve proper differentiation into chondrocytic lineage, one needs to
280 enable the mesenchymal condensation (precartilage condensation) to occur
281 (Fig. 7.1).
282 Pluripotent cell types (ESC and iPS) have to differentiate into multipotent MSCs
283 in order to form precartilage condensation required for efficient further differentia-
284 tion into chondrocytes. Chondrocytes, as fully differentiated cells, have lower dif-
285 ferentiation potential compared to fibroblasts, which can still be induced to direct
286 differentiation [50] as well as to conversion to iPS cells [51]. Adapted with permis-
287 sion of Springer from Gadjanski I, Spiller K and Vunjak-Novakovic G. Stem Cell
288 Reviews and Reports [52].
289 Mesenchymal condensation is a key event in the chondrogenic commitment,
290 after which tissue-specific transcription factors and structural proteins begin to
291 accumulate [52, 53]. Main coordinators of this process are transforming growth
292 factor-β (TGF-β) family proteins and Wnt/β-catenin signaling [54, 55].

In vitro, the mesenchymal condensation is mimicked through the self-assembly methods [56, 57] with TGF-β supplementation. Ng et al. used TGF-β and thyroxine for both cartilage maintenance and chondrocyte terminal (hypertrophic) differentiation, respectively [58]. Through such biomimetic recapitulation of physiological spatiotemporal signals, Ng and colleagues produced and maintained cartilage discs with functional and phenotypically stable hyaline cartilage with accompanying progressive deep-zone mineralization. The discs remained stable and organized following implantation [58]. Such recapitulation of both temporal and structural aspects of native development is the very essence of the biomimetic approach.

Bhumiratana and Vunjak-Novakovic report that clinically sized pieces of human cartilage with physiologic stratification and biomechanics can be grown in vitro by recapitulating some aspects of the developmental process of mesenchymal condensation [57, 59]. By exposure to TGF-β, MSCs were induced to aggregate into *condensed mesenchymal bodies* (CMBs) which then formed in vitro an outer boundary after 5 days of culture, as indicated by the expression of mesenchymal condensation genes and deposition of tenascin. Before setting of boundaries, the CMBs could be further fused into homogenous cellular aggregates, without using a scaffolding material, giving rise to well-differentiated and mechanically functional cartilage. The formation of cartilage was initiated by press-molding the CMBs onto the surface of a bone substrate. By image-guided fabrication of the bone substrate and the molds, the OC constructs were engineered in anatomically precise shapes and sizes. Importantly, the cartilage engineered in this way possessed physiologic compressive modulus and lubricative property (Young moduli >0.8 MPa, and friction coefficients <0.3). This method could be highly effective for generating human osteochondral tissue constructs, and for repairing focal cartilage defects to replace currently used dissociated chondrogenic cells [1].

7.1.2 Scaffolds as Biomimetic Systems Component

Even though the scaffold-less techniques are gaining impetus, particularly for generating self-assembling tissues [60], scaffolds are still one of the key components for OC tissue engineering. The biggest challenge is how to achieve similar degree of complex hierarchical structure as in the native OC unit, task particularly daunting for the zonal cartilage layer and the complex OC interface, for which many characteristics are still unknown.

Because of this, the prevalent approach is to use multicomponent systems and hybrid scaffolds combining the concepts mentioned earlier.

Scaffolds for the cartilaginous part are frequently hydrogel-based, fostering spherical morphology of the chondrocytes/chondrogenic cells due to hydrogel high water content [61]. Importantly, cell-laden hydrogels, or cell-hydrogel hybrid constructs, can be manufactured in patient-specific anatomical shapes [62]. Injectable hydrogels are particularly convenient materials for in vivo applications. An emerging class of bioinspired polymers for cartilage and bone tissue engineering are gly-

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copolypeptides that mimic naturally occurring glycoproteins, that have been processed into injectable hydrogels, by enzymatic cross-linking of glycopeptides in the presence of horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) [63]. However, hydrogels, due to their isotropic nature and poor mechanical characteristics, cannot fully mimic the zonal hierarchical structure of the native articular cartilage. This can be improved by adding nanofibers and microfibers, for which electrospinning and melt electrospinning writing techniques are particularly useful [64]. Nanoparticles can be added as well, and loaded with chondrogenic/osteogenic growth factors [65]. In fact, hydrogels with cells and growth factors are proving very useful in engineering OC interface and achieving biochemical gradients [62].

Regarding to above, recent study by Zhu et al. reports a method for rapid formation of tissue-scale gradient hydrogels as a 3D cell niche with tunable biochemical and physical properties. They used photocrosslinkable, multi-arm PEG hydrogel system as a backbone and chondroitin sulfate methacrylate, mixed with two cell-containing precursor solutions (chondrocytes and hMSCs), which, upon exposure to light, quickly formed insoluble cell-laden gradient hydrogels mimicking zonal structure of the native cartilage. The method enabled rapid (~2 min) formation of tissue-scale hydrogels (3 cm × 1 cm × 3 mm) with stiffness and/or ECM molecule gradient cues, while enabling homogeneous cell encapsulation in 3D [66].

Still, multilayered scaffolds can mimic stratified structure to a higher degree, especially important for treatments of full-thickness OC defects. Cartilaginous layer mechanical properties are obtained through the use of hydrogels or porous sponges, while more rigid, porous and fibrous scaffolds are implemented for the bone region [8, 67]. Native ECM components (proteins, GAGs, cell adhesion molecules) are mimicked via chemical functionalization either by chemical binding of peptides on a polymer scaffold [68] or by fabricating a 3D scaffold from self-assembling peptides [67].

The native biological cues are simulated through attached or encapsulated growth factors. To this aim, decellularized extracellular matrices (ECM) are receiving increasing interest as materials capable to induce cell growth/differentiation and tissue repair by physiological presentation of embedded cues [69]. Such ECM are derived from preexisting tissue (native ECM) after isolation and subsequent decellularization (demineralized bone matrix, Matrigel) [70], and, as recently described by Bourgine et al., through designed human cell lines serving as intrinsic tools to achieve efficient ECM deposition and decellularization, offering added possibility of targeted enrichment in the content and delivery of specific molecules. This interesting study reports engineering of ECM materials with customized properties, based on genetic manipulation of immortalized and death-inducible hMSCs, cultured within 3D porous scaffolds under perfusion flow. The strategy allows for robust ECM deposition and subsequent decellularization by deliberate cell-apoptosis induction. As compared to standard production and freeze/thaw treatment, this grants superior preservation of ECM, leading to enhanced bone formation upon implantation in calvarial defects [69].

Proper OC scaffold design should provide hierarchical structure, desired mechanical and mass transport properties (stiffness, elasticity, permeability, diffusion) and ability for processing into precise anatomical shapes [71]. Adequate

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porosity needs to be achieved as well. Pores of ≤400 μm are recommended by most groups for enhancing new bone formation and the formation of capillaries, and the minimum pore size of ~100 μm, as smaller pores limit cell migration and mass transport [72, 73].

Hierarchical organization needs to comprise all the levels—from nanoscopic to microscopic to macroscopic, in order to meet frequently conflicting requirements for mechanical function, mass transport, and biological regulation [74].

To this aim, various fabrication methods, particularly computer-aided additive manufacturing (CAM), in combination with finite element modeling (FEM) and computational fluid dynamics (CFD) are being developed and implemented [75, 76].

Probably the most utilized method out of CAM technologies is the 3D printing which enables generation of the architectural details that were previously impossible to fabricate. In addition, 3D printing techniques (stereolithography, fused deposition modeling, and selective laser sintering) allow incorporation of gradients into polymer scaffolds to achieve even higher degree of native-like structural, biochemical and mechanical environment. 3D printing can be combined with other approaches, such as self-assembly of nanoparticles [77]. An excellent recent review by Bracaglia et al. covers various 3D techniques for design and fabrication of polymer-based gradient scaffolds in detail [78], while Guo et al. in their review cover the applications of 3D printing for recapitulation of zonal structure of articular cartilage [79]. Importantly, anatomically shaped scaffolds can be made by CAM, tailored to the patient by using the CT images of the defect for creating the CAD (computer-aided design) model [71].

Regarding the use of CAM in OC TE, an interesting study by Hendrikson et al. analyzed the influence of additive manufactured scaffold architecture on distribution of surface strains and fluid flow shear stresses and expected osteochondral cell differentiation [80]. They compared four scaffold designs that only differed in the pore shape while the fiber diameter, spacing, and layer thickness remained constant. Different architectures were obtained by changing the angle of layer deposition and lateral shifting of the layers. Also, μCT-based models of the scaffolds were prepared, and stress and strain distributions within the scaffolds were predicted using CFD and FEM. The results show a distinct effect of the scaffold architecture on surface strains and fluid flow shear stresses under mechanical compression and imposed fluid flow. This implies that regions of the scaffold could be designed favoring specific cell differentiation stimuli. Coupling with biophysical loading regimes a priori in silico could accelerate the design of scaffolds and optimize the loading regimes [80].

One of the CAM methods is biofabrication or 3D bioprinting [81] that allows for the direct incorporation of the live cells in the scaffold fabrication process. There are three major types of 3D bioprinting techniques that are currently available: (1) inkjet bioprinting [82] (2) microextrusion bioprinting [83], and (3) laser-assisted bioprinting [84]. However, it is still challenging to bioprint clinically sized constructs, mostly due to the poor mechanical properties and limited structural integrity of the printed construct. To overcome this limitation, various modifications are tested, such as FRESH method where the tissue construct is built by embedding the printed

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424 cell-laden hydrogel within a secondary hydrogel that serves as a temporary, ther-
425 moreversible, and biocompatible support [85]. Other option is to combine multiple
426 processing methods, e.g., electrospinning with 3D bioprinting [86].
427 Currently, one of the main applications of 3D bioprinting is the fabrication of
428 mini-tissues for disease modeling [87]. Lozito et al. constructed an in vitro system
429 with 3D microtissues designed for biological studies of the osteochondral complex
430 of the articular joint [54]. The model was constructed by seeding hMSCs from bone
431 marrow and adipose tissue aspirates into photostereolithographically fabricated bio-
432 material scaffolds with defined internal architectures. Concerning OC disease-mod-
433 eling, hiPSCs are also frequently used in the so-called "disease-in-a dish" models.
434 Diseases to be modeled include of course osteoarthritis, but also the numerous
435 hereditary osteochondral dysplasias which result from genetic disorders causing
436 defective cartilage and bone differentiation, formation, and growth and for many of
437 which the disease-causing mutations are already known [88]. Reprogramming
438 patient-specific cells with a genetic predisposition and engineering disease-specific
439 genetic variations into healthy control hiPSC cell lines promises to recapitulate
440 "diseases in a dish" more realistically than immortalized human cell lines and will
441 be an invaluable complementation for animal models [89]. In addition to repro-
442 gramming patient-specific cells, novel gene editing methods, such as zinc-finger
443 nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and clus-
444 tered regularly interspaced short palindromic repeats (CRISPR)/Cas9 [90] allow
445 introducing genetic defects into well-characterized hiPSC lines [89]. Generating
446 stable hiPSC cell lines enables high-throughput drug screening and positions human
447 disease pathophysiology at the core of preclinical drug discovery [91], potentially
448 leading to personalized regenerative medicine therapies [92].

449 *7.1.3 Bioreactors as Biomimetic System Component*

450 Bioreactor is a necessary component for maintenance of differentiated cell pheno-
451 types and promoting the OC construct maturation by providing exchange of nutrients
452 and metabolites, control of environmental factors as well as biophysical signaling
453 and mechanical cues. In general, a bioreactor of OC TE should comprise two differ-
454 ent compartments—for cartilage and bone, while enabling the interface formation in
455 between. The compartments should allow for specific culture media perfusion as
456 well as biophysical and mechanical stimulation needed for the tissue in question.
457 Even though some studies report good results with chondrogenesis of undiffer-
458 entiated hMSCs in chondrogenic medium even in static culture [93], majority uti-
459 lizes dynamic loading with physiological frequency (1 Hz) to provide both the
460 mechanism for fluid transport through the tissue and the necessary biophysical
461 stimuli [94]. It has been detected that moderate amplitude strains (5%) applied at
462 1 Hz stimulate chondrogenesis of hMSCs and enable stable chondrocyte-like phe-
463 notype, while higher strains and lower frequencies have a negative effect on chon-
464 drogenesis [95].

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Bioreactor cultivation of the bone, as a tissue that should be vascularized, requires interstitial flow of culture medium through the tissue space, facilitating exchange of nutrients—particularly oxygen, metabolites, and regulatory factors to and from the cells, over minimal diffusional distances, while providing shear stress [96]. In the ideal scenario, the medium would be perfused through a network of channels with endothelial lining, serving as precursors of the vascular network to connect at a later point to the blood supply of the host. Such bioreactor systems are conceptually biomimetic, since they enable convective-diffusive mass transport similar to that occurring in vivo, between blood and tissue, along with dynamic hydrodynamic shear that is an important regulatory factor for bone development and maintenance [1, 96].

In the exemplary study on the effects of medium perfusion achieved through cultivation in a bioreactor, Grayson et al. showed that perfusion culture of predifferentiated osteoblasts or undifferentiated hMSCs with cocktail medium elicited the best osteogenic responses [93]. Bioreactors can be tailored to fit the specific shape, particularly important when engineering anatomically shaped constructs, to provide direct fluid flow through the tissue and/or gradients of biophysical/mechanical cues needed for spatiotemporal recapitulation of cell differentiation, assembly and ECM production [97, 98].

For the detailed overview of the principles of different bioreactor designs, and important parameters to mimic physiological phenomena in OC TE the work by Vunjak-Novakovic, Bhumiratana et al. and Martin et al. is recommended [3, 96, 99, 100].

7.1.4 Other Components in Biomimetic OC TE

7.1.4.1 Growth Factors

There are several key growth factors used in OC TE. These are members of the transforming growth factor-β (TGF-β) superfamily (including Bone Morphogenetic Proteins—BMPs, Growth and Differentiation Factors—GDFs [101]), fibroblast growth factor (FGF) family, insulin-like growth factor-1 (IGF-1), and platelet-derived growth factor (PDGF) [8]. Growth factors act through modulation of the local microenvironment (making it chondroinducive or osteoinducive), anabolic cellular effects, and increased matrix production. Additionally, some (e.g., PDFG) are important for vascularization, since they can induce angiogenesis and direct cell migration and support vessel maturation and stabilization [102].

The sequential addition of growth factors (GFs) to cell culture medium has proven useful in stimulating chondrogenesis in vitro [52]. GF addition in a sequence similar to native development, e.g., basic FGF (bFGF) or FGF2 followed by BMP2 or IGF1, TGFβ2 or TGFβ3, increased proliferation and subsequent chondrogenic differentiation [52, 103]. Similarly, exposure of chondrocytes seeded in agarose gels to TGFβ3 for 2 weeks followed by unsupplemented culture medium resulted in enhanced cartilage formation and mechanical properties compared to prolonged exposure to TGFβ3 [104]. The exposure of MSCs in poly(ethylene glycol) (PEG)

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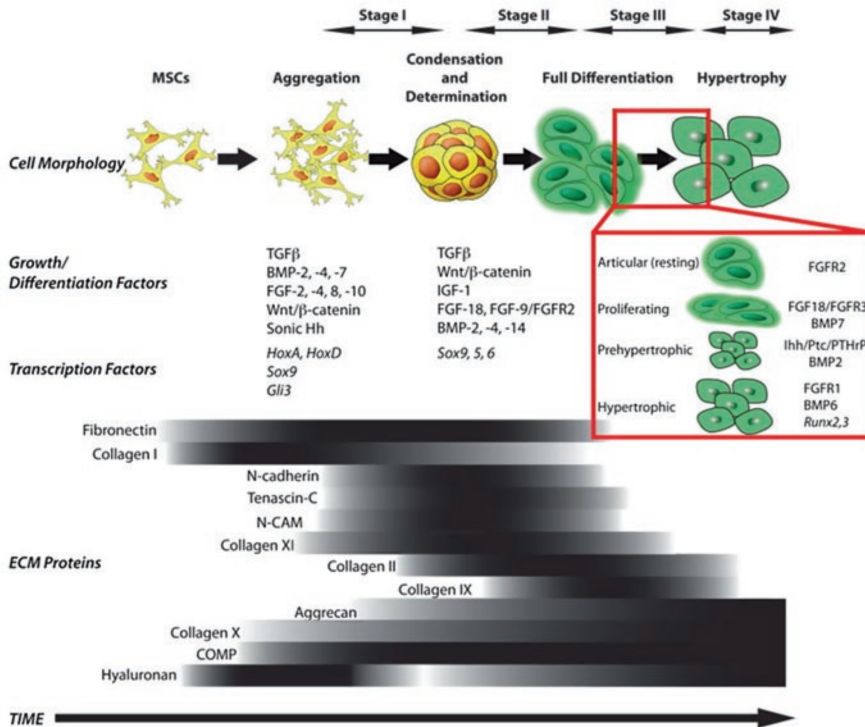

Fig. 7.2 Sequence of events and time-dependent involvement of growth factors during native chondrogenesis. Adapted with permission of Springer from Gadjanski I, Spiller K and Vunjak-Novakovic G. *Stem Cell Reviews and Reports* [52]

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505 hydrogels to TGFβ1 for just 7 days resulted in enhanced proteoglycan production
506 compared to prolonged culture, but decreased collagen production [105]. Figure 7.2
507 shows the sequence of events and time-dependent GF involvement in native chon-
508 drogenesis, which should be mimicked in OC TE.
509 Similarly, sequential GF application proved important for osteogenesis as well.
510 Aksel et al. showed that vascular endothelial growth factor (VEGF) addition in the
511 early phase rather than a continuous presence of both VEGF and BMP-2 enhanced
512 odontogenic/osteogenic differentiation of human dental pulp stem cells (DPSCs)
513 [106]. It was also shown that early delivery of an angiogenic factor (bFGF) com-
514 bined with sustained exposure to an osteogenic factor (Sonic hedgehog—Shh) can
515 recapitulate the critical aspects of natural bone repair [107]. These data emphasize
516 the importance of controlled duration of GF application.
517 Generally speaking, in the biomimetic OC construct, the chondrogenic growth
518 factors (e.g., TGF-β family) should be supplied in the cartilage phase (in combina-
519 tion with dynamic loading), while the osteogenic growth factors, e.g., BMPs (com-
520 bined with medium perfusion) should be applied in the bone phase.

7.1.4.2 Vascularization

Native bone tissue is highly vascularized, and its development and function are coordinated by synergistic interactions between the bone cells and vascular cells. The blood vessels supply oxygen and nutrients, as well as calcium and phosphate, the building blocks for mineralization [108]. To a certain degree, the emerging vasculature serves as a template for bone development. Following biomimetic approach, the bone phase in the OC construct should be engineered by synchronizing vascular and bone development in 3D scaffolds [71, 109]. Ideally, the OC construct would provide paracrine signaling between the bone and vascular cells, as well as larger vascular conduits that can help quickly connect the blood to the tissue and establish vascular perfusion following implantation of engineered tissue constructs [1]. However, in practice this is proving very difficult to achieve. Certain advancements have been made through harnessing the proangiogenic effects of immune cells.

7.1.4.3 Immunomodulation

Immune response is a major regulator of vascularization and overall functionality of engineered tissues, through the activity of different types of macrophages (proinflammatory M1 and anti-inflammatory M2 phenotype) and the cytokines they secrete [1]. Regarding their contribution to angiogenesis, human macrophages polarized to the M1 or M2 phenotypes behave in different ways. Spiller et al. showed that M1 macrophages express and secrete factors that promote the initiation of angiogenesis, especially VEGF. M2 macrophages secrete factors involved in later stages of angiogenesis, particularly PDGF-BB isoform, which recruits stabilizing pericytes [110]. In addition, M2 macrophages can express high levels of tissue inhibitor of metalloprotease-3 (TIMP3), which inhibits angiogenesis by blocking the actions of metalloproitenase-9 (MMP9) and VEGF [111] and prevents the release of the inflammatory cytokine TNFα [112]. TIMP3 also stabilizes vasculature formation from endothelial cells in vitro [113].

It is clear that coordinated efforts by both M1 and M2 macrophages are required for angiogenesis and scaffold vascularization [110]. hMSCs have been shown to promote macrophage differentiation toward an M2-like phenotype with a high tissue remodeling potential and anti-inflammatory activity, but also a protumorigenic function [114]. This is in line with previously mentioned hypothesis that many effects of the hMSCs used in regenerative medicine are due to their immunomodulatory effects and not to direct differentiation into specific cell types [115].

To harness immunomodulatory signals to the highest degree, researchers start using "smart" scaffolds that enable sequential release of immunomodulatory factors recruiting the waves of M1 and M2 macrophages [110]. Spiller et al. designed scaffolds for sequential release of pro-M1 (interferon-gamma; IFN-γ) and pro-M2 (interleukin-4—IL4) signals to achieve bone regeneration where IFN-γ was physically adsorbed onto the scaffolds, while IL4 was attached via biotin–streptavidin binding [116] (Fig. 7.3).

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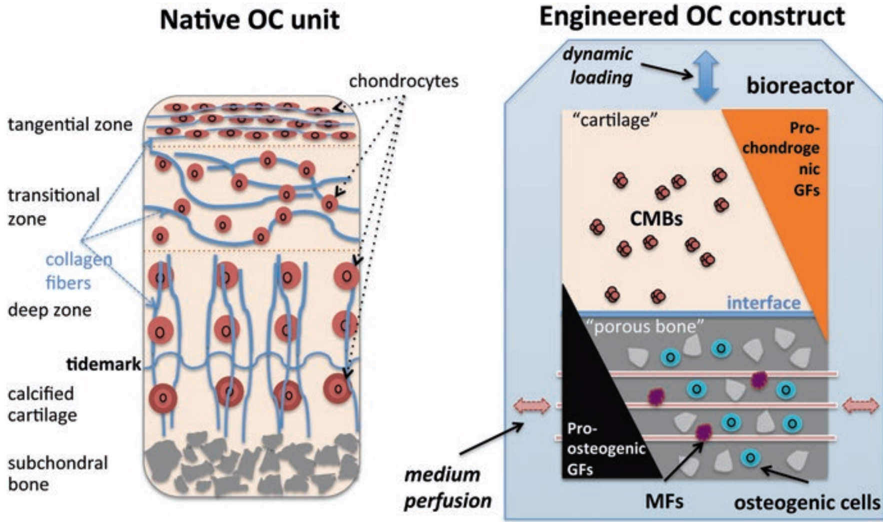

Fig. 7.3 Paradigm of the biomimetic approach in OC tissue engineering. *CMBs* condensed mesenchymal bodies, *GF* growth factors, *MF* macrophages. Detailed explanations in the text

562
7.1.4.4 Low Oxygen Tension Conditioning

563 Oxygen gradients are established early in embryonic development, since the grow-
564 ing tissues of the embryo rapidly deplete local oxygen and nutrient supplies pro-
565 vided via diffusion. During endochondral ossification, the cartilaginous anlage
566 develops into the fetal growth plate, becoming more hypoxic as it grows [117].
567 Articular cartilage remains hypoxic in adult stage, with spatial oxygen gradient of
568 <1% in the deepest layers, up to <10% at the cartilage surface. Chondrocytes are
569 very adapted to low oxygen tensions present in the avascular environment, but they
570 also promote (by secreting angiogenic stimuli) localized vascularization at the
571 periphery of the cartilage, the key process for the continued development and
572 growth of bone [118]. Oxygen levels and vascularization are connected through the
573 action of hypoxia-inducible factors (HIF) and vascular endothelial growth factor
574 (VEGF). Levels of oxygen in the tissue modulate HIF signaling cascades, the essen-
575 tial mediators of the complex homeostatic responses that enable cells to survive and
576 differentiate in low oxygen environment. VEGF is a downstream target of the HIF
577 pathway and a potent angiogenic factor. Osteoblasts express HIF-1α and HIF-2α,
578 which modulate bone development and homeostasis and angiogenesis. Some of the
579 effects of HIFs on bone and angiogenesis are mediated by VEGF. It is clear that
580 HIFs and VEGF have critical roles in skeletal development and bone homeostasis.
581 Such close spatial and temporal association of osteogenesis and vascularization is
582 now termed as *angiogenic-osteogenic coupling* [118, 119].
583 It is obvious from abovementioned data that ambient O_2 concentration of 21% O_2
584 represents hyperoxic environment for osteochondral cells and should not be used

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for the in vitro cultivation. Such hyperoxia disturbs the HIF-signaling pathways since when oxygen tension is >5%, the half-life of HIF-1α is very short (<5 min) [120]. This can impair normal anaerobic glycolysis in cartilage and posttranslational modifications of type II collagen [121].

Ambient O_2 concentrations are even more detrimental when considering stem/stromal cells that normally reside in "hypoxic" niches in vivo and are well adapted to low O_2 tensions. The "hypoxic", or more precisely physioxic (or in situ normoxic) oxygen concentration in adult tissues varies between 1 and 11% [122], while the O_2 concentration at the cellular level is estimated to be 1.3–2.5% or even <1% [21, 123]. Atmospheric 21% O_2 represents a hyperoxic environment for stem/stromal cells which start losing the phenotypic and molecular markers of stemness [21]. This is the reason why low oxygen conditioning—i.e., cultivation of cells in low O_2 tension environment is proving very efficient in stem/stromal based OC TE. If maintenance in low O_2 is technically challenging in long-term culture, it is beneficial to perform at least a transient preconditioning, followed by the switch to ambient O_2 concentration. Yodmuang et al. showed, using juvenile chondrocytes, that such transient culture of 5% O_2 increases expression of cartilaginous genes including COL2A1, ACAN, and SOX9 and increased tissue concentrations of GAG and type II collagen, with accompanying increase in the equilibrium Young's modulus [124]. Henrionnet et al. performed similar study on hMSCs and concluded that better chondrogenic differentiation is achieved when reduced oxygen tension (5% O_2) is applied during both expansion and differentiation times, avoiding the in vitro osteogenic commitment of the cells and subsequently the calcification deposition [125].

7.1.4.5 Purinergic Signaling

External mechanical stimulation leads to activation of mechanotransduction cascades that promote chemical signaling inside the cell [126]. These intracellular mechanotransduction pathways are still not fully defined [127]. Relatively recently, ATP (adenosine 5′-triphosphate) has been indicated as one of the first molecules to be released from chondrocytes into extracellular space in response to mechanical stimulation [128, 129], subsequently binding to purinergic P2 receptors and activating calcium signaling pathways [130]. Garcia and Knight suggested putative mechanism of ATP release via hemichannels (formed of connexin-43 subunits) in response to cyclic compression [131]. Since then a number of studies has shown that exogenous ATP supplementation, even in the absence of mechanical stimulation, can promote ECM biosynthesis and accumulation providing energy supply to fuel that process [132], increase mechanical properties, particularly through structural organization of the bulk phase and territorial ECM [133]. Exogenous ATP effects are proven to be dose- and time-dependent, where high doses can promote catabolic responses, necessitating the optimization of therapeutic dose range and application timing (e.g., transient vs. continuous) to the cell type and culture system [133–135]. Furthermore, MSCs have been shown to respond to extracellular ATP as well, even more receptively than the chondrocytes. Gadjanski et al. detected that

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exogenous ATP induced 72% vs. 16% increase in GAG content for human MSCs and chondrocytes, respectively [134], while Steward et al. showed that purinergic signaling regulates the TFG-β3-induced chondrogenic response of MSC [136]. A mathematical model was defined showing ATP release changes in loaded vs. unloaded cell constructs (chondrocytes and hMSCs) over time [137]. Such model can be of value in determining the potential for pharmacological manipulation of the purinergic mechanotransduction in the engineered osteochondral tissues.

Mechanosensitive purinergic signaling in bone has also been confirmed, where extracellular ATP has been shown to modulate multiple processes including cell proliferation, differentiation, function, and death [138]. Osteoblasts and osteoclasts have been reported to express nearly all the P2Y and P2X receptors to which the extracellular ATP can bind [139].

Additional important aspect to keep in mind is that any mechanical stimulus applied to cells in vitro, even as subtle as fluid movements after a medium change, can increase basal ATP release [140] which was recently again brought to attention in an informative review by Burnstock and Knight [141] who urge the researchers to always include this aspect in the interpretation of their data.

7.1.4.6 OC Interface Engineering

In order to engineer a native-like osteochondral interface and complex cell–cell communication between cartilage and bone, it is necessary to fine-tune scaffold properties such as graded molecular composition, structure, and biomechanics, i.e., to specify and precisely implement multiple gradients in scaffolding of the OC construct.

As mentioned several times, one approach is to build composite scaffolds through multilayered scaffold design, to generate structural templates for the cartilaginous layer, the tidemark and calcified cartilage, and the subchondral bone, while allowing the transitional interface layer to efficiently connect cartilage and bone. Usual method for fabrication of composites is by using two or more different materials [1]. Integration between layers (and with native tissue upon implantation) is achieved by suturing [142], cell-mediated ECM formation, use of fibrin and other glues [97], or simply by press fitting [143]. However, such layered composites are susceptible to delamination if the layers are not well connected. To overcome this, the gradient scaffolds are used, which sport gradual changes of physical and mechanical properties, ideally complemented with the biochemical gradients. Such scaffolds can achieve better transition between cartilaginous and osseous components.

Cross et al. present a fabrication method for a scaffold with graded mechanical properties. They used two natural polymers (gelatin methacryloyl (GelMA) and methacrylated kappa carrageenan (MκCA)) reinforced with 2D nanosilicates to mimic the native tissue interface. The addition of nanosilicates results in shear-thinning characteristics of prepolymer solution and increases the mechanical stiffness of cross-linked gradient structure [144]. D'Amora et al. formulated a method for achieving chemical gradients in which CAM and surface modification are combined. They first aminolyzed poly(ε-caprolactone) surface and subsequently cov-

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ered it with collagen via carbodiimide reaction. These 2D constructs were characterized for their amine and collagen contents, wettability, surface topography and biofunctionality. This functionalization treatment was extended to the 3D printed PCL scaffolds, demonstrating the possibility to manufacture 3D constructs with chemical gradients for OC interface engineering [145]. Dormer et al. achieved biochemical gradients by distributing the microspheres loaded with chondrogenic (TGF-β1) and osteogenic (BMP-2) factors into the two regions of a PLGA scaffold, to produce opposing growth-factor gradients for the formation of cartilage and bone [146]. In addition, therapeutic molecules can be surface-tethered to the microspheres [147]. Using "raw materials," i.e., components like chondroitin sulfate and bioactive glass, in 3D scaffolds was suggested for establishing continuous gradients of both material composition and signaling factors [148].

For now, the best approach seems to be to couple biochemical and structural gradients toward achieving native-like OC interface architecture and integration in large OC constructs intended for implantation [1, 149].

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Author Queries

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