Published in final edited form as:

Adv Biochem Eng Biotechnol. 2012 ; 126: 67–104. doi:10.1007/10_2011_107.

Harnessing Cell–Biomaterial Interactions for Osteochondral Tissue Regeneration

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Abstract

Articular cartilage that is damaged or diseased often requires surgical intervention to repair the tissue; therefore, tissue engineering strategies have been developed to aid in cartilage regeneration. Tissue engineering approaches often require the integration of cells, biomaterials, and growth factors to direct and support tissue formation. A variety of cell types have been isolated from adipose, bone marrow, muscle, and skin tissue to promote cartilage regeneration. The interaction of cells with each other and with their surrounding environment has been shown to play a key role in cartilage engineering. In tissue engineering approaches, biomaterials are commonly used to provide an initial framework for cell recruitment and proliferation and tissue formation. Modifications of the properties of biomaterials, such as creating sites for cell binding, altering their physicochemical characteristics, and regulating the delivery of growth factors, can have a significant influence on chondrogenesis. Overall, the goal is to completely restore healthy cartilage within an articular cartilage defect. This chapter aims to provide information about the importance of cell–biomaterial interactions for the chondrogenic differentiation of various cell populations that can eventually produce functional cartilage matrix that is indicative of healthy cartilage tissue.

Keywords

Biomaterials; Cartilage; Cells; Tissue engineering

1 Introduction

Biomaterials have been used in the medical field for several decades to address a variety of tissue defects and diseases. Initially, biomaterials applied for tissue defects were generally intended to be biologically inert so as to prevent an immune response. More recently, biomaterials have evolved to be bioactive and degradable by design so they can temporarily fill a defect space and serve as a conduit for tissue repair. Specifically, in the field of tissue engineering/regenerative medicine, biomaterials are commonly employed as cell transplantation vehicles or as conduits to support the infiltration of host cells for tissue

formation. Various progenitor cells can differentiate into distinctive lineages and are responsible for tissue maintenance. Biomaterials can act as a template and support structure for cell differentiation, cell proliferation, and the production of proteins and extracellular matrix (ECM). Additionally, biomaterials can be modified to promote cell adhesion or delivery of growth factors necessary to enhance tissue growth and maintenance. The field of tissue engineering has investigated the use of cells, growth factors, and biomaterials for regeneration of tissues from a variety of systems, such as cardiovascular, dental, endocrine, gastrointestinal, maxillofacial, nervous, ophthalmologic, and orthopedic. In this review, cell–biomaterial interactions will be highlighted within the context of cartilage tissue engineering applications.

2 Background

Articular cartilage is a tissue that is located on the surface of articulating joints and consequently may experience a significant amount of force and trauma. Since native cartilage tissues have a limited capacity to heal naturally, cartilage may accumulate damage over time, which can lead to discomfort and pain. As a result, surgical procedures are often applied to aid in repairing the injured cartilage tissue. Current surgical approaches for cartilage repair, including arthroscopic lavage/debridement, autologous chondrocyte implantation, microfracture, and osteochondral grafting, provide some pain relief and improved joint function but fail to fully restore cartilage that has the same biomechanical function as healthy cartilage. Accordingly, tissue engineers are investigating alternative strategies that involve the incorporation of cells, biomaterials, and/or biologically active factors to create a suitable initial support system for cartilage repair. To successfully realize a tissue engineering approach for cartilage regeneration, it is imperative to first understand the organization of native cartilage and the way in which the tissue components, especially cells and ECM molecules, interact with each other to allow proper cartilage tissue function. By studying this communication in native cartilage, researchers can determine the key components that are necessary to successfully recapitulate articular cartilage in repair strategies. This chapter will briefly review the biological aspects and structure of cartilage and discuss how cell–biomaterial interactions can be harnessed to aid in chondrogenesis and regeneration of cartilage.

3 Cartilage

Cartilage is located throughout the human body in sites such as the ears, elbows, knees, intervertebral discs, nose, and ribs. Articular cartilage, or hyaline cartilage, is primarily located on the surface of load-bearing articulating joints (e.g., ankles, elbows, knees, wrists) and enables the movement of the joints to occur smoothly. Articular cartilage, unlike most tissue, is alymphatic, aneural, and avascular. Consequently, the primary mechanism by which cells access nutrients and waste products are removed is by diffusion through the synovial fluid [1]. Accordingly, the transport of various molecules to and from cartilage is facilitated by the high water content of the tissue. Water composes approximately 60–85% of the total wet weight of cartilage [2]. When the tissue undergoes compression, some water is expelled from the tissue, providing lubrication to the articulating surface and allowing the

joints to move with low friction. The remainder of the cartilage weight comprises cells and the ECM. This section will briefly review the components and architecture of cartilage.

3.1 Chondrocytes

Chondrocytes are the primary cell type in cartilage and compose 1–10% of the total articular cartilage volume [3, 4]. They originate from embryonic mesodermal cells, which are responsible for limb development. Mesenchymal stem cells (MSCs) derive from the mesodermal cells and can differentiate into a variety of cell types, including chondrocytes. Chondrogenic differentiation occurs by a process known as cellular condensation. The process begins with degradation of the local ECM to allow the MSCs to aggregate and enhance the number of cell–cell interactions, which are necessary for chondrogenesis to occur [5–8]. Early differentiated chondrocytes are metabolically active, exhibit a high proliferation rate, and start to secrete ECM proteins. During this process, the chondrocytes delineate into two different zones: peripheral and central. In the peripheral zone, the characteristic zonal architecture of cartilage begins to develop, whereas in the central zone, endochondral ossification occurs [9]. The mature chondrocytes have a rounded morphology close to the subchondral bone, but at the cartilage surface, their shape is flatter and more discoidal. Mature chondrocytes have limited proliferative ability and are commonly surrounded by pericellular matrix [9, 10]. Chondrocytes interact with surrounding ECM through receptors, and they are primarily involved in maintaining the cartilage tissue by providing a balance between degradation and synthesis of ECM molecules.

3.2 Extracellular Matrix

The ECM makes up approximately 95% of the total cartilage volume and thus has a significant influence on the physical properties of cartilage [11]. The ECM comprises collagen, proteoglycans, and glycoproteins, with the dry weight of cartilage containing 60%, 25–35%, and 15–20% of these molecules, respectively [9]. There are multiple types of collagen such as types II, VI, and IX–XI [12, 13]. Type II collagen is the predominant collagen in cartilage (90–95% of collagen dry weight) [14]. The other collagens are fibrillar and tend to interweave throughout the ECM to create a framework that imparts tensile strength to the cartilage tissue [9]. Proteoglycans contain a protein core, which accounts for 5% of the molecule, with the rest being composed of branched glycosaminoglycans (GAGs) stemming from the protein core. [3]. GAGs are composed of long, nonrepeating disaccharides with negatively charged carboxylate/sulfate groups, such as hyaluronic acid (HA), decorin, biglycan, keratan sulfate, chondroitin sulfate (CS), dermatan sulfate, and heparin sulfate [9, 10]. Proteoglycans are found within the pericellular matrix and are known to aid in chondrocyte attachment to other matrix components [15]. Large proteoglycan monomers tend to aggregate, which results in the formation of aggrecan containing keratan sulfate and CS. Aggrecans can interact noncovalently with hyaluronan, which is stabilized by link proteins [16, 17]. The negative charge of GAGs and proteoglycans imparts the tissue with a high degree of hydrophilicity, and repulsion of these molecules has been linked to the compressive properties of cartilage [18]. Additionally, these aggregated molecules can prevent a large displacement of the cartilage matrix when undergoing compression, which confers cartilage with its resiliency and durability. Glycoproteins are simply polypeptides that are covalently attached to a carbohydrate group, such as link protein, fibronectin,

laminin, vitronectin, thrombospondin, and tenascin-C, and are distributed throughout the ECM [12, 15]. Fibronectin contains specific amino acid sequences that are responsible for cell binding such as Arg-Gly-Asp (RGD), Arg-Gly-Asp-Ser, Leu-Asp-Val, and Arg-Glu-Asp-Val [19]. Glycoproteins also contain adhesion sites for chondrocytes and multiple binding sites that are able to stabilize collagens and proteoglycans in the tissue [15]. Superficial chondrocytes produce lubricin, also known as proteoglycan 4 or superficial zone protein, which is another glycoprotein that has been found within the synovial fluid to facilitate joint lubrication [20].

3.3 Architecture

Cartilage has a zonal architecture with four main compartments: superficial/tangential, middle/transitional, deep/basal, and calcified. Overall, when looking from the superficial zone to the deep zone, one finds that the water content decreases, oxygen pressure decreases, compressive strength increases, and tensile strength increases [9, 21]. The superficial/tangential zone is located at the articulating surface and composes 10–20% of the total cartilage volume [22]. In the superficial/tangential zone, chondrocytes are flat and disc-shaped with collagen fibrils densely packed and parallel to the tissue surface. A limited number of proteoglycans are located in this area with high amounts of fibronectin [3]. The superficial/tangential zone receives the highest tension, compression, shear, and hydrostatic pressures [22]. A large amount of superficial zone protein has been isolated primarily in this zone [20]. The middle/transitional zone accounts for 40–60% of the total cartilage volume [22]. In this zone, chondrocytes are more rounded in shape and the proteoglycan content is greater compared with the superficial zone, with collagen fibrils being more dispersed throughout the tissue. There is no specific orientation of the cells and ECM in this zone. Within the deep/basal zone (30% of the total tissue volume), chondrocytes are rounded, clustered, and aligned into columns that are perpendicular to the subchondral bone, and the cells are more proliferative compared with those in the other zones [22, 23]. Additionally, the ECM molecules are aligned perpendicular to the joint surface. The ECM contains more collagen and GAG than in the superiorly oriented zones [21]. The collagen is larger in diameter in this zone and transcends the tidemark which delineates the deep zone from the calcified zone. The calcified zone is a thin layer that is located above the surface of the subchondral bone. Here, chondrocytes are rounded but smaller than in the other zones, and the cells are surrounded by calcified ECM [9]. The quantity of proteoglycans and collagen fibrils is lower in this zone, with the collagen fibrils anchored into the underlying calcified bone [9].

Chondrocytes are also surrounded by pockets of ECM proteins that are classified into three different regions defined as the pericellular matrix, territorial matrix, and interterritorial matrix. The pericellular and territorial matrices allow the chondrocytes to bind to matrix proteins and protect them during mechanical loading [10]. A chondrocyte surrounded by the pericellular matrix is known as a chondron, and the thickness of pericellular matrix is approximately 2 μm [12]. The pericellular matrix contains minimal collagen fibrils, with the exception of collagen type VI, and there are abundant proteoglycans, such as aggrecan, hyaluronan, decorin, and biglycan, and glycoproteins such as fibronectin, link protein, and laminin [10]. The territorial and interterritorial matrices function primarily for load bearing.

The territorial matrix surrounds the chondrocytes 2–5 μm from the membrane surface [12]. In this area, type II collagen is present with high amounts of aggrecan containing CS [12]. All these proteins wrap around the chondrocytes to protect them from the mechanical stresses. Collagen fibers from the territorial matrix adhere to the pericellular matrix. The interterritorial matrix is the largest portion of cartilage (more than 5 μm from the surface of chondrocytes) and is responsible for the zonal architecture of cartilage [9, 12]. This area contains the most type II collagen and the lowest amount of aggrecan [12].

4 Chondrocyte–ECM Interactions

Cell–cell and cell–matrix interactions play a key role in the differentiation of MSCs into chondrocytes during limb development. Initially, before condensation occurs, MSCs produce type I and type II collagen, hyaluronan, fibronectin, and tenascin C. These ECM components hinder the movement of the MSCs. However during condensation, the progenitor cells produce enzymes that breakdown local ECM components [24, 25]. The lower ECM density allows the cells to directly interact with each other via cell adhesion molecules, such as neural cadherin and neural cell adhesion molecule [26, 27]. Whereas neural cadherin and neural cell adhesion molecule need to be expressed during condensation, their expression must be lowered during the process of differentiation in order for chondrogenesis to successfully occur [12, 28]. During chondrogenesis, interactions of cells with the ECM once again become crucial for proper cartilage formation. One important ECM component that is not modulated during both the condensation and the chondrogenesis process is type II collagen [12]. The expression of the transcription factor *SOX9* by the cells allows the continual synthesis of type II collagen and is considered a major constituent in chondrogenesis, as the absence of *SOX9* gene expression has been shown to result in lack of cartilage development [29, 30]. Additionally, *L-SOX5* and *SOX6* have been linked with *SOX9* to further promote chondrogenesis and can upregulate cartilage matrix production of type II collagen and aggrecan [31, 32].

Mature chondrocytes are able to maintain healthy cartilage as they balance the degradation and synthesis of ECM components. The signals that chondrocytes receive from the surrounding environment help to define what is necessary to maintain the tissue. Chondrocytes interact with the ECM via receptors that are classified as non-integrin and integrin. Two common non-integrin receptors are annexin V/anchorin CII and CD44. Type II collagen binds to chondrocytes via the annexin V/anchorin CII receptor [33]. CD44 is a cell-surface glycoprotein that has a high affinity for hyaluronan in cartilage [34]. Integrins themselves are glycoproteins that function as heterodimeric transmembrane receptors with α and β subunits. Different types of α and β subunits can noncovalently associate to form receptors with a high affinity for various ligands. β_1 integrins with α_1 , α_2 , α_3 , or α_5 have been found to influence chondrocyte attachment to type II collagen [2]. Chondrocytes interact with type VI collagen by $\alpha_1\beta_1$ integrin and NG2/human melanoma proteoglycan receptors [35, 36]. The $\alpha_3\beta_1$ and $\alpha_5\beta_1$ integrins can mediate the binding of fibronectin [37].

5 Cell Sources for Cartilage Tissue Engineering

Chondrocytes are the primary cell type in cartilage and are an obvious cell source to be explored for cartilage regeneration. Indeed, a clinical product approved by the US Food and Drug Administration for articular cartilage repair, Carticel®, involves autologous chondrocyte implantation. The surgical procedure associated with the application of Carticel® involves isolation of chondrocytes from a non-load-bearing portion of cartilage, cell expansion, and subsequent implantation of the autologous cells into the defect area. Recent studies have reported that 68% of patients treated with Carticel® had graft failure, delamination, or tissue hypertrophy [38]. One possible cause of these complications is the chondrocytes themselves. In healthy tissue, chondrocytes are able to produce and breakdown the ECM in a balanced manner. However, in damaged cartilage, chondrocytes are generally unable to repair the tissue. The chondrocytes that are placed into the defect site might not be integrating with the surrounding tissue and may therefore hinder cartilage repair. Additionally, the limited quantity of chondrocytes in cartilage requires in vitro culture to expand the cell number to clinically viable levels. The simplest method to culture chondrocytes is to passage them in a monolayer, but this may alter the morphology and dedifferentiate the chondrocytes [39]. The cells tend to adopt a fibroblastic morphology and the levels of phenotypic expression markers such as aggrecan and type II collagen decrease with an increase in the level of type I collagen, indicating a fibroblastic phenotype [39]. Transformation of the chondrocytes in this manner may result in fibrocartilage tissue formation. Other studies have found that chondrocytes entrapped in a 3D hydrogel such as alginate or agarose are able to retain a spherical morphology and have shown their ability to redifferentiate [40, 41]. A disadvantage of this approach is that mature chondrocytes tend not to proliferate readily. Additionally, 3D constructs have diffusion limitations, with lower levels of nutrient and waste exchange occurring in the center of the scaffold. Consequently, the size of the construct is limited, which in the end affects how many cells can be entrapped within the system. Further, it is difficult to achieve clinically relevant cell numbers in 3D construct cultures as quickly as with monolayer cultures. Although understanding chondrocyte function is important to the development of approaches for cartilage repair, major challenges are associated with the application of chondrocytes in strategies for cartilage regeneration. These limitations have led to the investigation of alternative cell sources for promoting cartilage repair.

5.1 Stem/Progenitor Cell Sources

A variety of stem cell types can undergo chondrogenic differentiation and have been investigated for application in cartilage tissue engineering. Stem cells are used because they are able to differentiate along multiple cell lineages, can proliferate readily, and can be easily harvested.

5.1.1 Bone-Marrow-Derived Stem Cells—The bone marrow contains both hematopoietic stem cells and MSCs. The cloning abilities of MSCs along with their multilineage capacity suggest their potential for use in tissue engineering applications [42, 43]. The method used to culture MSCs and the associated culture conditions can affect the phenotype of the cells. Therefore, to identify a homogenous population of MSCs, the

Mesenchymal and Tissue Stem Cell Committee of the International Society of Cellular Therapy proposed minimal criteria for human designation of MSCs as follows: (1) MSCs must adhere to tissue culture plastic; (2) MSCs must be positive for CD105, CD73, and CD90 and negative for CD45, CD34, CD14, or CD11b, CD79a or CD19, and HLA-DR; (3) MSCs must be capable of differentiating into osteoblasts, adipocytes, and chondroblasts under standard in vitro conditions [44]. The ability of MSCs to differentiate into chondroprogenitor cells as well as to form cartilage in vivo has led to continued efforts to utilize MSCs for cartilage engineering [45–47].

As previously discussed, the process of condensation drives cell–cell interactions in vivo and initiates chondrogenesis. Therefore, MSCs have been cultured as pellets, aggregates, or spheroids to promote chondrogenic cell–cell interactions. MSCs are able to express type II collagen and aggrecan with low expression levels of type I collagen when they are in pellet culture [48, 49]. Cell culture conditions, such as the culture method and the presence of chondrogenic factors, play a key role in directing MSCs towards a chondrogenic lineage. For example, MSCs exposed to dexamethasone, an anti-inflammatory agent for MSC chondrogenesis, and transforming growth factor (TGF)- β_1 /TGF- β_3 together in pellet culture demonstrated an increased level of aggrecan and type II collagen expression compared with the presence of the individual chondrogenic factors [50, 51]. MSCs in pellet culture in the presence of dexamethasone and TGF- β_1 /TGF- β_3 have also shown increased levels of type X collagen expression and alkaline phosphatase activity, which indicates the pellet culture may induce hypertrophic chondrocyte differentiation under certain conditions [49, 52]. However, when MSCs were co-cultured with mature chondrocytes in a pellet culture with dexamethasone and TGF- β_3 , type II collagen expression was found to be significantly higher than with culture of chondrocytes alone [53]. Human MSC pellets cultured with dexamethasone and TGF- β_1 along with conditioned medium from human chondrocyte pellets induced type II collagen expression and lowered type X collagen expression, when compared with MSC pellets without conditioned medium from chondrocytes [51]. These co-culture systems provide a mechanism to expose MSCs to cartilage proteins and ECM molecules produced by chondrocytes, which may facilitate the initiation of chondrogenesis. Additionally, exposure of MSC pellets to 10 ng/mL parathyroid-hormone-related peptide has been shown to reduce type X collagen expression, which is responsible for inhibiting chondrocytes from transiting from a prehypertrophic to a hypertrophic state during condensation [32, 51]. Overall, these findings suggest that chondrogenic differentiation of MSCs promoted by cell–cell contact during in vitro culture can be enhanced by exposure to chondrogenic or cartilage-like factors.

The microenvironment has also been shown to greatly influence chondrogenic differentiation of MSCs. For example, when MSCs are entrapped in alginate gels, they are able to maintain a rounded morphology and express type II collagen in the presence of TGF- β_1 , and type X collagen expression was found to be lower than for MSCs grown in pellet culture [48, 54]. A lower level of type X collagen has been shown to be expressed by MSCs when ECM components are present, such as native cartilage-derived matrix with TGF- β_3 and/or bone morphogenetic protein (BMP)-6, versus culture in alginate alone [55], indicating the importance of cell–matrix interactions in MSC differentiation. Exposure of

MSCs to methacrylated HA has been found to upregulate type II collagen expression and, by also incorporating TGF- β_3 , higher levels of type II collagen, *SOX9*, and aggrecan are observed by 14 days of culture [56]. When the methacrylated HA constructs were implanted subcutaneously in mice, the presence of TGF- β_3 with MSCs in the constructs resulted in better neocartilage expression than observed with MSCs in poly(ethylene glycol) (PEG) hydrogels. Other work has shown that HA in poly(ethylene oxide) diacrylate hydrogels allows entrapped MSCs to express type II collagen and cartilage proteoglycans, but incorporation of TGF- β_3 was able to lower the expression of type I collagen [57]. Fibrin hydrogels with heparinized nanoparticles releasing TGF- β_3 were able to induce higher levels of type II collagen, aggrecan, and *SOX9* expression compared with fibrin hydrogels, fibrin hydrogels with TGF- β_3 , and fibrin hydrogels with nanoparticles alone. In a rabbit full-thickness articular cartilage defect, the MSCs in the fibrin construct containing the nanoparticles and TGF- β_3 showed the best neocartilage formation [58]. Overall, interactions of cells with growth factors and ECM molecules are essential for chondrogenic differentiation of MSCs.

5.1.2 Adipose-Derived Stem Cells—Adipose-derived stem cells (ADSCs) are part of the embryonic mesoderm and can be isolated from fat tissue, which is commonly removed from the body by liposuction. ADSCs have a fibroblastic morphology and are positive for cell markers CD90 and CD105 and negative for CD14, CD34, and CD45, which is similar to the criteria suggested for selection of human MSCs [59]. Additionally, ADSCs can be expanded for long periods of time with low levels of senescence. Cartilage nodules containing sulfated proteoglycan-rich matrix and type II collagen have been observed when ADSCs are cultured in a micromass culture [60]. The chondrogenic culture medium with dexamethasone containing TGF- β_1 , BMP-6, and/or TGF- β_3 has been found to be important to facilitate the differentiation of ADSCs into chondrocytes [55, 61].

ADSCs in direct contact with porous native articular cartilage ECM showed increased expression levels of type II collagen and aggrecan, with decreased levels of type X collagen in the absence of chondrogenic growth factors in the culture medium. These results indicate the importance of cell–matrix interactions for chondrogenic differentiation of ADSCs [62]. Additionally, the interaction of ADSCs with biomaterials can further influence chondrogenesis. For instance, biomaterials such as alginate and agarose, which naturally lack attachment sequences, allow ADSCs to maintain a rounded morphology. However, in fibrin or gelatin hydrogels, which contain cell binding sites, ADSCs tend to spread and become more fibrochondrogenic [63]. ADSCs entrapped within elastin-like polypeptides with repeating sequences of Val-Pro-Gly-Xaa-Gly, where Xaa is any amino acid except proline, were able to synthesize high levels of type II collagen, with low type I collagen formation in the absence of chondrogenic medium for at least 2 weeks [64]. Human ADSCs cultured on silanized hydroxypropylmethyl-cellulose hydrogels demonstrated higher type II collagen, *COMP*, and *SOX9* expression in 3D culture versus 2D culture without chondrogenic medium. However, to form cartilage matrix *in vivo*, culture of ADSCs in chondrogenic medium was necessary [65]. The preculture of ADSCs with chondrogenic medium has been found in other studies to be effective in the growth of cartilage ECM *in vivo*. When ADSCs are grown in a monolayer with chondrogenic medium and then

subcutaneously implanted with alginate, an increase in type II collagen, *COMP*, aggrecan, and *SOX9* expression was exhibited after 20 weeks, but there were low levels of type I and type X collagen [66]. ADSCs in micromass culture with TGF- β_1 have been grown in an atelocollagen honeycomb-shaped scaffold and placed into an osteochondral rabbit defect [67]. Although cartilage-like tissue formed in the scaffold with and without ADSCs, larger amounts of cartilage tissue and the histological scoring of the tissue indicated that the presence of ADSCs results in improved cartilage repair.

5.1.3 Embryonic Stem Cells—Human embryonic stem cells (ESCs) are pluripotent cells that are derived from the inner mass of the embryonic blastocyst. ESCs are self-maintaining and are able to proliferate indefinitely [68]. Even though the use of these cells is subject to ethical debate, there have been initial promising signs of their ability to differentiate into chondrocytes. Successful chondrogenesis of ESCs was initially thought to require the formation of embryoid bodies (EBs) [69]. Enzymatically dissociated EB cells cultured in 2% agarose with chondrogenic medium without any exogenous growth factors showed higher collagen and sulfated GAG content than native EBs [70]. Exposing ESCs to BMP-2, BMP-4, and BMP-7 has been found to aid in the expression of cartilage matrix, but TGF- β_1 can hinder chondrogenic differentiation of these cells [71–74].

Directed differentiation of ESCs to chondrocytes has also been accomplished by culturing the cells with exogenous growth factors that may allow them to first differentiate into primitive streak mesendoderm [fibronectin matrix, WNT3A, activin A, fibroblast growth factor (FGF)-2, BMP-4], then to differentiate into a mesoderm population (fibronectin matrix, FGF-2, BMP-4, neurotrophin-4, follistatin) [75]. Culturing the mesoderm cells with fibronectin and gelatin, and weaning them off BMP-4 while supplying FGF-2, neurotrophin-4, and growth differentiation factor 5, led the cells to express the chondrogenic marker *SOX9* as well as to produce type II collagen and sulfated GAGs [75]. Additionally, new approaches have begun to investigate how to bypass the formation of an EB, because of the lack of control of EB size and the associated cell number. Cell–cell interactions promoted by pellet or micromass culture of ESCs in combination with growth factors can further enhance the formation of type II collagen [73, 74, 76]. Co-culture of ESCs with chondrocytes can also aid in chondrogenic differentiation in vitro and in vivo [77, 78]. ESCs were initially co-cultured with irradiated chondrocytes and TGF- β_3 . Co-culturing these cells with fresh ESCs in Hyaff-11, a hyaluronan gel, and TGF- β_1 showed positive alcian blue–van Gieson staining for collagen and GAGs [78]. In addition, human ESCs cultured in RGD-modified PEG hydrogels showed an increase in synthesis of GAG and collagen as well as a stimulated gene expression level of link protein and type II collagen versus cells cultured in unmodified PEG hydrogels [79].

5.1.4 Other Stem Cells—Stem cells can be isolated from other tissues as well, such as the muscle and periosteum. These stem cells have shown the potential to become chondroprogenitor cells. Muscle-derived stem cells (MDSCs) are located in the muscle tissue, have been shown to be multipotent, and can proliferate quickly with limited senescence. New cartilage was able to form in full-thickness osteochondral defects in rats after they had been treated for 5 weeks with a type I collagen gel scaffold containing

MDSCs [80]. The presence of 10 μ g BMP-2 in a MDSC pellet culture in a diffusion chamber resulted in expression of type II collagen and aggrecan [81]. Additionally, when this pellet was placed in an in vivo rat patellar groove defect, the newly formed tissue covered the defect area with GAGs and collagen, as seen by histological sections stained with hematoxylin and eosin as well as with toluidine blue. MDSCs retrovirally transduced with a BMP-4 gene have been found to express type II collagen in vitro when cultured in chondrogenic medium supplemented with TGF- β_1 [82]. Injection of the MDSCs with acellular fibrin glue into a rat osteochondral defect resulted in glossy cartilage being formed after 24 weeks.

The periosteum is located on the surface of the bone cortex and contains two distinct layers: a fibrous outer layer and the cambium. Chondroprogenitor cells have been isolated in the thin, inner, cambium layer, which is located adjacent to the bone surface [83]. Surface markers that are present for MSCs have also been found in periosteum-derived stem cells (PDSCs) [84]. However, further research will be necessary to properly identify and locate chondrocyte precursors within the bulk PDSCs. The exposure of PDSCs to growth factors such as TGF- β_1 , TGF- β_3 , FGF-2, and insulin-like growth factor (IGF)-1 significantly influences chondrogenesis [85–87]. PDSCs and periosteum explants have been successfully cultured in alginate, agarose, and atelocollagen gels, but the presence of growth factors is necessary for successful neocartilage formation with these cells under these conditions [87, 88].

5.2 Fibroblasts

As an alternative to stem cells, fibroblasts are being investigated for cartilage engineering. Almost every organ in the human body contains fibroblasts, which originate from the mesoderm. An abundant source of fibroblasts can be isolated from skin biopsies and can be used for potential autologous transplantation. Fibroblasts have properties similar to those of MSCs, such as being able to adhere to tissue culture plastic, being positive for CD73 and CD105, and being negative for hematopoietic markers (e.g., CD14, CD34, CD45) [89]. Human dermal fibroblasts (hDFs) are able to produce cartilage-like matrices containing components such as chondroitin 4-sulfate and keratin sulfate when cultured on a collagen-sponge-demineralized bone matrix composite [90]. Treating adult hDFs with IGF-1 before culturing them on aggrecan led to production of type II collagen as observed by immunohistochemical staining [91]. Additionally, exposing entrapped neonatal hDFs in alginate beads to 5% oxygen with 100 ng/mL BMP-2 under 3 weeks of hydrostatic compression (1 Hz for 4 h/day) increased collagen production and aggrecan gene expression compared with static culture conditions [92]. Dermis-isolated, aggrecan-sensitive cells, which are isolated by culturing dermal fibroblasts in a monolayer on aggrecan, can be cultured as a micromass in an agarose gel [93]. The dermis-isolated, aggrecan-sensitive cells have been shown to produce a cartilage-like ECM. These results underscore the importance of cell–ECM interactions. Additionally, cell–cell interactions have been shown to be a significant parameter for fibroblasts to progress along a chondrogenic lineage. Co-culturing hDFs with porcine articular chondrocytes on poly(lactic acid)/poly(glycolic acid) as well as a micromass of hDFs with lactic acid has shown that these cells are able to synthesize type II collagen [94, 95]. Additionally, fibroblasts reprogrammed to progress towards a

chondrogenic lineage, such as BMP-7-transduced fibroblasts cultured on collagen hydrogels, retrovirally *SOX9* induced fibroblasts, and fibroblasts induced by cartilage-derived morphogenetic protein 1, have shown promising in vitro and in vivo results for the production of cartilage-associated ECM proteins [96–98].

6 Chondrogenesis in Biomaterials

6.1 Cell Homing

Biomaterials can be utilized as progenitor cell transplantation vehicles as well as to provide moieties that can aid in cartilage regeneration. Tissue engineering strategies may also involve leverage of biomaterials for the homing of progenitor cells, such as MSCs, from the host to the construct to facilitate cartilage repair. In general, the recruitment of endogenous host cells from a cell storage niche, such as the bone marrow, to an anatomic compartment is considered cell homing [99]. MSC homing is also specifically defined as a MSC population that is arrested within the vasculature of a tissue and transmigrated across the endothelium [100].

Natural healing and regeneration in defect tissues involves mobilization, homing, and subsequent reparative actions at the injured sites [101]. MSCs released from a cell storage niche first circulate in response to signals from distal injured tissues (mobilization), and vasculature arrestment as well as transendothelial migration (homing) occurs where MSCs will develop into mature healthy tissue [100, 101]. One of the early studies to investigate the origin and function of the progenitor cells involved in the repair of full-thickness defects of articular cartilage demonstrated that the repair was mediated entirely by proliferation and chondrogenic differentiation of primitive MSCs from the bone marrow [102]. It was also indicated by autoradiography after labeling with ^3H -thymidine and ^3H -cytidine that the chondrocyte population from the residual adjacent articular cartilage was not fully involved in defect repair. Therefore, this study emphasized the importance of bone marrow as a progenitor cell reservoir for articular cartilage regeneration and osteochondral tissue repair.

Owing to the potential drawbacks of clinical cell delivery, including undesired immune responses, pathogen transmission, and technical barriers associated with regulatory approval, cell homing to recruit MSCs from surrounding host tissues has been suggested [99]. Nonetheless, there are a limited number of publications investigating cell homing strategies for articular cartilage repair and chondrogenic tissue regeneration [101]. Owing to the lack of vasculature in a cartilage tissue, MSC homing strategies for cartilage tissue repair may need to target cell storage sources such as bone marrow and synovial fluid. One recent study has shown the potential use of synovium stem cells as a host cell source as well as the feasibility of a biomaterial-based cell homing strategy to induce articular surface regeneration [99]. The scaffold for the entire articular surface of the synovial joint was anatomically customized with poly(ϵ -caprolactone) (PCL) and 20% hydroxyapatite powder based on the surface morphology of a rabbit forelimb joint. An acellular scaffold containing interconnected microchannels (200–400 μm in diameter) with and without TGF- β_3 infusion was implanted in a rabbit model. TGF- β_3 was envisioned to serve as a cell homing molecule to accelerate functional recovery and hyaline cartilage regeneration. Compared with the scaffolds in the absence of TGF- β_3 , scaffolds infused with TGF- β_3 recruited a greater

number of cells to the injured site, exhibited mechanical properties similar to those of native cartilage, and contained higher concentrations of type II collagen and aggrecan. In addition, the microchannels in the scaffold functioned as conduits for cell homing, diffusion, histogenesis, and angiogenesis, thereby promoting tissue regeneration. In this study, it was proposed that some of the endogenous cells were recruited from the synovium, bone marrow, adipose tissue, and vasculature. The porous surface of the proximal end of the implanted scaffold and the presence of the microchannels may enable access to synovium stem cells and bone marrow progenitor cells, while providing a conduit for the migration of these cells. Therefore, this study demonstrated the possibility of utilizing a cell homing strategy to regenerate the entire articular cartilage surface, beyond focal defect healing, with the aid of growth-factor-loaded scaffolds.

6.2 Hydrogels

For cartilage tissue engineering, hydrogels are the most extensively investigated class of scaffold materials, as they provide a variety of advantages, including high water content and elastic properties that mimic native cartilage tissue, technical capacity for cell encapsulation, and effective transport of water and nutrients owing to their high equilibrium swelling [103]. Hydrogels may be tuned to mimic the function and architecture of native cartilage, which is composed of chondrocytes and ECM proteins [104, 105]. Therefore, success criteria for a functional hydrogel material for the delivery of cells and growth factors for cartilage regeneration include (1) biocompatibility, (2) an ability to encapsulate cells and support their viability and proliferation, (3) a capacity for sufficient hydration to provide effective diffusion of molecules in physiological conditions, (4) suitable mechanical properties, and (5) possible degradation to provide space for neotissue regeneration and remodeling. Naturally derived polymers such as alginate [106], fibrin [58, 107], silk [108, 109], chitosan [110, 111], and blends of these components [112, 113] as well as ECM components in native cartilage, including collagen, HA [56, 114], and CS [115], have been used to fabricate functional hydrogels for cartilage regeneration [116]. Although hydrogels comprising naturally derived materials generally exhibit excellent cytocompatibility and cell adhesion, synthetic polymers have the potential to overcome some of the limitations of naturally derived hydrogel materials, especially the general lack of tunability and insufficient mechanical strength. Synthetic polymers have been utilized in the fabrication of functional hydrogel systems owing, in large part, to their ability to modulate key physical properties of the hydrogels, such as the mechanical properties (e.g., elasticity and injectability), as well as the degradation kinetics. Surface properties of synthetically derived polymeric biomaterials can also be modulated to increase hydrophilicity, mobilize ECM-derived proteins, and fabricate micropatterned and nanopatterned surfaces [117]. In addition, it is also possible to fabricate hybrid composite gels by combining natural and synthetic polymers [110, 118] or by incorporating other molecules into the polymer structure [119]. Modification of hydrogel materials may be harnessed to provide enhanced functionality to stimulate encapsulated cell responses, particularly chondrogenic differentiation and matrix formation.

7 Modification of Biomaterials

Although synthetic polymeric materials generally lack biological moieties on the surface to interact with cells, an advantage of synthetic polymers is their tunable properties, which may be leveraged to conjugate a series of biologically active peptides. Other biological modifications of materials include delivery of exogenous growth factors through substrate materials or carrier molecules. Genetic modification by delivering genes encoding therapeutic growth factors could also be introduced to stimulate the production of growth factors from the seeded cell population without further modulation in a system. Moreover, intrinsic properties of biomaterials, including surface charge, roughness, topology, and scaffold architecture, could also be key parameters to modulate the cell–biomaterial interaction and influence chondrogenic differentiation of progenitor cells.

7.1 RGD Peptide Incorporation

Modification of biomaterials by utilizing biomimetic and bioactive motifs, such as short ligands, could improve the interaction between cells and materials [120]. Many studies have investigated a variety of bioactive peptides to improve cell adhesion, osteoinductivity of biomaterials, and maintenance of chondrocyte phenotype in a synthetic scaffold, including laminin-derived YIGSR [121], elastin-derived VPGIG [122], proteoglycan-binding peptide FHKIK [123], osteopontin-derived peptide [124], and matrix metalloproteinase (MMP)-derived peptide [125]. Among various peptides, adhesive peptides containing RGD sequences are known to function as a binding domain for cell integrins [117]. Many studies have demonstrated that the immobilized RGD peptides on the surface of scaffolds facilitate cell–biomaterial interactions, specifically cell binding and adhesion. Recent investigations have shown that the presence of RGD peptides on biomaterial surfaces or the proper bulk incorporation of RGD peptides within a 3D matrix can improve the chondrogenic differentiation of stem cells to facilitate the process of cartilage repair [126, 127]. As a cell-binding sequence, RGD peptides present on poly(hydroxyalkanoate) (PHA) scaffolds may improve cell survival and motility [126]. PHA scaffolds coated with PHA granule binding protein PhaP and fused with RGD peptide showed increased cell spreading, adhesion, proliferation, and chondrogenic differentiation of human MSCs [127]. This RGD-modified scaffold exhibited higher expression levels of chondrogenic differentiation markers, including type II collagen, aggrecan, and *SOX9*, as well as increased production of sulfated GAG and total collagen than a PhaP scaffold without RGD and a blank scaffold.

RGD peptides immobilized on macroporous alginate scaffolds have also been shown to stimulate chondrogenic differentiation of human MSCs [128]. Specifically, the positive effect of RGD modification of scaffolds upon chondrogenesis was illustrated in the observation that the TGF-induced Smad signaling pathway involved in chondrogenic differentiation of MSCs was more activated in the presence of RGD peptide under the conditions studied [128]. A western blot and its densitometric analysis showed that the phosphorylation of both SMAD2 and ERK1/2 was significantly higher in the RGD-incorporating scaffolds than in the control alginate scaffolds. This result is consistent with the observed upregulation of chondrogenic marker gene expression, including type II collagen and *SOX9*. A study using human articular chondrocytes also demonstrated that

bioactive RGD incorporation on a copolymer substrate of polystyrene/poly(L-lysine)/PEG improved GAG production and type II collagen messenger RNA (mRNA) expression compared with a blank polystyrene substrate [129]. Another study sought to mimic native RGD release by combining MMP-13 cleavage sites and demonstrated the importance of temporal regulation of integrin-binding peptides in chondrogenic differentiation [130]. In this study, human MSCs were encapsulated in PEG hydrogels with either an uncleavable RGD tether (CRGDSG) or a cleavable RGD tether (CPENFFGRGDSG). Both tethers were designed with MMP-13-specific cleavage sites. Once MMP-13 had been produced by encapsulated cells, RGD was released from the hydrogel system via the MMP-13 enzymatic cleavage of the tether. Released RGD induced greater chondrogenic differentiation of the MSCs than gels with uncleavable sequences, as indicated by higher GAG deposition and type II collagen staining. It has been suggested that RGD also functions as a mechanotransducer [131]. Under the mechanical loading environment, RGD ligands stimulated cartilage-specific gene expression and ECM protein synthesis. When dynamic compressive strains were applied to bovine chondrocytes encapsulated in a PEG hydrogel, the chondrocyte phenotype index (the expression ratio of collagen II and collagen I) and the proteoglycan synthesis were enhanced in RGD-incorporating gels relative to those without RGD incorporation.

ADSCs obtained from rats have also been used to investigate the influence of the integrin-binding peptides on chondrogenic differentiation [132]. RGD-chimeric protein with a cellulose binding domain in alginate beads resulted in increased gene expression of type II collagen, *SOX9*, aggrecan, and fibronectin, as well as the accumulation of chondrogenic matrix during TGF- β_3 -induced differentiation. The results also demonstrated that the mechanism of RGD-chimeric protein stimulation of chondrogenic differentiation might be associated with suppressed RohA activity in the early differentiation stage. Furthermore, human ESC-derived cells could also be encapsulated in RGD-incorporating hydrogels [79, 133]. Human ESC-derived MSCs were positive for MSC surface markers including CD29, CD44, CD109, and platelet-derived growth factor α [79]. These cells exhibited in vitro neocartilage formation with basophilic ECM deposition and upregulated chondrogenic gene expression in RGD-modified PEG diacrylate hydrogels. An in vivo study using predifferentiated human ESC-derived MSCs demonstrated the potential application of this cell type for osteochondral tissue repair, indicated by a smooth articular cartilage surface and architecture of repaired cartilage similar to that of normal cartilage [133].

Although RGD-incorporating hydrogel systems have shown the capacity to induce in vitro chondrogenesis and possible approaches to stimulate in vivo articular cartilage repair, there have also been some controversial results with use of RGD-modified materials [134, 135]. For example, bovine MSCs encapsulated in unmodified alginate gels have been shown to enhance chondrogenic gene expression and matrix accumulation, whereas this observation was not found in RGD-modified alginate gels [134, 135]. However, it was found that the inhibition of sulfated GAG synthesis was stimulated by increasing RGD density [134, 135]. Another study also reported apoptosis of chondrocytes and synovial cells could be induced by RGD peptides [135].

7.2 Growth Factor Incorporation

Chondrogenic differentiation of progenitor cells into mature chondrocytes, cartilage-specific ECM deposition, and articular cartilage regeneration require a dynamic interaction of various growth factors as soluble signaling molecules to initiate, stimulate, and maintain differentiated cell function. A number of studies have investigated the functions of specific growth factors with a given progenitor cell population in a synthetic environment as well as the influence of combinations of growth factors in a dynamic fashion. Members of the TGF- β superfamily, such as certain TGF- β s and BMPs, have been found to aid in the upregulation of type II collagen, *SOX9*, and aggrecan expression. Specific molecules that have shown promising results for chondrogenesis include TGF- β_1 , TGF- β_2 , and TGF- β_3 [49, 52, 136–141], IGF-1 [142–146], BMP-2, BMP-4, BMP-6, and BMP-9 [55, 147–151], and FGF [152–158]. Combinational and dynamic effects of these growth factors on chondrogenic differentiation and articular cartilage repair have also been investigated [144, 158–160]. Effective delivery of growth factors is of importance to induce the chondrogenesis of progenitor cells and enhance articular cartilage regeneration. In addition to selection of the growth factor and the combination of a series of growth factors, other parameters such as the dose and release kinetics are also of importance to augment cartilage repair. To this end, sustained, controlled growth factor delivery to defect sites by using various delivery vehicles has also been investigated.

7.2.1 Gelatin Microparticles—Among a number of available methods to incorporate a growth factor into a scaffold or matrix material, gelatin microparticles (GMPs) have been intensively investigated because of their ability to form electrostatic complexes with charged growth factors under physiological conditions (pH 7.4), depending upon the charge of the growth factor and the gelatin [161]. Through enzymatic degradation of gelatin, incorporated growth factor(s) can be released in a controlled manner by regulating tunable fabrication parameters, such as the extent of cross-linking. For instance, controlled release of TGF- β_3 from GMPs stimulated chondrogenic matrix production by MSCs in pellet culture [162].

Simultaneously, degradation of gelatin can also result in a porous inner morphology in a 3D matrix or hydrogel containing GMPs. Growth-factor-loaded GMPs can be incorporated with injectable hydrogels, such as systems based on oligo(poly(ethylene glycol) fumarate) (OPF) [163]. GMPs made of acidic gelatin with an isoelectric point of 5.0 can electrostatically complex with appropriately charged protein in aqueous solution under physiological conditions [164]. An initial in vitro release study revealed that the release profile of TGF- β_1 from GMPs in OPF hydrogels and degradation of the hydrogel composites could be modulated by altering key fabrication parameters, including the amount of loaded GMPs, the cross-linking extent of GMPs, and the molecular weight of OPF [165]. A study of TGF- β_1 release in the presence of the enzyme collagenase demonstrated that this OPF-GMP hydrogel system could be applicable to the cartilage wound healing environment [166]. In addition, dual growth factor loading using TGF- β_1 and IGF-1 was also evaluated for controlled and localized release [160]. To demonstrate the capability of this composite hydrogel system as a delivery vehicle for cells and growth factors, primary chondrocytes from the condyle of calf femurs [167] and rabbit bone marrow MSCs were embedded in the hydrogel [168]. Encapsulated chondrocytes in OPF hydrogels exhibited higher levels of

proliferation and GAG production in the presence of GMPs loaded with TGF- β_1 for 28 days of in vitro culture compared with control OPF gels as well as OPF gels with unloaded GMPs [167]. Chondrogenic differentiation of bone marrow MSCs derived from rabbit femurs was also investigated because of the potential application of MSCs as progenitor cells for cartilage tissue regeneration as mentioned previously [168]. Quantitative reverse transcription polymerase chain reaction data indicated that chondrogenic differentiation of bone marrow MSCs was upregulated with a medium dose of TGF- β_1 incorporation (10 ng/mL) at low cell seeding density (ten million cells per milliliter of gel). Dual growth factor delivery of TGF- β_1 and IGF-1 affected in vitro chondrogenic differentiation of encapsulated rabbit MSCs in the OPF-GMP composite hydrogels [145]. In this study, the dual-growth-factor-loaded group showed a significantly higher expression level of collagen type II and aggrecan on day 14, as determined by quantitative reverse transcription polymerase chain reaction, compared with the group with single growth factor incorporation. Moreover, the molecular weight of OPF was suggested to be a tunable parameter to potentially modulate the chondrogenic differentiation of MSCs, owing to the higher swelling ratio and larger mesh sizes of surrounding OPF hydrogels [169]. Therefore, in vitro chondrogenic differentiation of encapsulated bone-marrow-derived MSCs could be upregulated by modulating the formulation of OPF-GMP composite hydrogels.

7.2.2 Polymeric Microspheres—In addition to GMPs, polymeric microparticles could also be utilized as a delivery vehicle for growth factors and therapeutic agents. Poly(L,D-lactic-co-glycolic acid) (PLGA) is one of the most widely used polymeric materials for the fabrication of microsized carriers for growth factors [170]. Pharmacologically active microcarriers fabricated with fibronectin-coated PLGA microspheres have been applied to deliver TGF- β_3 in a controlled and sustained fashion for chondrogenesis [171]. Adsorbed fibronectin induced MSC adhesion onto the surface of the microspheres, and released TGF- β_3 stimulated chondrogenic differentiation of MSCs. In vitro differentiation of adherent MSCs was dependent on the amount of TGF- β_3 , as indicated by upregulated mRNA expression levels for aggrecan, type IIB collagen, and type X collagen. Qualitative histological image analysis of subcutaneous implantation of TGF- β_3 /PLGA microcarriers with adherent MSCs onto the surface in SCID mice also indicated high levels of type II collagen and aggrecan production as well as the formation of neotissue surrounding microspheres.

PLGA microspheres have also been applied for the delivery of other therapeutic factors, such as dexamethasone [172, 173]. Porous PLGA microspheres loaded with dexamethasone were incorporated within HA (4% w/v) based hydrogels and subcutaneously implanted in nude mice [172]. After 4 weeks, gene expression levels for cartilage-specific markers, including type II collagen and *SOX9*, were significantly higher in porous PLGA microspheres than in nonporous microspheres and the control treatment (bulk dexamethasone loading without PLGA carrier). Histological and immunohistochemical analyses also showed higher levels of GAG staining, as well as type II collagen synthesis, in the porous microsphere group. In addition, for the dual delivery of growth factor and dexamethasone, a more complex delivery system was introduced [173]. Dexamethasone-coated PLGA microspheres were conjugated with heparinized TGF- β_3 , and this complex

allowed dual release of dexamethasone and growth factor from the surface of the microspheres [173, 174]. Rabbit MSCs were cultured with these complexes and injected subcutaneously into the backs of nude mice. The dual delivery complex exhibited higher gene expression levels for chondrogenic differentiation markers, including type II collagen and aggrecan, than dexamethasone-coated PLGA microspheres as well as the blank PLGA control. In the presence of both dexamethasone and TGF- β_3 , the accumulation of proteoglycans and polysaccharides in the microspheres was observed.

7.3 Gene Delivery

In addition to exogenous growth factor incorporation during the hydrogel fabrication for localized and sustained delivery, gene transfer has also been explored to stimulate the encapsulated cell population to produce various chondrogenic growth factors [175–178]. Delivery of growth factor to the cartilage defect site in an animal model might be limited because of rapid clearance from the joint tissues [175]. Therefore, as an alternative means of delivery of exogenous growth factor, it has been hypothesized that overexpression of growth factors by transplanted chondrocytes or MSCs might enhance the repair of articular cartilage defects. Rabbit articular chondrocytes transfected with plasmid vectors encoding human IGF-1 complementary DNA by using nonviral and nonliposomal lipid formulations (FuGENE 6) demonstrated in vitro IGF-1 secretion from cells in the alginate construct [176]. In addition to the in vitro IGF secretion from transfected cells in alginate over a prolonged period (36 days), in vivo transplantation of the constructs led to enhanced articular cartilage repair and subchondral bone formation compared with what was seen in groups that were transfected with the *lacZ* reporter gene. This lipid-based transfection was also used for combined gene transfer for both human IGF-1 and human FGF-2 in vivo [177]. This study demonstrated that combined overexpression of both growth factors from NIH 3T3 cells encapsulated in alginate could accelerate the repair of full-thickness osteochondral cartilage defects, compared with the group receiving IGF alone and *lacZ* implants. In addition to nonviral gene delivery, viral vectors, including adenovirus, lentivirus, and retrovirus, could also be used as gene transfer agents [178–180]. In a study using adenovirus-mediated TGF- β_1 gene transfer to human MSCs, improved cartilage repair was observed 12 weeks after osteochondral implantation in a rat model [178]. FGF-2 has also been produced successfully by adeno-associated-virus-delivered transgene and improved in vivo cartilage tissue repair [179, 180]. Moreover, direct implantation of vector-laden, coagulated bone marrow aspirates (i.e., gene plug) has also been developed [175]. Typical ex vivo gene transfection techniques require a series of processes including expansion of cell number, transfection of cells with target genes, fabrication of cell/scaffold constructs, and surgical implantation [175]. By use of this alternative ex vivo protocol, implantation of bovine bone marrow aspirate transduced with adenoviral vector to deliver TGF- β_1 resulted in improved cartilage repair in partial-thickness defects of the medial condyle in mature sheep models.

7.4 Modulation of the Intrinsic Properties of Biomaterials

Other than the incorporation of biological moieties such as peptides and growth factors into hydrogel systems, there have been various investigations to enhance the level of cell–material interaction by altering surface properties of scaffolds, by modulating mechanical

stimulation, and by changing the architecture of biomaterial scaffolds. In addition to promotion of cell–material interactions by addition of biologically active stimuli, modulation of the intrinsic properties of biomaterial substrates can also influence the chondrogenic differentiation of cell populations as well as the in vivo tissue responses.

7.4.1 Surface Properties

Wetability: Surface hydrophilicity/hydrophobicity of the biomaterials can be one of the important parameters to regulate a series of cellular functions, including attachment, migration, cytoskeletal organization, and differentiation [181]. Although it is known that hydrophobic surface characteristics favor protein adsorption from the aqueous surrounding solution, a hydrophilic surface is also necessary to initiate cell attachment [117]. Therefore, controlling the optimum level of hydrophilicity/hydrophobicity of the material surface could induce positive cell responses. Technical methods to increase the hydrophilicity of hydrophobic synthetic polymeric biomaterials include grafting hydrophilic polymer through copolymerization [182], plasma treatment to increase the number of oxygen-containing groups such as –OH and –C=O [183, 184], and photooxidation to introduce peroxide groups onto the material surface with the aid of UV treatment [117]. For instance, increasing hydrophilicity in copolymeric hydrogels by increasing the hydrophilic PEG content relative to the hydrophobic PCL content resulted in higher proliferation of primary rabbit chondrocytes [182]. In addition, more hydrophilic gels (e.g., 14 wt% PEG and 6 wt% PCL) could be optimum to induce chondrogenic differentiation, as determined by stimulated gene expression levels of type II collagen, aggrecan, *SOX9*, and *COMP*. Plasma-treated electrospun PCL nanofibers also showed higher chondrocyte adhesion and proliferation than untreated hydrophobic PCL surfaces [184].

Roughness and Topography: Modulation of the hydrophilicity of the material surface can usually be related to changes in topography and roughness [181, 184–186]. Cell adhesion of both human MSCs and porcine chondrocytes mediated by integrin β was influenced by different topologies and surface roughness of PLGA-, PLA-, and PCL-coated plates [187]. In addition to initial cell adhesion, topographical changes of the material surface also affected chondrocyte aggregation (i.e., mesenchymal condensation) [188] and the osteoblastic signaling pathway [186]. Another study using a composite bone scaffold also demonstrated the related changes in increasing hydrophilicity and roughness by incorporation of hydroxyapatite nanoparticles into a hydrophobic poly(propylene fumarate) scaffold [185]. In this study, a mineral particle content of 20 wt% resulted in higher hydrophilicity and roughness, and the changes in the physicochemical properties of the composite material influenced osteogenic signal expression of rat bone marrow stromal cells.

7.4.2 Scaffold Architecture

Architectural Design for Cartilage Tissue Engineering: In addition to altering surface properties of biomaterials to improve cell–material interactions, modulation of the structure and architecture of a scaffold can also influence the cellular behaviors, since architectural changes, including pore size, porosity, interconnectivity, and morphology of the substrate surface, can affect subsequent cellular behaviors [189–192]. Architectural design is of

importance in fabrication of bone-tissue-engineered scaffolds to induce osteoblastic differentiation [190, 191], but the influence of structural parameters can also be observed in altering chondrocyte behaviors and differentiation for cartilage tissue engineering. One recent study demonstrated that morphological changes in four different cell-graft systems, including hyaluronan web, collagen fleece, collagen gel, and collagen sponge, affected chondrocyte distribution, morphology, and cell-scaffold interactions [192]. Passive chondrocyte distribution throughout the inner region of porous scaffolds might depend on porosity and structure, whereas changes in cell morphology may be correlated to fiber size. In addition, adhesion might be influenced by material composition through membrane receptors and adhesive matrix molecules.

Nanofiber Mesh Scaffold: Another example of architectural changes for cartilage tissue engineering scaffolds is a nanofiber mesh scaffold. Fiber meshes are commonly fabricated via an electrospinning technique [193, 194]. A potential advantage of electrospun nanofibrous scaffolds is the similarity of the fiber diameter to that of native collagen fibrils, which may provide an appropriate microenvironment for chondrogenic cell responses [194]. However, there are also some limitations to nanofiber scaffolds, such as insufficient control of pore size, inherent planar structure, and subsequent limited cell infiltration into the inner region of scaffolds [194]. Nevertheless, many researchers have shown that nanofiber mesh scaffolds can support chondrogenic differentiation of MSCs seeded on the scaffold [195–198] as well as multilineage differentiation, including osteogenesis and adipogenesis [199–201]. Composite fibrous scaffolds can also be fabricated by the dispersion of nanoparticles (e.g., hydroxyapatite minerals) in polymeric solution for electrospinning [197]. Some in vivo studies using nanofibrous scaffolds with MSCs revealed a promising method to repair cartilage defects [202, 203]. Six months after implantation, PCL nanofibrous scaffolds with both allogeneic chondrocytes and xenogeneic human MSCs in a swine model exhibited higher articular tissue regeneration over acellular PCL scaffold and the no-implant control [203]. Another in vivo study using periosteal cells from skeletally mature New Zealand White rabbits was also reported [202].

Varying the diameter of fibers in fibrous scaffolds could provide different architectures and morphologies of the substrate for cell interaction. Subsequent architectural changes such as specific surface area could be related to chondrocyte phenotype, ECM protein synthesis, and chondrogenic differentiation [192, 195, 198]. It has been demonstrated that a rounded morphology and disorganized cytoskeletal structure of cells were observed in nanosized fiber meshes, whereas well-spread chondrocytes with organized cytoskeletons were seen in microfibers [195]. This observation is correlated to another report demonstrating that rounded cell shape was retained when chondrocytes attached on fibers with a smaller diameter than the size of the cell [192]. In addition to morphological changes caused by altering the fiber diameter, GAG production and qualitative immunostaining for type II/IX collagen, aggrecan, and cartilage proteoglycan link protein were higher in nanofibrous poly(lactic acid) scaffolds than in microfiber scaffolds [195]. Moreover, type II collagen gene expression in a PCL fibrous scaffold with a diameter of 500 nm was also higher than in a PCL fibrous scaffold with a diameter of 1,000 nm [198]. Therefore, it can be speculated that changes in fiber diameter and subsequent modulation of architecture in nanofibrous

scaffolds can regulate the cell–material interaction and chondrocyte behavior can be optimized.

8 Osteochondral Tissue Regeneration

8.1 Zonal Cartilage Engineering

As previously described, articular cartilage is an avascular tissue with a single cell population and dense ECM that has a zonal organization. Each zone has a unique distribution of chondrocytes, biochemical composition, and mechanical properties [118, 204]. To closely mimic the native phenotype and formation across articular cartilage tissues, chondrocyte subpopulations in two or more distinct layers of hydrogel have been engineered.

Superficial and deep zone chondrocytes from bovine articular cartilage have been encapsulated in photopolymerized bilayered poly(ethylene oxide) diacrylate hydrogels [204]. In this bilayer co-culture system, deep zone cells produced more collagen and proteoglycan than superficial cells after 6 weeks of in vitro culture. In addition to the inhomogeneity of ECM production, deep zone cells also exhibited higher shear and compressive strength than the homogeneous cell control. This research showed the heterotrophic cell interaction and modulated biological/mechanical properties of engineered cartilage tissues. Another study also demonstrated that engineered agarose hydrogels containing zonal chondrocytes exhibited depth-varying cellular and mechanical inhomogeneity similar to that of native tissue [205]. Following 42 days of in vitro culture, the data indicated that production of GAG and collagen from superficial and middle/deep zone chondrocytes was enhanced when they were layered with the other subpopulation in a bilayered construct. One of the recent approaches to mimic the highly organized zonal architecture of articular cartilage investigated a variety of hydrogel formulations with a combination of CS, MMP-sensitive peptides, and HA in PEG hydrogels [118]. This study demonstrated that the unique mechanical properties and ECM composition of each formulation might direct a zonal-phenotype-specific chondrogenic differentiation. The result indicated that the PEG/CS/MMP-sensitive peptide group corresponded to the superficial zone, the PEG/CS group corresponded to the middle zone, the PEG/HA group corresponded to the deep zone, and the CS group corresponded to the calcified zone.

8.2 Bilayered Hydrogels

In addition to zonal engineering of articular cartilage, implantable bilayered hydrogel systems have also been investigated for osteochondral tissue regeneration with two distinct sublayers. Osteochondral tissue contains an articular cartilage surface at the top and subchondral bone underneath the cartilage tissue that provides mechanical support [206–208]. Therefore, biphasic layers with distinct biomechanical properties could simultaneously mimic the chondrogenic surface as well as the bony subchondral tissue. For the full-thickness joint defect, an approach using a bilayered structure of implantable hydrogel or scaffold could induce osteochondral tissue regeneration.

A composite bilayered hydrogel of OPF matrix and growth factor-incorporating GMPs has been studied as a functional model for osteochondral tissue regeneration [209–211]. An

early in vivo trial with this composite hydrogel demonstrated the support of healthy tissue growth in New Zealand White rabbit osteochondral defects by showing hyaline cartilage improvement in the chondral region and bone filling in the subchondral region at 14 weeks [211]. In this study, hydrogel composites of 3-mm diameter and 3-mm thickness were implanted in the full-thickness defect of a rabbit knee joint. The TGF- β_1 -loaded chondral layer exhibited a significant improvement in morphology of neoformed surface tissues among various histological scoring criteria compared with gels encapsulating blank GMPs without the growth factor. Additionally, the in vitro influence of the differentiation stage of an encapsulated cell population in this hydrogel system was also investigated [210]. A combination of MSCs with TGF- β_1 -loaded GMPs in the chondral (top) layer and osteogenically induced (6 days of culture in medium with osteogenic supplements) MSCs with blank GMPs in the subchondral (bottom) layer showed significantly higher mRNA expression levels of collagen type II and aggrecan compared with both nonosteogenic cells and TGF- β_1 -free groups. This study demonstrated that osteogenic cells in the subchondral region might produce chondrogenic-signaling molecules to induce chondrogenic differentiation of MSCs in the chondral layer. This observation was only seen in the presence of TGF- β_1 , which indicated the importance of the additional effect of growth factor on MSC differentiation in a hydrogel system. Similarly, precultured MSCs in medium with osteogenic supplements in the subchondral layer with the aid of TGF- β_3 induced a significantly higher level of in vitro chondrogenesis of MSCs in a chondral layer after 28 days of culture compared with groups without TGF- β_3 [209].

Although many in vitro studies have demonstrated that incorporation of growth factor within various cartilage scaffolds enhances chondrogenesis of progenitor cell populations and results in minor improvement after subcutaneous implantation in vivo, functional in vivo tissue regeneration in an articular cartilage surface remains a challenge. Some of the studies have shown only partial repair of cartilage defects and a minor level of improvement. Dual growth factor delivery using a bilayer osteochondral hydrogel was also investigated to demonstrate the interaction of growth factors in cartilage repair [159]. In this study, GMPs with TGF- β_1 , IGF-1, and both of them were incorporated in a chondral (top) layer of OPF hydrogels and the gels were implanted in rabbits. In vivo analysis indicated that single IGF-1 delivery showed minor chondral repair with GAG and cell content of the cartilage compared to other groups, whereas single TGF- β_1 and dual delivery did not show any improved tissue repair. A lack of any synergistic effect of dual growth factor delivery suggests the complexity of the dynamic process of cartilage repair and the existence of other parameters to investigate beyond a simple combination of growth factors.

In addition to growth factor delivery, functional remodeling of osteochondral tissue by MSC delivery remains a target for investigation. Despite various in vitro studies that indicate successful chondrogenic differentiation of encapsulated MSC populations in hydrogels with the aid of exogenous growth factor delivery, in vivo cartilage regeneration with complete cartilage repair remains a challenge. Rabbit MSCs in OPF hydrogels with or without TGF- β_1 incorporation did not show any significant improvement in cartilage tissue regeneration [212]. Both reduced cartilage thickness and improved surface regularity were observed with MSC-loaded gels. It might be hypothesized that faster subchondral bone formation in

OPF/MSCs provided sufficient mechanical support to the articular surface region and resulted in smoother articular surfaces. A smoother surface could also be obtained by the participation of implanted MSCs in cartilaginous matrix secretion and remodeling. A similar MSC/growth factor delivery in a rabbit in vivo model using a composite hydrogel made from the self-assembling peptide sequences (RADA)₄ and (KLDL)₃ showed inconsistent results with in vitro chondrogenesis and chondrocyte phenotypes [213]. Neither the addition of dexamethasone as well as the chondrogenic growth factors TGF- β ₁ and IGF-1 nor the combinational incorporation of these growth factors and bone-marrow-derived MSCs in a hydrogel led to any beneficial effect on cartilage repair. Fibrous tissue formation was even observed in the MSC/growth factor/hydrogel group. This study demonstrated a possibility to direct a single stem cell population to different zonal phenotypes within a 3D structure with multiple layers.

Other in vivo studies using tricopolymer scaffolds with gelatin, chondroitin 6-sulfate, and sodium hyaluronate demonstrated TGF- β ₁ release could help articular cartilage repair in the full-thickness defect (4 mm in diameter and 3 mm in thickness) in rabbits [214, 215]. An amount of 0.8 ng of TGF- β ₁ released from embedded GMPs induced chondrogenic differentiation of autologous MSCs loaded onto scaffolds [214]. Histological observation and semiquantitative scoring data indicated that a controlled TGF- β ₁ release using GMPs might be superior to stimulate cartilage repair to the absence of growth factor delivery to the cells. A hybrid PLGA scaffold with this tricopolymer also showed better in vivo cartilage regeneration [215]. After 24 weeks of implantation, histological grading revealed that cell morphology/matrix staining, surface regularity, and subchondral bone reconstruction were significantly better in the tricopolymer-incorporated PLGA scaffolds than in blank PLGA scaffolds.

9 Summary

The microenvironment plays a key role in engineering tissue. Therefore, special care and attention are necessary to create a successful combinatorial approach for tissue regeneration that involves the integration of cells, growth factors, and biomaterials. A variety of stem cells as well as fibroblasts have been investigated for cartilage regeneration. The process of chondrogenic differentiation requires the interaction of cells with growth factors and biomaterials. Biomaterials can be modified to control the release of bioactive molecules as well as to aid in cell adhesion to enhance cartilage formation. Ultimately, biomaterials can be used to recapitulate the cartilage architecture, which can enhance cellular function to successfully tissue-engineer cartilage as well as to potentially regenerate full-thickness osteochondral defects.

Acknowledgments

Work in the area of biomaterials science and cartilage tissue engineering is supported by the US National Institutes of Health (R01-AR048756, A.G.M. and F.K.K.; R01-AR057083, A.G.M.; and R21-AR056076, A.G.M.).

Abbreviations

ADSC	Adipose-derived stem cell
BMP	Bone morphogenetic protein
CS	Chondroitin sulfate
EB	Embryoid body
ECM	Extracellular matrix
ESC	Embryonic stem cell
FGF	Fibroblast growth factor
HA	Hyaluronic acid
hDF	Human dermal fibroblast
GAG	Glycosaminoglycan
GMP	Gelatin microparticle
IGF	Insulin-like growth factor
MDSC	Muscle-derived stem cell
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
OPF	Oligo(poly(ethylene glycol) fumarate)
PCL	Poly(ϵ -caprolactone)
PDSC	Periosteum-derived stem cell
PEG	Poly(ethylene glycol)
PHA	Poly(hydroxyalkanoate)
PLGA	Poly(_{L,D} -lactic-co-glycolic acid)
RGD	Arg-Gly-Asp
mRNA	Messenger RNA
TGF	Transforming growth factor

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