Cartilage Tissue Engineering: Scaffold, Cell, and Growth Factor-Based Strategies

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Abstract

The avascular, alymphatic, and aneural character of articular cartilage along with the reduced availability of chondrocytes/progenitors, its complex structure, and mechanics pose a major challenge for cartilage regeneration. State-of-the-art therapies for cartilage injuries can at best halt cartilage deterioration and are most often inadequate for promoting regeneration. The emerging field of tissue engineering has contributed significantly in regeneration of complex tissues including cartilage. The tissue engineering triads of scaffolds, cells, and growth factors have been investigated both independently and in combination for cartilage regeneration. This article focuses on the current developments revolving around these three components for the development of cartilage regenerative therapies. More specifically, we discuss about the influence of scaffold type, architecture, chemical/biochemical composition, and mechanical properties on chondrogenesis. Thereafter, different cell sources and types of growth factors that have been used for engineering cartilage tissue have been reviewed. Finally, the last section deals with various biomaterial-based approaches for controlled release of growth factors for cartilage tissue engineering.

Keywords

Cartilage regeneration • Hydrogels • Mesenchymal stem cells • Growth factor delivery and chondrogenesis

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Abbreviations

ADSC   Adipose-derived stem cell
BMP   Bone morphogenetic protein
ECM   Extracellular matrix
EDC   1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EGF   Epidermal growth factor
ESC   Embryonic stem cell
FGF   Fibroblast growth factor
GAG   Glycosaminoglycan
HA   Hydroxyapatite
hMSC   Human mesenchymal stem cell
HRP   Horse radish peroxidase
IGF   Insulin-like growth factor
iPSC   Induced pluripotent stem cell
LCST   Lower critical solution temperature
MMP   Matrix metalloproteinase
NHS   N-Hydroxysuccinimide
PCL   Polycaprolactone
PDGF   Platelet-derived growth factor
pDNA   Plasmid DNA
PEG   Polyethylene glycol
PLGA   Poly(lactide-co-glycolide)
PRP   Platelet-rich plasma
PVA   Polyvinyl alcohol
RGD   Arginine-glycine-aspartate
sGAG   Sulfated glycosaminoglycan
TGF   Transforming growth factor
UV   Ultra Violet
VEGF   Vascular endothelial growth factor
YAP   Yes-associated protein

14.1   Introduction

Articular cartilage is a dense connective tissue that lines bony surfaces of diarthro-dial joints. Its specialized structure not only provides a smooth and lubricated surface for friction less articulation of the bones but also helps in the effective transmission of loads. Healthy articular cartilage is largely composed of extracellular matrix (ECM) (>90% by tissue volume) and lacks blood/lymphatic vessels and nerve supply. A specialized class of cells known as chondrocytes, which occupy less than 10% cartilage tissue volume, are responsible for homeostasis of cartilage matrix in response to various physicochemical mediators such as growth factors, chemokines, and mechanical forces. While limited mechanical damage can be

compensated by increased matrix deposition by chondrocytes, large damage to the cartilage tissue often leads to progressive deterioration of the tissue function due to the limited self-repair ability [1]. The absence of progenitor cells and lack of vasculature are largely responsible for this limited self-repair ability of articular cartilage. As a result, large damage in articular cartilage in case of trauma and diseases like osteoarthritis often needs external interventions to initiate healing and restoration of joint function.

The current treatment protocols used in case of cartilage injury are most often symptomatic—these include the use of analgesics, physiotherapy, and arthroscopic chondroplasty (removal of loose bodies/cartilage fragments). Other surgical treatments like microfracture, mosaicplasty, and autologous chondrocyte implantation are successful to a limited extent as they are associated with problems such as fibrocartilaginous healing (mechanically inferior), donor site morbidity/lack of integration, and graft delamination/periosteal hypertrophy, respectively [2]. These limitations have provided an impetus to the development of new and improved treatment protocols such as tissue engineering strategies for cartilage repair and regeneration.
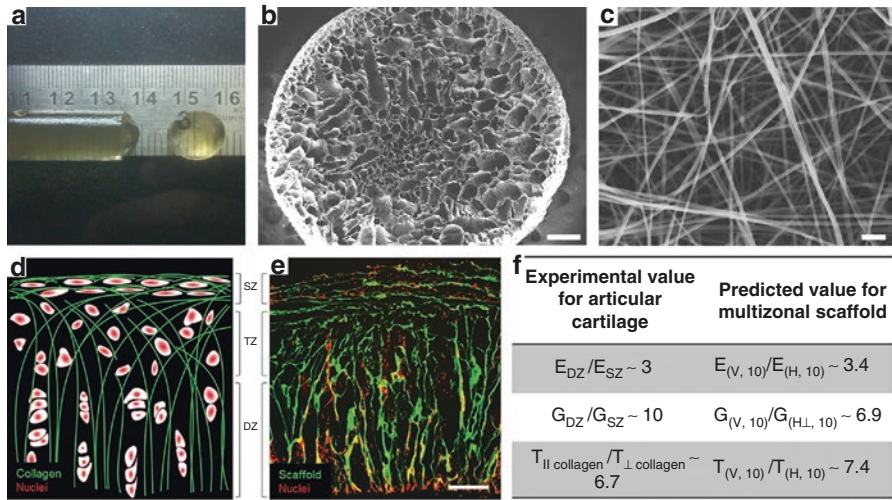
14.2   Cartilage Tissue Engineering

Cartilage tissue engineering seeks to restore cartilage function by using cells, scaffolds, and growth factors either alone or in various combinations. In the past decade or so, a range of strategies have been developed for the regeneration of cartilage tissue, and various studies have elucidated the influence of different parameters on the properties of engineered cartilage. This article summarizes the influence of scaffold properties, cell type, and growth factor type/mode of incorporation on chondrogenesis and cartilage repair/regeneration.

14.2.1 Scaffold Design in Cartilage Tissue Engineering

Scaffolds are generally three-dimensional structures that provide transient support to cells for enabling their growth and differentiation. Scaffolds for cartilage tissue engineering have been fabricated in various formats, of which sponges, hydrogels, and fibers are the most prominent (Fig. 14.1a). These scaffolds have been fabricated from different natural and synthetic polymers which include collagen, chondroitin sulfate, hyaluronic acid, gelatin, chitosan, polyethylene glycol, polyvinyl alcohol (PVA), poly(lactide-co-glycolide) (PLGA), etc. While synthetic materials provide high tailorability and reproducibility, they lack bioactive characteristics and need to be modified to modulate cell behavior. Whereas, natural materials are highly bioactive but associated with disadvantages like risk of disease transmission and batch to batch variability. Nevertheless, both synthetic and natural materials have been extensively explored for cartilage tissue engineering due to their independent merits. The following sections will describe the influence of various scaffold parameters

	Experimental value for articular cartilage	Predicted value for multizonal scaffold
	$E_{DZ}/E_{SZ} \sim 3$	$E_{(V, 10)}/E_{(H, 10)} \sim 3.4$
	$G_{DZ}/G_{SZ} \sim 10$	$G_{(V, 10)}/G_{(H\perp, 10)} \sim 6.9$
	$T_{II\ collagen}/T_{\perp\ collagen} \sim 6.7$	$T_{(V, 10)}/T_{(H, 10)} \sim 7.4$

Acronyms/ symbols - SZ: Superficial Zone; TZ: Transition Zone; DZ: Zeep Zone; E: Compressive elastic modulus; G: Shear modulus; T: Tensile modulus; (V/H, 10): of vertical/horizontal zone at 10% strain; II/ ⊥collagen: parallel/perpendicular to collagen alignment.

Fig. 14.1 Type of scaffolds employed for cartilage tissue engineering. (**a**) Gross image of tyramine-gelatin hydrogels cross-linked with hydrogen peroxide in the presence of horseradish peroxidase enzyme, (**b**) scanning electron micrograph of a macroporous sponge of gelatin fabricated using freeze-drying method (Scale bar: 400 µm), and (**c**) gelatin nanofibers fabricated using electrospinning method (scale bar: 2 µm) [unpublished data]. (**d**) Schematic representation depicting alignment of collagen fibers and cellular arrangement in different zones of the native articular cartilage, (**e**) vertical section of cell-seeded anisotropic multizonal scaffold fabricated using freeze-drying in conjunction with directional freezing (scale bar: 500 µm) (**d** and **e** are reproduced from Ref. 8 with permission from the publisher), and (**f**) table summarizing the anisotropic properties of different zones of articular cartilage with those of a multizonal scaffold. Permission has been obtained from Elsevier Limited, Oxford, UK to reproduce Fig. 8A and B from J Mech Behav Biomed Mater 2015:51:169–183

such as architecture, (bio)chemistry, and mechanical properties on chondrogenesis and cartilage regeneration; toward the end of this section we also discuss about the recent advances in development of injectable scaffolds for cartilage tissue engineering.

14.2.1.1 Role of Scaffold Architecture

Scaffold architecture is a crucial determinant of cell growth and differentiation and hence controls *neo*cartilage formation. A variety of scaffold architectures have been used for cartilage tissue engineering, including hydrogels, macroporous sponges, and fibrous materials [3].

Hydrogels are highly swollen physically or chemically cross-linked networks of one or more polymers. In general, the pore size of hydrogels is much smaller than cell size and hence is insufficient to allow cell infiltration into gels; thus cells need to be encapsulated in such systems. Various properties of hydrogels that influence architecture such as polymer concentration, cross-linking density, and susceptibility

to enzymatic degradation play a crucial role in determining cell fate in these hydrogels. While lower polymer concentration and cross-linking density are known to favor chondrogenesis and deter chondrocyte hypertrophy [4], reverse has been shown for matrix metalloproteinases (MMP) cleavable peptides, i.e., hydrogels with high presence of MMP cleavable sites may act as good scaffolds for chondrogenesis [5]. The major advantage of hydrogels is that they can be tailored to make them injectable thus making the procedure less invasive; however, their use is limited by their poor mechanical properties and reduced nutrient diffusion.

Sponges are macroporous solids that can be fabricated from a variety of materials including hydrophilic and hydrophobic polymers using different methods such as porogen leaching, cryogelation, freeze-drying, and gas foaming. Unlike classical hydrogels, these materials can be fabricated with pore size varying from few microns to >500 μm, thus facilitating cell infiltration. Pore size has been shown to have differential influence in different types of scaffolds, and a wide range of pore sizes have been shown to be permissive for effective chondrogenesis [6, 7]. For instance, in a recent study Matsiko et al. [7] demonstrated pore size >300 μm (when compared to 94, 130, and 300 μm) to be more suited for chondrogenesis in case of chitosan-hyaluronic acid scaffolds. Whereas, Stenhamre et al. [6] suggested better chondrogenesis in scaffolds with a pore size of <150 μm (when compared to <150, 300–500, and >500 μm) in case of polyurethane urea scaffolds. Thus, it may be concluded that while micro-architectural features such as pore size have an influence on cartilage formation, in the current setting it is difficult to generalize the optimum pore size for scaffolds with varying compositions. Pore orientation/aspect ratio has also emerged as a strong architectural feature that may influence cartilage formation/integration. To this end a recent study demonstrated the possibility to generate anisotropic multizonal scaffolds whose pore architecture closely mimics the collagen alignment of native articular cartilage (Fig. 14.1d and e). The results from this study demonstrated that the biomimetic arrangement of pores in these scaffolds led to a depth-wise variation in their bulk compressive, shear, and tensile properties similar to that of cartilage [8]. It may be speculated that this similarity in anisotropy of the scaffold and the recipient tissue may enhance the integration of these constructs in osteochondral defects.

Fibers have been used as scaffolds for cartilage tissue engineering as they mimic the fibrillar structure of native cartilage ECM. Fiber bonding, phase separation, and electrospinning are few of the popular methods available for fabrication of fibrous scaffolds for tissue engineering. Among these methods electrospinning has emerged as one of the most commonly used method as it not only allows fabrication of fibers from a variety of materials but also provides facile control over fiber diameter, alignment, and porosity. Architectural characteristics of fibers such as diameter, pore size, and alignment have been shown to influence chondrogenesis in vitro [9, 10]. While electrospun fibers provide very high surface to volume ratio and a physical mimic to collagen fibers, their use in cartilage tissue engineering is limited by poor control over third dimension of fiber meshes (mesh thickness), low pore size, and poor compressive mechanical properties.

14.2.1.2   Role of Scaffold (Bio)Chemistry

It is well established that chemical/biochemical composition of scaffolds has an overwhelming influence on cellular phenotype. Chemical moieties such as small functional groups most often alter the protein adsorption behavior of scaffolds which in turn modulates the extracellular microenvironment of the cells thereby influencing cell fate. However, biologically active motifs such as sugars and peptides can act both directly and indirectly on cells. Few studies have investigated the influence of simple chemical moieties in synthetic hydrogel environments on chondrogenesis. A study by Kwon et al. [11] demonstrated that the chondrogenic differentiation of pre-chondrogenic cells could be significantly enhanced by increasing anionic charge density on polyacrylamide gels by increasing the ratio of sulfonate to amine groups. Another study by Curran et al. [12] corroborated the importance of anionic groups in cellular microenvironment for chondrogenic differentiation as they demonstrated that hydroxyl and carboxyl groups facilitated chondrogenesis as compared to methyl, sulfahydryl, and amine groups over silane-coated glass surfaces.

In addition to small functional groups, more complex biologically active moieties have been shown to have a greater influence on chondrogenic differentiation and cartilage formation. Among the various biologically active moieties, ECM molecules and their mimics have been shown to play a crucial role. Arg-Glu-Asp (RGD) is one of the most common ECM mimetic peptides which is known to interact with integrin receptors and facilitate cell adhesion to scaffolds. Since synthetic hydrogels like those made up of PEG lack cell adhesion sites, they support poor cell adhesion and survival. Salinas et al. [13] surmounted this limitation of PEG hydrogels by incorporating RGD peptides in these hydrogels. This not only improved cell adhesion/survival but also led to significantly improved chondrogenic differentiation.

Another class of molecules that have been employed frequently is sulfated glycosaminoglycans (sGAG) and sGAG analogs. They can not only interact with cells but also have strong potential to modulate the activity of various chemokines and growth factors by forming ternary complexes. In one of the studies, it was demonstrated that incorporation of heparin in dextran hydrogels significantly improved secretion of cartilaginous matrix by chondrocytes along with enhanced cell survival [14].

14.2.1.3   Role of Mechanical Properties of Scaffolds

Matrix stiffness is one of the important physical cues that determine cell fate. In fact, it has been demonstrated that by varying the stiffness of the substrate alone, mesenchymal stem cells can be differentiated into diverse lineages such as neural, myogenic, and osteogenic [15]. Likewise, lineage commitment to chondrogenic fate has also been shown to be highly mechano-sensitive [16]. To prove this, Schuh et al. [16] cultured chondrocytes on polyacrylamide substrates of varying stiffness (4–100 kPa). They observed that chondrocytes maintained their rounded phenotype only on the softest substrate (4 kPa) and expressed high levels of collagen type II on these substrates. Contrastingly, cells on stiffer substrates showed a well-spread phenotype and low collagen type II expression. A similar response has also been demonstrated in the case of collagen-GAG sponges where cells on scaffolds with lowest

cross-linking density demonstrated best chondrogenic differentiation and vice versa [17]. More recently, Toh et al. [18] studied the influence of varying cross-linking density and in turn stiffness on chondrogenic differentiation of MSCs in injectable hyaluronic acid hydrogels. They demonstrated that hydrogels with least degree of cross-linking and an elastic modulus of 5 kPa promoted chondrogenesis over the stiffer hydrogels which facilitated differentiation of MSCs into smooth muscle cells. Overall, it can be concluded that substrates of lower stiffness which facilitate cell rounding also enable chondrogenic differentiation. This stiffness-mediated response has been shown to be regulated by the Yes-associated protein (YAP), which is known to show nuclear localization on stiff substrates and thereby negatively regulate chondrogenic differentiation [19].

14.2.1.4   Injectable Hydrogel Scaffolds in Cartilage Tissue Engineering

Initial approaches in cartilage tissue engineering employed preformed scaffold systems which not only require invasive surgeries for implantation but also need to overcome the challenge of filling irregular shaped defects. In order to meet these challenges, injectable hydrogel scaffolds are now being actively investigated as potential tissue-engineered constructs. In these systems, a polymer solution containing cells and/or growth factors is injected at the defect site which then cross-links in situ and form a hydrogel network. Based on their cross-linking mechanisms, injectable hydrogel scaffolds can be divided into photo-cross-linked, thermoresponsive, enzymatically cross-linked, and chemically cross-linked systems (Fig. 14.2).

Photo-Cross-Linked Hydrogel Scaffolds

Photo-cross-linked hydrogels utilize a polymer functionalized with highly reactive groups along with a photoinitiator. The photoinitiator generates free radicals upon excitation with light which further leads to cross-linking of the polymer chains to form a network [20]. A variety of photoinitiators such as eosin, rose bengal, riboflavin, and Irgacure have been widely used in conjunction with either visible or UV light sources to initiate photo-cross-linking of polymers functionalized with acrylate groups. In these systems light intensity and exposure time can be varied in order to control mechanical properties of gels and depth of gelling [20]. In a recent study, methacrylated chitosan-collagen II/chondroitin sulfate was employed in conjunction with riboflavin and blue light to synthesize photo-cross-linkable injectable hydrogels for cartilage regeneration [21]. In another study, photo-cross-linked methacrylated gelatin (mGL) scaffolds were used to successfully encapsulate human BMSCs and facilitate chondrogenesis. Unlike UV light-based cross-linking systems, visible light could cross-link mGL solution both in air and aqueous environment which suggested that it could be well suited for in situ tissue repair [22]. In addition to being less invasive, photo-cross-linked injectable systems also allow better spatial and temporal control over in situ gelation of polymers. However, photo-cross-linking reactions produce free radicals which can directly or indirectly (via reactive oxygen species) interact with cellular components with the possibility of causing cell damage. However, this disadvantage can be overcome by modulating
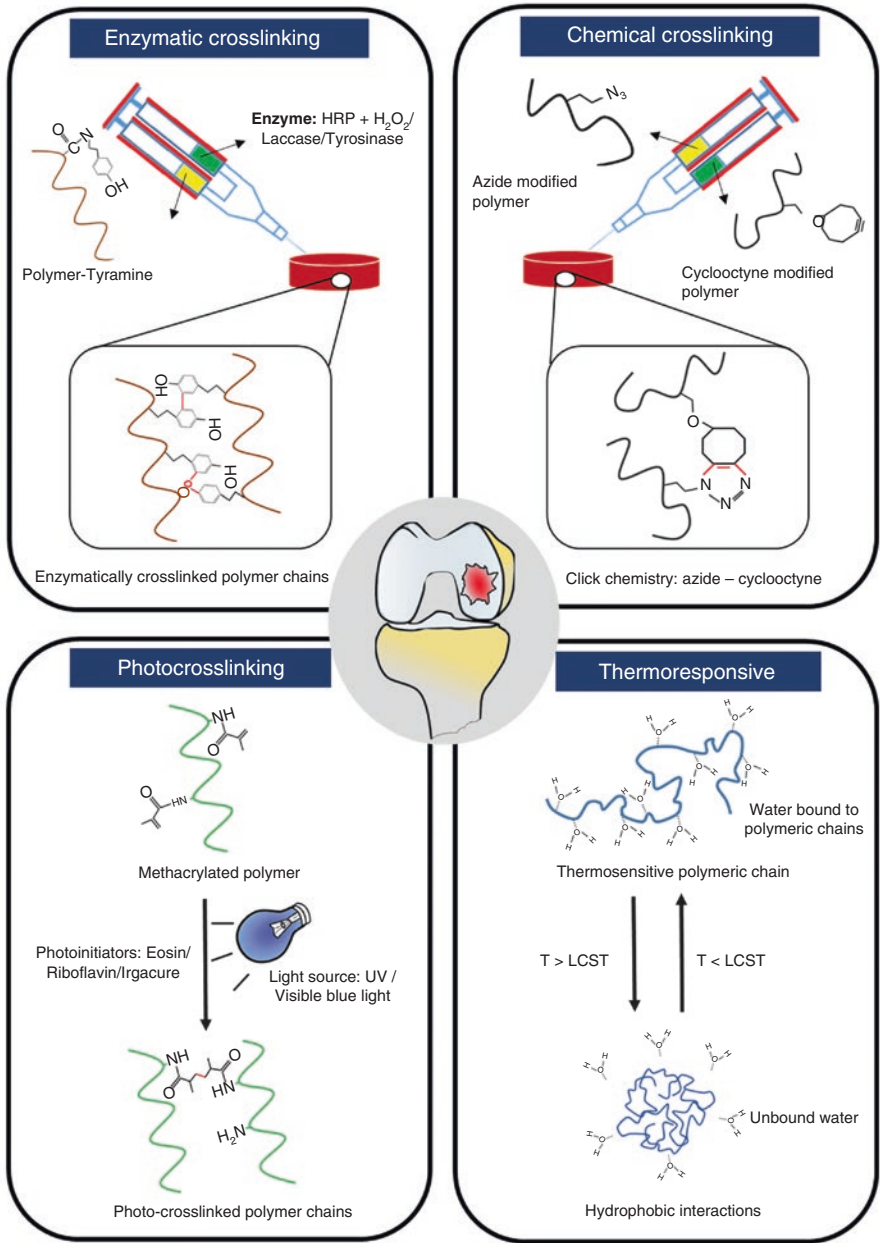
Fig. 14.2 Schematic depicting different strategies for fabrication of injectable hydrogel-based scaffolds for cartilage tissue engineering

the ratio of reactive groups to photoinitiator and light parameters. Furthermore, the use of photo-cross-linking may be partially compromised by the high light scattering tendency of concentrated cell suspensions and requirement of additional probes during surgery.

Thermoresponsive Hydrogel Scaffolds

Thermoresponsive hydrogels demonstrate a temperature-dependent gelation behavior and are often composed of polymers which have the property of reversible phase transition in response to change in temperature. These polymers have a "lower critical solution temperature" (LCST) (temperature above which the polymer loses its bound water to bulk solution) below 37 °C such that when the solution is injected into the body, it loses bound water to the bulk solution leading to solidification of the polymer solution [23].

For instance, BST-CarGel®, a commercialized thermoresponsive adhesive polymeric scaffold of chitosan, is delivered in conjunction with autologous blood and glycerol phosphate after bone marrow stimulation at the cartilage lesion site. The polymeric system gels at 37 °C and stabilizes the blood clot by preventing its retraction and increasing its residence time leading to hyaline cartilage-like tissue formation [24]. Until recently, thermoresponsive systems comprised only of physically cross-linked polymeric networks; however, more recently, thermally triggered release of horseradish peroxidase (HRP) enzyme from liposomes that enabled covalent cross-linking of hyaluronic acid in a thermoresponsive manner has also been explored [25]. However, the potential of this system in cartilage tissue engineering is yet to be established.

Enzymatically Cross-Linked Hydrogel Scaffolds

Enzymatic cross-linking-based methods are widely used to prepare artificial matrices for cartilage regeneration mainly because of the mildness of this type of reaction under physiological conditions. Unlike cross-linking by photoinitiators or organic solvents, enzymatically cross-linked reactions are highly specific for their substrates thus preventing unwanted side reactions. Dual syringe applicators have been used to inject the polymer solution and enzyme in situ, wherein the enzyme cross-links the polymeric chains and forms a 3D network. Toh et al. [18] utilized such an approach to synthesize tyramine-conjugated hyaluronic acid hydrogels cross-linked in the presence of HRP and H_2O_2. The authors demonstrated that the hydrogel properties could be tailored to modulate the extent of chondrogenesis simply by varying H_2O_2 concentration.

Though enzymatic cross-linking is a facile method for fabrication of hydrogels, this method usually results in the formation of mechanically weak hydrogels [26]. To circumvent this problem, a recent study used bienzymatic cross-linking approach to fabricate hydrogels with interpenetrating polymer networks of gelatin and chitosan. In this work, transglutaminase was used to catalyze amide bond formation between amine group of lysine and γ-carboxamide group of glutamine present on

adjacent gelatin chains, and HRP was used to cross-link phenol groups on adjacent chitosan chains modified with phloretic acid in presence of H_2O_2 [27]. Tyrosinase is another enzyme that catalyzes the oxidation of phenols into activated quinones which further reacts with amine and phenol groups resulting in cross-linking of the polymer chains. This enzyme was used successfully in fabricating chitosan-glycolic acid/tyrosine (CH-GA/Tyr) hydrogel scaffolds which showed high cytocompatibility and moderate mechanical strength [28].

Chemically Cross-Linked Hydrogel Scaffolds

A range of methods to fabricate injectable hydrogel scaffolds using chemical cross-linking have been developed. These methods are based on chemical reactions that can be performed under mild conditions permissive for cell encapsulation. One of the commonly used methods is based on the Schiff base formation, wherein hydrogels are prepared by cross-linking amine and aldehyde groups of the polymeric backbone and the strength of these hydrogels is determined by the number of amine and aldehyde groups present. Recently, Cao et al. [29] investigated viability, proliferation, and phenotype of chondrocytes encapsulated in Schiff base cross-linked hydrogels prepared from PEG and glycol chitosan. This study demonstrated that in situ-forming hydrogels supported chondrocyte viability and maintained their phenotypic characteristics. In future, this hydrogel system could provide a platform for cartilage tissue engineering provided the weak mechanical properties of such constructs are circumvented. Click chemistry is another versatile method that allows formation of hydrogels by joining small entities without the addition of initiators and through low-energy-consuming chemical reactions like Diels-Alder (DA) cycloaddition, azide-nitrile addition, thiol-ene addition, and azide-alkyne cycloaddition. Recently Takahashi et al. [30] synthesized HA hydrogels using azide-alkyne reaction by chemically modifying HA by azide and cyclooctyne groups. Due to high reactivity of these groups, they have the potential to be used for in situ gelation of polymeric solution. In another recent study by Yu et al. [31], hydrogels of furyl-modified HA and dimaleimide polyethylene glycol were synthesized using DA chemistry and were evaluated for chondrogenesis. Results from this study demonstrated that chondrocyte-seeded hydrogels exhibited significant increase in aggrecan and collagen type II expression. Furthermore, disulfide cross-linking, Michael-type addition reaction, and ionic cross-linking have also been used to fabricate in situ hydrogels for cartilage regeneration. While chemical cross-linking provides stable and irreversible hydrogels, these methods often utilize heavy metal catalyst which may have cellular toxicity. Moreover, chemical cross-linking provides poor control over gelation rate unlike enzymatic cross-linking where enzyme concentration can be modulated to vary gelation kinetics.

14.2.1.5   Scaffold-Free Approaches

In addition to scaffold-based approaches, recently scaffold-free methods have emerged as a new paradigm for engineering functional cartilage tissue. "Scaffold-free" tissue engineering approaches do not make use of any exogenous three-dimensional material for cells to adhere and proliferate. Such approaches mimic developmental process

of tissue formation following a sequence of processes of cell condensation, cell proliferation, cell differentiation, ECM production, and tissue maturation. Scaffold-free approaches exist in two broad categories: self-organization and self-assembly. Self-organization is a thermodynamic process in which external energy or force is required for cells to condense and attain a desired structure. The external energy is provided through centrifugation or rotational culture. This is in contrast to self-assembly approach which is spontaneous and works on the principle of minimizing free energy of the system with no external energy needed for cells to condense and attain a specific structure. The non-adherent agarose mold allows tissue formation of predictable and repeatable shape, size, and appearance [32]. Recently, self-organization of human mesenchymal stem cells was exploited to provide native-like environment to these cells which could lead to functional cartilage development. The condensed mesenchymal cells when pressed with a porous scaffold made of decellularized bone matrix led to formation of well-stratified cartilage interfaced with underlying bone. The utility of condensed mesenchymal cells was examined by filling these cells in an in vitro cartilage defect model where they integrated with the surrounding tissue [33]. Another study showed that articular chondrocytes when self-assembled on non-adherent agarose well plates led to the development of *neo*tissue with collagen II predominantly produced in these tissues [34].

14.2.2 Cell Sources for Cartilage Tissue Engineering

The ideal source of cells for cartilage tissue engineering should be the one that can be easily isolated and expanded in vitro and secrete cartilage-specific ECM which would lead to the formation of good-quality cartilage. Since last few decades, extensive search for appropriate cell type for cartilage regeneration is being performed; however, consensus on a suitable cell type is still elusive. Chondrocytes, being the only cellular component of cartilage, is the most obvious choice and has been extensively evaluated for regeneration of damaged cartilage. However, limited availability of chondrocytes has given impetus for finding alternative cell sources. Recently, mesenchymal stem cells (MSC) from bone marrow and adipose tissues are emerging as an alternative cell source for cartilage regeneration due to their ease of availability and high in vitro expansion ability. More recently pluripotent stem cells like induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) are being actively explored for cartilage tissue engineering (Fig. 14.3).

14.2.2.1   Chondrocytes
Chondrocytes, the resident cells of cartilage, are the first and foremost choice of cell type for repair and regeneration of damaged cartilage. Chondrocytes regulate anabolic and catabolic pathways of cartilage by secreting various factors thereby maintaining tissue homeostasis. Many studies have been performed to explore the potential of chondrocytes for regeneration of articular cartilage. In one such study, Wang et al. [35] used adult human chondrocytes with silk fibroin scaffolds for in vitro chondrogenesis and demonstrated that adult chondrocyte-seeded silk scaffolds supported
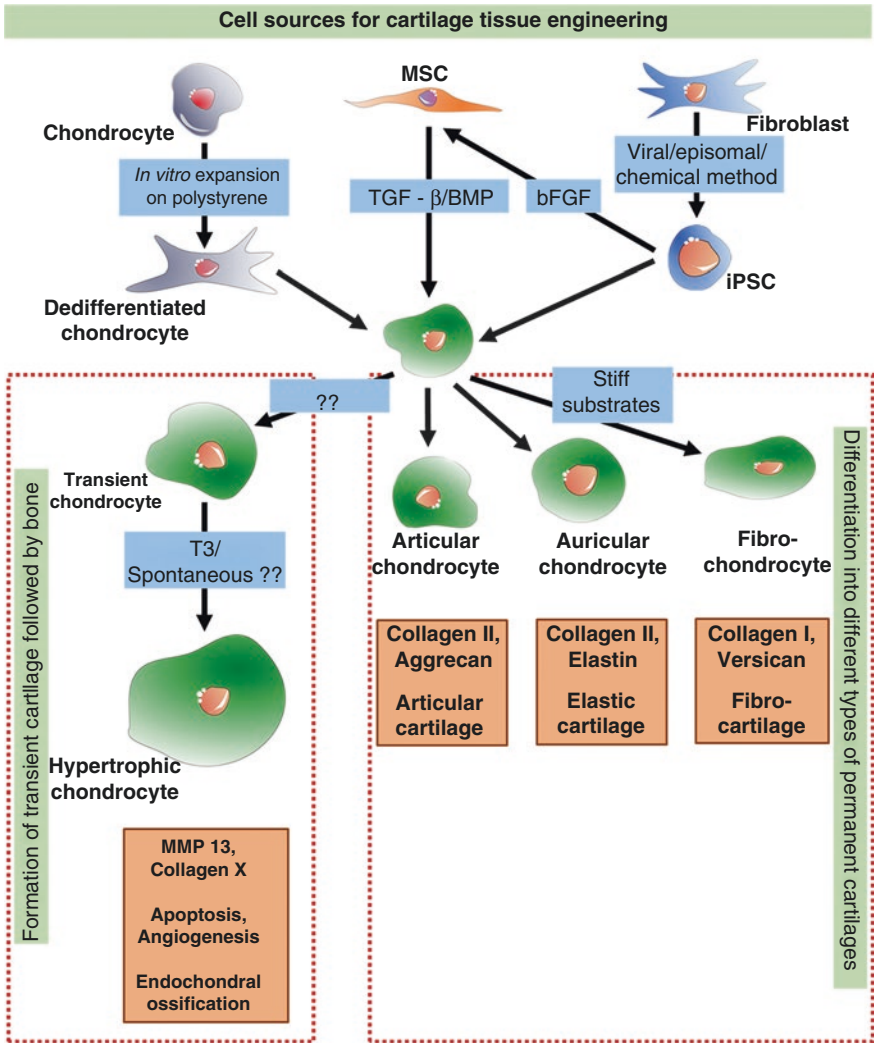
Fig. 14.3 Schematic depicting progression of progenitor cell types into chondrogenic lineage and their potential to differentiate into articular chondrocyte, fibro-chondrocyte, auricular chondrocytes, or hypertrophic chondrocytes

better chondrogenesis as compared to the same scaffolds seeded with MSCs. Similarly, in another study, Wolf et al. [36] used pre-aggregated human articular chondrocytes seeded on scaffolds for in vitro chondrogenesis. The results from this study indicated that seeding of pre-aggregated human articular chondrocytes on porous scaffold improved the quality of regenerated cartilage.

Other autologous chondrocyte sources like auricular, nasoseptal, and costal cartilage have also been investigated for cartilage repair and regeneration; however,

structural and functional differences between chondrocytes from different sources are markedly evident. Isogai et al. [37] have compared nasoseptal, costal, auricular, and articular chondrocytes, and they observed that costal chondrocytes expressed highest levels of collagen II and aggrecan compared to the other groups. Further, it is important to note that the availability of chondrocytes is scarce and that the resection of large amount of cartilage tissue may lead to donor site morbidity. Moreover, chondrocytes have limited expansion capacity and a tendency to dedifferentiate upon in vitro expansion. Taken together, these factors significantly reduce the translational utility of chondrocytes for cartilage regeneration.

14.2.2.2   Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) as an alternative cell source for cartilage regeneration have gained interest in recent years because they play a crucial role in homeostasis and regeneration of tissues. Moreover, they have very high expansion capacity, can differentiate into different lineages in vitro under appropriate stimuli, and are relatively more abundant than chondrocytes [38]. Different sources of MSCs have been explored in recent years for regeneration of cartilage which include but are not limited to bone marrow stem cells (BMSCs) and adipose-derived stem cells (ADSCs).

Bone Marrow-Derived Mesenchymal Stem Cells

BMSCs can be easily isolated and induced to chondrogenic differentiation in vitro using TGFβ supplementation. In past couple of decades or so, large numbers of studies have been performed in order to investigate the effect of BMSCs in chondrogenic differentiation and subsequent cartilage repair. Williams et al. [39] studied in vitro chondrogenesis of BMSCs by encapsulating the cells in a photopolymerizable hydrogel. This study demonstrated the ability of encapsulated MSCs to form cartilage-like tissues in vitro. In another study, BMSCs from osteoarthritic patients were seeded onto polyglycolic acid scaffolds in presence of TGFβ which led to extensive cartilaginous matrix deposition and hyaline cartilage-like tissue formation [40]. The major limitation of using BMSCs for cartilage tissue engineering is inferior mechanical properties of regenerated tissue and at times poor matrix deposition [41]. One of the possible explanations for this finding may be the high expression of hypertrophic markers during chondrogenic differentiation of BMSCs. Further, it has also been speculated that initial phase of in vitro chondrogenic induction of MSCs mimics endochondral ossification pathway leading to formation of mechanically inferior cartilage. Hence, more sophisticated approaches with better understanding of molecular events involved in chondrogenic differentiation and maintenance of chondrocyte phenotypes are probably required to regenerate hyaline cartilage using BMSCs.

Adipose-Derived Mesenchymal Stem Cells

ADSCs are emerging as an alternative to BMSCs in cartilage regeneration because they are relatively more abundant, can be isolated and expanded more rapidly, and possess a stable undifferentiated status. In recent years multiple studies have been performed to explore the potential of ADSCs in cartilage regeneration. In one such study,

Zheng et al. [42] used self-assembled peptide scaffolds to demonstrate in vivo chondrogenesis of ADSCs under the influence of recombinant fusion protein LAP-MMP-mTGFβ3 using lentiviral vectors in nude mice. The results from this study demonstrated that controlled release of TGFβ3 from peptide scaffolds facilitated chondrogenic differentiation of ADSCs in vivo. In another study, Kang et al. [43] demonstrated in vivo cartilage repair in a rabbit model using autologous ADSC-loaded decellularized ECM scaffolds. For this, 4 mm defects were created on patellar grooves of femur of both knees in a rabbit and implanted with cell-loaded scaffolds. The study demonstrated that cell-loaded decellularized ECM scaffolds led to cartilage repair that was comparable to native cartilage. However, for successful use of ADSCs in clinical practice, several key points need to be studied, including studies in large animal models and long-term safety and tumorigenicity studies [38].

Pluripotent Stem Cells

Apart from MSCs, various pluripotent stem cells like embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are also being investigated for cartilage tissue engineering. iPSCs have emerged as an exciting alternative to adult stem cells, as in this approach a small number of somatic cells can be used to generate a highly proliferative pluripotent cell population with high chondrogenic potential. In a study to demonstrate chondrogenic potential of iPSCs, Diekman et al. [44] used an in vitro cartilage defect model with chondrogenic pellet culture and showed that iPSCs synthesize cartilage-specific matrix with homogeneous matrix deposition. In another study, Ko et al. [45] compared chondrogenic ability of human iPSCs (hiPSCs) and human BMSCs under in vitro conditions and observed that hiPSCs support greater sGAG deposition and histologically closer cartilage formation with lacunae and abundant matrix formation. Further, when these hiPSCs were implanted in osteochondral defects, they showed significantly higher quality of cartilage repair as compared to BMSC controls indicating the potential of iPSCs for in vivo cartilage repair. Apart from iPSCs, ESCs have also been investigated for cartilage repair and regeneration; however, the use of ESCs is highly debated because of the ethical issues related to the source of ESCs.

While the use of pluripotent stem cells for cartilage tissue engineering seems highly promising because of their ability to recapitulate native cartilage-like phenotype, challenges like efficiency of iPSC production and safety concerns need to be addressed before their successful use in the clinic [46].

14.2.2.3   Coculture of Two or More Cell Types

Various strategies have been explored in the past decade or so to overcome limitations of chondrocytes and MSCs for achieving better chondrogenesis. Coculture of chondrocytes and MSCs is one such strategy that can overcome the disadvantages of chondrocyte/MSC monoculture for *neo*cartilage generation. In coculture systems, chondrocytes provide chondro-inducive signals to direct differentiation of MSCs into chondrocytes; on the other hand, MSCs secrete cytokines to facilitate proliferation of chondrocytes [47]. Moreover, it has been shown that chondrocyte-MSC coculture leads to reduction of hypertrophy [47] and calcification [48].

In recent years several studies have been directed toward exploring chondrocyte-MSC coculture for cartilage tissue engineering. In one such study, Yang et al. [49] tried to understand chondrocyte-driven differentiation of MSCs into chondrocytes using coculture. Coculture of juvenile chondrocytes with MSCs in vitro resulted in *neo*cartilage with cell morphology and behavior closer to articular chondrocytes and generated mechanically and structurally more robust *neo*cartilage than only chondrocyte-laden constructs when cultured in 3D agarose system. Similarly, in another study, chondrocytes and BMSCs encapsulated in a photo-cross-linked hydrogel implanted in a full-thickness defect in a rabbit knee resulted in the formation of hyaline cartilage with properties similar to native cartilage [50]. Apart from MSC-chondrocyte coculture, MSCs have also been cocultured with chondrons that resulted in better cartilage regeneration as compared to microfacture treatment in a goat model [51].

14.2.3 Growth Factors in Cartilage Tissue Engineering

Growth factors are highly potent biomolecules that regulate a variety of cellular processes like cell proliferation, migration, and differentiation. During development, different growth factors act in a spatiotemporal manner to bring about chondrogenesis and cartilage formation. Several growth factors play a crucial role in the maintenance of cartilage as well. Therefore, delivery of appropriate growth factor at the site of damage is a promising approach for cartilage tissue engineering. Anabolic growth factors mediating cartilage development and homeostasis stimulate synthesis of ECM components like proteoglycans and collagen II and facilitate MSC proliferation and differentiation toward chondrogenic lineage. These growth factors also play vital role in the reduction of catabolic activity of pro-inflammatory cytokines. Over the past few decades, various growth factors either alone or in combinations have been extensively investigated for regeneration of cartilage. These include different TGFβ and BMP subtypes, insulin-like growth factor (IGF), fibroblast growth factor (FGF), and platelet-rich plasma (PRP), each of which will be discussed in detail in the following sections.

14.2.3.1   Growth Factors

TGFβ
Several members of TGFβ superfamily are commonly explored for cartilage tissue engineering which mainly include TGFβ1 and TGFβ3. These factors are known to enhance anabolic activity of chondrocytes, maintain chondrocyte phenotype, and promote redifferentiation of cultured chondrocytes. Apart from their beneficial effect on chondrocytes, TGFβ isoforms also enhance MSC proliferation and their differentiation into chondrogenic lineage. Several studies in recent years have investigated the role of various isoforms of TGFβ in cartilage repair and regeneration in animal models. In one of the studies, TGFβ1 encapsulated in alginate beads was delivered to rabbit knee defects in order to investigate cartilage repair and regeneration. The

results from this study demonstrated that encapsulation of TGFβ in alginate beads resulted in its sustained release, without showing systemic side effects, leading to enhanced repair of cartilage defects [52]. In another study, hMSCs delivered through TGFβ3-loaded scaffolds in mouse and rabbit cartilage defects led to enhanced repair of cartilage defects with better quality of repaired cartilage [53]. In addition to the use of purified recombinant TGFβ, retrovirally transduced human chondrocytes expressing TGFβ1 have also been employed to investigate cartilage repair [54].

BMP

Bone morphogenetic proteins (BMP) belong to the TGF superfamily of proteins, and several of its isoforms have been shown to have strong chondrogenic potential. BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8, BMP-9, BMP-12, and BMP-14 have all been shown to have chondrogenic activity either in vitro or in vivo [55]. They have been shown to have activities varying from proliferative and pro-matrix deposition in chondrocytes to pro-chondrogenic effect in MSCs and fibroblasts [55]. Yang et al. [56] demonstrated that long-term delivery of BMP2 in conjunction with microfracture-based treatment of cartilage defects resulted in hyaline cartilage regeneration. In another study, Jung et al. [57] provided controlled release of BMP-7 from PLGA scaffolds and demonstrated successful regeneration of osteochondral defects. PLGA scaffolds and BMP-7 collectively provided best regeneration as compared to scaffolds alone or untreated group. Moreover, several different combinations of BMP with other growth factors have shown high success in enabling cartilage regeneration.

IGF

Insulin-like growth factor-1 (IGF-1) is an anabolic growth factor having similar protein sequence as that of insulin and is known to play a key role in cartilage homeostasis and maintenance of chondrocyte metabolism. IGF-1 is also known to reduce synovial inflammation thus decreasing catabolic responses in articular cartilage [58]. The level of free IGF-1 available for receptor mediated chondrogenic response is regulated by IGF-1-binding proteins present in synovial fluid which sequester free IGF-1. Furthermore, IGF-1 regulates chondrocyte proliferation and is responsible for synthesis of collagen II and proteoglycans. Many studies have investigated the effect of IGF-1 on the repair and regeneration of cartilage defects. In one such study, Longobardi et al. [59] demonstrated that IGF-1 enhanced the chondrogenic potential of mouse MSCs independent of TGFβ1. Another recent study in a rabbit defect model demonstrated that engineered cartilage constructs containing chondrocytes overexpressing IGF-1 gene when implanted in vivo markedly improved osteochondral defect repair along with reduction in cartilage damage at the adjacent sites [60].

FGF

Fibroblast growth factor (FGF) is a heparin-binding family of growth factors mainly responsible for proliferation and differentiation of many cell types. Among these, FGF-2 is known to have a potent role in maintaining homeostasis and anabolic

reactions in articular cartilage. In the past few years, many studies have investigated the effect of FGF-2 on chondrogenic differentiation potential of MSCs. In one such study, hMSCs supplemented with FGF-2 demonstrated enhanced proliferation and better chondrogenic phenotype as compared to the cells without FGF-2 treatment in vitro [61]. Similarly, Ishii et al. [62] demonstrated that delivery of FGF-2 via fibrin clots promoted the regeneration of articular cartilage and accompanying subchondral bone in full-thickness osteochondral defects in rabbit models. Contrastingly, a study by Im et al. [63] demonstrated that FGF-2 induces MMP-13 expression by human articular chondrocytes causing cartilage matrix degradation. In addition, FGF-2 is also associated with upregulation of aggrecans and has an antagonistic effect on proteoglycan synthesis [64]. Therefore, the role of FGF-2 in cartilage regeneration is not very clear, and more investigations are needed for better understanding of the same.

PRP

Platelet-rich plasma (PRP) is the autologous plasma sample with enriched platelet concentration and is regarded as platelet concentrate. PRP is known to regulate cartilage homeostasis and repair. In addition, PRP stimulates reduction in catabolic response and inflammatory cytokines in cartilage [65]. PRP consists of growth factors including PDGF, VEGF, TGFβ, EGF, and many bioactive proteins. PRP-based cartilage repair relies on the concept that when platelet concentrate is injected at the defect site, it forms a clot and allows stem cells to infiltrate into it leading to tissue repair when exposed to growth factors [66, 67]. Mishra et al. [68] studied the potential of PRP in enhancing MSC proliferation and its chondrogenic differentiation when added with media. An in vivo study by Sun et al. [69] assessed effect of PRP on repair of cartilage defects created in rabbit model. PRP with PLGA as a carrier when delivered at the defect site restored the damaged cartilage with promising mechanical properties.

Combinations of Growth Factors

Chondrogenic development is a very complex process which requires interplay between different biochemical signaling pathways. Several growth factors like TGFβ, BMP, IGF, FGF, etc., play a crucial role to bring about chondrogenesis [70]. Therefore, it is less likely that delivery of single growth factor will be able to recapitulate functions of all these factors necessary for cartilage regeneration. Thus, sequential or simultaneous delivery of multiple growth factors is considered to be a more rational approach for repair and regeneration of cartilage defects. There have been quite a few studies directed toward delivery of multiple growth factors for cartilage regeneration. In one such study, Park et al. [71] delivered TGFβ1 and IGF-1 in combination along with MSCs which led to higher expression of chondrogenic markers after 14 days of in vitro culture. Although TGFβ1 and IGF-1 worked well in in vitro culture, study by Holland et al. [72] demonstrated that co-delivery of TGFβ1 and IGF-1 does not have any additional benefit for cartilage repair in vivo. Other combinations of growth factors that are being investigated for cartilage regeneration include TGFβ with BMP-7, TGFβ with parathyroid hormone, and IGF with BMP-7 and FGF.

14.2.3.2   Controlled Release of Growth Factors for Cartilage Tissue Engineering

Multiple growth factors, as discussed in the above sections, have shown high success in enabling differentiation of progenitor cells into chondrogenic phenotype and thus generating *neo*cartilage in vitro. However, translation of these growth factors into a regenerative therapy necessitates long-term presentation of these factors to cells at the site of injury. The fact that long-term presentation of chondrogenic growth factors like TGFβ is necessary was demonstrated in a study by Kim et al. [73]. They demonstrated that continuous exposure of TGFβ to MSCs was necessary for cartilage-like intense collagen II and sGAG deposition. Shorter durations even up to 10 days only led to compromised cartilaginous matrix deposition.

Bolus delivery of growth factors such as intra-articular injections leads to rapid diffusion of growth factor into off-target sites which may result in the need of multiple injections making the regimen significantly invasive and expensive. Moreover, loose growth factors are associated with poor proteolytic stability and off-target side effects. Collectively these factors give rise to the need of developing growth factor delivery systems which not only prolong growth factor presentation to cells but also prevent their proteolytic degradation and reduce off-target effects. Based on these needs, a repertoire of growth factor delivery systems (Fig. 14.4) has been developed for cartilage tissue engineering; these include those based on (1) physical encapsulation, (2) ionic complexation and affinity binding, (3) covalent binding, and (4) gene delivery.
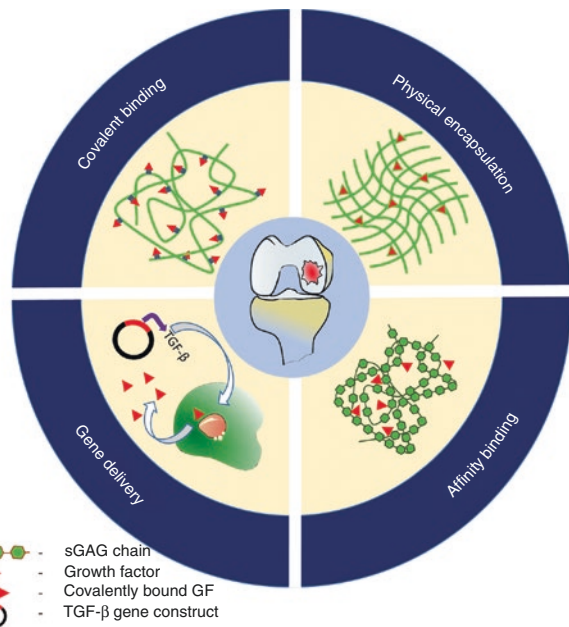
Fig. 14.4 Schematic depicting different strategies for the delivery of growth factors in articular cartilage defects

Physical Encapsulation

During physical encapsulation of growth factors, the factors of interest are mixed with a carrier material (e.g., synthetic/natural polymer solutions) before gelation/drying/cross-linking. Growth factors encapsulated physically show a slow release profile where the release kinetics is determined by diffusion of the factors and degradation kinetics of the carrier material. The release kinetics of encapsulated growth factors can be modulated by altering the size and geometry of carrier device, cross-linking density, and material properties (molecular weight, susceptibility/rate degradation, mode of degradation, and swelling properties). Growth factors can be incorporated in scaffolds using a wide variety of fabrication techniques where harsh fabrication conditions are not utilized, such as solvent casting and particulate leaching, freeze-drying, electrospinning, emulsion solvent evaporation, in situ polymerization, and gas foaming. For tissue engineering strategies, the growth factors may be incorporated directly into scaffolds or may be incorporated indirectly such that the scaffolds are loaded with growth factors encapsulated in microspheres. Kopesky et al. [74] used the former approach to incorporate TGFβ in self-assembling peptide-based hydrogels. These hydrogels allowed a sustained release of TGFβ for 21 days and thereby led to improved cell proliferation and cartilaginous matrix deposition. In another study, the latter approach was used—where IGF-1-loaded PLGA microspheres were incorporated in PVA hydrogels. This system allowed a controlled release of the factor for 6 weeks, thereby enabling significantly better cartilage formation as compared to blank hydrogels. The *neo*cartilage tissue in IGF-1-loaded hydrogels not only showed better matrix deposition but also significantly better mechanical properties [75].

A recent advancement in physical encapsulation of growth factors is in the area of stimulus responsive growth factor release. These systems utilize polymers that either swell reversibly (increasing pore size) or irreversibly break down in response to a stimulus such as heat, pH, ionic concentration, light, presence of enzymes, etc., to release encapsulated molecules only when stimulated. One example of these systems is use of MMP-sensitive peptides as cross-linkers in synthetic hydrogels. In such a system, the molecules encapsulated in the hydrogel are released whenever there is increased presence of MMPs. Though multiple of these systems have been utilized in other tissue regeneration applications, not much work has been done in the area of cartilage tissue engineering using these systems.

Ionic Complexation and Affinity Binding

It is interesting to note that native cartilage tissue sequesters large amounts of active growth factors and this phenomenon is a result of the strong ionic interactions between heavily sulfated GAGs like heparan sulfate and the highly basic growth factors. Taking inspiration from this study, ionic- and affinity-based interactions between basic growth factors and natural or synthetic GAGs for their controlled presentation have been examined. It has been shown that this interaction between growth factor and GAGs not only restricts spatial localization of growth factors but

also potentiates their activity and improves their proteolytic stability. For example, it was recently demonstrated that exogenous heparan sulfate promotes TGFβ3-mediated chondrogenic differentiation of mesenchymal stem cells via improved TGFβ/Smad2/3 signaling [76]. A recent study by Jha et al. [77] demonstrated the possibility of controlled release of TGFβ from heparin-containing hyaluronic acid hydrogels. They demonstrated that the rate of release of TGFβ from these gels was inversely correlated with the molecular weight and concentration of heparin. Taking inspiration from heparin, Re'em et al. [78] synthesized alginate sulfate as its mimic and demonstrated controlled release of TGFβ from macroporous alginate scaffolds containing alginate sulfate. Unlike control alginate scaffolds which released 90% growth factor in 24 h, alginate sulfate containing hydrogels provided controlled release for over 7 days. Moreover, it was shown that this led to Smad2 activation in MSCs for 14 days and high deposition of collagen type II. Apart from heparin and heparin analogs, cartilage-derived matrices which are extremely rich in a variety of sulfated GAGs have also been utilized for prolonged presentation of TGFβ to cells during chondrogenic differentiation [79].

Covalent Binding

Covalent conjugation of growth factors to scaffolds has also been proposed as a strategy for prolonged presentation of growth factors to cells. In addition to prolong the presentation, this method may provide an ability to precisely control the spatial distribution, density, and amount of growth factors in the matrix. In a recent work, Sridhar et al. [80] used thiol-ene chemistry to covalently conjugate TGFβ1 to PEG for long-term stimulation of chondrocytes. They demonstrated that the conjugated TGFβ1 not only retained its bioactivity but also performed significantly better over soluble factor in terms of maintaining chondrocytic phenotype of cells. In another study, Bertolo et al. [81] conjugated FGF and TGFβ on collagen microcarriers for improving expansion and chondrogenic differentiation of MSCs, respectively. They compared two conjugation chemistries for this purpose, and it was observed that while EDC/NHS seemed to be best suited for FGF conjugation, riboflavin/UV proved to be more desirable for the conjugation of TGFβ. While some success has been achieved in covalent binding of growth factors to scaffolds, a larger set of studies need to be performed to identify suitable chemistries for conjugation of growth factors to tissue engineering scaffolds. Also in 3D culture the fact that covalently bound factor cannot diffuse may act as a disadvantage as this factor will be accessible only to the cells in proximity and not to other cells that are not directly interacting with the scaffold surface.

Gene Delivery

The use of therapeutic proteins including growth factors is associated with certain limitations which include high cost of production, poor in vivo stability, and the need to maintain the final product in a cold chain. To circumvent these issues in tissue engineering strategies, growth factor gene delivery has been proposed as an alternate to growth factor protein delivery. Both viral and nonviral gene delivery methods are being pursued for this purpose. While viral vectors such as adeno, adeno-associated,

retro-, and lentiviral vectors show high transfection efficiency, safety issues associated with them remain a major hindrance for their translation to the clinic. In contrast, nonviral vectors are generally regarded to be safe but show significantly lower transfection efficiencies.

Brunger et al. [82] used lentiviral system to deliver gene encoding for TGFβ3 to mesenchymal stem cells. They immobilized lentivirus on poly-L-lysine-coated polycaprolactone scaffolds and demonstrated that these viruses could effectively transduce MSCs seeded on the scaffolds leading to high expression of TGFβ3. This in turn led to improved chondrogenic differentiation of MSCs and cartilaginous matrix deposition. In another study, Tomas et al. [83] used nano-hydroxyapatite (nHA) as a carrier for plasmid DNA (pDNA) encoding for TGFβ3 and BMP-2. The authors demonstrated that pDNA complexed with nHA when encapsulated in alginate gels along with MSCs transfected the MSCs much more efficiently as compared to free pDNA. Finally, they observed efficient chondrogenesis and matrix production in groups where pDNA for both TGFβ3 and BMP-2 were delivered by complexation with nHA. An alternative to gene delivery through scaffolds is the use of cells which are already transfected with the gene of interest. He et al. [84] used such an approach where MSCs were transfected with pDNA coding for TGFβ using pullulan spermine as the transfection reagent. These cells were then implanted in osteochondral defects using gelatin sponges. The results of this study demonstrated that transfected MSCs significantly outperformed control MSCs in terms of osteochondral regeneration.

Conclusions

In the past several decades many studies, including basic and translational, have been conducted with the objective of generating functional tissue-engineered cartilage. Though these studies have enhanced our understanding about the role of scaffolds, cells, and growth factors in cartilage regeneration and repair, there has been only partial success in terms of regenerating complex cartilage structure with high strength and mechanical properties in vivo. While a variety of scaffold-free approaches and scaffolds including isotropic and anisotropic sponges, fibers, and injectable hydrogels have shown success in hyaline cartilage regeneration, porous sponges and injectable hydrogels stand out as the most promising options. Among the different cell sources, MSCs and MSCs cocultured with chondrocytes seem to possess high translational applicability for cartilage regeneration. Furthermore, the optimal combination of growth factors to provide cells with cues that could recapitulate the developmental process of chondrocytes from MSCs still needs to be studied. Although significant progress has been taken place independently in identifying the best suited scaffold/cell/growth factor, ideal combinations of these need to be developed before successful translation of regenerative therapies to clinic. In addition, it is important to select approaches which can not only overcome bottlenecks of existing approaches but also integrate seamlessly with the current healthcare setup to make them clinically successful.

References

1. Mankin HJ, Mow VC, Buckwalter JA, et al. Articular cartilage structure, composition and function. In Buckwalter JA, Einhorn TA, Simon SR, editors. Orthopaedic basic science. 2nd ed. American Academy of Orthopaedic Surgeons (AAOS): 2000. p. 443–70.
2. Mollon B, Kandel R, Chahal J, et al. The clinical status of cartilage tissue regeneration in humans. Osteoarthr Cartil. 2013;21:1824–33.
3. Chung C, Burdick JA. Engineering cartilage tissue. Adv Drug Deliv Rev. 2008;60:243–62.
4. Bian L, Hou L, Tous E, et al. The influence of hyaluronic acid hydrogel crosslinking density and macromolecular diffusivity on human MSC chondrogenesis and hypertrophy. Biomaterials. 2013;34:413–21.
5. Feng Q, Zhu M, Wei K, et al. Cell-mediated degradation regulates human mesenchymal stem cell chondrogenesis and hypertrophy in MMP-sensitive hyaluronic acid hydrogels. PLoS One. 2014;9(6):e99587.
6. Stenhamre H, Nannmark U, Lindahl A, et al. Influence of pore size on the redifferentiation potential of human articular chondrocytes in poly (urethane urea) scaffolds. J Tissue Eng Regen Med. 2011;5:578–88.
7. Matsiko A, Gleeson JP, O'Brien FJ. Scaffold mean pore size influences mesenchymal stem cell chondrogenic differentiation and matrix deposition. Tissue Eng Part A. 2014;21:486–97.
8. Arora A, Kothari A, Katti DS. Pore orientation mediated control of mechanical behavior of scaffolds and its application in cartilage-mimetic scaffold design. J Mech Behav Biomed Mater. 2015;51:169–83.
9. Noriega SE, Hasanova GI, Schneider MJ, et al. Effect of fiber diameter on the spreading, proliferation and differentiation of chondrocytes on electrospun chitosan matrices. Cells Tissues Organs. 2011;195:207–21.
10. Schneider T, Kohl B, Sauter T, et al. Influence of fiber orientation in electrospun polymer scaffolds on viability, adhesion and differentiation of articular chondrocytes. Clin Hemorheol Microcirc. 2012;52:325–36.
11. Kwon HJ, Yasuda K, Ohmiya Y, et al. In vitro differentiation of chondrogenic ATDC5 cells is enhanced by culturing on synthetic hydrogels with various charge densities. Acta Biomater. 2010;6:494–501.
12. Curran JM, Chen R, Hunt JA. The guidance of human mesenchymal stem cell differentiation in vitro by controlled modifications to the cell substrate. Biomaterials. 2006;27:4783–93.
13. Salinas CN, Cole BB, Kasko AM, et al. Chondrogenic differentiation potential of human mesenchymal stem cells photoencapsulated within poly (ethylene glycol)-arginine-glycine-aspartic acid-serine thiol-methacrylate mixed-mode networks. Tissue Eng. 2007;13:1025–34.
14. Jin R, Teixeira LSM, Dijkstra PJ, et al. Chondrogenesis in injectable enzymatically crosslinked heparin/dextran hydrogels. J Control Release. 2011;152:186–95.
15. Engler AJ, Sen S, Sweeney HL, et al. Matrix elasticity directs stem cell lineage specification. Cell. 2006;126:677–89.
16. Schuh E, Kramer J, Rohwedel J, et al. Effect of matrix elasticity on the maintenance of the chondrogenic phenotype. Tissue Eng Part A. 2010;16:1281–90.
17. Vickers SM, Squitieri LS, Spector M. Effects of cross-linking type II collagen-gag scaffolds on chondrogenesis in vitro: dynamic pore reduction promotes cartilage formation. Tissue Eng. 2006;12:1345–55.
18. Toh WS, Lim TC, Kurisawa M, et al. Modulation of mesenchymal stem cell chondrogenesis in a tunable hyaluronic acid hydrogel microenvironment. Biomaterials. 2012;33:3835–45.
19. Zhong W, Li Y, Li L, et al. YAP-mediated regulation of the chondrogenic phenotype in response to matrix elasticity. J Mol Histol. 2013;44:587–95.

20. Lum L, Elisseeff J. Injectable hydrogels for cartilage tissue engineering. In Ashammakhi N, Ferretti P, editors. Topics Tissue Eng. 2003;3:1–25.
21. Choi B, Kim S, Lin B, et al. Cartilaginous extracellular matrix-modified chitosan hydrogels for cartilage tissue engineering. ACS Appl Mater Interfaces. 2014;6:20110–21.
22. Lin H, Cheng AWM, Alexander PG, et al. Cartilage tissue engineering application of injectable gelatin hydrogel with in situ visible-light-activated gelation capability in both air and aqueous solution. Tissue Eng Part A. 2014;20:2402–11.
23. Tekin H, Sanchez JG, Tsinman T, et al. Thermoresponsive platforms for tissue engineering and regenerative medicine. AICHE J. 2011;57:3249–58.
24. Shive MS, Hoemann CD, Restrepo A, et al. BST-CarGel: in situ chondroinduction for cartilage repair. Oper Tech Orthop. 2006;16:271–8.
25. Ren CD, Kurisawa M, Chung JE, et al. Liposomal delivery of horseradish peroxidase for thermally triggered injectable hyaluronic acid–tyramine hydrogel scaffolds. J Mater Chem B. 2015;3:4663–70.
26. Teixeira LSM, Feijen J, van Blitterswijk CA, et al. Enzyme-catalyzed crosslinkable hydrogels: emerging strategies for tissue engineering. Biomaterials. 2012;33:1281–90.
27. Zhang Y, Fan Z, Xu C, et al. Tough biohydrogels with interpenetrating network structure by bienzymatic crosslinking approach. Eur Polym J. 2015;72:717–25.
28. Jin R, Lin C, Cao A. Enzyme-mediated fast injectable hydrogels based on chitosan–glycolic acid/tyrosine: preparation, characterization, and chondrocyte culture. Polym Chem. 2014; 5:391–8.
29. Cao L, Cao B, Lu C, et al. An injectable hydrogel formed by in situ cross-linking of glycol chitosan and multi-benzaldehyde functionalized PEG analogues for cartilage tissue engineering. J Mater Chem B. 2015;3:1268–80.
30. Takahashi A, Suzuki Y, Suhara T, et al. In situ cross-linkable hydrogel of hyaluronan produced via copper-free click chemistry. Biomacromolecules. 2013;14:3581–8.
31. Yu F, Cao X, Li Y, et al. Diels–alder crosslinked HA/PEG hydrogels with high elasticity and fatigue resistance for cell encapsulation and articular cartilage tissue repair. Polym Chem. 2014;5:5116–23.
32. DuRaine GD, Brown WE, Hu JC, et al. Emergence of scaffold-free approaches for tissue engineering musculoskeletal cartilages. Ann Biomed Eng. 2015;43:543–54.
33. Bhumiratana S, Eton RE, Oungoulian SR, et al. Large, stratified, and mechanically functional human cartilage grown in vitro by mesenchymal condensation. Proc Natl Acad Sci U S A. 2014;111:6940–5.
34. Ofek G, Revell CM, Hu JC, et al. Matrix development in self-assembly of articular cartilage. PLoS One. 2008;3:e2795.
35. Wang Y, Blasioli DJ, Kim HJ, et al. Cartilage tissue engineering with silk scaffolds and human articular chondrocytes. Biomaterials. 2006;27:4434–42.
36. Wolf F, Candrian C, Wendt D, et al. Cartilage tissue engineering using pre-aggregated human articular chondrocytes. Eur Cell Mater. 2008;16:92–9.
37. Isogai N, Kusuhara H, Ikada Y, et al. Comparison of different chondrocytes for use in tissue engineering of cartilage model structures. Tissue Eng. 2006;12:691–703.
38. Veronesi F, Maglio M, Tschon M, et al. Adipose-derived mesenchymal stem cells for cartilage tissue engineering: state-of-the-art in in vivo studies. J Biomed Mater Res A. 2014;102:2448–66.
39. Williams CG, Kim TK, Taboas A, et al. In vitro chondrogenesis of bone marrow-derived mesenchymal stem cells in a photopolymerizing hydrogel. Tissue Eng. 2003;9:679–88.
40. Kafienah W, Mistry S, Dickinson SC, et al. Three-dimensional cartilage tissue engineering using adult stem cells from osteoarthritis patients. Arthritis Rheum. 2007;56:177–87.
41. Kock L, van Donkelaar CC, Ito K. Tissue engineering of functional articular cartilage: the current status. Cell Tissue Res. 2012;347:613–27.
42. Zheng D, Dan Y, Yang SH, et al. Controlled chondrogenesis from adipose-derived stem cells by recombinant transforming growth factor-β3 fusion protein in peptide scaffolds. Acta Biomater. 2015;11:191–203.

43. Kang H, Peng J, Lu S, et al. In vivo cartilage repair using adipose-derived stem cell-loaded decellularized cartilage ECM scaffolds. J Tissue Eng Regen Med. 2014;8:442–53.

44. Diekman BO, Christoforou N, Willard VP, et al. Cartilage tissue engineering using differentiated and purified induced pluripotent stem cells. Proc Nat Acad Sci U S A. 2012;109:19172–7.

45. Ko JY, Kim KI, Park S, et al. In vitro chondrogenesis and in vivo repair of osteochondral defect with human induced pluripotent stem cells. Biomaterials. 2014;35:3571–81.

46. Bernhard JC, Vunjak-Novakovic G. Should we use cells, biomaterials, or tissue engineering for cartilage regeneration? Stem Cell Res Ther. 2016;7:56.

47. Qing C, Wei-ding C, Wei-min F. Co-culture of chondrocytes and bone marrow mesenchymal stem cells in vitro enhances the expression of cartilaginous extracellular matrix components. Braz J Med Biol Res. 2011;44:303–10.

48. Fischer J, Dickhut A, Rickert M, et al. Human articular chondrocytes secrete parathyroid hormone-related protein and inhibit hypertrophy of mesenchymal stem cells in coculture during chondrogenesis. Arthritis Rheum. 2010;62:2696–706.

49. Yang YH, Lee AJ, Barabino GA. Coculture-driven mesenchymal stem cell-differentiated articular chondrocyte-like cells support neocartilage development. Stem Cells Transl Med. 2012;1:843–54.

50. Ko CY, Ku KL, Yang SR, et al. In vitro and in vivo co-culture of chondrocytes and bone marrow stem cells in photocrosslinked PCL–PEG–PCL hydrogels enhances cartilage formation. J Tissue Eng Regen Med. 2016;10(10):E485–96.

51. Bekkers JE, Tsuchida AI, van Rijen MH, et al. Single-stage cell-based cartilage regeneration using a combination of chondrons and mesenchymal stromal cells comparison with microfracture. Am J Sports Med. 2013;41(9):2158–66. doi:10.1177/0363546513494181.

52. Mierisch CM, Cohen CB, Jordan LC, et al. Transforming growth factor-β in calcium alginate beads for the treatment of articular cartilage defects in the rabbit. Arthroscopy. 2002;18:892–900.

53. Park JS, Woo DG, Yang HN, et al. Chondrogenesis of human mesenchymal stem cells encapsulated in a hydrogel construct: neocartilage formation in animal models as both mice and rabbits. J Biomed Mater Res A. 2010;92:988–96.

54. Noh MJ, Copeland RO, Yi Y, et al. Pre-clinical studies of retrovirally transduced human chondrocytes expressing transforming growth factor-beta-1 (TGF-beta1). Cytotherapy. 2010;12:384–93.

55. Miljkovic N, Cooper G, Marra K. Chondrogenesis, bone morphogenetic protein-4 and mesenchymal stem cells. Osteoarthr Cartil. 2008;16:1121–30.

56. Yang HS, La WG, Bhang SH, et al. Hyaline cartilage regeneration by combined therapy of microfracture and long-term bone morphogenetic protein-2 delivery. Tissue Eng Part A. 2011;17:1809–18.

57. Jung MR, Shim IK, Chung HJ, et al. Local BMP-7 release from a PLGA scaffolding-matrix for the repair of osteochondral defects in rabbits. J Control Release. 2012;162:485–91.

58. Schmidt M, Chen E, Lynch S. A review of the effects of insulin-like growth factor and platelet derived growth factor on in vivo cartilage healing and repair. Osteoarthr Cartil. 2006;14:403–12.

59. Longobardi L, O'Rear L, Aakula S, et al. Effect of IGF-1 in the chondrogenesis of bone marrow mesenchymal stem cells in the presence or absence of TGF-β signaling. J Bone Miner Res. 2006;21:626–36.

60. Madry H, Kaul G, Zurakowski D, et al. Cartilage constructs engineered from chondrocytes overexpressing IGF-1 improve the repair of osteochondral defects in a rabbit model. Eur Cell Mater. 2013;25:229.

61. Solchaga LA, Penick K, Goldberg VM, et al. Fibroblast growth factor-2 enhances proliferation and delays loss of chondrogenic potential in human adult bone-marrow-derived mesenchymal stem cells. Tissue Eng A. 2009;16:1009–19.

62. Ishii I, Mizuta H, Sei A, et al. Healing of full-thickness defects of the articular cartilage in rabbits using fibroblast growth factor-2 and a fibrin sealant. J Bone Joint Surg. 2007;89:693–700.

63. Im HJ, Muddasani P, Natarajan V, et al. Basic fibroblast growth factor stimulates matrix metalloproteinase-13 via the molecular cross-talk between the mitogen-activated protein kinases and protein kinase cδ pathways in human adult articular chondrocytes. J Biol Chem. 2007;282:11110–21.

64. Ellman MB, An HS, Muddasani P, et al. Biological impact of the fibroblast growth family on articular cartilage and intervertebral disc homeostasis. Gene. 2008;420:82–9.
65. Sundman EA, Cole BJ, Karas V, et al. The anti-inflammatory and matrix restorative mechanisms of platelet-rich plasma in osteoarthritis. Am J Sports Med. 2014;42:35–41.
66. Foster TE, Puskas BL, Mandelbaum BR, et al. Platelet-rich plasma from basic science to clinical applications. Am J Sports Med. 2009;37:2259–72.
67. Marx RE. Platelet-rich plasma: evidence to support its use. J Oral Maxillofac Surg. 2004;62:489–96.
68. Mishra A, Tummala P, King A, et al. Buffered platelet-rich plasma enhances mesenchymal stem cell proliferation and chondrogenic differentiation. Tissue Eng Part C. 2009;15:431–5.
69. Sun Y, Feng Y, Zhang C, et al. The regenerative effect of platelet-rich plasma on healing in large osteochondral defects. Int Orthop. 2010;34:589–97.
70. Lam J, Lu S, Kasper FK, et al. Strategies for controlled delivery of biologics for cartilage repair. Adv Drug Deliv Rev. 2015;84:123–34.
71. Park H, Temenoff JS, Tabata Y, et al. Effect of dual growth factor delivery on chondrogenic differentiation of rabbit marrow mesenchymal stem cells encapsulated in injectable hydrogel composites. J Biomed Mater Res A. 2009;88:889–97.
72. Holland T, Bodde T, Cuijpers V, et al. Degradable hydrogel scaffolds for in vivo delivery of single and dual growth factors in cartilage repair. Osteoarthr Cartil. 2007;15:187–97.
73. Kim HJ, Kim YJ, Im GI. Is continuous treatment with transforming growth factor-beta necessary to induce chondrogenic differentiation in mesenchymal stem cells? Cells Tissues Organs. 2008;190:1–10.
74. Kopesky PW, Byun S, Vanderploeg EJ, et al. Sustained delivery of bioactive TGF-β1 from self-assembling peptide hydrogels induces chondrogenesis of encapsulated bone marrow stromal cells. J Biomed Mater Res A. 2014;102:1275–85.
75. Spiller KL, Liu Y, Holloway JL, et al. A novel method for the direct fabrication of growth factor-loaded microspheres within porous nondegradable hydrogels: controlled release for cartilage tissue engineering. J Control Release. 2012;157:39–45.
76. Chen J, Wang Y, Chen C, et al. Exogenous heparan sulfate enhances the TGF-β 3-induced chondrogenesis in human mesenchymal stem cells by activating TGF-β/SMAD signaling. Stem Cells Int. 2015;2016
77. Jha AK, Mathur K, Svedlund FL, et al. Molecular weight and concentration of heparin in hyaluronic acid-based matrices modulates growth factor retention kinetics and stem cell fate. J Control Release. 2015;209:308–16.
78. Re'em T, Kaminer-Israeli Y, Ruvinov E, et al. Chondrogenesis of hMSC in affinity-bound TGF-beta scaffolds. Biomaterials. 2012;33:751–61.
79. Almeida HV, Cunniffe GM, Vinardell T, et al. Coupling freshly isolated CD44+ infrapatellar fat pad-derived stromal cells with a TGF-β3 eluting cartilage ECM-derived scaffold as a single-stage strategy for promoting chondrogenesis. Adv Healthc Mater. 2015;4:1043–53.
80. Sridhar BV, Doyle NR, Randolph MA, et al. Covalently tethered TGF-β1 with encapsulated chondrocytes in a peg hydrogel system enhances extracellular matrix production. J Biomed Mater Res A. 2014;102:4464–72.
81. Bertolo A, Arcolino F, Capossela S, et al. Growth factors cross-linked to collagen microcarriers promote expansion and chondrogenic differentiation of human mesenchymal stem cells. Tissue Eng Part A. 2015;21:2618–28.
82. Brunger JM, Huynh NP, Guenther CM, et al. Scaffold-mediated lentiviral transduction for functional tissue engineering of cartilage. Proc Natl Acad Sci U S A. 2014;111:E798–806.
83. Fernandez TG, Tierney EG, Cunniffe GM, et al. Gene delivery of TGF-β3 and BMP2 in an MSC-laden alginate hydrogel for articular cartilage and endochondral bone tissue engineering. Tissue Eng Part A. 2016;22:776–87.
84. He CX, Zhang TY, Miao PH, et al. TGF-β1 gene-engineered mesenchymal stem cells induce rat cartilage regeneration using nonviral gene vector. Biotechnol Appl Biochem. 2012;59:163–9.