Chapter 9
Cartilage Regeneration

Yuankun Dai and Changyou Gao

Abstract The treatment of damage to cartilage represents one of the most challenging clinical tasks due to the limited spontaneous healing and regenerative capability of cartilage. Clinically applied protocols for cartilage regeneration are still faced with various obstacles. The cartilage tissue engineering combines scaffolds, cells, and bioactive molecules, achieving cartilage engineering in vitro and cartilage regeneration in vivo. More recently, the controversy and difficulty in regulatory application of various cells and bioactive molecules gradually push forward the emergence of in situ inductive cartilage regeneration by recruiting endogenous regenerative cells. With these perspectives, we aim to present an overview of existing cartilage regeneration technologies with emphasis of recent progresses, development, and major steps taken toward the structure and functional regeneration of cartilage. In this chapter, essential elements of various protocols and their advantages and disadvantages and challenges and future perspectives of cartilage regeneration are discussed.

Keywords Tissue engineering · Cartilage regeneration · Scaffolds · Chondrocytes · Stem cells · In situ inductivity

9.1 Introduction

Articular cartilage is a highly developed connective tissue for weight-bearing and friction-reducing. Chondrocyte is the only type of cells in mature articular cartilage, occupying 1–10% of the tissue volume. Seventy to 80% of weight of articular cartilage is water. Collagen, proteoglycans, matrix glycoproteins, and small amount

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of elastin and phospholipids contribute the other 20–30% of the weight [1, 2]. Figure 9.1a shows the composition and structure of articular cartilage [3]. Cells and extracellular matrix (ECM) in cartilage distribute laterally in the superficial, randomly in the middle, and vertically in the deep layers of cartilage, respectively.

The avascular structure in the articular cartilage determines that the chondrocytes can only get nutrients from the synovial fluid [4]. After maturation of cartilage, chondrocytes have low ability to migrate and proliferate. Hence, articular cartilage has low possibility of self-healing when lesion occurs. The intrinsic migration of bone marrow mesenchymal stem cells (BMSCs) into cartilage defect always leads to the formation of fibrocartilage [4].

Articular cartilage defects caused by arthritis and trauma severely affect the healthy life of human being. In order to treat cartilage defects, different protocols such as autologous chondrocyte implantation (ACI), mosaicplasty, microfracture, autologous matrix-induced chondrogenesis (AMIC), and cartilage tissue engineering have been developed, as shown in Fig. 9.1b [5].

ACI utilizes autologous chondrocytes grown in culture, which are reimplanted in a second-stage procedure to repair large chondral defects [6]. Mosaicplasty is indicated for the treatment of smaller defects, less than 2–4 cm^2 in size, primarily on the femoral condyles. The treatment of larger lesions is limited by donor site morbidity, and the use in the patellofemoral joint is controversial [7]. To overcome these challenges, cartilage tissue engineering has been developed to realize the structural and functional regeneration of damaged cartilage [8]. As shown in Fig. 9.2a, the cells, scaffolds, and bioactive molecules are defined as three essential elements for the traditional cartilage tissue engineering [9, 10]. Various chondrogenetic cell sources are available for the cartilage tissue engineering. The chondrogenesis capability of these cells can be induced or enhanced with many biochemical or biomechanical stimulation in vitro. After culture in vitro, scaffold-based or scaffold-free engineered cartilage could be obtained and implanted for cartilage regeneration in vivo. Hence, cartilage tissue engineering involves direct intra-articular delivery of progenitor cells, progenitor cell delivery on scaffolds, or cell-free scaffolds coated with biological factors to recruit endogenous cells for articular cartilage defect repair [10]. The implantation of biomaterials or cartilage constructs is always accompanied by injury through the surgical procedures.

Inflammatory response takes a pivotal role in tissue repair and regeneration, since injury to the tissue always initiates an inflammatory response to the biomaterials. Moreover, the implantation of engineered cell–material hybrids elicits an adaptive immune reaction toward the cellular component, which in turn influences the host response to the material component [11]. When degradable biomaterials are applied, the immune response is additionally affected by the degradation products and surface changes of the biomaterials. Chronic inflammation in osteoarthritis develops as inflammatory stimuli persist at the implant site with macrophages, representing the driving force in perpetuating immune responses. Monocytes arriving at the implantation site undergo a phenotypic change to differentiate into macrophages. Their activation leads to further dissemination of chemo-attractants. Macrophages

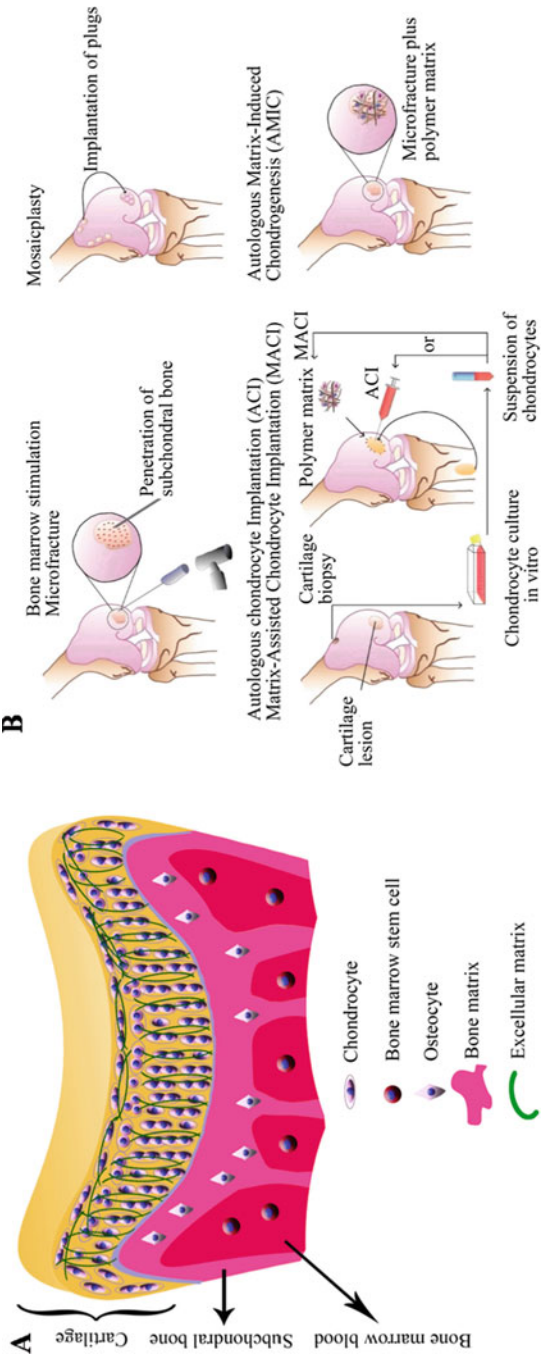

Fig. 9.1 (**a**) Composition and structure of articular cartilage and (**b**) various clinical strategies for regeneration of cartilage. (Reprinted from [5] with permission)

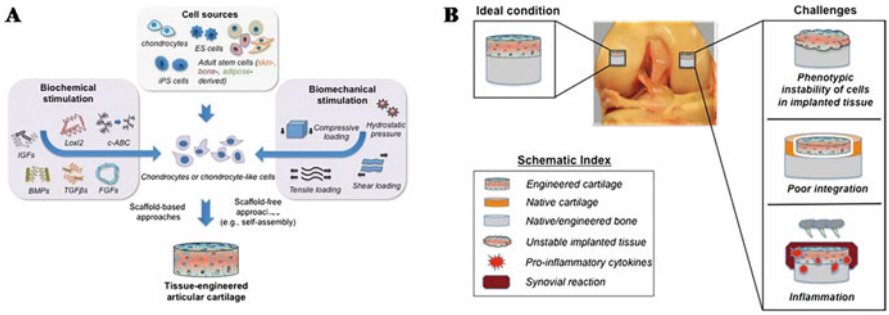

Fig. 9.2 (**a**) Articular cartilage tissue engineering involving the formation of three-dimensional tissues in vitro by seeding cells into scaffolds or through scaffold-free approaches in the presence of biochemical and biomechanical stimuli. (**b**) Challenges in cartilage tissue engineering. (Reprinted from [10] with permission)

attached to the biomaterials can foster invasion of additional inflammatory cells by secreting chemokines [12]. Taking these concerns into consideration, challenges of articular cartilage tissue engineering are shown in Fig. 9.2b. In summary, difficulty in the regulation and maintenance of cell chondrogenetic phenotype, poor integration between the implanted and the host tissues, and immunoregulation of the implanted biomaterials are the main issues that impede the development of cartilage tissue engineering [10].

9.2 Traditional Cell-Loaded Constructs for Cartilage Regeneration

9.2.1 Biomaterials for Cartilage Regeneration

An ideal cartilage tissue engineering scaffold should preserve the following characteristics: biocompatible, biodegradable, highly porous, suitable for cell attachment, proliferation and differentiation, osteoconductive, noncytotoxic, flexible and elastic, and nonantigenic. Generally, biomaterials used for cartilage tissue engineering can be divided into two categories: natural polymers and synthetic polymers. Each kind of these materials has their own advantages and shortcomings [13]. The natural materials are hydrophilic and bioactive, which enhance the cell–material interactions and facilitate the cells' chondrogenesis to the same extent. Collagen [14–21], fibrin [22–27], silk fibrin [28–32], hyaluronic acid (HA) [33–46], alginate [47–51], gelatin [40, 52–57], chitosan [58–64], etc. have been broadly invested in tissue engineering. The scaffolds based on these natural polymers are usually in a format of hydrogels, either with single or multicomponents. Examples of cartilage tissue engineering scaffolds based on native materials are shown in Fig. 9.3.

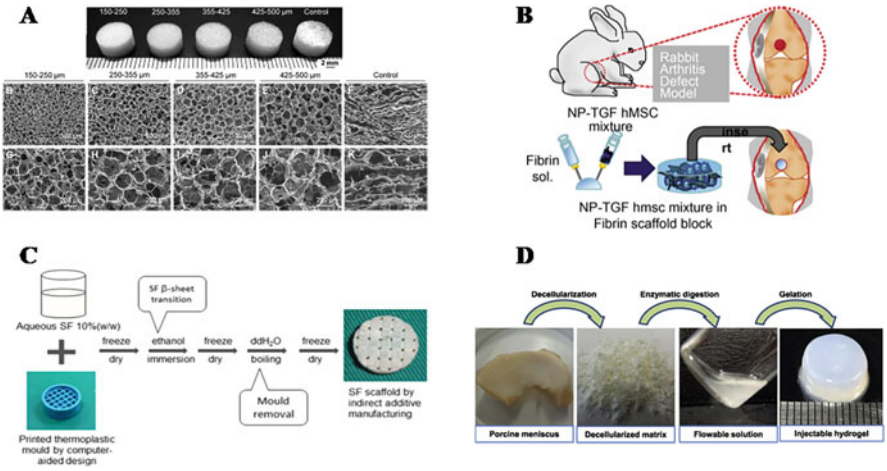

Fig. 9.3 Examples of cartilage tissue engineering scaffolds based on native materials. (**a**) Collagen porous scaffold. (**b**) BMSCs-loaded fibrin glue. (**c**) Silk fibroin scaffold. (**d**) Acellular cartilage matrix. (Reprinted from [29, 65–67] with permission)

9.2.1.1 Natural Materials

Collagen, which constitutes the major part of the extracellular matrix (ECM) and is the essential component and mechanical building block of various physiological systems including cartilage, is highly recommended in cartilage tissue engineering. Collagen has many advantages including favorable biocompatibility and high density of the RGD sequences and other sequences facilitating cell adhesion and cell differentiation [19]. Macroporous scaffolds of collagen can be fabricated conveniently by freeze-drying and chemical cross-linking (Fig. 9.3a) [67]. Vickers et al. prepared a chemically cross-linked collagen type II and glycosaminoglycan (GAG) scaffold with a low cross-linking density. Culture of bone marrow stem cells in the scaffold for 4 weeks in vitro found cell-mediated contraction, increased cell number density, and a greater degree of chondrogenesis [68]. Levingstone et al. fabricated a multilayer scaffold consisting of a bone layer composed of collagen type I and hydroxyapatite, an intermediate layer composed of collagen type I and type II and hydroxyapatite, and a superficial layer composed of collagen type I and HA [69]. The scaffolds were implanted into osteochondral defects created in the medial femoral condyle of the knee joint of New Zealand white rabbits, resulting in tissue regeneration with a zonal organization, repair of the subchondral bone, formation of an overlying cartilaginous layer, and evidence of an intermediate tidemark.

Fibrin gel has several features including biocompatibility and biodegradability. The fibronectin-rich fibrin glue is an essential protein in cartilage matrix for chondrocytes-ECM interaction [26]. Fibrin gel could serve as a delivery system for chondrogenetic cells and/or bioactive molecules to facilitate cartilage regeneration (Fig. 9.3b) [65]. Fibrin gel loaded with human bone marrow-derived

mesenchymal stem cells (hMSCs) and growth factor could realize full regeneration of cartilage defects in rabbits [65]. Park et al. fabricated a hybrid hydrogel composed of fibrin and HA, into which chondrocytes were implanted for culture in vivo [23]. Cartilage-like tissues were formed in the hybrid hydrogel, showing higher amounts of the ECM components, GAG, and collagen.

Hyaluronic acid (HA) is one of the most extensively studied natural materials for cartilage tissue engineering. HA is a linear polysaccharide found natively in adult articular cartilage that is involved in many cellular processes, including proliferation, morphogenesis, inflammation, and wound repair. Furthermore, HA is also important to cartilage formation and is differentially regulated during limb bud formation and mesenchymal cell condensation. HA hydrogels support chondrocyte matrix deposition and chondrogenic differentiation of mesenchymal stem cells (MSCs) [70]. HA is widely used to functionalize hydrogels or scaffolds for regeneration of cartilage defects. Sheu et al. fabricated a hydrogel based on oxidized HA and resveratrol, into which chondrocytes were implanted for culture in vitro, resulting in upregulated expression of collagen type II, aggrecan, and Sox9 genes and downregulated inflammatory factors [39].

Alginate is a natural anionic and hydrophilic polymer obtained primarily from brown seaweed and bacteria. It is composed of β-D-mannuronate and α-L-guluronate residues [71] and has been widely applied in many biomedical fields due to its excellent biocompatibility, low toxicity, and the mild gelation condition required to form a cross-linked structure [49]. Alginate can be easily modified through chemical and physical reactions to obtain derivatives and can be processed into three-dimensional scaffolds such as hydrogels, microspheres, microcapsules, sponges, foams, and fibers. Studies prove that the alginates would support the chondrogenesis [72, 73]. The cells-alginate constructs are widely used for the regeneration of articular cartilage defects, and some of the researches demonstrate quite positive results. Igarashi et al. delivered BMSCs in an ultra-purified alginate gel into articular cartilage defects in rabbit knees, resulting in complete regeneration of the defects [74].

Gelatin is a denatured collagen, but has relatively low antigenicity compared with collagen. Recently, gelatin-based biomaterials have been widely studied in tissue engineering. However, it is difficult to use pure gelatin scaffold for hard-tissue regeneration such as bone and cartilage due to its weaker mechanical strength. Hence, many studies focus on preparing pure gelatin scaffolds by using proper cross-linking methods [75] or hybrid scaffolds based on gelatin [40, 54, 55, 76]. Some natural materials such as HA, fibrin, chitosan, and synthetic materials have been extensively incorporated to obtain hybrid scaffolds, which not only preserve higher mechanical property but also retain the bioactivity of natural materials.

Chitosan is obtained by deacetylation of chitin which is an abundant natural material. The positive charge in the molecular chain may protect GAGs from hydrolysis [61]. However, the positive charge may also limit the proliferation of chondrocytes. Meanwhile, weaker mechanical property of wet chitosan also limits its application in cartilage tissue engineering [62]. Therefore, the hybrids of one or

more materials are always adopted for the application of chitosan in tissue engineering.

Silk fibroin extracted from silkworm cocoons is composed of fibrous protein (fibroin), containing amino acids and glue-like protein (sericin). Silk fibroin is widely used natural material for tissue regeneration taking into consideration of their excellence in biocompatibility, degradability, and mechanical properties [77, 78]. Scaffolds based on silk fibroin for cartilage regeneration can be fabricated through a template/solution-casting method as reported (Fig. 9.3c) [29]. Biphasic scaffolds with a cartilage phase constituting of bovine cartilage matrix biofunctionalized fibroin and differentiated autologous prechondrocytes, and a bone phase (decellularized bovine bone) has been fabricated to promote cartilage regeneration in a model of joint damage in pigs [79]. Cao et al. developed a multifunctional silk-based hydrogel incorporated with metal-organic framework nanozymes, which showed enhanced cell viability as well as antioxidant and antibacterial properties. In the full-thickness osteochondral defect model of rabbit, the hydrogel displayed successful regeneration of osteochondral defect [80].

ECM materials have become more popular because the matrices retain the structure of native cartilage, which preserve mechanical and chemical signals that can induce cell differentiation and recruitment without additional biologic additives. Cartilage ECM can be obtained from either cell-derived matrices secreted during culture in vitro or from native cartilage (Fig. 9.3d) [66]. Decellularization is an effective way to fully remove all cellular components and nucleic acids or to kill the remnant cells within the matrix [79–84]. The scaffolds based on the decellularized cartilage ECM regenerate hyaline cartilage when combined with rabbit MSCs after transplantation into weight-bearing area of patellar grooves in rabbits for 12 weeks [85]. Dai et al. prepared an acellular bone matrix scaffold using iliac bone of pigs [86]. The scaffold implantation combined with microfracture was used to treat full-thickness articular defects (9 mm in diameter) without destroying the subchondral bone of pigs. 24 weeks after surgery, the defects were repaired with hyaline-like neocartilage which has the similar mechanical properties to the normal cartilage. Ayariga et al. developed a decellularized ECM scaffolds from avian articular cartilage [87]. The obtained scaffolds registered an interconnected and porous architecture, as well as stiffness comparable to the native cartilage tissues. Meanwhile, human chondrocytes survived, proliferated, and interacted with the scaffolds, showing that the decellularized scaffolds are suitable for cartilage regeneration. Das et al. prepared a cartilaginous ECM-derived biomaterial from goat ears [88]. MSCs showed obvious chondrogenic differentiation with increasing amount collagen and GAGs in the decellularized scaffolds. Upon implantation of the IGF-1-loaded cell-free scaffolds in rabbits' osteochondral defects for 3 months, the histological and micro-CT evaluation revealed significant enhancement and regeneration of neocartilage and subchondral bone. Oh et al. prepared full-thickness porcine cartilage-derived ECM, and then fabricated mechanically reinforced ECM scaffolds by combining salt-leaching and crosslinking methods [89]. Chondrocytes showed higher levels of cartilage-specific markers in the scaffolds compared to that in the ECM scaffolds prepared by simple freeze-drying [90]. Antler decellularized

cartilage-derived matrix (AdCDM) rich in collagen and GAGs was prepared by combining freezing-thawing and enzymatic degradation. Treatment of osteochondral defects with the AdCDM showed a flat and smooth surface of the neocartilage at the surgery site. Meanwhile, compared to porcine decellularized cartilage-derived matrix, AdCDM could lead to better osteochondral regeneration with higher international cartilage repair society scores (ICRS). Decellularized ECM bioinks, derived from specific native tissues or organs, have been used to fabricate 3D-printed tissues and organs. Zhang et al. developed a crosslinker-free bioink with silk fibroin and decellularized articular cartilage extracellular matrix of goat [91]. The silk fibroin and decellularized ECM interconnect with each other through physical crosslinking and entanglement, which bypass the toxicity inherent in the chemical crosslinking process of most bioinks. In vitro test proved that BMSCs highly expressed chondrogenesis-specific genes in the 3D-printed scaffold using this bioink.

9.2.1.2 Synthetic Materials

Synthetic polymers are also widely applied in cartilage tissue engineering, but the relatively low cell adhesive ability limits their applications. The widely used synthetic materials include poly(lactide-*co*-glycolide) acid (PLGA) [40, 57, 92–94], polycaprolactone (PCL) [95–99], poly(ethylene glycol) (PEG) [34, 100–108], etc. The scaffolds composted of solely synthetic materials can hardly realize good tissue regeneration. Therefore, the natural materials such as collagen, gelatin, fibrin, HA, and acellular ECMs, as mentioned before, can be compounded or incorporated into the synthetic polymeric scaffolds. Examples of cartilage tissue engineering scaffolds based on synthetic materials are shown in Fig. 9.4.

PCL is a semicrystalline polymer. It belongs to a family of poly α-hydroxyl esters and is one of the most widely used biodegradable polyesters for medical applications because of its biocompatibility, biodegradability, and flexibility [111]. It is widely used to prepare scaffolds for cartilage tissue engineering as well [40, 46, 47, 92, 112–114]. For example, Kim et al. prepared a PCL scaffold constructed with layers of electrospun and salt-leaching PCL membrane, into which chondrocytes were seeded by using an injectable heparin-based hydrogel (Fig. 9.4a). In vivo transplantation of the construct into partial-cartilage defects demonstrates significant cartilage formation with good integration to the surrounding cartilage [95]. Lebourg et al. modified PCL scaffolds with cross-linked HA to grant PCL more hydrophilic and biomimetic microenvironment. Complete regeneration of chondral defects in rabbits in vivo was confirmed by implanting the scaffolds for 24 weeks [38].

PLAG is usually synthesized via ring-opening copolymerization of lactide and glycolide, which has prominent advantages such as adjustable molecular weight and degradation rates, good mechanical properties especially toughness, and excellent processability [115]. It has been widely used to prepare scaffolds to engineer tissues including cartilage, bone, nerve, etc. [116–121]. Chang et al. seeded endothelial progenitor cells into a highly porous PLGA scaffold and implanted into the

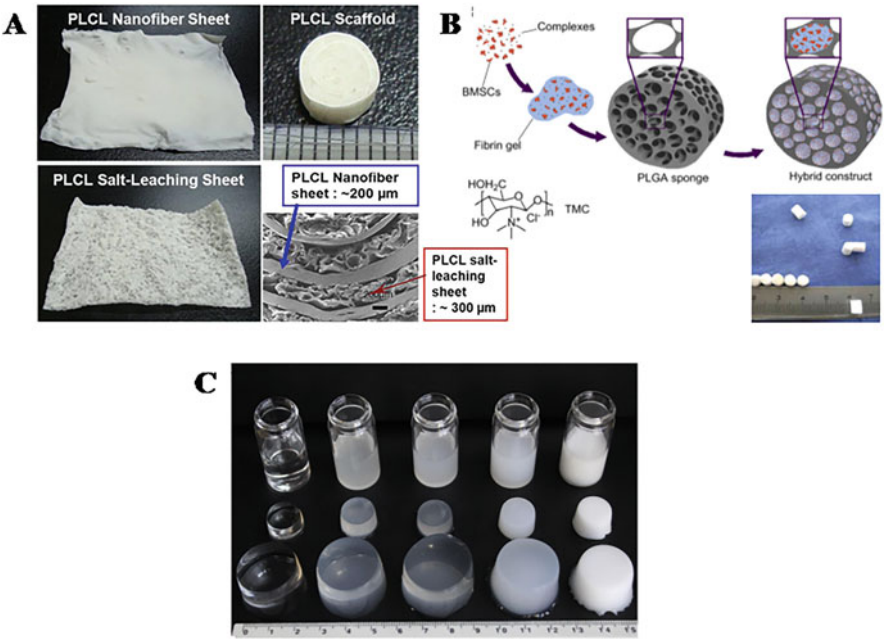

Fig. 9.4 Examples of cartilage tissue engineering scaffolds based on synthetic materials. (**a**) PLCL scaffold. (**b**) PLGA scaffold. (**c**) PEG hydrogel. (Reprinted from [95, 109, 110] with permission)

osteochondral defect in the medial femoral condyle of rabbits. After 12 weeks, the defects were regenerated with hyaline cartilage, showing a normal columnar chondrocyte arrangement, higher Sox9 expression, and greater contents of GAG and collagen type II [122]. In order to enhance the bioactivity of PLGA scaffolds, bioactive materials such as HA, gelatin, collagen, and fibrinogen can be usually incorporated. PLGA/fibrin gel-based constructs combined with MSCs and TGF-β1 chondrogenic genes could facilitate the in vivo regeneration of full-thickness cartilage defects in a rabbit model (Fig. 9.4b) [109, 123, 124]. The PLGA scaffold is fabricated by a gelatin porogen leaching method, into which fibrinogen containing cells and plasmid TGF-β1 gene complexes is infiltrated and then gelated. The chondrocytes cultured in vitro distribute evenly and maintain a round morphology in the hybrid scaffold as that in the normal cartilage [125]. The implantation of PLGA/fibrin gel/N,N,N-trimethyl chitosan chloride (TMC)/pDNA-TGF-β1 construct into osteochondral defects for 12 weeks in vivo results in regenerated cartilage with smooth surface and well integration with its surrounding tissue and subchondral bone [109].

PEG hydrogel has received wide attention due to its injectability, noncell-adhesive property, cell compatibility, and low immunogenicity. Meanwhile, PEG hydrogel could be prepared for cartilage regeneration (Fig. 9.4c) [110]. The nondegradability of PEG in physiological environment limits its application in tissue

engineering, although the PEG molecules of lower molecular weight, like PEG-400, have been proved to metabolize via renal or intestine pathways [126]. Biodegradable segments such as oligo(lactic acid), oligo(ε-caprolactone), oligo(trimethylene carbonate), and phosphate groups have been introduced into the PEG-based macromers. Fan et al. developed a microcavitary hydrogel via photopolymerization of biodegradable oligo(trimethylene carbonate)-poly(ethylene glycol)-oligo(trimethylene carbonate) diacrylate macromers [106]. The cavitary structure in the hydrogel would accelerate degradation of the hydrogel. Compared with noncavitary hydrogel, the cell density and total contents of collagen and GAG are significantly higher. The hydrolytically biodegradable PEG hydrogels offer a promising platform for chondrocyte encapsulation and for tuning degradation of cartilage tissue engineering scaffolds. Skaalure et al. prepared a semi-interpenetrating network of bioactive HA and oligo(lactic acid)-PEG hydrogel, into which chondrocytes were encapsulated and cultured for 4 weeks. In this way, the contents of collagen and GAG are significantly increased [34].

9.2.2 Cells for Cartilage Regeneration

Chondrocytes in the cartilage produce cartilage ECMs and therefore have been the first choice for cartilage tissue engineering [127]. They are isolated from various sources such as articular cartilage, nasal septum, ribs, and ear cartilage and are extensively used for the study of cartilage regeneration in vitro and in vivo. However, one of the major limitations of chondrocytes is their instability in the culture in vitro, leading to the loss of expression of cartilage matrices such as collagen type II and aggrecan. Recently, multipotent MSCs have been gained increasing interest in cartilage tissue engineering as an alternative to autologous chondrocytes due to their ease in isolation and high expansion capacity in vitro. MSCs exhibit the potential to differentiate into chondrocytes [128], tenocytes [129], ligament cells [130], neuronal cells [131, 132], cardiomyocyte [133, 134], osteoblasts [135], and other cell types [136]. In particular, bone marrow-derived stem cells (BMSCs), adipose-derived stem cells (ADSCs), and embryonic stem cells (ESCs) are most widely applied in cartilage tissue engineering.

9.2.2.1 Chondrocytes

Chondrocytes are metabolically active cells that synthesize a large spectrum of ECM components such as collagen, glycoproteins, proteoglycans, and HA [127]. Since the chondrocytes are the only type of cells in articular cartilage, they are used for the regeneration of cartilage defects in priority both in vitro and in vivo [127, 137–144]. It is believed that the use of chondrocytes would lead to the formation of neotissue with exactly the same ECMs with the native cartilage [145]. The activity of chondrocytes is altered by many factors present within their chemical and

mechanical environment. However, the use of chondrocytes for cartilage repair suffers from chondrocyte dedifferentiation. A proper culture and delivery of chondrocytes, including the use of chondrogenetic culture medium, growth factors, and mesenchymal stem cells, need to be well adjusted in order to keep the phenotype of chondrocytes [140]. Three-dimensional scaffolds can better mimic the native microenvironment of chondrocytes in cartilage tissue, promoting cell–cell and cell–matrix interactions and enforcing round chondrogenetic cell morphology and thereby maintaining their phenotype. Xu et al. encapsulated chondrocytes in alginate gel beads and cultured in spinner flasks in chondrogenic and chondrocyte growth medium and then subcutaneously implanted the cells-loaded beads to evaluate the ectopic chondrogenesis [142]. The results prove high deposition of glycosaminoglycan and expression of cartilage-specific genes. Lohan et al. precultured chondrocytes in polyglycolide (PGA) scaffolds for 3 weeks, which were then implanted into critical-sized osteochondral defect of rabbit knee femoropatellar groove [138, 141]. Twelve weeks after implantation, neocartilage was formed in vivo in the PGA constructs seeded with chondrocytes. The results are significantly better than those of the cell-free PGA scaffolds and empty defects.

9.2.2.2 Bone Marrow-Derived Stem Cells (BMSCs)

BMSCs have been extensively used for chondrogenesis in a three-dimensional culture in vitro with addition of chondrogenetic factors and regeneration of cartilage defects in animal models in vivo [33, 146–149]. BMSCs can be isolated via plastic adhesion or negative selection from bone marrow aspirate that includes a highly heterogeneous cell population such as hematopoietic cells, endothelial cells, and adipocytes [150]. However, there are some limitations of BMSCs. The relative number of BMSCs in the marrow blood is rather small, and their differentiation ability decreases significantly with age [151]. Meanwhile, the constructs of cartilage containing BMSCs can raise many problems such as fibrosis, vascularization, the "hollow" phenomenon, and shrinkage likely due to the incomplete differentiation of BMSCs, deterring the clinical translation of tissue-engineered cartilage [149]. Hence, chondrogenetic bioactive factors are always applied to promote chondrogenesis differentiation of BMSCs. Li et al. fabricated a bilayered poly (vinyl alcohol)/gelatin/vanillin (PVA/Gel/V) and nanohydroxyapatite/polyamide-6 (n-HA/PA6) scaffold, into which BMSCs were implanted. The obtained constructs were used for the regeneration of cartilage and subchondral bone defects in rabbits in vivo [152]. With BMSCs loading, the two different layers of the composite biomimetic scaffolds provide a suitable microenvironment for cells to form respective tissues.

9.2.2.3 Adipose-Derived Stem Cells (ADSCs)

ADSCs are becoming more and more attractive because they can be easily isolated from adipose tissues and cultured in vitro for an extended period of time with stable expansion and low levels of senescence [153]. Adipose tissue contains a large proportion of MSCs and is easily accessible in all individuals. Compared with BMSCs, the ADSCs are relatively abundant and can be easily available. In vitro and in vivo studies confirm the chondrogenetic ability of ADSCs and the ability of cartilage regeneration [154–160]. In the presence of platelet-rich plasma (PRP) and cartilage-specific extracellular molecules, the expression of collagen type II and aggrecan can be significantly upregulated [159, 160]. Wang et al. proved different chondrogenic degrees of ADSCs being cultured in hydrogels composed of chondroitin sulfate, HA, and heparin sulfate, respectively [157]. This chondrogenetic potential of ADSCs makes them a promising candidate for restoration of cartilage defects in vivo. Wang et al. implanted ADSCs into acellular cartilage matrices and used the cell-loading constructs to restore the articular cartilage defects of rabbits [158]. After 12 weeks of implantation, the defects are filled with neotissues, showing a smooth surface, highly expressed collagen type II and GAG, and chondrocyte-like cells in the recesses. TEM analysis confirms plenty of secretary matrix particles in the neotissue.

9.2.2.4 Embryonic Stem Cells (ESCs)

Recently, several studies have demonstrated the regeneration of cartilage defects in vivo by using ESC progenitor cells [161–164]. ESCs can be obtained from the blastocyst and are able to self-renew for a prolonged period of time without differentiation and, most importantly, can be differentiated into a large variety of tissues derived from all three germ layers. Although the application of ESCs would bring problems such as immunologic incompatibility, possible development of teratomas, and ethical issues in human, the in-depth study of ESCs would promote their applications in healing human diseases. For the cartilage regeneration, ESCs are also a promising choice [161, 163, 165–167]. Pilichi et al. demonstrated a positive result of application of nondifferentiated ESCs in vivo for osteochondral regeneration without tumorigenic and teratoma formation [164]. They treated osteochondral defects in a sheep model with ESCs for 24 weeks, proving the regeneration of articular cartilage defects with hyaline cartilage, without signs of immune rejection or teratoma. Toh et al. used TGF-β1 to induce chondrogenic differentiation of ESCs, explored the potential of these ESC-derived chondrogenic cells to produce an ECM-enriched cartilaginous tissue construct when cultured in HA hydrogel, and further investigated the cartilage regenerative ability in an osteochondral defects in a rat model [162]. Twelve weeks after implantation, a hyaline-like neocartilage layer is formed, showing good surface regularity and complete integration with the adjacent host cartilage and a regenerated subchondral bone.

9.2.2.5 Induced Pluripotent Stem Cells (iPSCs)

iPSCs may be generated from somatic cells through reprogramming, enabling them to possess embryonic-like properties. Shinya Yamanaka's group initially derived the iPSCs in 2006 by reprogramming mouse fibroblasts, and human fibroblasts in the following year [168, 169]. iPSC may differentiate into other cell linages and be maintained in a nondifferentiated state for an extended period of time to cultivate cells, known as the self-renewal process. The iPSCs are similar to ESCs but less of an ethical dilemma [170]. Nam et al. obtained human iPSCs from cord blood mononuclear cells using the Sendai virus [171]. The iPSCs were differentiated into chondrogenic lineage with pellet culture and maintained for 30 days. The generated pellets showed high expression of chondrogenic gene and deposition of cartilage extracellular matrix proteins. Yamashita et al. reported that differentiation of iPSCs into hyaline cartilaginous particles and implantation of the particles into joint surface defects realized the repair of cartilage defects, and neither tumor nor ectopic tissue formation was observed [172]. Kotaka et al. labeled iPSCs magnetically with nanoscale iron particles, and delivered the cells specifically into cartilage defects in nude rats using a magnetic field [173]. The histological grading proved useful and safe for cartilage repair using the mentioned iPSCs. Liu et al. fabricated a polycaprolactone/gelatin scaffold using two separate electrospinning processes [174]. After seeded with mouse iPSCs derived from mouse dermal fibroblasts, the iPSCs-scaffolds were implanted into osteochondral defects of rabbits, resulting in an enhanced gross appearance and histological improvement, higher cartilage-specific gene expression and protein levels as well as subchondral bone regeneration.

9.2.2.6 Dental Pulp Stem Cells (DPSCs)

DPSCs are a type of self-renewal MSCs residing within the perivascular niche of the dental pulp [175]. DPSCs are a promising source of stem cells for tissue-engineering therapies because of their low cost and easy accessibility. DPSCs can differentiate into several different cell types, including neurons, odontoblasts, osteoblasts, adipocytes and chondrocytes [176]. Mata et al. cultured DPSCs in 3% alginate hydrogel, and implanted the hydrogel in a rabbit model of cartilage damage [177]. Three months post surgery, the cartilage defects were well regenerated. Yanasse et al. reported a successful regeneration of full-thickness articular cartilage defects in rabbits using DPSCs-loaded platelet-rich plasma scaffolds [178].

9.2.2.7 Umbilical Cord Mesenchymal Stem Cells (UCMSCs)

Human UCMSCs can be derived from various parts of human umbilical cord, including Wharton's jelly, cord lining, and the perivascular region [179]. hUCMSCs are advantageous because of their high expansion capacity, noninvasive harvesting,

and hypoimmunogenicity. hUCMSCs possess the same potential of chondrogenic differentiation regardless of the portion of the umbilical cord from which they are isolated [180]. According to the research of Fong et al., the chondrogenic potential of hUCMSCs is thrice that of BMSCs in producing collagen [181]. Zheng et al. fabricated polycaprolactone/hydroxyapatite (PCL-HA) scaffolds using fused deposition modeling 3D-printing technology [182]. Furthermore, rabbit UCMSCs and chondrocytes with a ratio of 3:1 were seeded on the prepared PCL/HA scaffolds. After 8 weeks of implantation into rabbits' femoral trochlear defects, the ICRS scores of the repaired defects for the UCMSCs and chondrocyte-seeded PCL-HA scaffolds were significantly higher than the unseeded PCL/HA scaffolds. 125 patients were included in a clinical study to evaluate cartilage regeneration by implanting allogenic hUCMSCs with concomitant high tibial osteotomy (HTO) [183]. Second-look arthroscopy and ICRS grade evaluation proved the effectiveness of this treatment for patients with medial compartment osteoarthritis and various deformities. Another clinical research including 176 patients also confirmed that implantation of allogenic hUCMSCs with concomitant HTO could provide clinical outcomes in terms of pain relief, functional scores, and quality of life [184].

9.2.2.8 Other Cells

Besides BMSCs, ADSCs, ESCs, iPSCs, DPSCs, UCMSCs, other types of stem cells from muscle, synovium, and periosteum can also be used for the cartilage regeneration [185–188].

Several works report that synovium-derived MSCs (SMSCs) show a higher colony-forming efficiency than BMSCs. Because the SMSCs display a great potential to differentiate into chondrocytes, they are one of the best candidates for the repair of cartilage defects [189]. SMSCs have the potential for both cartilage tissue engineering in vitro and cartilage regeneration in vivo. With appropriate stimulation, SMSCs are capable of migrating into articular cartilage defects and differentiating to chondrocytes [189–194]. Fan et al. explored therapeutic chondrogenesis of rabbit SMSCs encapsulated in photopolymerized hydrogels with the treatment of TGF-β1, resulting in positive SMSC chondrogenesis. Meanwhile, SMSCs may be a type of tissue-specific stem cells, because they can respond to signaling in the joint and promote cartilage defect regeneration [195]. Pei et al. isolated SMSCs from synovial tissue of rabbit knee joints and mixed SMSCs with fibrin glue, followed by seeding into a nonwoven PGA mesh. After the constructs were prematured for 1 month in vitro, they were implanted into rabbit knees to repair osteochondral defects. Six months later, the cartilage defects were full of smooth hyaline-like cartilage with high expressions of collagen type II and GAG and were well integrated with the surrounding native cartilage. No detectable collagen type I and macrophages were found [196].

9.2.3 *Bioactive Signals for Cartilage Regeneration*

The cell growth factors are typical bioactive molecules, which can stimulate or inhibit cellular proliferation, differentiation, migration, and gene expression [198]. There are a number of essential growth factors that have regulatory effects on chondrocytes or stem cells in terms of chondrocyte maturation and cartilage formation. The candidate growth factors include transforming growth factor β (TGF-β), insulin-like growth factor-1 (IGF-1), bone morphogenic proteins (BMPs), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), etc. [199]. Each growth factor plays a different role in the migration, proliferation, and differentiation of cells as summarized in Fig. 9.5. However, it is difficult to precisely define the function of each growth factor due to the functional overlaps in temporal scale [197].

9.2.3.1 TGF-β

So far four types of TGF-β superfamily, namely, TGF-β1, TGF-β2, TGF-β3, and BMP, have been found in cartilage [198]. Activated TGF-β not only increases the synthesis of proteoglycan but also prevents degradation of cartilage ECM by inhibiting matrix metalloproteinase (MMP). These TGF-β isomers play an important role in the late stage of chondrocyte differentiation and may participate in bone formation as well. TGF-β1 induces early stage of chondrogenesis and increases

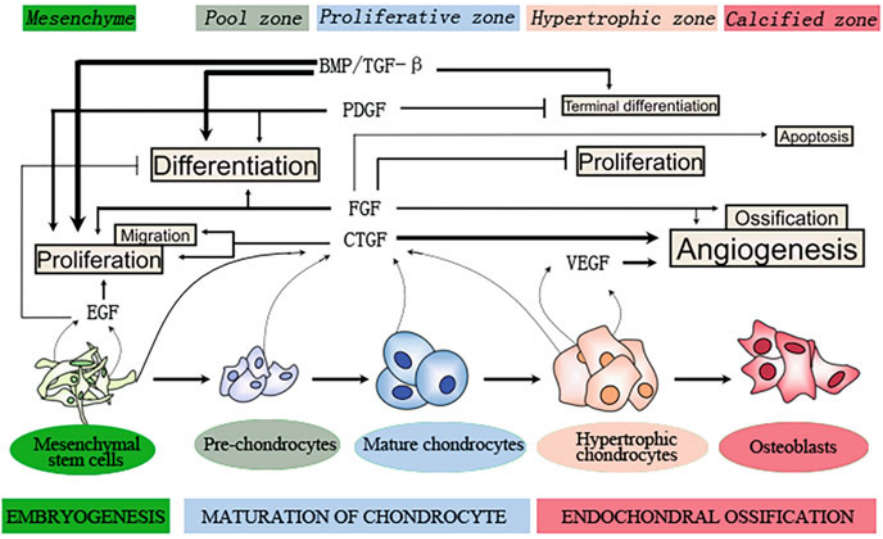

Fig. 9.5 Schematic overview of the role of growth factors at different stages of chondrogenesis. (Reprinted from [197] with permission)

the production of aggrecan and collagen type II [200]. TGF-β3 plays a role in the maturation of chondrocytes [201]. The TGF-β has been extensively used for the regeneration of cartilage defects in vitro and in vivo [122, 202–211]. For example, Yin et al. fabricated a TGF-β1-immobilized scaffold by incorporating TGF-β1-loaded gelatin microspheres into PLGA framework and evaluated the ADSC differentiation in the scaffold in vitro and regenerative ability of cartilage defect in vivo. The cell proliferation and GAG deposition in the TGF-β1-immobilized scaffold are significantly increased, and the cartilage regeneration is promoted in the defective articular cartilage in vivo [211]. Lu et al. engineered ADSCs with a baculovirus system that confers prolonged and robust TGF-β3/BMP-6 expression. Culture for 2 weeks in vitro in a porous scaffold leads to the formation of cartilaginous constructs with improved maturity and mechanical properties. After implantation into full-thickness articular cartilage defects in rabbits, these engineered constructs regenerate neocartilages that resemble native hyaline cartilage in terms of cell morphology, matrix composition, and mechanical properties. The neocartilages also have cartilage-specific zonal structures without signs of hypertrophy and degeneration and integrate well with the native cartilages [187].

9.2.3.2 IGFs

IGFs have a polypeptide sequence similar to proinsulin that allows cells to communicate with their physiologic environment. IGF-1 is well known to promote cell proliferation and inhibit apoptosis. IGF-1 is expressed in developing cartilage, mature cartilage, and synovial fluid of the joint. Both of in vitro and in vivo studies confirm that IGF-1 can induce chondrocyte differentiation and proliferation of MSCs and enhance proteoglycan and collagen type II synthesis [212–219]. Spiller et al. encapsulated IGF-1 in degradable PLGA microparticles and embedded the particles in PVA hydrogel. The PGA fiber scaffolds with chondrocytes were wrapped around the hydrogels and were implanted subcutaneously in athymic mice. Histology analysis proves enhanced cartilage formation in the layers surrounding the hydrogel with increased content of ECMs, mechanical properties, and integration between the cartilage layers and the hydrogels [218]. The regeneration of cartilage and subchondral bone in vivo was confirmed by injecting IGF-1 suspended HA solution to the temporomandibular in a rabbit model. Twelve and twenty-four weeks after the injection, the defects were well repaired, and nearly normal microarchitectural properties of the subchondral cancellous bone were found in the defects [217].

9.2.3.3 BMPs

BMPs are able to induce the formation of the cartilage and bone, which are required for the formation of prechondrogenic condensation and differentiation into chondrocytes. Meanwhile, they can increase the expression of the specific chondrocyte markers such as type X collagen [139, 205, 220–227]. BMP-2, a potent

regulator of chondrogenic expression, has received considerable attention in cartilage and osteochondral tissue engineering. Jeong et al. investigated the influence of BMP-2 on the production of cartilage matrix and subsequent bone matrix by using primary chondrocytes seeded on designed three-dimensional PCL scaffolds with chemically conjugated BMP-2. The chemically conjugated BMP-2/PCL scaffolds can significantly promote better cartilage regeneration without particularly accelerating endochondral ossification both in vitro and in vivo compared with those non-BMP-2-treated scaffolds [139].

9.2.3.4 FGF-2

FGF-2 is known as a chondrocyte mitogen found in normal cartilage and has great potential for clinical applications. It can stimulate chondrocytes to synthesize cartilaginous matrix [228–233]. Maehara et al. impregnated a porous hydroxyapatite/collagen scaffold with FGF-2 and used the scaffolds to repair large osteochondral defects in a rabbit model. With the addition of FGF-2, the neotissue in the defects displays not only the most abundant bone regeneration but also cartilage regeneration with hyaline-like appearance [232].

9.2.3.5 PDGF

PDGF is a glycolytic protein released by platelets and other cells, which stimulates the growth of cells of mesenchymal origin, for example, the cartilage [234–237]. Meanwhile, the released PDGF-AA from hydrogel being filled in the full-thickness cartilage defects greatly promotes BMSC recruitment into the hydrogel. This confirms the ability of PDGF to recruit BMSCs besides promotion of cell proliferation [237].

9.2.3.6 Exosomes (Exos)

Exos are extracellular vesicles with 30–150 nm in diameter that are produced by cells through the paracrine pathway, which contain various types of nucleic acids and proteins [238]. Recently, Exos have been regarded as important carriers for transmitting biological signals between cells instead of waste products of cells. Exos derived from stem cells are considered as ideal substitutes for stem cells in "cell-free" cartilage regeneration [239]. Jiang et.al. combined Exos derived from human Wharton's jelly-derived MSCs with scaffold of acellular porcine articular cartilage [240]. 6 months' experiment in vivo proved that the Exos can promote osteochondral regeneration in a "cell-free" condition. Shao et. al. revealed that Exos derived from infrapatellar fat pad MSCs can significantly promote the proliferation as well as the expression of Sox-9, Aggrecan, and Collagen II relative genes of chondrocytes in vitro [241]. Furthermore, Shao et.al. created a rabbit articular cartilage defect

with 4 mm in diameter and 1.5 mm in depth, and then treated with the Exos suspension. 12 weeks after the treatment, the defected cartilage was effectively regenerated with a hyaline morphology. In spite of these positive results using Exos to facilitate the regeneration of articular cartilage defect, the underlying mechanism of action remains unknown. Additionally, the low yield of Exos leads to a higher cost of Exos therapy than stem cell therapy, which might be the potential limitation to move the Exos therapy forward from bench to bedside [242].

9.2.3.7 Platelet-Rich Plasma (PRP)

PRP is a kind of autologous derivative of the whole blood, which is rich in growth factors. PRP could stimulate the migration and chondrogenic differentiation of human subchondral progenitor cells [243]. Meanwhile, PRP would counteract effects of an inflammatory environment on genes regulating matrix degradation and formation in human chondrocytes [244, 245]. Recently, PRP has commonly been utilized in the repair and regeneration of damaged articular cartilage. Lu et al. prepared an injectable hydrogel with hyaluronic acid (HA), fucoidan (FD) and gelatin (GLT), which was further cross-linked with genipin (GP) [246]. The PRP-loaded injectable hydrogel was prepared by adding PRP in the hydrogel before gelation. It could facilitate the sustained release of PRP growth factors, and promote cartilage regeneration in rabbits. Singh et al. developed a hybrid scaffold by embedding PRP/alginate-based hydrogel in porous 3D scaffold of chitosan/chondroitin sulfate/silk fibroin [247]. The hybrid construct could provide PRP-based cocktails of growth factors, which facilitates chondrogenic ECM deposition and enhanced expression of cartilage tissue-specific collagen type II and aggrecan. Autologous chondrocytes-loaded hybrid scaffolds possess the superior potential to regenerate hyaline cartilage defect of thickness around 1.10 ± 0.36 mm and integrate with surrounding tissue at the defect site.

9.2.4 Methods for Cartilage Tissue Engineering

9.2.4.1 Preculture In Vitro for Cartilage Tissue Engineering

Functional repair of focal cartilage defects requires filling the space with neotissue that has compressive properties comparable to native tissue and integration with adjacent host cartilage. One of the main issues in cartilage tissue engineering is represented by the ideal maturation of the construct before implantation in vivo, in order to optimize matrix quality and integration [248]. Considerable progress has been made toward the in vitro tissue engineering of neocartilage with compressive properties approaching native levels [249–253]. In 1997, Cao et al. reported a human ear-shaped tissue-engineered construct by using bovine articular chondrocytes and a nonwoven PGA scaffold [254]. Deponti et al. studied the difference of cartilage

maturation with or without preculture. Articular chondrocytes were embedded in fibrin glue with preculture in vitro for 1 week and implanted subcutaneously in rat, proving better tissue maturation compared with the constructs without preculture [249]. Pei et al. mixed synovium-derived stem cells with fibrin glue, which were then seeded into nonwoven PGA mesh. After 1-month incubation with growth factors, the premature construct was used to repair osteochondral defects in a rabbit model. Six months later, the defects were full of smooth hyaline-like cartilage with high expression of collagen type II and GAG, which integrated well with the surrounding tissue too [196].

Culture of constructs in a dynamic environment involving fluid flow or agitation is beneficial for cartilage synthesis compared to the static culture conditions [255]. Therefore, various bioreactors have been applied for cartilage tissue engineering, offering advantages such as better control over culture conditions, reduced diffusional limitations for delivery of nutrients and metabolites, enhanced oxygen transfer, and exertion of mechanical and hydrodynamic forces influencing cell and tissue development [256]. Shahin et al. precultured chondrocytes in PGA scaffold for 5 weeks within a bioreactor, confirming improved GAG retention in the scaffolds [257].

9.2.4.2 Regeneration of Cartilage Defects In Situ

With the deep acknowledge of cell behavior regulation and bioactive molecule functions, the in situ regeneration of cartilage defects with direct implantation of cartilage tissue engineering constructs based on biomaterials, cells, and bioactive growth factors has been extensively studied. The scaffolds based on native and/or synthetic materials play a role in supporting the viability of cells and deposition of neo-ECMs, while the bioactive growth factors regulate cell differentiation and physiological activity. Numerous studies give positive regenerative results by using the bioactive constructs in repair of articular cartilage defects. As described early, cells (chondrocytes, BMSCs, ADSCs, ESCs, etc.) and bioactive growth factors (TGF-β, IGF-1, BMPs, FGF, PDGF, etc.) are loaded into scaffolds (hydrogels, porous scaffolds, etc.), which are then implanted into the cartilage defects without prematuring. Li et al. implanted a PLGA scaffold filled with fibrin gel, mesenchymal stem cells (MSCs), and poly(ethylene oxide)-b-poly(L-lysine) (PEO-b-PLL)/pDNA-TGF-β1 complexes into osteochondral defects, resulting in full in situ regeneration of the defect [123]. However, the application of constructs containing cells and bioactive molecules is still faced with obstacles like source, amount, and phenotype maintenance of MSCs during culture, immune reaction against foreign cells, as well as feasibility of clinical translation considering the ratio of performance to price [258].

Injectable hydrogels have a greater potential to promote articular cartilage regeneration considering their tailorable structural and mechanical capabilities. Importantly, the free-flowing property makes it convenient for the loading of drugs, growth factors and cells into the injectable hydrogel by simple dissolution procedures

[38]. Zheng et.al. fabricated an injectable hydrogel based on silk fibroin, chitosan and thermal-sensitive glycerophosphate [259]. With the incorporation of TGF-β1 and BMSCs, the prepared injectable hydrogel could promote the regeneration of partial-thickness cartilage defect on knees of SD rats. Dong et.al. developed a physiochemical dual crosslinking injectable hydrogel using catechol-modified gelatin, dopamine-modified oxidized hyaluronic acid, and dendritic mesoporous organic silica nanoparticles with Fe^{3+} layers for the encapsulation of dexamethasone [260]. The obtained hydrogel was injected into osteochondral defects of 3.5 mm in diameter and 5 mm in thickness of SD rats. Post implantation for 8 weeks revealed the efficacy of the treatment on cartilage defects by the effective removal of the ROS and the inhibition of TNF-α and IL-6. Dong et.al. fabricated an injectable chitosan/silk fibroin hydrogel containing SDF-1 and PLGA microspheres loaded with Kartogenin [261]. The SDF-1 released from the hydrogel facilitated the recruitment of BMSCs in vivo, and the slowly released Kartogenin promoted the chondrogenesis of MSCs. After the hydrogel was injected into the cartilage defects (4 mm in diameter and 1.5 mm in depth) of rabbits combined with microfracture for 12 weeks, the subchondral bones and superficial cartilage were reconstructed, which were similar to the natural tissues.

9.3 Cell-Free Constructs for Cartilage Regeneration In Situ

Based upon the principles of tissue engineering, the stem cells and chondrocytes are usually used for cartilage regeneration. However, the controversy of using cells in tissue engineering still exists because of the uncertainty of dose, time point, as well as side effects [262]. In fact, stem cells are abundant in bone marrow and adult organs such as the brain, peripheral blood, skin, teeth, etc. Once tissues get damaged, endogenous stem/progenitor cells will migrate to the injured site through peripheral blood by responding to the immune cell-secreted biochemical signals [263, 264]. Therefore, homing of endogenous cells for tissue regeneration in situ would be a promising new therapeutic option to bypass the controversial of cell usage. Compared to that of the traditional cartilage tissue engineering, the recruitment of cells into cartilage defect to realize the regeneration in situ still remains rare [265]. Nonetheless, the cell-free scaffolds combined with anti-inflammatory molecules and BMSC-attractive chemokines would have positive influence on the regenerative outcome of cartilage defects. For example, Park et al. studied the in situ recruitment of BMSCs into cartilage defects by transplantation of polylactide/β-tricalcium phosphate (PLA/β-TCP) scaffolds containing IL-8 or MIP-3α [8]. Compared to those scaffolds without chemokines, the scaffolds with IL-8 or MIP-3α can highly facilitate the restoration of cartilage with a smoother surface and higher deposition of collagen. Wang et al. fabricated an anti-inflammatory scaffold composed of resveratrol-grafted polyacrylic acid and atelocollagen [266]. The scaffolds were transplanted into osteochondral defects without the employment of cells. After implantation for 12 weeks, the

proinflammation genes such as IL-1, MMP13, and COX-2 were downregulated, while the cartilage-related genes were upregulated, leading to efficient regeneration of cartilage defects. For the sake of easier application clinically, a widely accepted biomaterial instead of a brand-new one would be the best choice for fabricating the scaffold. Dai et al. fabricated a macroporous fibrin scaffold with high Fg content and mechanical strength through a porogen leaching method by using PCL microspheres as the porogen. Together with the excellent bioactivity of Fg, the cell-free fibrin scaffold could efficiently regenerate full-thickness cartilage defects in rabbit knees, resulting in neocartilage with a smooth surface, well integrity with surrounding tissue, highly deposited GAGs and collagen type II, and higher expression of cartilage-related genes and proteins, which ensure the great potential for clinical application of Fg scaffold to achieve in situ inductive cartilage regeneration [267]. A PLGA scaffold with oriented pores in its radial direction was implanted into rabbit articular osteochondral defect for 12 weeks, confirming obvious tide mark formation, and abundant chondrocytes distributing regularly with obvious lacunas in the cartilage layer [268]. A scaffold with oriented pores in radial direction can be prepared by using methacrylated hyaluronic acid via controlled directional cooling, and followed with structure-stabilization via post photocrosslinking, and further infiltrated with PLGA to enhance the mechanical strength [269]. In vivo test proved that the composite without loading cells can facilitate simultaneous regeneration of both cartilage and subchondral bone. Meanwhile, the cell-free scaffolds can facilitate cartilage regeneration in clinic too. Roessler et al. implanted a cell-free collagen type I matrix for the treatment of large cartilage defects (mean defect size 3.71 ± 1.93 cm^2, range 1.20–9.00) of the knee and conducted a short-term follow-up after the implantation. Significant pain reduction was achieved after implantation for 6 weeks, while the activity of patients was highly improved and nearly reached to preoperative value after 12 months [270].

9.4 Simultaneous Regeneration of Cartilage and Subchondral Bone

Articular cartilage defects can be divided into two forms, full-thickness cartilage defects without subchondral bone damage and osteochondral defects involving both the cartilage and the underlying subchondral bone [271]. Subchondral bone plays a pivotal role in supporting cartilage and will suffer from deterioration once cartilage is damaged. When damage of subchondral bone occurs, the neocartilage has poor integration with the subchondral bone, leading to negative regeneration of the articular cartilage defects [272]. Hence, the regeneration of structure and functions of the articular cartilage defects can be realized only if both cartilage and subchondral bone are simultaneously regenerated with good interface binding [273]. There are several problems that should be overcome for the regeneration of osteochondral defects, including the construction of different layers of scaffolds,

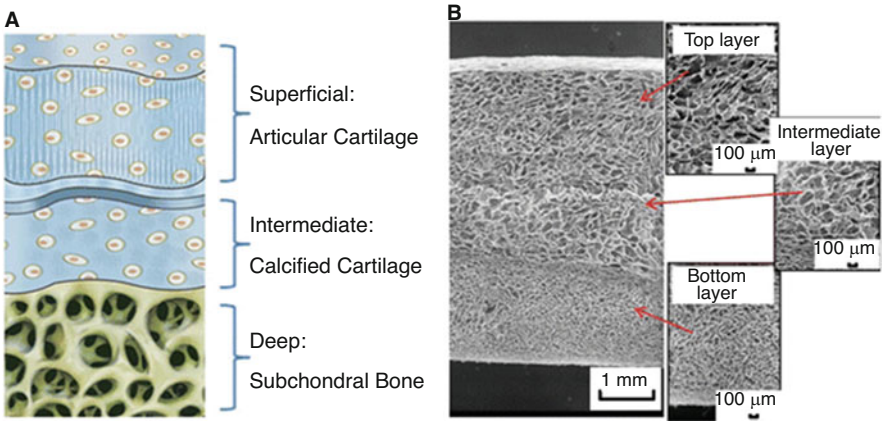

Fig. 9.6 (**a**) Schematic design of multilayered scaffolds for osteochondral defect regeneration. (**b**) Three-layered collagen scaffolds. (Reprinted from [69] with permission)

well integration of the neoformed tissues with native tissues, and the effective binding of neoformed cartilage and subchondral bone [274]. Schematic design of multilayered scaffolds for osteochondral defect regeneration and typical multilayered collagen scaffolds is shown in Fig. 9.6 [69]. Osteochondral tissues encompass cartilage layer, calcified cartilage, and subchondral bone layers in the spatial scale (Fig. 9.6a). The scaffolds with a biphasic structure based on different materials and different chemical or mechanical properties are designed for the regeneration of cartilage and subchondral bone, respectively (Fig. 9.6b) [275–279]. The evaluation of the regenerative ability of the scaffolds in vivo has found some positive results [280–283]. For example, the biphasic PEG/hydroxyapatite scaffold with cartilage- and subchondral bone-like hierarchical nanoroughness, microstructure, and spatiotemporal bioactive cues can be prepared by the 3D-printing technology. In vitro culture proves osteochondral differentiation of BMSCs in the scaffold [284]. The bilayered scaffold composed of PLCL, PLGA, and β-tricalcium phosphate (β-TCP) has been prepared by a sintering method and a gel pressing method. The PLGA/β-TCP layer has osteoconduction activity for bone regeneration, while the elastic PLCL scaffold has mechanoactive properties for cartilage regeneration [285]. The biphasic scaffold composed of aragonite-hyaluronic acid (Ar-HA) layers shows full regenerative ability of osteochondral defects with a critical size of 6 mm in diameter and 10 mm in depth in the load-bearing femoral condyle of goats [286].

Recently, 3D-printing technology has emerged as a promising strategy to fabricate scaffolds for osteochondral defects. 3D-printing provides many advantages, including well-controlled architecture (size, shape, interconnectivity, and orientation). The 3D-printed scaffold would provide structural and mechanical support, and sufficient nutrient supply, leading to regeneration of functional cartilage akin to native tissue. Depending on the biomaterials, 3D-printed scaffolds for cartilage regeneration can be classified as natural, synthetic, and inorganic scaffolds.

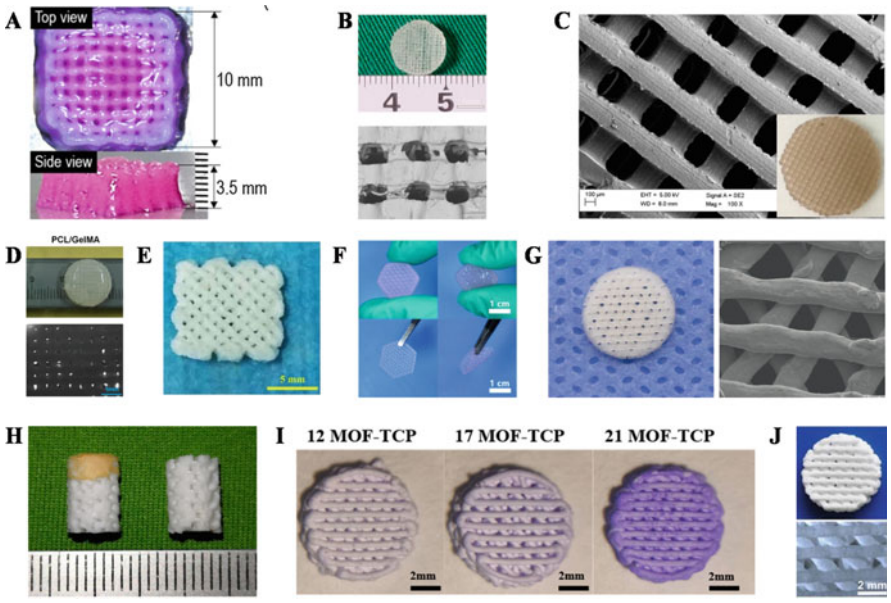

Fig. 9.7 3D-printed scaffolds of (**a**) cell-laden collagen, (**b**) modified PEG and gelatin, (**c**) modified PEG and gelatin incorporating with graphene, (**d**) polycaprolactone and modified gelatin, (**e**) short electrospinning gelatin/PLGA fibers and cartilage decellularized matrix, (**f**) gellan gum with Li-Mg-Si bioceramics, (**g**) polycaprolactone and hydroxyapatite, (**h**) bone layer (polycaprolactone / hydroxyapatite) and cartilage layer (chitosan/silk firoin), (**i**) Zn/Co-MOF-β-TCP, **(j)** Mo-doped bioactive glass ceramic. (Reprinted from [287, 291, 296, 299, 295, 303, 182, 302, 316, 317] with permission)

The natural 3D-printed scaffold is mainly designed in a form of hydrogel, by using proteins (collagen (Fig. 9.7a) [287–289], gelatin (Fig. 9.7b) [290–301], fibrin [292], and silk fibroin [298, 302]), polysaccharides (gellan gum [303], cellulose [303], chitosan [302], hyaluronic acid [294, 304, 305], alginate [290, 297, 303, 306–309], chondroitin sulfate [291]), and acellular matrix [295, 310, 311]. Compared with the traditional hydrogel with submicro- or nano-sized gel network, the 3D-printed hydrogel could be granted with macropores which facilitate the supply of oxygen and nutrients and the proliferation and differentiation of encapsulated cells. Li et al. fabricated a macroporous hydrogel with silk fibroin and tyramine-substituted gelatin by extrusion-based low temperature 3D printing [292]. The internal structure of the hydrogel could be well designed to improve the retention of stem cell aggregates and promote the articular cartilage repair. A bilayered hydrogel was fabricated using gellan gum, cellulose and sodium alginate [303]. Bioceramic particles were incorporated into the lower part of the hydrogel to mimic the subchondral bone. The hydrogel loaded with stem cells in the lower part, and with chondrocytes in the upper part could facilitate simultaneous regeneration of both cartilage and subchondral bone. Hydrogel with interpenetrating polymer network could be fabricated by 3D-printed technology using polyethylene

glycol diacrylate, gelatin methacryloyl, and chondroitin sulfate methacrylate through photocrosslinking [291]. The designed hydrogel possessed not only adequate mechanical strength but also maintained a suitable 3D microenvironment for differentiation, proliferation and extracellular matrix production of stem cells.

Compared with natural biomaterials, synthetic biomaterials are favored by researchers because of their strong controllability and mechanical properties. Up to now, several biodegradable synthetic polymers, including polyethylene glycol (PEG) (Fig. 9.7c) [291, 296], polyvinyl alcohol (PVA) [312], polyurethane [304, 311, 313], poly (lactic-*co*-glycolic acid) (PLGA) (Fig. 9.7e) [295], and polycaprolactone (PCL) (Fig. 9.7d) [182, 299, 302, 308, 309, 314, 315], have been used in 3D-printed scaffolds for cartilage regeneration. For instance, Zhou et al. developed a graphene oxide-doped, gelatin methacrylate and poly (ethylene glycol) diacrylate (PEGDA)-based 3D-printing ink, in which the PEGDA could greatly improve the printability performance of the ink [296]. Because of the strong hydrogen bonding interaction in the PEGDA solution, there exists severe extrusion swelling of the pure PEGDA solution during the most common nozzle printing process, which greatly restricts the development of 3D printing of PEGDA hydrogel. Meng et al. improved the printing accuracy of PEGDA solution by adding graphene oxide and hydroxyapatite, and realized the 3D printing of a PEGDA-based hydrogel with a biomimetic pore size gradient [312]. Inspired by the architecture of collagen fibers in native cartilage tissue, Cao et al. fabricated a tri-layered scaffold with pore size gradient based on polycaprolactone and methacrylated alginate hydrogel encapsulating stem cells [308]. The stem cells-loaded gradient 3D-printed scaffolds showed excellent cell survival, proliferation and morphology, collagen II deposition, and hopeful chondrogenic differentiation.

Moreover, the scaffolds for osteochondral repair based on bioresorbable ceramic, including hydroxyapatite (Fig. 9.7g, h) [182, 288, 297, 301, 302, 307], β-tricalcium phosphate(β-TCP) [316], and bioactive glass ceramic (Fig. 9.7f) [303, 317] can be fabricated by 3D-printing technology. Hydroxyapatite is one of the essential inorganic components from bones and teeth, which is widely applied in biomedical engineering due to their excellent biocompatibility, bioactivity, osteointegrity, and osteoconductive properties [318]. Hsieh et al. prepared a biomimetic scaffold consisting of hydroxyapatite/polycaprolactone and glycidyl-methacrylate-hyaluronic acid for healing osteochondral defects [319]. The scaffolds were implanted in the knees of a miniature pig for a period of 12 months, and realized the regeneration of hyaline cartilage. β-TCP is a typical bioresorbable ceramic for bone tissue regeneration [320]. Shu et al. prepared a 3D-printed β-TCP scaffold, which was further functionalized with zinc-cobalt bimetallic organic framework (Zn/Co-MOF) (Fig. 9.7i) [316]. The hybrid scaffolds preserve excellent antioxidative and anti-inflammatory properties to protect cells from reactive oxygen species invasion, and induce the osteogenic and chondrogenic differentiation simultaneously in vitro. Moreover, in vivo studies prove that the Zn/Co-MOF-TCP scaffolds could accelerate the integrated regeneration of cartilage and subchondral bone in severe osteochondral defects induced by osteoarthritis. Dang et al. prepared a series of molybdenum-doped bioactive glass ceramic through combining a sol-gel

method with 3D-printing technology (Fig. 9.7j) [317]. The scaffold not only significantly stimulated the proliferation and differentiation of both chondrocytes and stem cells in vitro, but also showed bi-lineage bioactivities for regeneration of articular cartilage and subchondral bone tissues in vivo.

9.5 Histological Grading of Cartilage

Histological quality of repaired cartilage is one of the most important evaluations of success in cartilage regeneration. Up to present, various histological scoring systems are used to evaluate the quality of cartilage tissues. Basically, a scoring system should be comprehensive but also applicable to researchers with limited knowledge of cartilage histology. In summary, the systems are divided into three categories to describe the osteoarthritic, in vivo repaired, and in vitro engineered cartilage, respectively [321].

Scoring systems for osteoarthritic cartilage focus on the degenerative features of healthy or diseased cartilage. Histological-Histochemical Grading System (HHGS) is the first system for the evaluation of osteoarthritic cartilage [322]. It evaluates the cartilage structure, cell distribution, Safranin-O staining, and tidemark integrity to classify the level of cartilage damage. HHGS is applied in the grading of both human and animal cartilages [323, 324]. Although widely used, HHGS is not efficient to evaluate the specific extent of cartilage deterioration [325]. Osteoarthritis Research Society International (OARSI) developed an Osteoarthritis Cartilage Histopathology Assessment System for better evaluation of the severity and the extent of cartilage surface damage during the arthritic process [326]. The OARSI system is more adequate for the assessment of mild osteoarthritis and could be more conveniently used by less experienced observers [325].

Many scoring systems are developed to evaluate the regeneration of cartilage defect in vivo. O'Driscoll score, Pineda scale, Wakitani score, OsScore, Knutsen score, and International Cartilage Repair Society (ICRS) score are widely used [321]. O'Driscoll is the first scoring system to assess the repaired cartilage in vivo and is frequently used for cartilage analysis in animal studies [327]. However, many different subitems make it a bit lengthy and complicated to use. Pineda scale is developed to simplify the assessment and is applied to evaluate the self-healing ability of cartilage defect in rabbit at the first beginning [328]. After that, Wakitani introduced a modified scoring system based on Pineda scale, which is extensively applied to evaluate animal cartilage repair in vivo [329]. O'Driscoll, Pineda scale, and Wakitani score are mainly used to evaluate cartilage repair in animal models. In contrast to the animal studies, the study of cartilage repair in human is hard to evaluate due to the infeasible harvest of large biopsy. Considering that, Robert et al. proposed a scoring system for small biopsy of repaired human cartilage, which is named as OsScore [330]. Moreover, ICRS introduced ICRS I and ICRS II scoring systems for more easy and reliable histological evaluation of repaired cartilage [331, 332]. ICRS scoring systems are based on a catalogue of repaired cartilage as

a reference for scoring. Distinguished from other systems, ICRS scoring enables discrimination of each subitem, instead of summarizing all the subitems to create a total score. Compared with the ICRS I, the ICRS II contains additional categories, making it more comprehensive. Especially when a scaffold is used in cartilage repair, a category of inflammation can be included to the ICRS II [333].

Scoring system for engineered cartilage should focus on the quality of newly generated cartilage after engineering in vitro. Few histological scoring systems are available for the evaluation of engineered cartilage. O'Driscoll introduced a simple scoring system to evaluate the density of GAGs in the engineered cartilage [334]. This system is not sufficient since many other characteristics, for example, cell morphology, are not included. Another grading system, Bern score, was validated for the evaluation of engineered cartilage [335]. In contrast to O'Driscoll score, Bern score has a broader score range, which gives more information about the characteristics of tissue [336].

9.6 Challenges and Perspectives

Although the cartilage tissue engineering has been investigated for over two decades, rather limited success is achieved to develop clinically relevant outcomes. Nonetheless, significant strides have been made to select optimal cell sources; to identify suitable chemistry, morphology, and compliance of scaffold materials; and to optimize culture conditions and dose and delivery of soluble factors, which are of great importance to develop models of cartilage development in vitro and regeneration of cartilage defects in vivo. Meanwhile, many efforts have been made to overcome the limitations in cell harvesting and to establish culture and implantation techniques in vitro. Novel methods of manufacture such as 3D printing have opened new horizons for constructing personalized constructs for cartilage regeneration. A thorough understanding of the biological processes at both cellular and molecular levels will ensure the safety and effectiveness of these innovations. With the deep understanding of pathological and healing principles under cartilage defects, cell homing and in situ inductive regeneration of both cartilage and subchondral bone are also full of prospects. All these developments, taken together, may in the future lead to the successful and cost-effective translation from the bench top to the bedside by using novel cell/biomaterial constructs in cartilage regeneration.

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