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Chapter 3

Three-dimensional Bioprinting for Cartilage Regeneration

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Abstract: Articular cartilage is hyaline cartilage which has very limited self-repairingcapacity after its degeneration or injury. Recently, three-dimensional (3D) bioprinting provides a promising method for repair and regeneration of articular cartilage. Significant progress has been made in 3D bioprinting for cartilage regeneration, particularly in printing hydrogels in combination of cells and growth factors. In this chapter, we reviewed recent progress in cartilage 3D bioprinting, including the use of various cell sources and growth factors for cartilage formation. We also discussed the challenges and the future research directions of cartilage regeneration.

Keywords: Bioprinting, bioink, cartilage, tissue engineering, regenerative medicine.

1. Introduction

Articular cartilage is hyaline cartilage, which is rich in collagen type II and proteoglycan, and plays an important role in joint activities through bearing the mechanical load or lubricating joints. Unlike most tissues, articular cartilage does not have blood vessels, nerves, or immune response, and shows limited capacity for self-repair after degeneration or injury.[1] Currently, there are four main surgical approaches to treat articular cartilage lesions: microfracture, autologous chondrocyte implantation (ACI), mosaicplasty and osteochondral allograft.[2,3] These treatments often result in fibrous repair tissue that is rich in collagen type I. As the hyaline cartilage lacks mechanical properties, their fibrous repair may lead to degenerative changes and arthritis.[4,5]

A conventional scaffold-based tissue engineering method is based on random cell-seeding and growth factor administration.[6] In this approach, cells can only attach on the surface of the scaffold; their distribution inside and the inner composition of the product cannot be controlled.[7] Conventional scaffolds have obvious disadvantages that affect their clinical applicability, e.g. limited cell-seeding efficiency and control over spatial distribution and localization.[6,7]

Recently, cartilage tissue engineering (CTE) provides a promising method for cartilage repair and regeneration. Many reports have demonstrated the success of these methods in growing chondrocytes or undifferentiated cells alone or in combination with various types of three-dimensional (3D) scaffolds and hydrogels fabricated from natural or synthetic materials.[8–10] However, it is still difficult to obtain the long-term outcome of cartilage repair. With the boom of 3D bioprinting and new engineering technologies to create scaffolds of different materials and shape, there has been a wide development of printers and machines. Several "additive manufacturing" technologies that allow the fabrication of customized parts and devices with geometrically complex structures have been applied in the field of biofabrication.[11] These include fused deposition modeling (FDM),[12,13] pneumatic extrusion printing, stereolithography,[14–16] extrusion printing gels,[17] inkjet printing,[18–21] and selective laser sintering (SLS).[22,23] With regards to cartilage regeneration, hydrogel-based scaffolds are the main materials used, given their inherent compatibility with

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chondral tissue. And inkjet and pneumatic extrusion printers are the most commonly used machines in this field of tissue engineering.

3D bioprinting is a promising biofabrication method for cartilage regeneration. This emerging technology has overcome many limitations of current CTE method. This process combines cells and biomaterials in an ordered and predetermined way. Because the cells are immerging into the printing composition, it allows for the accurate positioning of cells and fabricating the construct in a layer-by-layer deposition process. In this chapter, we review the recent advances in cartilage bioprinting, classify the cell sources for cartilage formation, identify the current challenges and discuss the directions for future developments in cartilage regeneration.

2. Advances in Cartilage Bioprinting

2.1. Thermal Inkjet-Based Bioprinting

Inkjet printing is a non-contact printing technique that reproduces digital pattern information onto a substrate with tiny ink drops. Air bubbles generated by heating in the printhead collapse to provide pressure pulses to eject ink drops with various volumes from 10 pL to 150 pL. Bioink prepared for thermal inkjet printing is usually water based in order to minimize the clogging of the printhead. In 2012, Cui *et al.* developed an inkjet-based bioprinting system with simultaneous photopolymerization capable for 3D CTE (Fig. 1).[24]

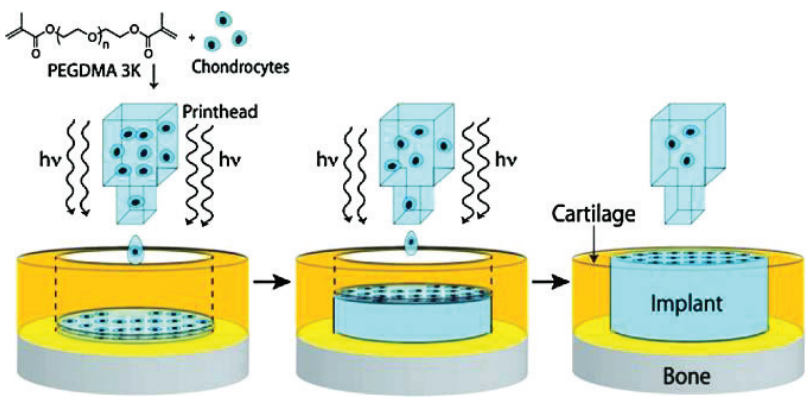

Fig. 1. Schematic of bioprinting cartilage with simultaneous photopolymerization process. PEGDMA, poly (ethylene glycol) dimethacrylate; hv, UV light energy.[24]

A polymerizable bioink was prepared by combing a polyethylene glycol dimethacrylate (PEGDMA) with a photoinitiator and a suspension of human chondrocytes. Printed cell-laden hydrogel firmly integrated with native tissue in 3D biopaper maintaining cell phenotype with consistent gene expression analysis and biochemical data.[24]

2.2. Extrusion-Based Bioprinting

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Extrusion-based printing allows the deposition of cell-laden filaments and is regarded as the most suitable technique for the 3D bioprinting of viable constructs of several centimeters in size and with high cell densities. Consequently, for the printing of cartilage constructs, extrusion-based printing techniques are most often considered. Hydrogel prepared for extrusion printing must be viscous enough to keep its shape during printing and must have cross-linking abilities allowing for it to retain the 3D structure after printing. Cross-linking can be induced chemically (e.g. calcium ion to cross-link alginate), thermally, or using UV or visible light with the addition of appropriate initiators. For bioprinting, these cross-linking methods can be used separately or combined with each other.

Cartilage contains predominantly collagen, proteoglycans, water, and low numbers of chondrocytes. To mimic the environment for chondrocytes growth, a lot of studies are focused on natural polymers, such as collagen (Col), gelatin (Gel), hyaluronic acid (HA), chondroitin sulfate (CS) and alginate (AL). Gelatin is a water-soluble protein obtained by the denaturation of collagen. Functionalization of gelatin with unsaturated methacrylamide groups results in gelatin–methacrylamide (GelMA), which can form covalently cross-linked hydrogels in the presence of a photoinitiator and light. However, GelMA solutions have a low viscosity at 37°C which is incompatible with extrusion-based bioprinting. In 2013, Schuurman *et al.*[25] improved the printability of GelMA by adding HA. HA and CS are two of the most abundant glycosaminoglycans (GAGs) in cartilage Schuurman *et al.* They can be functionalized with methacrylic anhydride to become photocrosslinkable.[25] In 2014, Levett *et al.* developed a biomimetic extracellular matrix (ECM) for CTE centered on photocurable gelatin, HA and CS. They

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encapsulated human chondrocytes in GelMA-based hydrogels, and showed that with the incorporation of small quantities of photocrosslinkable hyaluronic acid methacrylate (HAMA), and to a lesser extent chondroitin sulfate methacrylate (CSMA), chondrogenesis and mechanical properties can be enhanced.[26] In the study by Levett PA *et al.* they investigated in detail the role of HAMA in the developed mechanical properties of engineered cartilage constructs. Their result showed that combinations of GelMA and HAMA are promising candidates for CTE. Encapsulated chondrocytes display a predominantly rounded morphology, and secreted ECM that increases the compressive modulus by up to three-fold over eight weeks culture.[27] In 2016, Costantini *et al.* presented an innovative method based on a coaxial-needles extruder for 3D printing and bioprinting alginate and ECM analogues-based bioinks.[28] They showed that by blending alginate with photocurable polymers such as GelMA, CSMA and HAMA, it was possible to formulate ECM biomimetic inks that can be used for CTE. All the employed hydrogels exhibited an enhanced chondrogenic differentiation of bone marrow-mesenchymal stem cells (BM-MSCs) after 3 weeks of culture in chondrogenic medium. Among the formulated bioinks, the one composed of alginate, GelMA and CSMA turned out to be the best candidate in neocartilage formation with the highest collagen type II/collagen type I and collagen type II/collagen type X ratios.[28]

2.3. Hybrid Bioprinting

Despite the ability to mimic the native properties of tissues, printed 3D constructs that are composed of naturally-derived biomaterials still lack structural integrity and adequate mechanical properties for use *in vivo*, thus limiting their development for use in load-bearing tissue engineering applications, such as cartilage.

The use of synthetic polymers such as poly (e-caprolactone) (PCL) and poly (D,L-lactic-co-glycolic acid) (PLGA) for scaffolding has yielded higher mechanical strengths, higher process ability, and controllable degradation rates. These synthetic polymer scaffolds can provide a biologically favorable, highly hydrated 3D structure similar to natural cartilage matrix. Therefore, combining both hydrogel and

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polymeric components into a hybrid construct can mimic the biologically and structurally supportive properties of cartilage, offering promise for optimizing CTE strategies.

In 2015, Kundu *et al.* used a multihead deposition system (MHDS) to fabricate 3D cell-printed scaffolds through layer-by-layer deposition of PCL and chondrocyte cell-encapsulated alginate hydrogel.[29] The 3D cell-printed scaffolds of PCL–alginate gel were implanted in the dorsal subcutaneous spaces of female nude mice. Histochemical (Alcian blue and haematoxylin and eosin (H&E) staining) and immunohistochemical (collagen type II) analyses of the retrieved implants after 4 weeks revealed enhanced cartilage tissue and type II collagen fibril formation in the PCL–alginate gel (+TGFβ) hybrid scaffold.[29]

In 2016, Izadifar *et al.* demonstrated that bioprinting 3D hybrid constructs of PCL and cell-impregnated alginate hydrogel is a promising approach for CTE (Fig. 2).[30] They evaluated the heat distribution of printed PCL strands and the rheological property and structural stability of alginate hydrogels at various temperatures and

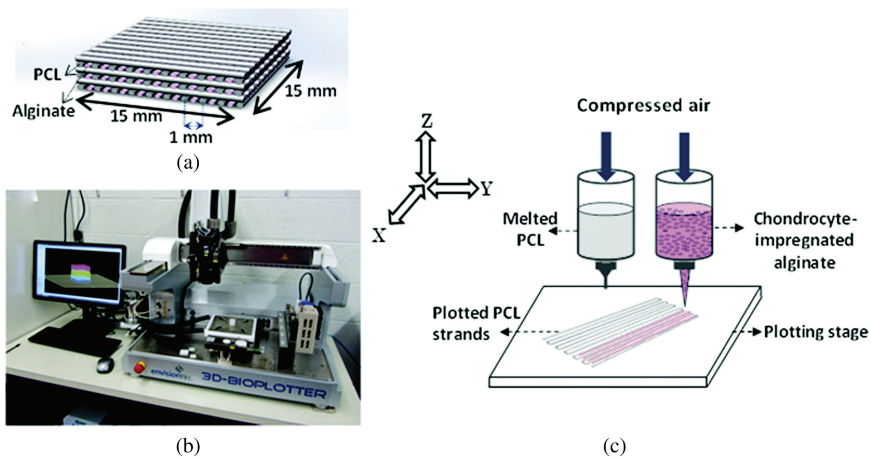

Fig. 2. Design and 3D bioprinting of hybrid constructs with structural and biological features. (a) Schematic of designed 3D hybrid construct with alternating strands of polycaprolactone (PCL) and chondrocyte-impregnated alginate in each layer, (b) 3D bioplotter system employed for biofabrication of designed hybrid constructs, and (c) hybrid biofabrication using pneumatic dispenser heads.[30]

concentrations. The cell viability, proliferation, and cartilage differentiation were observed at high levels in hybrid constructs.[30] Their data suggest that this hybrid fabrication method may improve engineering of cartilage and other tissues. Complex structural and biological properties could be designed into constructs that mimic the zonal characteristics of articular cartilage. Such biomimetic tissue constructs may promote more natural ECM formation.[30]

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2.4. Optimization of Cartilage Bioprinting, from Photoinitiator to Formulation of Photocurable Bioinks

The most popular system for cartilage 3D printing is the combination of GelMA with other materials (HA, HAMA, Alginate...) in the presence of a photoinitiator to cross-link under UV light (320–365 nm). 2-hydroxy-1-[4-(2-hydroxyethoxy) phenyl]-2-methyl-1-propanone (I2959) is the most commonly used photoinitiator. However, the use of UV light has shown that it can influence chromosomal and genetic instability in cells.[31] Therefore, other photoinitiators that absorb in the visible light range may offer significant advantages for bioprinting. Fairbanks *et al.* have synthesized a initiator, lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), and explored its potential for application to photo-encapsulation of living cells.[32] The initiator demonstrated remarkable advantages over I2959, including greater water solubility, increased polymerization rates with 365 nm wavelength light, and absorbance above 400 nm that enables efficient visible light polymerization. Cell survival of fibroblasts encapsulated in LAP-initiated PEG diacrylate hydrogels was 95% or greater for every condition evaluated.[32] The survival rate of cells during the printing is affected by many factors. Billiet *et al.* examined the influence of needle type (conical vs. cylindrical), needle internal diameter, and dispensing pressure on the viability of cells.[33] They found that the highest cell viabilities, >97%, were observed at low dispensing pressures (1 bar) using a conical needle type ($\Phi = 200\ \mu$m). In addition, ALMA has maintained the characteristic of alginate in the formulation, which can be cross-linked by calcium ion.[33] In our own study, we have investigated different formulations of photocurable bioinks

made of GelMA+HAMA, GelMA+ALMA and GelMA+HAMA+CSMA. Our primary data showed that viscosity of 5% GelMA+3% ALMA is suitable for our bioprinting system.

2.5. Bioprinting *In Situ*

A major advantage of the bioprinting approach is the ability to tailor implants to the anatomy of the defect and/or specific lesion by using medical imaging data to inform implant design. However, surgical approaches to chondral injury repair nominally require an initial debridement step to remove excess fibrous tissue around the defect. This means the size and shape of the final defect to be filled may not be accurately known prior to surgery and so prefabrication of the construct may not represent the best approach. Potentially, cartilage defects could be filled *in situ*, by printing the implant directly into the lesion.[34] In 2016, O'Connell *et al.* described the development of a handheld biofabrication tool, dubbed the "biopen", which enables the deposition of living cells and biomaterials in a manual, direct-write fashion.[35]

2.6. Bioprinting with Bioactive Material

Although 3D print scaffolds with cells offer much promise for articular cartilage repair, this kind of cell-based procedure is much more complex and must go through a very strict approval process from drug regulatory authority before applying in the clinic. Bioprinting with bioactive material, without live cells for cartilage regeneration, may simplify the process and get into clinical practice earlier.

Microfracture, the most widely used procedure for the repair of cartilage defects, so far, enhances migration of MSCs from bone marrow to the site of a cartilage defect, which provides a good cell source for the bioactive scaffold. However, microfracture often results in the formation of fibrocartilage that is biochemically and biomechanically inferior to hyaline articular cartilage. To induce MSCs to differentiate to hyaline cartilage, print scaffolds with sustained release of growth factors will provide favorable condition.[36] In 2010, Lee *et al.*

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fabricated an anatomically correct bioscaffold using a composite of poly-ε-caprolactone and hydroxyapatite, infused with transforming growth factor β3 (TGFβ3) to induce MSCs to chondrocytes. Their results suggest that the entire articular surface of the synovial joint can regenerate without cell transplantation.[37] In 2016, Shi *et al.* described a novel strategy by utilizing an ultraviolet (UV) light-reactive, rapidly cross-linkable matrix integrated with KGN-loaded nanoparticles to obtain the natural hyaline cartilage with a simple procedure. Their data shows that after a convenient one step procedure, this KGN-based release strategy could efficiently and persistently promote chondrogenesis.[38]

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3. Cells for Cartilage Bioprinting

3.1. Cell Source

3.1.1. Chondrocyte

In 1994, Brittberg *et al.* introduced the autologous chondrocyte transplantation for the first time. They obtained healthy chondrocytes from the injured knee in the process of arthroscopy and cultured the chondrocytes for 14–21 days, and then injected chondrocytes to the defective area. This method reduced pain and swelling in all patients, and 2 years later, 14 of the 16 patients showed good to excellent results.[39] This result consists with Peterson *et al.* who evaluated 94 patients with 2–9 years follow-up after autologous chondrocyte transplantation. They found that 53 patients showed good repair tissue fill, and histologic analysis of 37 biopsies showed a correlation between hyaline like tissue and good to excellent clinical results.[40]

3.1.2. Mesenchymal stem cells

Human MSCs can be isolated relatively easily from a variety of adult mesenchymal tissues, have extensive proliferation potential and are easily expanded without loss of their multilineage differentiation potential within several passages. Therefore, MSCs are perceived as a

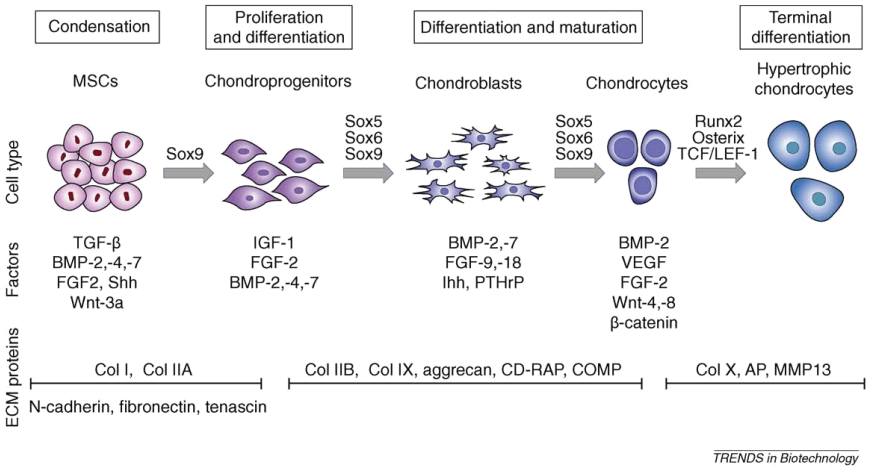

Fig. 3. Sequence of events leading to the differentiation of MSCs toward chondrocytes. alkaline phosphatase (AP), cartilage-derived retinoic acid-sensitive protein (CD-RAP), Col, collagen; cartilage oligomeric protein (COMP), matrix metalloprotease (MMP), vascular endothelial growth factor (VEGF).[47]

good cell source for regeneration of cartilage.[41] Recently, there are more and more studies focusing on the chondrogenic potential of MSCs from bone marrow,[42–45] adipose,[43–45] synovium,[44,45] periosteum,[44,47] umbilical cord[46] and muscle.[44,45] In 2005, one group compared the yield, expandability, differentiation potential, and epitope profile among MSCs from five different tissue sources (bone marrow, synovium, periosteum, muscle and adipose). Their results indicate that there are significant differences in MSC properties according to tissue source, further than donor and experimental variations. Synovium-derived cells particularly had the greatest ability for chondrogenesis.[44] Figure 3 illustrates the whole differentiation of MSCs towards chondrocytes.

3.1.3. Embrgonic stem cells/induced pluripotent stem cells

Currently, there are three methods for the differentiation of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs)

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toward chondrocytes, each method has advantages along with disadvantages.

Many research groups have published articles on the coculture of human embryonic stem cells (hESCs) and chondrocytes; they found that chondrocyte-secreted morphogenetic factors can promote the differentiation of hESCs. Coculture with primary chondrocytes can induce hESCs to differentiate toward the chondrocyte lineage. This coculture system formed colonies and secreted ECM containing GAG.[48] Furthermore, this result is confirmed by gene expression and immunostaining analysis. In the meantime, during monolayer expansion of the chondrogenically-committed cells, a dynamic expression profile of chondrocyte-specific genes was observed.[49] One obstacle of human pluripotent stem cells (hPSCs) clinical application is its tumorigenicity, but Karlsson. *et al.* take demonstrated that no teratoma formation was detected after transplantation of cocultured hESCs under the kidney capsule of SCID mice.[50]

The second chondrogenic differentiation method involves the formation of EBs from ESCs/iPSCs. For example, human iPS cells from fetal neural stem (FNS) cells can be successfully subjected to *in vitro* chondrogenic differentiation by EBs formation to form functional cartilaginous tissue.[51] Comparison shows that self-assembly of cells obtained by enzymatic dissociation of EBs is superior to self-assembly of EBs.[52] When chondro-induced human iPSCs (hiPSCs) were implanted in osteochondral defects created on the patellar groove of immunosuppressed rats and evaluated after 12 weeks, the defects showed a significantly better quality of cartilage repair than the no-treatment control, and the majority of cells in the regenerated cartilage consisted of implanted hiPSCs.[53]

Recently, a three-stage protocol has been developed for the differentiation of hESCs toward chondrocytes, driving the differentiation of hESCs through primitive streak–mesendoderm and mesoderm intermediates to a chondrocyte population. Gene expression analysis suggests that the hESCs progress through primitive streak or mesendoderm to mesoderm, before differentiating into a chondrocytic culture comprising cell aggregates which also express cell surface CD44 and aggrecan, and deposit a

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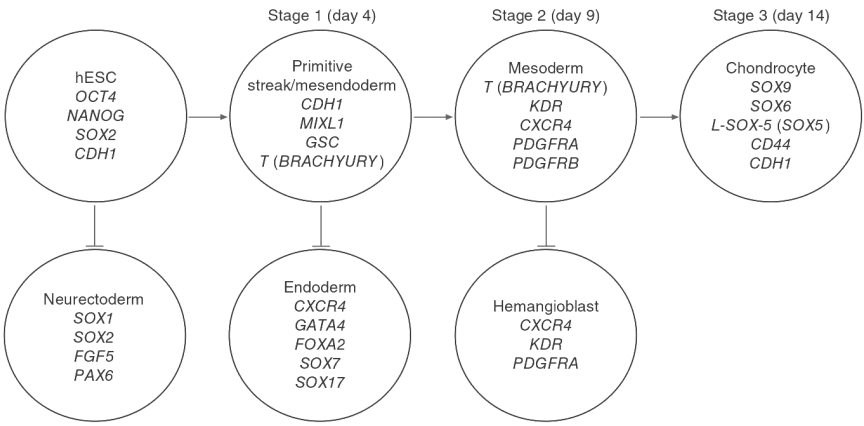

Fig. 4. Schematic of directed differentiation protocol in three stages. In stage 1, pluripotent hESCs are directed toward a primitive streak–mesendoderm population; in stage 2, differentiation proceeds to a mesoderm population; and in stage 3, toward chondrocytes. As some genes are expressed in different cell lineages and at different stages, the developmental status of each cell population was characterized by expression of panels of marker genes including SOX2, which is expressed by both pluripotent hESCs and cells derived from the neurectoderm germ layer, CDH1, expressed on pluripotent and mesendoderm cells and CXCR4, used to identify cell lineages from both the endoderm and mesodermal-derived hemangioblast.[54]

sulfated GAG and cartilage-specific collagen II matrix. At this final stage, 74% (HUES1 cells) and up to 95–97% (HUES7 and HUES8 cells) express the chondrogenic transcription factor SOX9.[54] Figure 4 schematically shows their differentiation protocol in three stages.

3.2. Growth Factors for Chondrogenic Differentiation

3.2.1. The transforming growth factor-β superfamily

The transforming growth factor-β (TGF-β) family comprises transforming growth factor-β, bone morphogenetic proteins (BMPs),[55] activins and inhibins.[56] TGF-β members direct the mesenchymal stem cell fate into the chondrogenic and osteogenic directions.[57]

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3.2.1.1. *TGF-β*

The TGF-β family includes five members (TGF-β1–5) which are predominantly produced in bone and cartilage.

TGF-β1 at a concentration of 5 ng/mL has the most pronounced effect to stimulate bone marrow-derived MSCs for their chondrogenic differentiation in a dose-dependent relationship.[58] When transferred to the full thickness articular cartilage defects, it can be restored with hyaline cartilage from *in vivo* differentiated autologous MSCs, which was superior to implantation of *in vitro* differentiated autologous MSCs, as evidenced by a better surface zone repair and reconstitution. All results indicated TGF-1 was able to induce the MSCs into chondrocytes *in vivo* and prevent the deterioration of newly formed cartilage with time.[59]

hESCs also respond to TGF-β1, the hESC-derived cells exhibited growth factor–dependent matrix production in pellet culture but did not produce tissue characteristic of cartilage morphology. But when these cells were encapsulated in RGD modified poly(ethylene glycol) hydrogels, they formed neocartilage with basophilic ECM deposition within 3 weeks of culture, and produced cartilage-specific gene up-regulation and (ECM) production.[60]

3.2.1.2. *Bone morphogenetic proteins*

Bone morphogenetic proteins (BMPs) play a role in many stages of chondrogenic differentiation, initiating chondroprogenitor cell determination and differentiation of precursors into chondrocytes, and also at the stage of chondrocyte maturation and terminal differentiation.

Recombinant human BMP-2, -4 and -6 can enhance *in vitro* cartilage formation of MSCs from bone marrow. All the BMPs tested, increased chondrogenic differentiation as assayed by immunohistochemistry and by the size and weight of the cartilage synthesized.[61] Other group transfected BMP-4 into adipose-derived stem cells (ADSCs) by nanoparticles and evaluated the cartilage repair effect in a rabbit model. The results showed that the collagen type II protein and aggrecan expression was up-regulated *in vitro*.[62] The similar results were obtained when hESCs were cultured as an aggregate in a pellet culture system with BMP-7 treatment.[63]

3.2.2. Fibroblast growth factor family

The fibroblast growth factor (FGF) family comprises 22 structurally related proteins that bind one of four FGF receptors (FGFRs).

By forcing the expression of FGFR3in a pluripotent murine mesenchymal stem cell line (C3H10T1/2), Hoffmann *et al.* found that FGFR3 is adequate enough for chondrogenic differentiation, indicating an important role for FGF-signaling during the manifestation of the chondrogenic lineage in this cell line.[64] Meanwhile, another murine genetic-based model revealed that FGF18 signals through FGFR3 to promote cartilage construction.[65]

Allison *et al.* studied the effect of FGF-2 on bone marrow-derived MSCs and demonstrated that the use of 100 ng/mL of FGF-2 significantly increased MSCs pellet DNA and GAG content. Collagen type II content of the pellet was also increased by use of 10 ng/mL and 100 ng/mL of FGF-2. Collagen type II and aggrecan mRNA levels were increased by treatment with FGF-2 too.[66] FGF2 seems to have a positive effect on hESCs in the process embryoid bodies (EBs) formation and can induce greater numbers of osteogenic and chondrogenic lineage cells.[67]

3.2.3. Insulin-like growth factor family

Insulin-like growth factor-1 (IGF-1) significantly increased chondrogenesis in a dose-dependent manner when administered continuously throughout the culture period. *In situ* hybridization for type II collagen showed that continuous IGF-1 maintained type II collagen mRNA expression throughout the cambium layer from 2 to 6 weeks.[68] When entrapped in silk fibroin scaffolds, IGF-1 can stimulate chondrogenic differentiation of hMSC whereas no chondrogenic responses were observed on unloaded control scaffolds.[69]

3.2.4. Hedgehog family

The impact of Sonic hedgehog (Shh), a member of hedgehog family, on adult stem cells was tested on human bone marrow-derived MSCs. It showed expression of cartilage markers aggrecan, Sox9, CEP-68,

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and collagen types 2 and 10 within 3 weeks of Shh stimulation. Only r-Shh treated cells showed a very strong cell proliferation and much higher BrdU incorporation in cell assay systems.[70]

3.3. Biophysical Stimuli

3.3.1. Oxygen tension

Cartilages are mostly avascular tissues, with synovial fluid providing oxygen and nutrients that diffuse through the ECM. The lack of blood supply creates a hypoxic environment, with reports of oxygen levels that range from 1% to 8%, depending on the location of the tissue and depth inside.[71]

After exposure of mouse stromal ST2 stem cells to 1% oxygen, Robins *et al.* demonstrated that exposure to low oxygen levels induces genotypic and phenotypic changes consistent with differentiation along a chondrocyte pathway.[72] This conclusion agreed with Wang *et al.* who induced a two-fold increase in the rate of protein synthesis and a three-fold increase in total collagen synthesis in human adipose-derived adult stem cells.[73]

Hypoxic differentiation conditions enhanced the chondrogenic potential of hESCs. Koay *et al.* used the human ESC cell line H9, which showed significant increase in collagen II production by hypoxic conditions. Their result also indicates the possibility of generating a spectrum of different cartilages.[71]

3.3.2. Mechanical stimuli

Articular cartilage is primarily composed of type II collagen and proteoglycans,[74] the collagen type II provides stiffness to the tissue in tension, and the proteoglycans, which are negatively charged, attract water resulting in the provision of stiffness in compression.[75]

Dynamic compressive loading on chondrogenesis was performed on human MSCs. After 70 days of culture, dynamic compressive loading increased the mechanical properties of human MSCs encapsulated HA hydrogels, as well as the GAG and collagen contents.[76] The same result was obtained by Huang *et al.* who applied long-term dynamic

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compression to MSC-seeded constructs. Their results demonstrate that dynamic compressive loading initiated after a sufficient period of chondroinduction and with sustained TGF-β exposure enhances matrix distribution and the mechanical properties of MSC-seeded constructs.[77]

Shear force was another factor that had been widely investigated. The potential enhancing effect of surface shear on chondrogenic differentiation of hMSCs was studied by Schatti *et al.* They found that the application of shear superimposed upon dynamic compression led to significant increases in chondrogenic gene expression.[78]

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4. Current Challenges and Future Directions

4.1. Choices of Cell Sources

In Sec. 2.1, multiple cell types have been discussed for their applications in bioactive cartilage implants. So far, in the field of cartilage bioprinting research, the focus has been predominantly on the use of chondrocytes. Nevertheless, when using autologous chondrocytes, obtaining sufficient cell numbers remains a challenge, since the differentiated chondrocytes have limited proliferative capacity. Allograft of cartilage has been proved to be safe due to cartilage immune privilege, and allogeneic juvenile chondrocytes was shown to produce more ECM than adult chondrocytes.[79] But it is hard to use juvenile chondrocytes as a major cell source for cartilage regeneration due to limitations of donors.

An alternative cell type for cartilage repair is MSC, which can be derived from multiple tissues, relatively easier to expand and can be differentiated into chondrocyte-like cells in the presence of specific growth factors (see details in Sec. 2.2). Scaffolds with growth factors are also being investigated in combination with the surgical method of microfracturing to coax the patient's own bone marrow MSCs to form articular cartilage. However, adequate cues to control MSC fate have to be provided, as these cells have the tendency to progress into hypertrophic chondrogenesis and to give rise to bone formation via the endochondral pathway once implanted *in vivo*.[80]

Furthermore, the induced pluripotent stem cells (iPSCs), which show unlimited self-renewal as ESCs and can be generated from

numerous cell types (i.e. keratinocytes), remain an interesting cell source for cartilage regeneration. Although there are several publications which claimed iPSCs-derived chondrocytes' safety, more cases accretion and longer time observation are needed.

For clinical application of human iPSCs and ESCs, there is a requirement to minimize the risk of contamination with animal components. It requires that not only the culture media is chemically defined, but the culture plate coating material is xeno-free. In 2015, we have developed xeno-free coating substrate, which can be stored at 4°C and is ready to be used upon request, may serve as an easier way to amplify hESCs/iPSCs for clinical applications.[81]

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4.2. Hybrid 3D Bioprinting

Cartilage 3D bioprinting can make the transition in the clinic from non-living personalized 3D printed implants toward biologically active living implants. Although chondrogenic cell-laden bioinks can be solidified in seconds, it may take months of transition time to function as articular cartilage. It is a challenge to remain the right shape, size and position for those soft cell-laden filaments during their transition. Absorbable and biocompatible polymers, e.g. PLA and PCL, can be the right choice for scaffold to provide a temporary support for chondrocyte-laden hydrogel. Kundu *et al.* have brought in an excellent example of the hybrid printing approach (see Sec. 1.3, Refs. 29,30). It is expected that this supporting scaffold will gradually retreat when printed cartilage grows and regains its function. However, to synchronize two speeds of scaffold degradation and cartilage growth, many factors need to be optimized on choices of cell type, cell density, and bioinks and its formulation.

4.3. Functional Repair of Cartilage Damage

Functional recovery of damaged cartilage is harder to achieve, but more beneficial for patients. To reach such a goal, unique cartilage architecture needs to be rebuilt.

It is believed that there is an appropriate zone-specific compositional and mechanical heterogeneity present in articular cartilage, and

restoration of this zonal organization will improve integration and performance of the construct at the defect site.[82,83] 3D bioprinting may be a unique way to achieve this zonal construction, but it is a challenge to remain such organization after printing. We believe that mechanical loading regimes (i.e. periodical impact pressure) are important factors to rebuild the zonal organization *in vitro*, but to rebuild *in vivo,* a good rehabilitation strategy may play the trick after cartilage transplant surgery.

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4.4. Cartilage 3D Bioprinting Through Bio-Pen

Many orthopedic surgeons may like the idea of using a hand-held 3D printing device (bio-pen) to cope with variable cartilage defects in operation rooms. Although printing directly into a defect is an exciting idea, it becomes a challenge to keep the 3D bioprinter in small size as a mobile device, and retain its complexity and accuracy as a standard 3D bioprinter. This approach has been exemplified by the direct *ex vivo* printing into osteochondral plugs or femurs.[34–36] We believe that for further development of biopen, the following factors should be taken in consideration: flexible single small tube combining bioink injection and light source, safety control for air pressure and light, and integration of defect scanning and bioprinting *in situ*.

4.5. Clinical Regulatory Standards for 3D Bioprinting Cartilage Implants

For clinical application, all materials for 3D bioprinting the cartilage implants, e.g. cells, growth factors and scaffolds must follow a set of regulations from drug administration authority. To guaranty the safety of bioprinted living cartilage implants, all the cells and bioinks have to meet their quality requirements, for example, sterility, endotoxin-free, safety and reproducibility, etc. For fabrication of bioinks, the whole process needs to be incorporated in a good manufacturing practice (GMP) facility and quality management system (ISO 9001:2000). For the final clinical application, the printer itself and all

its components should be sterile and able to be handled in a sterile environment, such as in biosafety cabinets or operation rooms.

Although research on cartilage 3D bioprinting is growing rapidly, there are still lots of challenges. No approach to date has produced a regenerated hyaline cartilage with long-term stability and functional recovery. It is generally accepted that for stable long-term reconstruction and function repair, the therapy should not only address the cartilage but also focus on reconstructing the underlying bone and reestablishing joint homeostasis. In order to create constructs for successful cartilage regeneration, we need pay attentions not only on the mechanical strength, cell survival, and functionality, but also on deeper understanding of cartilage regeneration in general, including cell types, biological cues, and organization.

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