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Review

Recent Development of Biomaterials Combined with Mesenchymal Stem Cells as a Strategy in Cartilage Regeneration

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Abstract: Osteoarthritis leads to the progressive decay of articular cartilage. Due to its intrinsic avascular character, cartilage shows an inadequate capacity for regeneration. Cartilage loss may result in chronic pain, movement disorder and morbidity, which lack effective treatments except for joint replacement for late-stage osteoarthritis. To overcome this challenge, tissue engineering has emerged as a promising method. Scaffolds provide mechanical and biochemical support to stem cells that undergo differentiation and secrete a cartilage-specific matrix, and this strategy has been proven to have positive results. However, there is still a gap between the current strategy and perfection. Researchers are confronted with difficulties such as poor cell survival, insufficient differentiation, hypertrophy and endochondral calcification of neocartilage, and inadequate integration into the host tissue. The current research focuses on modifying scaffold parameters, including composition, stiffness, pore size, surface morphology, hydrophilicity and electric charge. On the other hand, cell regulation is another focus, including predifferentiation, gene editing, dynamic mechanical stimulus, and hypoxia. This review aims to provide a comprehensive discussion of existing challenges, scaffold types and properties, practical methods to improve chondrogenic potential and an outlook on future trends in cartilage bioengineering.

Keywords: osteoarthritis; cartilage repair; tissue engineering; biomaterial; mesenchymal stem cells

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1. Introduction

Articular cartilage functions by reducing joint friction and resisting mechanical load. The native articular cartilage is divided into the superficial zone for lubrication, the middle/deep (transitional) zone for resistance, and the calcified zone for load transmission to the underlying bone tissue. Cartilage fibrils have a parallel arrangement to the articular surface in the superficial zone, random arrangement in the middle/deep zone, and perpendicular arrangement in the calcified zone. Osteoarthritis (OA) mainly jeopardises cartilage and represents not only a leading cause of disability worldwide but also a common degenerative disease in people of all ages, with a high socioeconomic impact. It affects 14% of adults aged over 25 and nearly 34% of those aged over 65 [1]. Cartilage has low self-renewal ability due to its intrinsic physiologies [2,3], which are avascular, aneural, the lack of a lymphatic system [4], low cellularity in adult tissue, and a dense hydrated ECM hampering resident chondrocyte or progenitor cell migration to the defect site to secrete a reparative matrix [3]. Currently, the traditional treatment approaches focus on relieving symptoms in late-stage osteoarthritis and have not yielded effective disease-modifying outcomes.

Mesenchymal stem cells (MSCs) have recently been considered a promising cell source and have been widely investigated because of their multilineage differentiation potential, roles in modulating the immune response [5–7], release of trophic exosomes through paracrine signaling [8], and relative ease of isolation [9]. A next-generation therapy is focused on combining biomaterials with MSCs to improve pharmacological and therapeutic effects in cartilage repair. Researchers have developed three-dimensional (3D) scaffolds

for this purpose; in general, 3D scaffolds with complex porous nanotopography outperform two-dimensional (2D) structures because of better differentiation properties and maintenance of cellular lineages [10].

2. The Sources of MSCs

MSCs are abundant in the human body and can be obtained from multiple tissues. There are three primary sources: human bone marrow-derived MSCs (hBMSCs), human adipose-derived MSCs (hASCs) and MSCs derived from Wharton's jelly (hWJSCs) of the human umbilical cord. Among the above three, hBMSCs are the most commonly used, and they undergo chondrogenesis upon stimulation with GFs. However, one of the problems of hBMSCs is possessing intrinsic hypertrophy and endochondral ossification potential, clearly higher than that of hASCs [11]. Hence, scientists have found an alternative source of MSCs coming from adipose tissues, which are considered the largest reservoir. hASCs showed age-independent differentiation capacity in comparison with other stem cells [12]. However, some properties of hASCs, such as proliferation and apoptosis rates, are unstable and vary among donors [13]. It is also indicated that hASCs showed inferior chondrogenic potential compared to hBMSCs [14]. Compared to other types of MSCs, hWJSCs are isolated from Wharton's jelly of the umbilical cord and are much closer to embryo-derived stem cells, have low immunogenicity and have no risk of tumorigenesis [8,15]. In a comparison of other types of MSCs, Fong et al. [16] reported that hWJSC-seeded scaffolds strongly expressed collagen and GAGs, much higher than hBMSC-laden scaffolds, and chondrogenic genes, including *SOX9*, collagen type II and cartilage oligomeric matrix protein (*COMP*), were also highly expressed in hWJSC-laden scaffolds. Huang et al. [17] found that hWJSCs produced higher chondrogenic markers and matrix accumulation than hASCs on chitosan (CS)-based membranes upon chondrogenic induction. Thus, hWJSCs may possess a higher proliferative capacity and chondrogenic potential than hBMSCs and hASCs. This point of view still needs further investigation and more evidence to determine the optimal source of MSCs.

3. Types of Scaffolds

There are two main types of scaffolds: natural polymers and synthetic polymers. On the one hand, natural polymers are proteins (e.g., collagen, SF) and polysaccharides (e.g., Alg, CS, and HA derivatives). Natural polymers already have a long history of application in wound treatment. They are the closest substances to human tissue and show biocompatibility and biodegradability without toxic byproducts, and their technologies and properties have been widely investigated. Furthermore, in the form of hydrogels, they can retain a great amount of water. However, natural polymers are normally poor in mechanical strength. On the other hand, synthetic polymers have different properties. They allow the better control of formation, surface morphology, mechanical strength and physicochemical properties than natural polymers. Among them, poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactic-co-glycolic acid) (PLGA), poly(ε-caprolactone) (PCL) and poly(urethanes) (PU) are the most popular candidates in osteochondral regeneration. The limitations of synthetic polymers are poor hydrophilicity, proinflammatory degradation byproducts, and unmatched degradation rates [18]. However, it is noticeable that these two types of polymers are not independent. Several groups have tried to combine natural polymers and synthetic polymers to overcome the shortcomings of both, simultaneously maximising their advantages [19]. A summary of the advantages and disadvantages of natural and synthetic materials is provided in Table 1.

Table 1. Summary of advantages and disadvantages of natural and synthetic materials.

Type of Scaffold		Advantages	Disadvantages	References
3.1. Natural polymers	3.1.1. Type I/II collagen	Enough strength and stability, intrinsic bioactivity, rich in water content.	Fast degradation rate.	[11,20–30]
	3.1.2. Alginate	Similar structure to native ECM, hydrophilicity, biocompatibility, biodegradability, and nonimmunogenicity.	Fast degradation rate and insufficient mechanical properties.	[14,22,31–33]
	3.1.3. Agarose	Good biocompatibility, mechanical strength, and elasticity.	Poor degradation.	[33–35]
	3.1.4. Hyaluronic acid	Intrinsic bioactivity, high water content, outstanding elasticity, good biodegradability, and suitable for chemical modification.	Lower mechanical properties than collagen.	[17,36–40]
	3.1.5. Silk fibroin and cellulose	Balance among mechanical strength, toughness, and elasticity. Controllable slow degradation rate and good biocompatibility.	Expensive and time-consuming harvest process.	[41–46]
	3.1.6. Chitosan	Rich sources, good biocompatibility, bioactivity, biodegradability, and biodegradability.	Insufficient mechanical properties.	[17,18,23,30,47–49]
	3.1.7. Decellularised extracellular matrix	Closest to the native tissue. Intrinsic bioactivity, excellent biocompatibility and biodegradable.	Time-consuming and costly	[50–54]
3.2. Synthetic polymers	3.2.1. Polycaprolactone	Biocompatible and porous. Relatively slow degradation rate and harmless byproducts.	Hydrophobic, limited cell attachment.	[9,12,55–60]
	3.2.2. Poly(lactic-co-glycolic acid)	Easy processability, good mechanical strength, biocompatible and controlable degradation.	Poor cell attachment.	[38–40,61,62]
	3.2.3. Polyurethane	Good biocompatibility, flexibility and exceptional mechanical strength	Poor thermal capability. Utilise toxic isocyanates during synthesis. Flammable.	[63–65]
	3.2.4. Polyethene glycol and Polyethersulfone	Low cytotoxic and low immunogenicity. Suitable mechanical strength, thermal and chemical resistance. Good biocompatibility.	Weak biological activity	[66–72]
	3.2.5. Hydroxyapatite and graphene oxide	Outstanding mechanical properties. Forming composites with other materials.	Lack of bioactivity in monomer. Slow biodegradability.	[73–78]

3.1. Natural Polymers

3.1.1. Type I/II Collagen

Collagen is the most abundant protein in the human body and the primary component of ECM in cartilage. Collagen is frequently chosen as the material of scaffolds and displays excellent performance; it has gone through in vitro, in vivo and small-scale long-term follow-up clinical trials, which makes it one of the most promising materials. For type

I collagen, in vitro, Filardo et al. [24] recently fabricated 3D bioprinting type I collagen scaffolds, utilising a microvalve-based inkjet dispensing technique. This 'cell-friendly' type I collagen bioink allowed MSCs to be homogeneously suspended in the bioink priming for printing and then to manufacture anatomical and patient-specific constructs. Their results show that collagen-based hydrogels enhanced the proliferation and chondrogenesis of hBMSCs by providing biochemical signals and revealed a predominant clinical translation potential. Calabrese et al. [11] and Chen et al. [79] drew a similar conclusion that both hACSs or hWJSCs embedded in type I collagen cultured in chondrogenic medium had significant increases in chondrogenic marker gene expression and type II collagen and GAG formation. Moreover, another study indicated that hBMSCs embedded in cross-linker-free type I collagen microspheres exhibited chondrogenic matrix accumulation in vitro, and cartilage-like tissue formed after being subcutaneously injected into mice, and finally witnessed calcification [25]. Similarly, type II collagen scaffolds combined with quickly released chondroitin sulfate successfully guided hBMSCs' chondrogenesis in the absence of GFs, and this scaffold demonstrated excellent biocompatibility and properties [26].

In vivo large animal experiments revealed that predifferentiated autologous ovine MSC-seeded type I collagen hydrogels implanted within sheep medial femoral condyle defects induced hyaline cartilage, type II collagen and better ICRS histologic scores than undifferentiated MSC-seeded hydrogels at six months postimplantation [27].

Regarding the clinical trial, although it is still in the small-scale phase, it has revealed some encouraging results. Five patients who had isolated medial meniscal tears were treated with hBMSC-laden type I collagen scaffolds. At the time the follow-up ended, three patients were asymptomatic and had clinical improvement in knee function scores at 24 months without magnetic resonance imaging evidence of recurrent tears. Two required subsequent meniscectomy due to retear or nonhealing of the meniscal tear at approximately 15 months after implantation. No other adverse events occurred [28]. Another clinical trial carried out by Sadlik et al. [8] indicated that five patients with femoral condyle chondral defects received hWJSC-embedded porcine type I/II collagen scaffold implantation, and all of them achieved significant pain relief in their knees without adverse effects. Furthermore, two patients with lateral femoral condyle cartilage defects received type I collagen scaffolds and autologous hBMSCs implantation and witnessed great defect filling and incorporation into the adjacent cartilage after 30–31 months of long-term follow-up [29]. These positive clinical results warrant further large-scale investigation in the future to assess the repair capability of MSC-embedded collagen scaffolds.

3.1.2. Alginate (Alg)

Alg is a linear polysaccharide extracted from brown algae. It has a structure similar to native ECM, with hydrophilicity, biocompatibility, biodegradability and nonimmunogenicity, and can be used as a scaffold for regeneration bioengineering. Alg can absorb a large amount of water, which allows the rapid diffusion of nutrients and metabolites and is thus capable of being used as a scaffold for embedding MSCs. Nevertheless, Alg displays limitations such as a fast degradation rate and insufficient mechanical properties [22]. In vitro, hBMSCs embedded in Alg hydrogels and filled in osteochondral explants for four weeks were associated with hyaline cartilage, consistent with high *COL2* and *ACAN* gene expression and GAG content [24]. In another study, D1 murine MSCs were grown in Alg scaffolds in the absence of GFs, and this composition successfully induced chondrogenesis [31]. To enhance the mechanical properties of the sponge-like Alg structure, a more solid CS could mix with Alg, and this combination demonstrated better chondrogenic differentiation of hBMSCs and higher GAG and total collagen production than Alg alone [14]. Yang and coworkers [32] tried another mixed formulation in which they fabricated porous gelatin-Alg scaffolds and implanted them together with murine BMSCs into SCID® mice. However, in vivo experiments demonstrated that both osteogenesis and chondrogenesis were suppressed compared to in vitro culture, which revealed different responses between

in vitro and in vivo. Additionally, neocartilage only formed on the scaffold surface due to the small pore size (90 μm) and insufficient interconnectivity.

3.1.3. Agarose (AG)

AG is a polysaccharide containing residues of L- and D-galactose harvested from marine algae. It has been used as an analogue to mimic proteoglycans of cartilage and has been verified to increase the production of cartilage-specific pericellular matrix. AG scaffolds have mostly been investigated in vitro. hBMSC-seeded AG microbeads doped with 10% type II collagen could promote better chondrogenesis than pure AG matrices, demonstrating that incorporating type II collagen could enhance the cartilage regenerative abilities of AG [34]. In another study, different cell types (differentiated/delifferentiated chondrocytes or TGF-β3-pretreated calf MSCs) seeded on AG scaffolds were tested in vitro, demonstrating that differentiated chondrocyte-embedded AG scaffolds showed the highest quality of integration, amount of accumulated ECM and biomechanical properties compared to scaffolds seeded with all other cell types. TGF-β3-pretreated calf MSC-laden scaffolds also displayed sustained chondrogenesis and superior ECM deposition and integration compared to dedifferentiated chondrocytes [35]. AG hydrogels seeded with porcine MSCs were injected into defects created in cartilage explants and showed an abundance of type II collagen and GAG accumulation after six weeks of culture [33]. Because of the poor degradation of AG, it has scarcely been studied in vivo.

3.1.4. Hyaluronic Acid (HA)

HA is a class of GAG that exists in the human body, particularly in bone joints. It carries a negative charge and has hydrophilicity, anti-abrasive and compressive-resistant properties in the joints. HA enfolds chondrocytes and absorbs water molecules through negatively charged chains, which in turn contributes to the resilience of the cartilage [36]. HA has weaker mechanical strength than collagen, but it can be utilised as a scaffold since it is one of the significant components in ECM and plays a crucial role in regulating chondrogenesis [80,81]. The cross-linking density has a significant impact on HA hydrogels, and a low cross-linking density showed better chondrocyte morphology, while a high cross-linking density led to fibrocartilage and calcification [82,83]. To date, many commercial HA scaffolds have been tested in small animals. For instance, using commercial Hyalofast® HA scaffolds together with hBMSCs + cartilage pellets (CPs) supported faster cartilage regeneration in vivo in full-thickness tibial articular defects of rabbits. Neocartilage close to normality was evidenced by an intact superficial layer, typical chondrocyte arrangement, tidemark and cartilage matrix staining in histology, along with the highest International Cartilage Repair Society (ICRS) score (75%) and magnetic resonance observation of cartilage repair tissue (MOCART) score (76.26) compared to using HA scaffolds alone or HA combined with either hBMSCs or CP [36]. Rabbit BMSC-HA scaffolds (Hyaff®-11) were utilised to treat the rabbit OA model, and the results revealed that the BMSC-HA group produced hyaline-like cartilage, which was proven by morphological, histological, and immunohistochemical data. The regenerated cartilage was significantly thicker in the BMSC-HA group compared to the HA scaffold alone and the untreated control group at six months, as reported by Grigolo et al. [37]. These results may support the industrialisation of HA scaffolds.

3.1.5. Silk Fibroin (SF) and Cellulose

Generally, SF was extracted from silkworm *Bombyx mori* cocoons. In comparison to other natural polymers, SF displays a balance among suitable mechanical strength, toughness, and elasticity due to its crystallinity, hydrogen bonding, and numerous small β-sheet crystals [42]. SF has great biocompatibility and a controllable slow degradation rate with nontoxic amino acids and peptides as byproducts. In addition, SF can withstand common sterilisation techniques because of its high thermal stability. Cellulose is the most abundant natural linear polysaccharide comprising a linear homopolymer of glucose (C6H10O5)n, with n ranging from 500 to 5000. It is biocompatible, degradable,

mechanically robust and able to be easily fabricated into various shapes [43]. In vitro, the recent literature indicates that SF films/scaffolds fabricated by air-drying or freezing were seeded with canine ASCs, and both SF films and SF scaffolds showed cartilage-like tissues. The chondrogenic markers *SOX9* and *ACAN* were statistically significantly upregulated on SF films without the addition of GFs in comparison to the negative control, while the author failed to evaluate mRNA on SF scaffolds [44]. SF could be mixed with a bundle of polymers to acquire better performance. Different SF proportions have various efficiencies; for instance, SF made from 12% w/v concentration seeded with hADSCs showed the most effective promotion of chondrogenic differentiation, compared to 8% w/v and 12% w/v [45]. Jaipaew et al. [41] tested SF/HA scaffolds in different ratios (w/w) seeded with hWJSCs. The results indicate that the 80 SF:20 HA and 70 SF:30 HA groups possessed spherical cell shapes and expressed cartilage-specific markers, along with an accumulation of ECM. Higher SF concentrations also increased the mechanical strength of scaffolds [45]. Another study revealed that a 75 cellulose:25 SF scaffold coated with fibronectin (FN) significantly upregulated *SOX9*, *ACAN* and *COL2* without adding GFs. Ch chondrogenesis was undetected in the cellulose/SF 50:50 blend composition [43]. Furthermore, a blend composed of 40 fibrin:8 Alg (w/w) also induced chondrogenic differentiation; the fibrin fraction offered flexibility and improved cell proliferation, while the Alg fraction enhanced biostability and upregulated the expression of chondrogenic genes, GAGs and type II collagen [46].

3.1.6. Chitosan (CS)

CS is the second most abundant natural linear polysaccharide after cellulose, derived from partial deacetylation of chitin, which can be commonly isolated from crab and shrimp exoskeletons [23]. CS has appealing biocompatibility, bioactivity, nonimmunogenicity and biodegradability. In particular, CS has a similar molecular structure to HA, which facilitates osmotic swelling and resistance within cartilage [47]. Thus, it has been used as a scaffold in cartilage regeneration and showed positive results [30]. Recently, CS microspheres with an ECM-mimicking nanofibrous structure were fabricated via a physical gelation process and microfluidic technology. When CS microspheres were cocultured with rabbit primary chondrocytes, they displayed enhanced cell attachment and proliferation. Additionally, the microsphere–cell mixtures could form a macroscopic 3D cartilage-like composite with mechanical elasticity, as reported by Zhou and colleagues [48]. However, natural CS does not have adequate mechanical strength, so it is often combined with other stiffer materials to enhance stability. For example, Meng et al. [18] fabricated CS scaffolds combined with a demineralised bone matrix and E7 peptide sequence, which revealed increased cell viability and ECM production and improved chondrogenic differentiation ability of murine BMSCs in vitro. In parallel, the scaffolds induced hyaline cartilage after four weeks of implantation in vivo. Combining CS with other natural polymers also resulted in improved chondrogenesis. CS mixed with HA improves chondrogenesis due to the interaction between HA and CD44, which enhances cell–cell signalling. CS-HA membranes could induce faster spheroid shape formation of MSCs than CS alone and help prevent dedifferentiation. CS-HA membranes exhibit higher levels of *SOX9*, *ACAN* and *COL2* gene expression and higher GAG and type II collagen contents than CS alone and cells cultured in plates [17]. When combined with collagen, collagen offers abundant binding sites for cells. MSC adhesion, matrix production, and chondrogenic gene expression were improved in type II collagen-coated CS scaffolds, according to Ragetly and colleagues [49].

3.1.7. Decellularised Extracellular Matrix (dECM)

In recent years, dECM scaffolds derived from in vitro cultured cells have drawn researchers' interest. The dECM has the advantages of possessing intrinsic native GFs and biological features. There is evidence that the dECM could contribute to the stability of MSC stemness after long-term expansion [50]. Usually, the ECM is first deposited by hBMSCs on tissue culture plates and then subjected to a decellularisation process to remove hBMSCs, after which they are seeded with chondrocytes. Yang et al. [51]

reported that chondrocyte-embedded decellularised hBMSC-ECM scaffolds exhibited a significantly enhanced proliferation rate, better robust chondrogenesis, suppression of chondrocyte hypertrophic genes and chondrocyte phenotype maintenance in vitro than chondrocytes cultured on plates, in parallel with similar in vivo results of hBMSC-ECM scaffolds implanted into SCID® mice. Cai et al. [52] fabricated hBMSC-ECM scaffolds mimicking the early stage of chondrogenesis, which could promote the chondrogenesis of hBMSCs. Additionally, Lu et al. [53] tested hBMSCs or chondrocyte-derived ECM scaffolds, and the results reveal that these scaffolds could facilitate hBMSC adhesion, proliferation, chondrogenesis, and cartilage formation compared to hBMSCs in pellet culture. Aside from hBMSCs, hWJSCs also showed enhanced chondrogenesis on decellularised chondrocyte-derived ECM in a pellet culture system [54]. The dECM scaffolds have some limitations. For example, the preparation of ECM deposition incurs additional time costs and expenditure, and the exact mechanism by which the hBMSC-ECM enhances chondrogenesis is not yet fully understood. In the future, researchers may need to investigate signalling pathways and vital bioactive factors.

3.2. Synthetic Polymers

3.2.1. Polycaprolactone (PCL)

PCL belongs to a family of poly(α-hydroxyl esters), and it is a flexible, biocompatible and biodegradable synthetic polymer that can be fabricated into fibres or porous structures via many different methods. PCL has a relatively slow degradation rate and harmless byproducts and thus has become the most widely used polyester in many fields of medicine. PCL-based scaffolds are widely studied both in vitro and in vivo, and they can be fabricated via 3D weaving or electrospinning.

In vitro, 3D woven PCL hemispherical scaffolds were seeded with human ASCs transfected with lentiviral vectors containing interleukin-1 receptor antagonist (IL-1Ra) transgenes. When constructs were cultured for 28 days, they displayed a decrease in matrix metalloprotein production induced by the proinflammatory molecule IL-1 and an increase in total collagen and GAGs, and smooth ECM evenly infiltrated the interior and exterior of the scaffolds [55]. Electrospun PCL scaffolds with a nonwoven mesh structure provide a larger surface-to-volume ratio for cell attachment and infiltration. Moreover, coating PCL with natural polymers exhibits enhanced chondrogenic ability. Liao et al. [57] manufactured electrospun PCL coated with acellular ECM composite scaffolds containing GAGs and collagen. The composite upregulated *ACAN* and *COL2* expression, suppressed the fibroblastic phenotype of differentiated rabbit BMSCs and displayed an enhanced response to TGF-β1 treatment. The hBMSC-seeded PCL/Pluronic F127 scaffolds with surface treatment of type I collagen supported cell survival and chondrogenic differentiation, and the PCL/F127/collagen scaffolds produced the highest SOX9 and COL2A1 mRNA levels compared with PCL/F127, PCL/collagen and PCL alone. Additionally, the PCL/F127/collagen and PCL/collagen scaffolds showed abundant matrix deposition and suppressed hypertrophy. These results reveal that both F127 and collagen enhanced chondrogenic gene expression and that collagen was more effective [58].

In vivo, a 3D-printed PCL artificial trachea combined with rabbit chondrogenic predifferentiated BMSCs and respiratory epithelial cells revealed successful induction of neocartilage formation in a tracheal defect rabbit model [60]. Another small animal study tested 3D woven PCL scaffolds both in vivo utilising a nude murine subcutaneous pouch model and in vitro under simulated conditions, and indicated that the PCL scaffold was highly positive in promoting rapid hBMSC infiltration, both chondrogenesis and osteogenesis [9]. A large animal study was carried out by Vahedi et al. [12]. They treated sheep knee defects using sheep ASCs coincubated with gold precoated PCL scaffolds and demonstrated hyaline cartilage-like tissue, as well as the highest *ACAN*, *SOX9* and *COL2* gene expression, compared to a thinner layer of cartilage-like tissue and lower gene expression in the single ASC group and single PCL scaffold group.

3.2.2. Poly(Lactic-Co-Glycolic Acid) (PLGA)

PLA, PGA and their copolymer PLGA are widely used in tissue engineering. Among them, PLGA has drawn more attention in recent years. Liu et al. [38] manufactured a roll-up PLGA scaffold wrapped in a rabbit BMSC macroaggregate sheet, which gradually degraded with the increasing formation of cartilage and finally successfully produced an artificial trachea after four weeks of in vitro incubation. This result indicated that PLGA bulk scaffolds possessed potential in cartilage regeneration.

However, pure PLGA generally shows a poor ability to promote cell adhesion and proliferation due to its negative charge on the surface, which impedes cell attachment. Thus, PLGA is usually doped with bioactive molecules or materials to enhance affinity. Moreover, aside from being made into bulk scaffolds, a growing number of studies are investigating the potential of PLGA microparticles. Go and coworkers [39] developed novel magnetic microbeads composed of PLGA bodies and amine-functionalised magnetic nanoparticle (MNP)-coated surfaces. The microbeads successfully loaded D1 murine MSCs to 2D/3D target sites using external magnetic fields and induced MSC proliferation and chondrogenic differentiation in vitro. More importantly, these microbeads can be injected into synovial fluid via syringe, which makes minimally invasive surgery possible. However, this method requires a wearable magnetic device postoperation to assist in maintaining the local attachment of magnetic microbeads, which raises concerns regarding compliance, reliability and convenience. Furthermore, in some cases, a large-scale defect may require a large dose, so another issue is that the acceptable dose of MNPs has not yet been determined. Nevertheless, magnetic microbeads have a high application value and are worth improving. PLGA microbeads with hydroxyl (−OH) groups also displayed chondrogenic differentiation potential without adding GFs [40]. Another type of microbead is composed of PLGA-poloxamer 118 (P118)-PLGA, hBMSCs and the controlled release of TGF-β3. The composition could enhance the proliferation and expression of specific chondrogenic markers in vitro in the absence of any GFs [61], and the following in vivo series experiment showed successful induction of cartilage-like neotissues and protection of endogenous murine cartilage degradation in a mouse knee OA model by PLGA-P118-PLGA [62]. In vivo, hBMSCs and PLGA microspheres coated with an FN surface and engineered to release TGF-β3 were implanted into SCID® mice, and the results revealed the formation of neocartilage stained positive for type II collagen and aggrecan. This complex allowed MSCs to quickly adhere and differentiate on the surface of the microspheres while under chondrogenic induction from controlled released GFs.

3.2.3. Polyurethane (PU)

PU is among the most popular synthetic polymers because of its biocompatibility, flexibility and exceptional mechanical strength. Most of the conventional synthetic scaffolds displayed static stiffness, while dynamic mechanical changes persistently exist in native cartilage, so the scaffolds need to adapt to this environment. According to this, Wu et al. [63] developed a poly(urea-urethane) (PUU)-polyhedral oligomeric silsesquioxane (POSS) polymer (PUU-POSS) with a thermal responsive 'stiffness memory' ability via a 3D printing-guided thermally induced phase separation (3D-TIPS) technique. The PUU-POSS scaffold can transition to a soft rubbery phase at body temperature without noticeable shape change because the hard segments are responsible for the permanent shape, and the soft segment of PUU chains that soften by reverse self-assembly at a specific transition temperature is responsible for the temporary shape. The biological properties of this scaffold were investigated in vitro by seeding with human dermal fibroblast cells [63] or hBMSCs [64], showing promoted adhesion and proliferation of both cells and facilitating the osteochondral synthesis of hBMSCs. The PUU-POSS scaffold provides a wide range of tunable, dynamic physical and mechanical properties with little change to the microstructure. Before their stiffness relaxation, the PUU-POSS scaffold reached a maximum compression modulus of 0.80–0.10 MPa, which makes them potential candidates for cartilage regeneration. In vivo, rabbit ASC-seeded 3D-printed PU/HA scaffolds incorporating the small

molecule drug Y27632 were implanted into a rabbit femoral condyle defect model. The composite significantly promoted GAG and type II collagen synthesis [65].

3.2.4. Polyethylene Glycol (PEG) and Polyethersulfone (PES)

PEG is a low cytotoxic and low immunogenic polymer, but it has weak biological activity and lacks cell adhesion sites, so pure PEG has no apparent positive effect on MSC adhesion and chondrogenic differentiation [66]. Nevertheless, adding RGD peptides or ECM molecules to PEG can enhance the cell response. In vitro, researchers tested different concentrations and different peptide modifications for PEG hydrogels. Screening results showed that the 6.5% (*w*/*v*) PEG constructs cross-linked with the GPQGIWGQ peptide and containing the RGD peptide sequence sustainably facilitated cellular viability, proliferation and chondrogenic differentiation of human periosteum-derived cells (hPDCs) and murine ATDC5 cells [67]. Ravindran et al. [68] incorporated RGD peptide into PEG microspheres and cocultured them with hBMSCs, illustrating that cells aggregated in the presence of RGD-PEG microspheres, while PEG microspheres without RGD peptide failed to adhere to cells. Moreover, the hBMSCs/RGD-PEG microspheres showed higher *COL2A1* expression than the hBMSCs pellet culture. Nevertheless, it is noteworthy that the existence of microspheres may impede cell–cell adhesion and paracrine or cell–matrix interactions.

PES nanofibers show suitable mechanical strength, thermal and chemical resistance, and remarkable biocompatibility [71]. There is evidence that the nanosized structures imitating the biomechanical and biological structure of ECM play a critical role in promoting cell attachment, function, proliferation and infiltration. Mahboudi et al. [72] reported a PES nanofibrous scaffold prepared by electrospinning, and its surface was modified by plasma treatment and collagen grafting and then seeded with hBMSCs. The results show that hBMSCs-PES scaffolds appeared to display a cartilage-like morphology, containing abundant ECM, as seen by SEM and immunocytochemistry, and the level of cartilage-specific genes in the hBMSCs-PES scaffold group was higher than that in the scaffold-free group. These results support the suggestion that nanofibrous PES scaffolds successfully improve hBMSCs chondrogenesis.

3.2.5. Hydroxyapatite (HAp) and Graphene Oxide (GO)

HAp is usually combined with natural polymers to provide suitable stiffness in scaffolds with osteochondral regeneration ability. Yu and colleagues [74] tested Alg/HA and Alg/HAp scaffolds combined with hWJSCs in vitro and found that Alg/Hap scaffolds showed better cell viability, and both types of scaffolds displayed equivalent ECM production at day 30. Zhou and coworkers [75] fabricated a bilayer scaffold with an upper collagen layer and a lower collagen/Hap layer that could induce hBMSCs into chondrocytes and osteocytes, respectively. In a third study, the HAp-collagen matrix induced rabbit BMSCs into the osteogenic lineage, while the HAp-synthetic hydrogel matrix favoured chondrogenesis. This result is consistent with another earlier in vivo study that tested these two materials subcutaneously in rabbits [76]. Clinical case reports are sporadic. One patient with a large osteochondral knee defect and postseptic arthritis was treated with interconnected porous HAp ceramic and hBMSCs, and cartilage-like tissues were successfully regenerated [77]. A clinical study of level III evidence was carried out, and trilayer scaffolds composed of collagen and HAp were tested in 33 patients with 'complex cases' in their knees and achieved positive results, but they were cell-free scaffolds without the engagement of MSCs [78].

GO can absorb substantial transforming growth factor β3 (TGF-β3) through electrostatic attraction and protect TGF-β3 from being enzymatically degraded. Moreover, GO showed remarkable GF-retaining properties, releasing <0.35% TGF-β3 over 72 h and <1.72% in 28 days, which ensured long-term sustained chondrogenic stimuli without GF supplementation [73]. Based on this, in vitro, Zhou et al. [73] reported a collagen hydrogel incorporated with GO flake-adsorbed TGF-β3 and cocultured with encapsulated hBMSCs in the same gel, inducing higher chondrogenic gene expression and a more significant cartilage matrix in 28 days compared to exogenously adding TGF-β3 to media.

4. Optimal Properties of the Scaffold

The ideal 3D scaffolds need to have versatile properties to mediate cell–cell signalling and cell–matrix interactions for controlling the cellular behaviour of MSCs, specifically: (1) sufficient mechanical strength; (2) biocompatibility; (3) suitable surface morphology for cell attachment; (4) appropriate porosity and pore size to allow the cells to infiltrate as well as nutrients and waste to diffuse; (5) promoting cell proliferation, differentiation and maintenance of a chondrogenic phenotype of seeded cells; (6) capability of integrating with native tissues; and (7) controlled degradation without toxic byproducts [12,19]. All of these criteria are for a successful scaffold that promotes sustained ECM deposition and integrates neocartilage into the surrounding native cartilage. Scientists have studied how to improve the chondrogenic properties of scaffolds by means of various methods and have obtained some encouraging results. However, the correlating mechanisms are not fully understood. Until now, the optimal material and technique have yet to emerge. To obtain a clear outline of scaffold properties, Table 2 summarises their major influences and ideal conditions, and this information is elaborated in the following subchapters.

Table 2. Summary of the influences and ideal conditions of different scaffold properties.

Scaffold Properties	Influences	Ideal Conditions	References
4.1. Composition	Contribution to the very fundamental microenvironment for cells, and affects cells comprehensively.	Mimicking the natural composition of ECM.	[26,41,84–88]
4.2. Stiffness	Mainly applies influence on cell fate (differentiation).	Scaffold stiffness matches with that of natural tissues.	[26,74,82,89–98]
4.3. Porosity, pore size and pore shape	Cell attachment, proliferation, and migaration.	For chondrogenesis, at least 50% porosity and pore size of 200 μm to 500 μm is recommended.	[14,21,22,26,32,45,63,95,99–102]
4.4. Surface properties	Cell attachment and differentiation.	Rich in RGD or chemical groups, with patterned topography.	[18,40,43,72,93,95,103–109]
4.5. Hydrophilicity and electric charge	Cell attachment and proliferation.	Hydrophilic and positive charged.	[10,23,39,44,61,62,110–114]
4.6. Anisotropic structure	Cell differentiation.	Anisotropic and ordered topography/structure.	[115–117]

4.1. Composition of the Scaffold

The composition of the ECM that encloses stem cells illustrates the pivotal influence on directing cell differentiation. Matrices offer biochemical and physical cues to drive stem cells to a particular lineage [84]. For example, cells produced type II collagen on type II scaffolds and type I collagen on type I scaffolds [26]. The possible mechanism by which the composition modulates differentiation is cell–matrix interactions, including integrin expression and cytoskeleton organisation. First, different types of ECM stimulate cells to express different integrins on the cell membrane and transmit various chemical and mechanical signals into cells through integrins, which elicits a cascade of gene translational events, thus influencing cell adhesion, migration, proliferation and differentiation [85,86]. Second, cytoskeletal organisation regulates chondrogenesis via changes in microfilaments (consisting of actin) and microtubules (consisting of tubulin) [86]. Hence, the properties of scaffolds can benefit greatly from mimicking the natural composition of ECM. This strategy leads researchers to find a suitable material from or similar to the ECM. Articular cartilage is predominantly composed of type II collagen and GAGs. Knowing this, many researchers have utilised these materials to fabricate scaffolds to direct MSC differentiation. Murphy et al. [87] investigated murine BMSC-seeded scaffolds made of collagen and two types of GAGs, either chondroitin sulfate or HA. The collagen-HA scaffolds pro-

moted higher *SOX9* expression than collagen-chondroitin sulfate scaffolds; in contrast, collagen-chondroitin sulfate scaffolds expressed higher *RUNX2* expression than collagen-HA scaffolds, which indicated that HA had a chondrogenic influence, while chondroitin sulfate had an osteogenic influence on murine BMSCs. In some cases, the mixed composition did not increase ECM production; for example, incorporating HA into chitosan scaffolds displayed no significant impact on enhancing chondrogenesis. This phenomenon may come down to a low dose of HA (0.01%) added to the mixtures [88]. Nevertheless, materials with high water uptake and swelling ratios act as physical cues to promote chondrogenic differentiation [41].

4.2. Stiffness of the Scaffold

When MSCs anchor onto the substrate surface through integrin-mediated adhesion, the substrate stiffness reorganises ligands and modulates integrin binding. Meanwhile, cells reshape cytoskeletal organisation by sensing substrate stiffness and transferring mechanical signals into cells via nonmuscle myosin II [89,90]. A study reported that MSCs exhibited different morphologies and behaviours on gels with different stiffnesses; MSCs aggregated into clusters in round shapes on soft gels, spread out in elongated shapes, proliferated rapidly on stiff gels, and partially aggregated on medium-stiff gels [90]. The condensation and spherical shape are highly relevant to chondrogenesis [91]. Similarly, Wang et al. [92] reported that chondrogenesis relied on the interaction of matrix stiffness and biochemical cues. Wu et al. [93] also indicated that chondrogenesis and ECM accumulation depended on matrix stiffness, and soft scaffolds promoted better chondrogenesis in a dose-dependent manner, and vice versa. These findings illustrate that MSCs are extremely sensitive to stiffness, which could significantly affect stem cell fate [26,89], especially for the first 1–2 weeks of the early stage of chondrogenic differentiation. As mentioned above, the compression modulus of cartilage is ~1 MPa, with dynamic compressive stiffness at ~10 MPa. Hence, a soft scaffold with a similar compressive stiffness would promote chondrogenesis, and the mismatch of scaffold stiffness and adjacent native tissues may result in unexpected differentiation and the failure of the long-term integration of implants. For instance, the literature revealed that MSCs differentiated into osteoblasts when cultured on a matrix that stiffened at later time points [94].

Regarding some natural polymers that have insufficient stiffness, adding some synthetic polymers can improve mechanical properties. For example, the incorporation of HAp into Alg improved the integral stiffness of the scaffold [74]. In another aspect, a study illustrated that scaffold stiffness is integrally influenced not only by the robust elastic modulus of the composition but also by the porosity and topology (interconnection and shape of pores) [95]. Another study reported that not only would the cross-link density increase stiffness and result in the formation of fibrocartilage [82], but also that newly deposited cartilage matrices would gradually heighten the stiffness [96]. When researchers are designing scaffolds, they should consider these parameters and situations.

Unlike differentiation, whether stiffness impacts cell proliferation remains controversial. Wu et al. [93] found that the proliferation rate remained similar across PCL, PLA and PGA scaffolds with different stiffnesses. In contrast, some studies showed a higher proliferation rate and larger spreading area on stiffer substrates [97,98].

4.3. Porosity, Pore Size and Pore Shape of the Scaffold

Porosity has also been known as a regulator of cellular behaviours. It is an essential parameter because it guarantees the viability of the cells on the scaffold before expansion and differentiation. High porosity and interconnected inner structure ensure that nutrients and gases diffuse inward into the deep zone of the scaffold and remove metabolic wastes inside-out [26], allowing the cells to migrate deep into the scaffold. Porosity was found to decrease with increased cross-link density, but the pore size was not affected [32]. It has been reported that a minimum of 50% porosity is adequate for the attachment, migration

and proliferation of cells on scaffolds [63,99], but a more significant porosity (more than 90%) is more favourable [14].

The pore size affects initial cell adhesion and subsequent events, including proliferation, migration and differentiation. If the pore size far exceeds the dimensions of MSCs, it will influence MSC migration ability and speed. On the flip side, the pore size should not be too small; otherwise, it would be easily blocked by expanded cells, leading to a limitation of cell infiltration and apoptosis [21]. For cartilage regeneration, an article pointed out that collagen scaffolds with pore sizes of 50–300 μm are generally favourable to stimulate cartilaginous tissue formation [100], consistent with another study indicating that collagen-HA scaffolds with pore sizes of 90–300 μm promoted chondrogenesis. In addition, among them, the largest mean pore size (300 μm) displayed significantly higher cell proliferation, cartilage-specific gene expression, cartilage-like matrix deposition, and compressive modulus compared to other smaller sizes [101]. The reported maximum pore diameter was approximately 500–550 μm in SF scaffolds, which showed not only the best cell adhesion and cell proliferation but also facilitated chondrogenic differentiation [22,45]. Hereto, the literature showed that the pore size suitable for chondrogenic differentiation is not limited to a specific figure, but rather a wide range from 50 μm to 550 μm is acceptable. We presume that the optimal pore size may change from material to material, and the possible mechanism for this may be that porosity and pore size simultaneously affect both substance exchange and mechanical properties [102] to different extents in different materials, finally showing an overall effect on cell behaviour. Collectively, we recommend a pore size between 200 and 500 μm for chondrogenesis. For microparticles, the literature usually recommends a bead diameter in the range of 100–500 μm [62,94], which ensures that the maximum substance diffusion distance is within the range of metabolically active tissues [95].

4.4. Surface Properties of the Scaffold

MSC attachment to the surface of the scaffold is the first step prior to subsequent cellular activities. Apart from the surface roughness facilitating cell adhesion, nonreceptor mediation (weak chemical bonding), such as electrostatic, hydrogen or ionic bonding, also achieves adhesion. However, this type of adhesion lacks cell–matrix signal transmission, which is vital for the viability of cells and ECM secretion. In contrast, receptor-mediated adhesion via ECM molecules, including FN or collagen, allows cells to receive physiological signals [95]. These specific adhesion motifs on ECM molecules contain at least three amino acids symbolised by Arg-Gly-Asp (RGD) [103]. RGD is commonly used to assist cells in adhering to scaffolds without intrinsic binding sites. When RGD is integrated onto the surface of the scaffold, it can work as a ligand and specifically bind with integrin of receptor cells. In this way, cells can anchor to the surface of the scaffold and sense cell–matrix signal transmission. Scaffolds made from natural materials (e.g., collagen, AG, and fibrin) naturally possess RGD sequences, but synthetic polymers (e.g., PCL, PLA, and PLGA) may require deliberately incorporating RGD through protein adsorption or other methods [95]. The literature reported that hPDC-embedded PEG hydrogels combined with RGD promoted higher GAG deposition and chondrogenic gene expression than RGD-free PEG hydrogels [104]. Another study indicated that the E7 peptide (an RGD sequence) could significantly enhance murine BMSC aggregation, viability and chondrogenic differentiation [18]. RGD density is another crucial indicator that influences MSC focal adhesion, spreading and proliferation. Lower RGD density has been shown to enhance chondrogenesis of hMSCs on electrospun methacrylated HA scaffolds [105].

Apart from RGD, selective specific chemical groups can be an alternative. Commonly, plasma surface modification is used to introduce chemical groups onto scaffolds. Some chemical groups, such as the carboxyl (−COOH) group and −OH, present on the scaffold surface have been shown to upregulate chondrogenic marker gene expression in MSCs in the absence of GFs [40,106], while $–NH_2$ facilitates osteogenesis [40]. For instance, cellulose comprising three −OH groups per repeat could facilitate chondrogenic differentiation [43].

PLGA scaffolds that originally had poor bioactivities were activated by introducing –COOH groups and heparin onto the surface. This method finally formed –CONH– and showed binding affinity to MSCs and TGF-β1. As a result, modified PLGA scaffolds with GFs sharply increased the expression of cartilage-specific markers, along with type II collagen production [107]. The plasma surface treatment of scaffolds with N_2, O_2 and NH_3 endowed the construct surface with more hydrophilicity and more bioadhesion [72].

The abovementioned RGD or chemical groups belong to surface chemistry. On the other hand, surface physics and the nanoscale topography, including patterns of the surface, influence chondrogenesis. They affect cellular processes through changes in focal adhesion and the actin cytoskeleton. Currently, nanoscale surface modification is attracting increasing interest in tissue engineering. Specifically, a nanopillar surface facilitated hyaline-like cartilage, while a nanograting surface tended to induce fibro/superficial zone-like cartilage [93]. BMP-2-coated TiO_2 nanotubes 100 nm in size strongly supported chondrogenic differentiation, while 15 nm nanotubes greatly facilitated osteogenic differentiation, which showed fewer focal contacts and stress fibres but allowed cell aggregation to facilitate chondrogenesis [108]. Nevertheless, matrix stiffness still showed a far more dominant effect than surface patterns on differentiation [93,109].

4.5. Hydrophilicity and Electric Charge of the Scaffold

The moderate hydrophilicity and positive charge of the scaffold are considered to represent the optimal adhesive properties for cells [110]. The ability to retain water and a high swelling rate have been shown to promote cell infiltration, proliferation and differentiation [23]. The underlying mechanism is that adhesion molecules are adsorbed in a favourable geometry in this situation, making it easier for ligands to bind with cell receptors [111]. SF is quite hydrophobic in the dry state, but it becomes hydrophilic when wetted with a water contact angle of 0°, which successfully induces chondrogenesis [44]. Synthetic polymers usually exhibit intrinsic hydrophobicity that goes against cell attachment [112]. For instance, the hydrophobicity of PLGA limits the adhesion and proliferation of osteoblasts, chondrocytes and MSCs [10].

The cell membrane has a negative charge that results in a difficulty in attaching to negatively charged materials but an affinity to positively charged surfaces [113]. CS showed an intrinsic high positive charge density in acidic solution due to primary amine groups. Thus, CS can easily facilitate cell adhesion and consequent chondrogenic differentiation [114]. To enhance the affinity of intrinsically negatively charged materials such as PLGA, it needs to integrate another cationic or absorb some specific proteins onto the scaffold surface, to which the cells attach via integrin receptors [39]. For example, after combining P188, PLGA-P188-PLGA microbeads showed a positive charge, which facilitated the adhesion of cells [61,62].

4.6. Anisotropic Structure of the Scaffold

Anisotropic structures of collagen in cartilage have remarkable effects on the mechanical properties of the cartilage [115] and the differential fate of MSCs, similar to scaffolds. For instance, adding aligned nanofibers parallel to the direction of the hydrogel surface would significantly enhance the superficial zone, similar to the differentiation of hBMSCs [116]. Chou et al. [117] indicated that a bovine type I collagen scaffold containing parallel micrometre-wide channels displayed enhanced compressive properties (elasticity modulus ranged from 1.2 to 2.1 MPa) compared to control constructs without these channels in mechanical testing, along with extensive GAG and type II collagen deposition. These channels were in favour of cell localisation, aggregation and rounding, facilitating aligned neo-cartilage formed perpendicularly along the length of guidance channels, similar to the deep zone of native articular cartilage. Scaffolds fabricated using natural materials through gelatinisation and other methods typically form unoriented and disarrayed matrices. On the other hand, synthetic materials are more accessible to form oriented structures via pre-

cisely controlled 3D printing or woven methods, which endows an advantage to synthetic materials for customisation.

5. Enhancement of Chondrogenic Differentiation via MSC Regulation

Apart from modifying scaffolds themselves to a better status, another target naturally focuses on MSCs, and many methods independent of scaffolds can be used. Using GFs is the most common way to induce MSC differentiation, while other approaches, such as bioreactors, gene therapy, and hypoxia, have gained growing attention, and more methods have been explored to enhance MSC chondrogenic potential. These strategies come from the idea that in native human joints, MSCs receive more than biochemical cues. The microenvironment simultaneously imposes mechanical loads, low oxygen concentrations and other complex factors on MSCs. Thus, comprehensive stimulations should be applied to MSCs to stimulate them to their maximum potential. Table 3 outlines the strategies that can be used to enhance MSCs on scaffolds, followed by elaboration in the subchapters.

Table 3. Summary of strategies used to enhance chondrogenic MSC differentiation.

Strategies	Ideal Conditions	References
5.1. Growth factors (GFs)	A combination of synergetic GFs. High concentration of GFs at the first week, followed by a progressive release.	[2,10,56,61,72,116,118–130]
5.2. MSCs and chondrocytes coculture	MSCs cocultured with chondrocytes. For the clinical purpose, a single-step coculture procedure is recommended.	[2,36,131–134]
5.3. Chondrogenic predifferentiation of MSCs	Currently ambiguous. Short-period chondrogenic stimulation might be beneficial.	[27,28,60,135–137]
5.4. Bioreactor and dynamic loads	Bioreactors applying both dynamic compression and shear forces.	[138–145]
5.5. Gene therapy	Transfecting MSCs with anti-inflammatory genes via viral or nonviral methods.	[55,114,146]
5.6. Oxygen tension	Controversial. Hypoxia does not always show a positive impact on chondrogenesis.	[147–151]
5.7. High cell density	At least a seeding density of 10^6 cells/mL is needed, while 10^7 cells/mL is more commonly documented.	[14,67,79,87,104,116,118,137,141,152–157]

5.1. Growth Factors (GFs)

To induce MSC chondrogenic differentiation, first, GFs are introduced. The major chondrogenic GFs include members of the TGF-β superfamily, insulin-like growth factor (IGF), and fibroblast growth factor (FGF) [10,72]. The TGF-β superfamily includes TGF-βs, bone morphogenetic proteins (BMPs), activins and inhibins. Among them, TGF-β1, -β2 and -β3, BMP-2, -4, -6, -7, -13, and -14, and IGF-1 are the most commonly used to facilitate chondrogenesis [2,118,119]. Numerous studies have reported that the TGF-β superfamily is a potent stimulator in chondrogenic differentiation, even at tiny doses. For biomolecule mechanisms, a study identified that the TGF-β superfamily induces chondrogenesis by activating the Smad signalling pathway and upregulating the downstream gene *SOX9* [119,120]; the latter is the main transcriptional regulator of type II collagen [121], and *SOX9* subsequently activates the *COL2A1* and *ACAN* genes [11,122]. Another function of TGF-β is downregulating type I collagen and stabilising the chondrogenic phenotype [123]. BMPs are also responsible for chondrogenesis by enhancing the synthesis of type II collagen and aggrecan. For example, BMP-6 regulates the onset of chondrogenic development [124]. BMP-2 may lead to endochondral ossification when applied in ectopic localisation, which should be considered and avoided [125]. IGF is an analogue of insulin, and two ligands, IGF-1 and IGF-2, correspond to two receptors, IGF1R and IGF2R, on the cell membrane.

IGF-1 is the most studied aspect of cartilage repair; it binds to correlative IGF1R and triggers a signal cascade to promote MSC chondrogenic differentiation along with the synthesis of aggrecan, proteoglycans, and type II collagen [126]. FGFs are heparin-binding proteins that affect differentiation and proliferation in cells. The FGF family contains 22 proteins with a molecular mass from 12 to 34 kDa. FGF-2 accelerated MSC proliferation and induced chondrogenesis [2]. Researchers also found that FGF-2 combined with IGF-1 improved cartilage regeneration in rabbit knee defects [127].

After selecting the candidate GFs, the optimal dose of each category needs to be determined. As reported, TGF-β1 and BMP-7 are responsible for superficial zone cartilage maintenance, while a combination of TGF-β1 and IGF-1 had a synergistic dominating effect on chondrogenic differentiation of hBMSCs to middle/deep zone characteristics. Regarding the calcified zone, TGF-β1 and HAp signalling show dominant effects [116]. Hence, TGF-β1 has alterable functions throughout all cartilage layers, ranging from 3 ng/mL in the superficial zone to 30 ng/mL deep in the calcified zone. However, BMP-7, IGF-1 and HAp only restrictively function in the superficial, middle/deep and calcified zones, respectively [116]. TGF-β has potent chondrogenic stimulation at a low dose (such as 1 ng/mL) while inducing hypertrophy at a sustained high content [56,128], but its mechanism remains unknown. Until now, the existing evidence has been insufficient to indicate the optimal combination and dosage of each GF, so it is still unclear and needs more investigation.

However, something that can be confirmed is that determining the rough 'optimal' dosage of incorporated GFs is not effective enough, and a consistently high concentration of GFs in the whole differentiation period can result in osteophyte formation [129]. Thus, a more precise progressive release system is considered. The literature has documented that burst GF exposure at the initial culture (the first week) followed by a relatively low maintenance dose is the best strategy in vitro. Namely, a high concentration of GFs in the first week triggers chondrogenic progression, and a maintenance dose afterwards protects differentiated MSCs from hypertrophy and calcification [61,130]. In a study reported by Morille and colleagues [61], a strategy of 32 ng/mL/day TGF-β3 for the first week and 2–3 ng/mL/day for the following culture could induce hyaline cartilage. However, the in vivo situation is more complicated because GFs may face diffusion, immune response, and the negative influence of proinflammatory factors. Moreover, GFs have a different impact within the specific cartilage hierarchy. For the middle/deep zone, GFs played a more critical role in the differentiation of hBMSCs than matrix stiffness and orientation. In contrast, stiffness had the most dominant effect on cell hypertrophy in the calcified zone, even in the absence of GFs [116]. Although GFs are widely investigated in vitro and in vivo, there is no sufficient validated evidence supporting their positive influence in humans.

5.2. MSCs and Chondrocyte Coculture

Coculture of MSCs and chondrocytes appears to be an effective way to facilitate neocartilage. The idea of coculture coming from multisignal events from various stimuli could better mimic the in vivo microenvironment of cartilage than mono-culture [131]. Indeed, a combination of both MSCs and chondrocytes on HA scaffolds demonstrated better cartilage regeneration than the use of HA combined with either MSCs or chondrocytes alone [36]. Bekkers et al. [132] carried out a comprehensive study to evaluate chondrogenic abilities by coculturing goat MSCs and chondrocytes in vitro and in both small (nude mice) and large animal (goats) models. The results demonstrate that the addition of MSCs to chondrocyte culture produced higher GAG production and cartilage-specific gene expression than 100% chondrocyte cultures, consistent with superior microscopic, macroscopic, and biochemical cartilage regeneration compared with microfracture treatment. Coculture also exhibited a more stable cell phenotype, reduced hypertrophy and heightened sensitivity to TGF-β3 compared to mono-culture [133]. The positive response of coculture could be attributed to the vital protein IGF-1 and FGF-1 released by hBMSCs [2,131], by which hBMSCs stimulate chondrogenesis of chondrocytes instead of differentiating into chondrocytes by themselves [134]. For clinical purposes, coculture shows promise as a single-step procedure;

that is, hBMSCs and chondrocytes can be isolated from the same patient who undergoes surgery and then loaded onto a scaffold, followed by implantation into the defect site.

5.3. Chondrogenic Predifferentiation of MSCs

The existing literature has no consensus on whether predifferentiating MSCs in the chondrogenic medium before loading to scaffolds is favourable for better chondrogenic differentiation remains disputable. On the one hand, growing evidence has documented that predifferentiation enhances chondrogenesis [135]. For example, predifferentiated rabbit BMSCs exhibited higher GAG accumulation and chondrogenic gene expression than undifferentiated BMSCs in vitro [60]. Zscharnack and colleagues [27] also indicated that predifferentiated ovine MSCs outperformed undifferentiated ovine MSCs in terms of the histologic quality of repaired tissues after six months in vivo, and a differentiation duration of 14 days in vitro was considered optimal. Similarly, findings from Marquass and colleagues [136] suggest that predifferentiated ovine MSCs had better histologic outcomes than undifferentiated ovine MSCs and chondrocytes within distal femur osteochondral defects in sheep after 12 months in vivo. On the other hand, some claimed that predifferentiation of MSCs is not a necessary step for tissue repair, and undifferentiated MSCs have sufficient capability for cartilage repair [28]. Another study reported contrary results that predifferentiated rabbit BMSCs-collagen microspheres showed inferior type II collagen, GAG production and cartilage thickness compared to the undifferentiated group, probably due to prolonged in vitro chondrogenic differentiation (21 days) resulting in increased apoptosis and weakened chondrogenic potential [137]. Above all, predifferentiation should have a positive influence on chondrogenesis in the condition of short-period chondrogenic stimulation, while prolonged predifferentiation may not be helpful. This hypothesis warrants further investigation.

5.4. Bioreactor and Dynamic Loads

In the natural articular cavity microenvironment, cartilage undergoes comprehensive dynamic mechanical stress, including hydrostatic pressure (HP), tension, compression and shear. Many works in the literature have indicated that these stimuli play a crucial role in determining MSC fate and matrix production [138–141], although the underlying mechanism is not precise. Bioreactors can mimic the physiological loading conditions of cartilage and shape MSCs to a chondrogenic fate in vitro.

In the research of cyclic HP applied in a bioreactor, the protocol was applying an amplitude of 10 MPa at a frequency of 1 Hz four hours per day five days per week for three weeks on porcine BMSC-seeded fibrin or agarose hydrogels. The results show enhanced GAG accumulation and reduced type I collagen and alkaline phosphatase levels in fibrin hydrogels. In contrast, MSCs had almost no response to HP in agarose hydrogels [142]. These results demonstrate that HP not only induces cartilage regeneration but also maintains the chondrogenic phenotype. Apart from HP, alternatively, 15% cyclic compressive strain was regarded as upregulating chondrogenic markers [139]. This result was recently further confirmed by Horner and colleagues [143]. They investigated different magnitudes applied on hBMSCs-PCL scaffolds, and the dynamic load was set as compressive strains of 5, 10, 15, or 20% at a frequency of 1 Hz for two hours daily for up to 28 days in osteogenic media. Their in vitro results revealed that chondrogenic gene expression and GAG synthesis were upregulated with increasing magnitude and reached peak GAG synthesis along with the most exceptional ECM alignment at the 15% strain. Interestingly, 20% strain inversely reduced chondrogenic genes and matrix expression, which illustrated that overstimulation might jeopardise chondrogenesis due to possible inflammation. Controversially, Schätti and coworkers [144] indicated that chondrogenic mechanical stimulation must have the engagement of both dynamic compression and shear forces, and either compression or shear alone is insufficient for chondrogenesis. Cochis et al. [145] used a bioreactor to apply a combination of compression and shear forces for 21 days on methylcellulose scaffolds

seeded with hBMSCs, which induced higher chondrogenic gene expression, GAGs and type II collagen accumulation than the negative control.

5.5. Gene Therapy

The application of gene therapy, such as transfecting MSCs with anti-inflammatory genes via viral or nonviral methods, has emerged as a powerful approach to treat the inflammatory microenvironment in the OA articular cavities, enhancing the chondrogenic effect [55] and avoiding protein denaturation problems. A nonviral method using microparticles to deliver plasmid DNA (pDNA) to encode TGF-β1 and BMP-2 within porcine BMSCs was reported. These BMSCs were then subjected to 3D aggregate culture and displayed an endochondral ossification process [146], but the initial phase of endochondral ossification was chondrogenic differentiation. Thus, this method has the potential to be used for chondrogenesis. The nonviral method minimises the risks of mutation and immunogenicity caused by the virus but raises the costs and decreases the transfection efficiency. Cao and coworkers [114] created a 3D nanoparticle gene delivery system comprising collagen/chitosan scaffolds, calcium phosphate nanoparticles and plasmid TGF-β1-transfected rat BMSCs. This gene delivery system successfully induced chondrogenic differentiation and could continuously release approximately 10 ng/mL TGF-β1 for two weeks in vitro. Another study carried out an in vivo small animal (rabbits) experiment in which the TGF-β1-implanting CS/gelatin scaffold was seeded with rabbit BMSCs. The composite remarkably promoted chondrogenesis and hyaline cartilage formation, and more importantly, the neocartilage was firmly integrated into the host cartilage at ten weeks postoperation. pDNA uptake by MSCs offers an alternative approach to enhance chondrogenesis without adding exogenous GFs.

5.6. Oxygen Tension

Articular cartilage is an avascular tissue, so oxygen tension may be particularly relevant to articular cartilage. The superficial zone contains approximately 6% O_2, and it declines to 1% in the deep zone [147], which is much lower than that of standard cell culture (20% O_2). Oxygen tension is known to regulate MSC differentiation fate mainly through the hypoxia-inducible factor HIF1-α and the PI3K/Akt pathway. However, hypoxia does not always show a positive impact on chondrogenesis. The literature is contradictory. According to one study, hypoxia has been identified as having a powerful positive effect on porcine BMSC proliferation, enhancing collagen and GAG deposition, but the type of scaffold (PCL, PCL-HA or PCL-Bioglass®) simultaneously modulated this effect. Specifically, hypoxia promoted cell proliferation in PCL-Bioglass® and PCL-HA scaffolds but merely had a positive effect on ECM secretion in PCL and PCL-HA scaffolds [148]. Conflicting with the study mentioned above, Wise et al. [149] found that hypoxia had no effect on either osteogenic or chondrogenic differentiation in collagen-CS hydrogel microbeads. Zhu and colleagues [150] found other bidirectional results, which indicated that hypoxia inhibited hMSC hypertrophy and calcification in low-HA concentration (1.5% w/v) hydrogels, whereas hypoxia showed the reverse effect on high-HA concentration (5% w/v) hydrogels irrespective of their cross-linking density. Some studies even demonstrated negative results; for example, in hypoxic conditions, bovine BMSCs produced little hyaline cartilage with hypertrophy and eventually calcification on PCL scaffolds, according to Meretoja et al. [151]. The duration of hypoxia also influences differentiation. An initial hypoxic 3-day culture of rat BMSCs enhanced cell survival and proliferation, but 21-day constant hypoxia did not further promote differentiation [149]. In conclusion, these adverse effects of hypoxia to some extent respond to the material of scaffolds, as well as the concentration of oxygen, duration of hypoxia, source of cells, seeding densities, and other factors [149], which are still not fully understood.

5.7. High Cell Density

For chondrogenesis, many studies have stated that MSCs' aggregation and conversion to spherical shapes represent a crucial process before chondrogenic differentiation [152–154]. High cell density can induce chondrogenesis via strong cell–cell interactions (such as N-cadherin and neural cell adhesion molecules). A study showed that high cell density could result in chondrogenic differentiation and cartilage-specific matrix deposition in vivo, regardless of the differentiation status of MSCs [137]. Normally, centrifuging to obtain condensed MSC pellets with the addition of chondrogenic GFs is defined as a standard protocol for in vitro chondrogenesis, although the mechanism is not fully understood. However, conventional pellet structures may tend to guide MSCs towards hypertrophy and ultimately endochondral ossification [155]. A novel aggregating method is condensing magnetic particle-labelled MSCs to form 3D tissues via a magnetic field, which displayed a significant increase in type II collagen and aggrecan [141]. For the specific cell density, studies adopted MSC densities of 1.0 to 4.8 $\times$ 10^7 cells/mL on scaffolds in vivo [118,156]. An earlier animal experiment indicated that 5.0 $\times$ 10^7 cells/mL on type I collagen gels showed chondrogenesis, while 1.0 $\times$ 10^6 cells/mL failed and exhibited a low cell proliferation rate and apoptosis trend [157]. Currently, the optimal quantity of seeded MSCs per unit volume remains unknown. According to the vast literature, a density of at least 10^6 cells/mL is presumably the essential requirement [79,87], while 10^7 cells/mL is more commonly documented to be used for chondrogenic differentiation [14,67,104,116]. This speculation relies on previous experience. The reason could be attributed to superimposed influences from substrate materials, source of MSCs, culture conditions, and GFs, which makes determining the optimal MSC seeding number for each situation overcomplicated.

6. Conclusions and Future Perspectives

The treatment of OA and cartilage defects remains a challenge due to the weak cartilage self-repair capacity. Bioengineering combined with stem cells is a promising way to achieve better repair of articular cartilage. Some studies have reported positive results from both fields of natural polymers and synthetic polymers; however, there is still a lack of sufficient clinical evidence. The diverse categories of materials and unclear underlying mechanisms necessitate a massive body of work in the future. Currently, many types of scaffolds have shown potential for cartilage regeneration, but their properties are not optimal and require further modification.

In the future, multilayer scaffolds may have promising efficacy in repairing osteochondral lesions [118]. To fabricate a bulk scaffold with multilineage differentiation abilities, presumably, we could emphasise the properties of stiffness or GFs or matrix orientation for each layer because different layers of cartilage have different major influencing factors. This strategy could lead to the precise control of producing layered neocartilage.

A remarkable breakthrough in the last decade is that researchers have found that MSCs function through the paracrine pathway. MSC-derived exosomes have biological functions similar to those of stem cells and have many other advantages, such as bypassing the risk of pathological transformation, uncontrollable cell differentiation, or immune activation for allogeneic preparations [158]. Thus, exosomes may be chosen to incorporate into scaffolds, taking the place of MSCs. For clinical translation and application, injectable microparticles make minimally invasive implantation possible, which has promising application value. They have a small size and short diffusion distance for easier access to oxygen and nutrients, resulting in higher cell viability than bulk scaffolds [34]. On the other hand, 'release-free' scaffolds are also appealing since they can avoid repeatedly supplementing expensive exogenous GFs or exosomes or designing controlled release systems, which benefits cost reduction, long-term storage at room temperature, and translation into off-the-shelf industrial production.

Finally, although abundant individual parameters, such as stiffness, pore size, GFs, and hypoxia, have been widely investigated, current tools are insufficient to integrate so many superimposed influence factors and accurately predict consequences in vivo. The

current research strategy involves picking several factors out and running through every possible combination to test the hypothesis, which results in an onerous workload and a lengthy experimental period. Perhaps a computer-assisted algorithm is able to simulate the effect of all possible combinations. Currently, a feedback system control method using a differential evolution algorithm has been developed for drug screening and has been successfully applied to high-efficiency screening osteogenic cocktails composed of various extrinsic GFs [159,160]. Similar software is urgently needed to greatly reduce the workload.

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Abbreviations

Abbr.	Full Name
OA	osteoarthritis
GFs	growth factors
PRP	platelet-rich plasma
ACI	autologous chondrocyte implantation
MACI	matrix-induced autologous chondrocyte implantation
MSCs	mesenchymal stem cells
hBMSCs	human bone marrow-derived MSCs
hASCs	human adipose-derived MSCs
hWJSCs	human Wharton's jelly derived MSCs
hPDCs	human periosteum-derived cells
2D	two-dimensional
3D	three-dimensional
3D-TIPS	3D printing-guided thermally induced phase separation
dECM	decellularised extracellular matrix
GAGs	glycosaminoglycans
SF	silk fibroin
HA	hyaluronic acid
Alg	alginate
HAp	hydroxyapatite
COMP	cartilage oligomeric matrix protein
CS	chitosan
PLA	poly(lactic acid)
PGA	poly(glycolic acid)
PLGA	poly(lactic-co-glycolic acid)
PCL	poly(ε-caprolactone)
PU	poly(urethanes)
PUU	poly(urea-urethane)
POSS	polyhedral oligomeric silsesquioxane
PES	polyethersulfone
PEG	polyethene glycol
AG	agarose
CP	cartilage pellet
ICRS	International Cartilage Repair Society
MOCART	magnetic resonance observation of cartilage repair tissue
FN	fibronectin
MNPs	magnetic nanoparticles
GO	graphene oxide

RGD	Arg-Gly-Asp
TGF	transforming growth factor
BMP	bone morphogenetic protein
IGF	insulin-like growth factor
FGF	fibroblast growth factor
−OH	hydroxyl group
−COOH	carboxyl group
HP	hydrostatic pressure
pDNA	plasmid DNA

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