Engineered mesenchymal stem cells for cartilage repair

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Healthy cartilage is a highly robust tissue, and is resilient against the stringent mechanical and biological contraints imposed upon it. Cartilage defects are common features of joint diseases, but current treatments can rarely restore the full function of native cartilage. Recent studies have provided new perspectives for cartilage engineering using mesenchymal stem cells (MSCs). However, the sequential events occurring during chondrogenesis must be fully understood before we are able to reproduce faithfully the complex molecular events that lead to MSC differentiation and long-term maintenance of cartilage characteristics. Here, we focus on the potential of MSCs to repair cartilage with an emphasis on the factors that are known to be required in inducing chondrogenesis.

Keywords: bone marrow, chondrocyte, joint, stromal stem cell, treatment

future medicine

Articular cartilage damage frequently results from injury or diseases, such as rheumatoid arthritis or osteoarthritis. It has limited intrinsic healing capacity because of its avascularity, the immobility of chondrocytes and the limited ability of mature chondrocytes to proliferate [1]. Different clinical approaches have been proposed to repair injured articular cartilage, including microfracture, abrasion arthroplasty [2], transplantation of chondrocytes [3], perichondrium and periosteum graft [4,5], meniscal allograft [6] and osteochondral graft [7]. However, there is no known treatment that enables full restoration of the original characteristics of articular cartilage following damage or injury.

Tissue engineering based on cell and gene therapy offers some of the most promising strategies of tissue repair, and this includes repair of articular cartilage. This approach involves the use of different cell types to act as chondroprogenitor cells and/or as gene-delivery vehicles that produce a therapeutic protein. Mesenchymal stem cells (MSCs) offer new potential for cartilage repair, as they may be induced to differentiate toward various lineages, and in this instance, toward chondrocytes. The fate of these cells within tissues is determined by specific cell–cell and cell–matrix interactions, which are controlled by extracellular signaling molecules and their respective receptors, and by intracellular events to control gene transcription in a cell-specific manner. Various differentiation factors, such as bone morphogenetic proteins (BMPs), fibroblast growth factor (FGF) and Wnt molecules, have been shown to be required but not specific for chondrogenesis [8]. These factors promote both cartilage and bone formation *in vivo* [9] and the exact molecular pathways governing each specific lineage are still under investigation. In this review, we highlight the potential of MSCs for cartilage engineering and discuss the specificity of differentiation factors to exploit these cells.

Poor intrinsic capacity of cartilage to repair

Articular cartilage is a highly organized tissue composed of chondrocytes that secrete various macromolecules (particularly Type II collagen and aggrecan) to form the extracellular matrix, which confers the architectural structure and biomechanical strength of the tissue. Mature chondrocytes rarely divide, and their number decreases with age [10]. This explains, at least in part, why most lesions do not spontaneously heal. In the case of partial thickness defects, the subchondral plate remains intact, with no access to the vascular system. By contrast, full-thickness chondral injuries are generally associated with a violation of the subchondral plate, exposing the lesion to the vascular system via the marrow space. This results in a migration of MSCs into the injured site, where they undergo chondrogenic differentiation [11]. In most instances, however, the repaired tissue consists of fibrocartilage composed predominantly of Type I collagen fibers, which do not fulfil the criteria for a functional tissue. Thus, disruption of the subchondral bone stimulates chondral and bony repair, but it rarely restores an articular surface with normal biological and mechanical properties.

Current clinical approaches

Stimulation of MSCs to migrate to the site of injury and to differentiate into mature chondrocytes has been employed as a therapeutic

approach, using the techniques of microfractures or subchondral drilling. Although significant relief has been obtained, these procedures have been shown to produce a repaired tissue that generally consists of fibrocartilage. In the same way, the technique of osteochondral transplantation has been widely employed, with functional and symptomatic joint improvement. However, both methods are limited to relatively small defects, and the isolation of the osteochondral graft is associated with donor-site morbidity and may initiate or exacerbate a degenerative process. In the case of substantial loss of cartilage tissue, more sophisticated therapeutic strategies are required. Transplantation of osteochondral cylinders from an unaffected cartilaginous region of the joint to the lesion and autologous chondrocyte implantation (ACI) [12] have led to promising results in some clinical studies [13–16]. In humans, ACI was first described by Brittberg and colleagues for the repair of deep cartilage defects in the femorotibial articular surface of the knee joint [3].

Although ACI can offer long-term symptomatic relief and is an established clinical technique, its contribution to the overall structural integrity of the repair cartilage is poorly understood. Although in the canine model implanted chondrocytes do not appear to be required for the effective repair of cartilage defects, their absence in the rabbit model leads to complete failure of the ACI procedure [3,12]. More recently in a goat model, it has been reported that fluorescently labeled chondrocytes implanted into cartilage defects were involved in the regenerated tissue as denoted by the expression of Type II collagen in the region populated with the fluorescent chondrocytes [17]. Moreover, in humans, this procedure involves the suture of a periosteal flap on top of the defect, and it is still unclear whether the cells involved in the neotissue are the implanted chondrocytes or the MSCs arising from the overlapping periosteal tissue. Therefore, although these treatments have been shown to be beneficial in certain cases, no procedure is yet available to supplant established methods.

Use of MSCs for cartilage repair

The use of MSCs provides an attractive alternative to mature chondrocyte transplantation owing to their potential to differentiate towards a chondrocytic phenotype following *in vitro* culture in a 3D system and serum-free medium [18–20]. MSCs are highly replicative cells with multilineage differentiation capacities. Most surface markers have been found inadequate to identify MSCs specifically because they are also present on other cells. However, MSCs are believed to be uniformly positive for markers, such as CD44, CD73, CD90, CD105 and CD106, and negative for markers of the hematopoietic lineage, including CD14 and CD45. MSCs are accessible from many tissues including adipose tissue, synovium, synovial fluid, periosteum, deciduous teeth, umbilical cord blood and blood vessels [21–28], but those isolated from the bone marrow are the most commonly studied.

Although implantation of unmodified MSCs has been reported to repair full-thickness cartilage defects in the rabbit [29], delivery of uncommitted MSCs to cartilaginous lesions does not yield a reproducible or satisfactory regenerated tissue but usually leads to the formation of fibrocartilage. One possible explanation is insufficient local stimulation of implanted cells by the factors necessary to drive their *in situ* differentiation as observed after the alteration of the biomechanical environment during violation of the subchondral layer [30]. Many studies have reported the use of various scaffolds to improve the quality of the neotissue. Such scaffolds have been based mainly on the use of hyaluronic acid, polylactic acid and/or polyglycolic acid, which may help in maintaining the cells inside the defect and provide a chondroinductive matrix, mimicking the natural tissue geometry. However, biodegradable scaffolds, such as fibrin, may not fulfil the biomechanical requirements for the joint resurfacing of the knee. Indeed, Shao and colleagues have shown that MSCs seeded in a fibrin-glue matrix were able to form a cartilage-like neotissue at 3 months. However, at 6 months prominent fissures and splits appeared on the cartilage surface in combination with poor integration [31].

Natural materials, such as agarose, alginate, gelatin and collagen derivatives, are inferior to synthetic and hybrid materials owing to poor resistance to mechanical stress, and their clinical usefulness is severely limited [32]. The biomechanical qualities and the biodegradability of synthetic biomaterials are more easily modified than natural polymers. Thus, Li and colleagues have developed a nanofibrous scaffold (NFS) based on the synthetic biodegradable polymer poly-caprolactone (PCL), and they have examined its ability to support the chondrogenesis of MSCs *in vitro*. This unique form has a similar microstructure to the native fibrillar matrix of collagens, and chondrogenesis was found to be

superior to that seen in pelleted cell culture. In addition, this study reported zonal morphologies within the neocartilage similar to those observed in the native type [33]. Gao and colleagues used a two-phase composite material composed of injectable calcium phosphate (ICP) and a hyaluronan derivative loaded with MSCs to attempt the repair of osteochondral defects [34]. By 12 weeks, the zonal features of the repair tissue became distinct; chondrocytes were arranged in a columnar array, which integrated with surrounding native cartilage and the new bone tissue that formed within the ICP. More recently, Frosch and colleagues reported that round titanium implants seeded with MSCs and inserted into an osteochondral defect resulted in satisfactory regeneration of the subchondral bone layer after 6 months [35]. The titanium implants provided the biomechanical support for the differentiation of cells into hyaline-like cartilage, but slow bone and cartilage regeneration and incomplete healing in half of the MSC-coated implants was observed. This highlights the need to improve the scaffolds and the disadvantage of using a material, such as titanium, that does not fulfil one of the requisite criteria for a tissue engineering composite: resorbability. These data suggest that scaffolds permitting anchorage, support of cell differentiation and maintenance of a mature phenotype, combined with the use of stem cells, offer promising prospects for the regeneration of fully functional tissue. The development of the scaffolds must also take into account that the chondrogenic potential of MSCs is favored by hypoxia and is not only dependent on hydrostatic pressure but also on the cell density inside the matrix and the presence of growth factors [36].

Transcription factors involved in chondrogenesis

Identification of key transcription factors implicated during chondrogenesis arose mainly from studies of developmental biology. Bapx1 appears to be an important transcription factor involved in chondrogenesis and has been reported to mediate Sonic Hedgehog (Shh) signaling to induce chondrogenic differentiation in the sclerotome [37]. The signaling mediated by Shh targets Pax1 and Pax9, which are markers on chondrifying mesenchyme, has been shown to activate Bapx1 in sclerotomal cells [38]. Many lines of evidence have also shown that Sox proteins, in particular Sox-9, are necessary for chondrogenesis. Sox-9, Sox-5 and Sox-6, are members of the Sox family of transcription factors that are characterized by a high-mobility-group (HMG)-box DNA-binding domain [39]. During embryogenesis in mice, Sox-9 has been reported to regulate positively the proliferation of chondrocytes and negatively the terminally differentiated hypertrophic state [40]. Moreover, mice lacking Sox-9 display a severe generalized chondrodysplasia, similar to that in Sox-5/Sox-6 double-null mutant mice [40]. Recently, in adult MSCs, Ikeda and colleagues showed that this Sox family is not only necessary for chondrogenesis, but in combination, the Sox trio (Sox-5, Sox-6 and Sox-9), is sufficient for the process [41]. Despite its importance for chondrogenesis, the mechanisms by which Sox-9 regulates cartilage-specific transcription are poorly understood. However, recent work shows that peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1α) and CREB-binding protein (CBP/p300) act as coactivators for Sox-9 to regulate chondrogenesis [42,43].

The Twist subfamily includes transcription factors, such as Twist, Scleraxis, Dermo-1, Paraxis and HAND2, that function as transcriptional enhancers [44–46] or repressors [47]. During embryogenesis, Paraxis expression precedes that of Scleraxis in the region of the somite fated to form the axial skeleton and tendons. Moreover, in the absence of Paraxis, Pax-1 is no longer expressed in the somites and presomitic mesoderm. These results suggest that Paraxis acts upstream of Scleraxis and regulates early events during chondrogenesis by positively directing transcription of sclerotome-specific genes [48]. Scleraxis was first shown to be expressed in developing chondrogenic cell lineages during embryogenesis. Later studies based on the overexpression of Scleraxis in osteoblastic cells have shown that this factor transactivates the expression of aggrecan through binding to its high-affinity binding site in the promoter [49]. On the contrary, the repressor activity of some members of the Twist family, in particular eHAND, has been studied in transient transfection assays of the C3H10T1/2 cells [50]. In this case, eHAND inhibits MyoD-dependent skeletal muscle cell differentiation and expression of the muscle-specific myosin heavy chain protein.

Chondrogenic factors

Some growth factors acting as inductive signals for chondrogenesis have been identified, mainly from studies in developmental biology. Although

Table 1. Summary of the main factors involved in the transition from mesenchymal stem cell to chondrocytes.

	In vivo		*In vitro*	Ref.
	Condensation phase	***Differentiation phase***		
TGF-β_1	+	-	+	[54]
β_2	+	-	+	[53]
β_3	+	-	+	[53]
BMP-2	+	+	+	[9]
4	+	+	+	[51]
6			+	[19]
7	+	+	+	[62]
9	+	+	+	[60]
14	-	+	+	[51]
Wnt 3a	+	-	-	[76]
4	-	+		[73]
5a	+	-	+	[73]
5b	+	-	+	[73]
7a	-	-	-	[74]
FGF-2	+	+		[51]
4	+	-		[51]
8	+	-		[51]
10	+	-		[51]
18	+	+	+	[51,65,70]
Sox-5	-	+	+	[41,51]
6	-	+	+	[41,51]
9	+	+	+	[41,51]

+: Indispensable; -: Dispensable.

BMP: Bone morphogenetic protein; FGF: Fibroblast growth factor; TGF: Transforming growth factor.

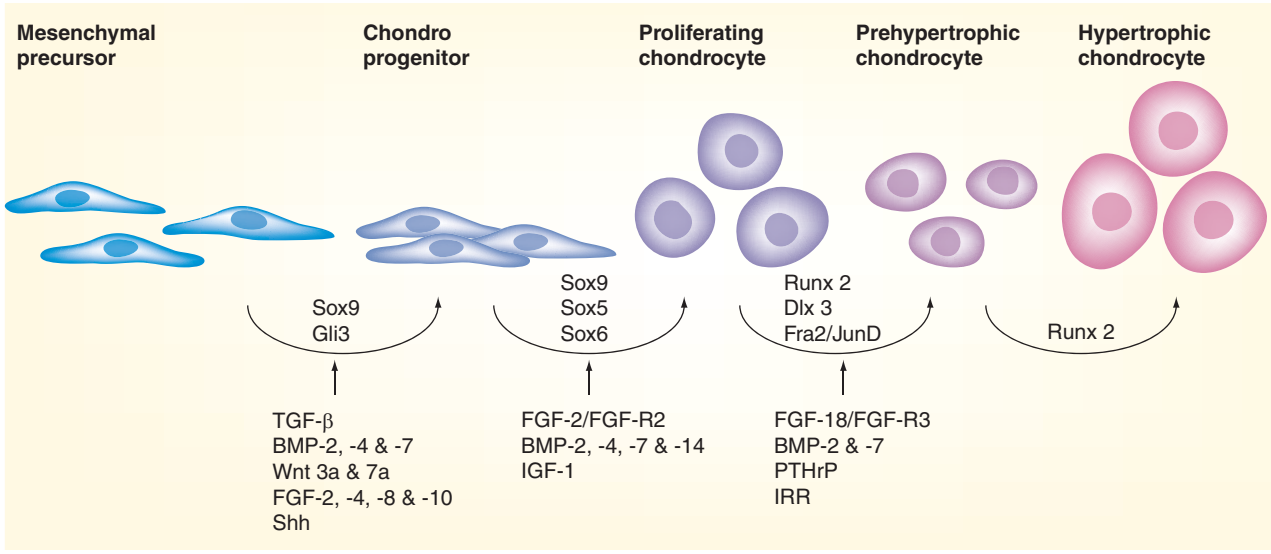
the findings cannot be directly extrapolated to the formation of adult tissues, the role of most of the genes has been studied in adult stem cells, with some discrepancies that probably result from the experimental systems or the animal models used. Apart from the members of the insulin growth factor (IGF) and hedgehog (Hh) families (reviewed in [51]), we focus here on the genes that are the most studied in animal models and, in particular, on the genes belonging to the transforming growth factor (TGF)-β, Wnt and fibroblast growth factor (FGF) family.

The development of *in vitro* systems of chondrogenesis has been important to the identification of factors that can promote chondrocyte differentiation of adult MSCs and improved cartilage repair *in vivo*. In a defined medium containing dexamethasone and members of the TGF-β family, chondrogenesis is induced in MSCs when cultured as aggregates. In this system, the aggregates synthesize an extracellular matrix characteristic of cartilage, containing proteoglycans and Type II collagen [52]. In comparison with TGF-β_1, the evaluation of the chondrogenic potential of other TGF-β family members, including TGF-β_2 and TGF-β_3 has also been reported. A similar cellular content was observed over 3 weeks, with TGF-β_2 and TGF-β_3 producing significantly more proteoglycans and Type II collagen than TGF-β_1 [53]. TGF-β_1 is involved in the early stage of the process stimulating chondrogenesis via transition from an initial N-cadherin-contributing stage to a succeeding fibronectin-contributing stage during the process of chondrogenesis in MSCs [54]. In de-differentiating chondrocytes, the gene for TGF-β_1 was constantly expressed, while the gene for TGF-β_2 was never expressed. TGF-β_2 was shown to enhance *in vitro* proliferation and redifferentiation of chondrocytes and participate in adult and embryonic growth and development [55].

Recently, Jin and coworkers reported that TGF-β_3 upregulates the expression of Wnt5a, and that Wnt5a, at least in part, mediates the chondro-stimulatory effect of TGF-β_3 by modulating PKC-α and p38 mitogen-activated protein kinase (MAPK) activity in chick wing bud mesenchymal cells [56]. Furthermore, they showed that the protein levels of cell adhesion molecules, such as fibronectin and integrin α5, were consistently increased in the presence of TGF-β_3 and Wnt5a. These results indicate that upregulation of Wnt5a signaling by TGF-β_3 promotes expression of cell adhesion molecules through the activation of p38 MAPK in early stage during chondrogenesis and that, at least in human cells, TGF-β_3 may be the most potent inducer of chondrogenesis.

Because BMPs were reported to play a crucial role early in the formation of the joints in the limb [57,58], this signaling pathway has been studied extensively both *in vitro* and *in vivo* using adult MSCs. Indeed, BMP signaling is required both for the formation of precartilaginous condensations and for the differentiation of precursors into chondrocytes [59]. BMP-2, -9 and -13 serve as potent anabolic factors for juvenile cartilage, which contains chondroprogenitors, but not for adult cartilage, whereas BMP-7 has been demonstrated to have a strong anabolic activity in both young and adult cartilage [60]. Adenoviral-BMP-2 infected MSC aggregates showed more intense staining for proteoglycans and Type II collagen than adenoviral-TGF-β_1 aggregates [61]. BMP-2 is known to induce the formation not

Figure 1. Schematic representation of the sequence of events occurring during chondrogenesis.



The temporal patterns of growth and differentiation factors are indicated below the arrows while main transcription factors are indicated above the arrows. The representation includes most of the factors referenced in the text.
BMP: Bone morphogenetic protein; FGF: Fibroblast growth factor; IGF: Insulin-like growth factor; IRR: Insulin receptor-related receptor; Shh: Sonic Hedgehog; TGF: Transforming growth factor.

only of new cartilage, but also of bone tissue, thus demonstrating a true capability of BMP-2 to induce both chondrogenic and osteogenic pathways [8]. Furthermore, Knippenberg and colleagues have shown that at day 4, a short treatment of MSCs with low concentration (10 ng/ml) of BMP-2, but not BMP-7, stimulated runx-2 and osteopontin gene expression, and at day 14 BMP-7 downregulated expression of these genes and stimulated aggrecan gene expression [62]. Therefore, MSCs triggered with BMP-2 or -7 in specific conditions may provide a feasible tool for both bone and cartilage tissue engineering. BMP-4 and -6 have also been shown to promote Type II collagen production and to assist in differentiation [19,63]. A recent study underlines the role of BMP-14 in the cellular recruitment and chondrocyte differentiation in the early stages of fracture repair. The authors support the hypothesis that BMP-14 deficiency leads to a delay in fracture healing and highlight the importance of examining more closely the role of BMP-14 in normal fracture healing and the mechanism by which it works [64]. Taken together, the data show that, among the members of the BMP family, BMP-2, -4, -6 and -7 are the most potent in triggering the differentiation of MSCs towards chondrocytes.

The role of the members of the FGF family in skeletal development is poorly defined probably because many genes have essential functions in other tissues during embryogenesis and functional redundancy of some members has been observed. However, it has been clearly described that the congenital absence of either FGF-18 or FGFR3 results in a similar expansion of the growth plate in fetal mice [65]. However, FGF-2 seems to play a dual role in postnatal chondrogenesis. On the one hand, FGF-2 has been shown to enhance TGF-β_1-induced periosteal chondrogenesis [66] and on the other it may induce the proliferation of MSCs prior to chondrogenesis [67–69] and inhibit the synergetic effect of Shh and BMP-2 on transfected prechondrogenic cells [66]. More recently, Davidson and colleagues have reported that FGF-18 signals through FGFR3 to promote chondrogenesis [70].

The members of the Wnt family are important regulators of several developmental processes, including skeletogenesis. After the binding of Wnt to the Frizzled family of receptors and the LRP5/6 family of coreceptors, the canonical Wnt signaling pathway will stabilize the β-catenin, which translocates to the nucleus and interacts with members of the TCF/LEF families to activate target genes. Whereas inactivation of β-catenin causes ectopic formation of chondrocytes at the expense of osteoblast formation, the canonical Wnt pathway leads to enhanced ossification and suppression of chondrocytes owing to the transcriptional downregulation of Sox9 [71,72]. Indeed, Church and colleagues have shown that Wnt4 blocks the

initiation of chondrogenesis and accelerates terminal chondrocyte differentiation *in vitro* while Wnt5a and Wnt5b promote early chondrogenesis and inhibit terminal differentiation *in vivo* [73]. Whereas it has been clearly demonstrated that Wnt7a blocks chondrogenesis [74,75], the exact role of Wnt3a is more controversial. Indeed, Wnt3a has the capacity to enhance BMP-2-mediated chondrogenesis of mesenchymal micromass cultures through the regulation of N-cadherin-mediated adhesion, the inhibition of GSK-3β kinase activity and the nuclear signaling of β-catenin and LEF-1 [76]. More recently, another study has shown that Wnt3a inhibits chondrogenesis by stabilizing cell–cell adhesion leading to the dedifferentiation of chondrocytes by activating the β-catenin-T-cell factor/lymphoid enhancer factor (TCF/LEF) transcriptional complex and the c-Jun/AP-1 pathway [77]. This discrepancy could be explained in part by the differences in the experimental systems particularly in the choice of cells. These studies illustrate that Wnt/β-catenin signaling plays an essential role in MSCs by controlling the osteoblastic and chondrocytic differentiation. However, some redundancies between members of this family, as well as the presence of various agonists and antagonists, add complexity for evaluating the individual role of each factor.

Engineered MSCs for cartilage repair

Gelse and colleagues have investigated the repair capacity of MSCs by combining gene transfer of growth factors and cell transplantation in partial-thickness lesions created in rat articular cartilage. MSCs infected with recombinant adenoviral vectors expressing BMP-2 or IGF-1 were able to repair the cartilage of hyaline morphology containing a Type II collagen-positive and Type I collagen-negative proteoglycan-rich matrix, which restored the articular surface in most lesions [78]. However, excessive cells were partially relocated to the joint margins, leading to osteophyte formation if BMP-2-expressing cells were used. However, the adverse effects were not observed with IGF-1-expressing cells. In another study, the authors have shown that the implantation of genetically modified MSCs expressing BMP-7 or Shh in articular cartilage lesions significantly enhanced the quality of the repair tissue, resulting in a more hyaline-appearing cartilage, as compared with untransduced MSCs [79]. There was, however, a noticeable difference in the persistence of the cartilage phase between the groups. The subchondral compartment seemed to remodel with bone much faster in the group that received the *BMP-7* gene. Together with the growth factor, the quality of the repaired cartilage depends on the delivery scaffold. The most encouraging results were found when BMP-7 was combined with a collagen matrix, while linking BMP-7 to *N*,*N*-dicarboxymethyl chitosan led only to partial healing of the articular surface [80]. However, although hyaline-appearing cartilage was formed, bone was also obtained. These results underline the necessity to identify chondral specific factors that could be used for tissue engineering.

Conclusion

Cartilage engineering through the use of MSCs is a promising approach in different pathological situations. Ultimately, this approach could supplant current treatments, which rarely restore the full functions of the tissue to its previous state. However, to achieve stem cell-based repair, it is possible that far more sophisticated strategies will be required to faithfully reproduce the complex molecular events of the chondrogenic process and the long-term maintenance of the articular cartilage phenotype. Several experimental approaches are currently under investigation that may prove useful, such as the local implantation of genetically modified MSCs into cartilage defects [78,79,81]. Regardless of the approach, the challenge at present is the characterization of candidate gene products that could direct the chondrogenic process specifically *in vivo*.

Executive summary

- Articular cartilage presents limited intrinsic healing capacity.
- Current treatments for cartilage repair, including periosteum/perichondrium grafts, osteochondral implantation or autologous chondrocyte implantation, have shown encouraging results but no procedure has been proved to supplant the overall methods.
- Mesenchymal stem cell-based therapy may be a suitable alternative to other surgical procedures.
- Identification of growth/differentiation factors specific for the chondrocytic lineage is of great interest for stem cell-based therapy.
- Defining a combination of a suitable scaffold, a specific chondrogenic factor and well characterized mesenchymal stem cells will be a pre-requisite for cartilage engineering.

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