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Review

Cartilage tissue engineering: Molecular control of chondrocyte differentiation for proper cartilage matrix reconstruction ☆

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ABSTRACT

Background: Articular cartilage defects are a veritable therapeutic problem because therapeutic options are very scarce. Due to the poor self-regeneration capacity of cartilage, minor cartilage defects often lead to osteoarthritis. Several surgical strategies have been developed to repair damaged cartilage. Autologous chondrocyte implantation (ACI) gives encouraging results, but this cell-based therapy involves a step of chondrocyte expansion in a monolayer, which results in the loss in the differentiated phenotype. Thus, despite improvement in the quality of life for patients, reconstructed cartilage is in fact fibrocartilage. Successful ACI, according to the particular physiology of chondrocytes in vitro, requires active and phenotypically stabilized chondrocytes.
Scope of review: This review describes the unique physiology of cartilage, with the factors involved in its formation, stabilization and degradation. Then, we focus on some of the most recent advances in cell therapy and tissue engineering that open up interesting perspectives for maintaining or obtaining the chondrogenic character of cells in order to treat cartilage lesions.
Major conclusions: Current research involves the use of chondrocytes or progenitor stem cells, associated with "smart" biomaterials and growth factors. Other influential factors, such as cell sources, oxygen pressure and mechanical strain are considered, as are recent developments in gene therapy to control the chondrocyte differentiation/dedifferentiation process.
General significance: This review provides new information on the mechanisms regulating the state of differentiation of chondrocytes and the chondrogenesis of mesenchymal stem cells that will lead to the development of new restorative cell therapy approaches in humans. This article is part of a Special Issue entitled Matrix-mediated cell behaviour and properties.

1. Introduction

Articular cartilage is made up of dense, elastic connective tissue localized at the junction of several locations in the skeleton. It covers the surface of the joints to ensure that joints and bones move together. It is an avascular tissue that is not innervated and is composed primarily of a single cell type, the chondrocyte, which synthesizes an abundant extracellular matrix (ECM). Chondrocyte has a hypoxic (physioxic)

Abbreviations: ACI, autologous chondrocyte implantation; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motif; AGEs, advanced glycation end products; AP-2, activator protein-2; BM MSC, bone marrow mesenchymal stem cell; BMP, bone morphogenetic protein; BMPR, bone morphogenetic protein receptor; Cbfa1/Runx2, core-binding factor subunit alpha-1/Runt-related transcription factor 2; CD, cluster of differentiation; CS, chondroitin sulfate; ECM, extracellular matrix; ESC, embryonic stem cell; GAG, glycosaminoglycan; HA, hyaluronic acid; HAC, human articular chondrocyte; HIF, hypoxia-inducible factor; HtrA1, high temperature requirement A; IGF, insulin-like growth factor; IL, interleukin; iPSC, induced-pluripotent stem cell; KS, keratan sulfate; MMP, matrix metalloproteinase; MSC, mesenchymal stem cell; NF-κB, nuclear factor-kappa B; NO, nitric oxide; OA, osteoarthritis; PEG, polyethylene glycol; PG, proteoglycan; PGA, poly(glycolic acid); PLA, poly(lactic acid); PLGA, poly(ε-caprolactone); PTHrP, Parathyroid Hormone-related Protein; ROS, reactive oxygen species; Sox, SRY-type HMG box; TGF-β, transforming growth factor-beta; TNF, tumor necrosis factor; T3, triiodothyronine; T4, thyroxin; UCB MSC, umbilical cord blood mesenchymal stem cell; VEGF, vascular endothelial growth factor

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metabolism and produces mainly type II collagen and aggrecan, the two major phenotypic markers specific to articular cartilage. This collagen isoform imparts resistance to compression forces in cartilage. Aggrecan is a proteoglycan (PG) composed of many sulfated glycosaminoglycan (GAG) chains, such as chondroitin sulfate (CS) and keratan sulfate (KS) and, as a result, it can retain a significant amount of water, providing flexibility and viscoelasticity to the musculoskeletal system. The viscoelastic nature of cartilage tissue confers the ability to absorb pressure and distribute it throughout the articular surface. Additionally, the three-dimensional organization and the nature of ECM molecules are different depending on how deep they are in the cartilage, adding to the complexity of cartilage tissue.

Osteoarthritis (OA), a degenerative disease of articular cartilage, is characterized by the degradation of the ECM, associated with increased secretion of matrix metalloproteinases (MMPs) and aggrecanases [1,2]. Increased intra-articular expression of these ECM-degrading enzymes is triggered by the secretion of pro-inflammatory cytokines in the synovial fluid, such as interleukin-1β (IL-1β) or tumor necrosis factor-α (TNF-α). These catabolic molecules disrupt the integrity of the ECM and decrease the response and sensitivity of chondrocytes to external anabolic signals [3]. In addition, the OA process induces chondrocyte dedifferentiation characterized at least in part by increased synthesis of type I collagen, an atypical isoform in articular cartilage [4]. Moreover, due to the poor intrinsic healing capacity of articular cartilage, there is currently no treatment to restore the chondrocyte phenotype and, in the most advanced stages of OA, the joint must be replaced with a prosthesis, requiring surgery.

Therefore, various drug and surgical treatments have been developed in an attempt to prevent the destruction of cartilage which, in light of their relative success, then lead to new, improved therapeutic strategies. One of the most promising approaches is based on articular cartilage tissue engineering based on the procedure described by Brittberg et al. using autologous chondrocyte implantation (ACI) [5]. Applied in the earliest stages of OA or chondral lesions, ACI is based on the use of chondrocytes from a healthy, non-bearing region of the diseased joint. The cells are then amplified in monolayer culture and then re-implanted in the lesion. However, amplification of autologous chondrocytes in two-dimensional culture mimics, at least in part, some of the characteristics of the OA process and is accompanied by cell dedifferentiation leading to the formation of non-functional fibrocartilage.

The numerous pharmaceutical approaches and surgical techniques developed to repair cartilage lesions have revealed their limitations. Ideally, traumatic cartilage lesions should be treated earlier to prevent OA and postpone prosthetic surgery. In the interest of preventing OA, cartilage cell therapy has proven to be a pivotal approach for repairing damaged tissue. Cell therapy consists not only in filling the cartilage lesion with healthy chondrocytes, but also in reconstituting the structure, the physico-chemical properties and the functionality of the hyaline matrix. The transplantation of autologous chondrocytes is the foundation of cell therapy and there have been several generations of ACI, each improving on the previous one. However, even the most recent ACI techniques are beginning to show limitations.

Consequently, research efforts are now focused on improving this technique to obtain, after amplification, a differentiated and stable chondrocyte phenotype using new types of biomaterials, molecules known for their chondrogenic activity, RNA interference strategies and different cell sources. Human articular chondrocytes (HACs) and/or human mesenchymal stem cells (MSCs) are employed to restore or maintain the chondrocyte phenotype and induce chondrogenesis, respectively. Today, adult MSCs hold much promise for biomedical research because they are able to reform many tissues, including cartilage. However, the culture conditions used to obtain a differentiated chondrocyte phenotype must be well defined. The nature of the medium, the amount of oxygen or the presence of pro-chondrogenic factors, the use of different biomaterials, and the gene therapy strategy are all parameters that need to be considered to stabilize the differentiated chondrocyte phenotype.

As a result, we present some of the most recent advances in the field of cartilage engineering and the critical parameters for which more research is required to better understand the mechanisms regulating the state of differentiation of cartilage cells and chondrogenesis of adult MSCs. Knowledge in these areas will most probably lead to new restorative cell therapies for human medicine.

2. Articular cartilage

2.1. Adult articular cartilage homeostasis

Adult articular cartilage is composed of chondrocytes which are encapsulated in a dense pericellular matrix. It contains a high concentration of PGs and type VI collagen for anchoring cells to the ECM [6]. Chondrocytes do not divide and apoptotic activity is very low [7,8]. In adult articular cartilage, chondrocytes are not organized in columns, but appear to be uniformly isolated in the tissue. New chondrocytes are probably produced by successive mitosis and not via the initial chondrogenesis process. In addition, the metabolic activity of chondrocytes from adult cartilage is decreased. Articular cartilage had long been considered as a tissue containing only one cell type, the chondrocyte. However, we now know that MSCs and/or progenitor cells are also present in this tissue and have the capacity to differentiate into mature articular chondrocytes [9]. These cells may thus also contribute to the local repair of micro-lesions in articular cartilage [10].

Within the cartilage, chondrocytes are subjected to numerous mechanical and environmental factors that regulate their metabolic activity and phenotype. Thus, according to the different signals they perceive, chondrocytes are responsible for the production, organization and maintenance of the integrity of cartilage ECM.

2.1.1. The extracellular matrix (ECM) of cartilage

The nature of the ECM in adult articular cartilage depends on the location of chondrocytes. Chondrocytes are surrounded by a pericellular matrix covered in turn by a territorial matrix. These two matrix layers bind chondrocytes to the interterritorial matrix and serve to protect against potential cartilage injuries. The interterritorial matrix fills the cell-free matrix space and occupies the greatest volume in cartilage. The chondrocyte is anchored to the ECM via cartilage PGs and membrane proteins such as syndecan [11] or glypicans [12]. Transmembrane proteins are also involved in anchoring the chondrocyte to its pericellular space, including anchorin CII, which interacts with cells possessing type II collagen, and integrins, which attach chondrocytes to collagen molecules and fibronectin [13].

Cartilage ECM is mainly composed of collagen (60% of dry weight), PGs (5–10%), including in particular a specific marker, aggrecan, and non-collagenous proteins. Among the different types of collagen, the type II isoform, a homotrimer composed of an α1 (II) chain, is the most abundant isoform in articular cartilage, representing 80% of collagen. The remaining isoforms include type IX and XI collagens (15%) and 5% of other collagen types (types III, XII, VI, etc.) [14]. Given its abundance in the ECM space, type II collagen is the leading marker specific to chondrocytes along with aggrecan. Its role in ECM homeostasis is critical. Its loss induces perturbation of the physico-mechanical properties of cartilage and leads to OA. Moreover, type II collagen −/− mice have a disorganized ECM, without any collagen fibers. The mice are smaller and have a much less developed skeleton [15]. Large amounts of water molecules (65–80%) are found in the ECM and the dry weight of the ECM represents 20–40% of cartilage volume. Chondrocytes are responsible for maintaining cartilage homeostasis but the nature of the ECM can also affect their behavior and phenotype. Alteration of the ECM or degradation of its components inevitably leads to chondrocyte dedifferentiation. The arrangement of molecules in this three-dimensional network thus maintains chondrocytes in a differentiated

state and gives the cartilage its visco-elasticity properties and resistance to compression forces.

2.1.2. The chondrocyte, a delicate balance between anabolism and catabolism

Chondrocytes are responsible for the regulation of ECM turnover by synthesizing and secreting cartilage-specific matrix markers such as type II collagen and large aggregating PGs, called aggrecans. However, these cells represent only 1–2% of the total matrix volume [14]. In adulthood, these large round cells no longer divide, and are called post-mitotic. Therefore, the cartilage has a low turnover rate and a very limited ability for self-repair. Articular cartilage is avascularized, and chondrocytes therefore function with a hypoxic (physioxic) metabolism and nutrients and oxygen are supplied to the cells primarily by diffusion from the synovial fluid and subchondral bone. From the most superficial to the deepest layers, it is estimated that the oxygen gradient ranges from 10 to 1%, respectively. In these conditions of hypoxia, chondrocytes metabolize glucose into energy substrates via the anaerobic glycolysis pathway [16]. Glucose is a sugar essential for the proper functioning of cellular machinery and is a nutrient essential for cartilage because it is especially required for the synthesis of GAGs (a main component of aggrecan). Like oxygen, it diffuses from the synovial fluid and into the chondrocyte through glucose transporters called GLUT (GLUT1, 3, 5, 9, 10 and 11) [17].

The chondrocytes maintain the surrounding matrix by modulating the balance between the synthesis and degradation of the various components in physiopathological situations. This balance is controlled by the relative amounts of cytokines and growth factors in the cartilage or synovial fluid. Chondrocytes synthesize ECM molecules, but also factors involved in ECM degradation such as metalloproteinases (MMPs) [18], hyaluronidases [19] and aggrecanases [20]. As a result, the chondrocyte regulates cartilage homeostasis by maintaining a delicate balance between anabolism and catabolism (Fig. 1).

However, the cellular microenvironment directly affects the anabolic and/or catabolic activities. It is characterized by soluble factors (vitamins, hormones, cytokines, growth factors, inflammation and/or differentiation factors), oxygen levels, the anchoring to the tridimensional matrix and even mechanical stress [21]. Cartilage cells do not directly interact with each other but are linked to the ECM by surface receptors such as integrins. The chondrocyte therefore has a close relationship with its microenvironment and is sensitive to changes in it.

2.1.3. Transcription regulation of type II collagen and alternative splicing

In humans, type II collagen is encoded by a single gene, *COL2A1*, which contains 53 exons. Many activating and inhibitory transcription factors as well as cofactors are involved in regulating the transcriptional activity of the *Col2a1* gene by binding to the promoter and/or in the intron-specific enhancer (Fig. 2).

Sox9, L-Sox5 and Sox6 stimulate chondrogenesis and also the transcriptional activity of the *Col2a1* gene by interacting with the first intron specific enhancer [22]. Taken individually, Sox9 significantly increases the expression of type II collagen whereas L-Sox5 and Sox6 slightly stimulate the transcriptional activity of the *Col2a1* gene in the absence of Sox9. When all three Sox are overexpressed, a higher transactivation of *Col2a1* occurs, demonstrating cooperation between them [23]. Moreover, the presence of L-Sox5 and Sox6 is essential for the proper development of mouse cartilage because L-Sox5 −/− and Sox6 −/− genotypes lead to general chondrodysplasia [24].

The collagen Krüppel-box (C-Krox), also called the "zinc finger and BTB domain containing protein 7B" (ZBTB7B) is a zinc finger protein [25,26], inhibits the expression of type II collagen via a short promoter region of −266 bp [27,28] in differentiated and dedifferentiated chondrocytes. In addition, during chondrocyte dedifferentiation, its protein levels and DNA-binding activity are decreased.

Sp1 and Sp3 are ubiquitous zinc finger proteins. Sp3 shares 90% homology with Sp1 but, unlike Sp1, it encodes four protein isoforms that seem to have distinct transcriptional activities [29]. Sp3 can act both as an activator and an inhibitor of transcription, whereas Sp1 most often acts as a transcriptional activator. These two factors bind to consensus GGGCGG DNA sequences, known as the GC box. Thus, the stimulatory action of Sp1 on the expression of target genes can be modulated by Sp3 because both proteins can bind to the same cis-regulatory sequences [30]. On the other hand, their close homology indicates that they potentially recruit the same transcriptional cofactors.

Sp1 and Sp3 play an important role in regulating the transcriptional activity of the *COL2A1* gene and their ratio is different according to the

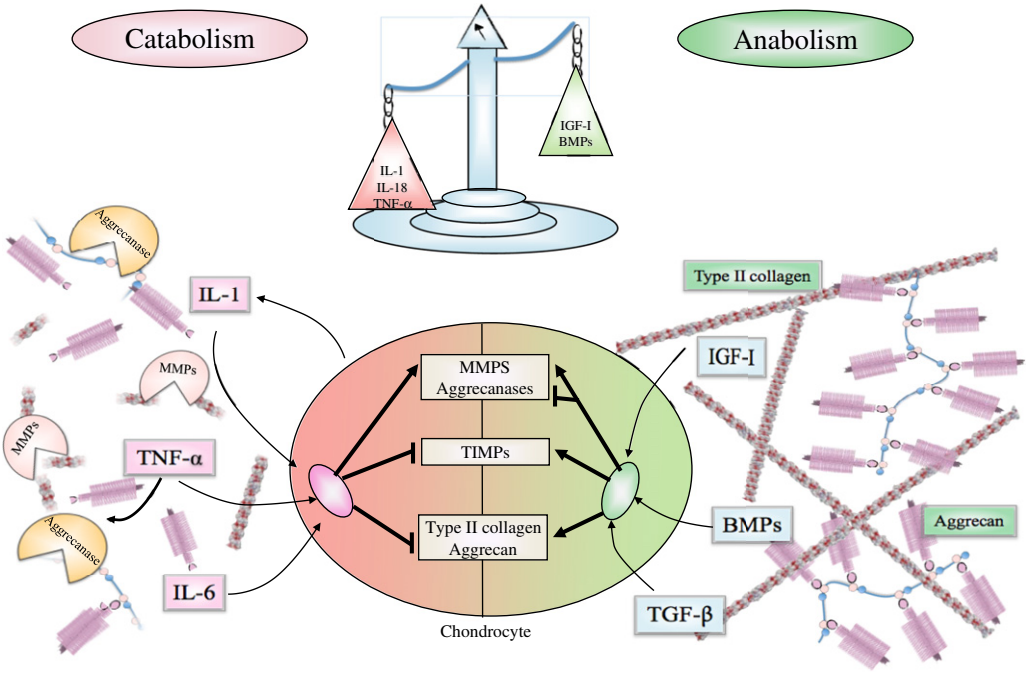

Fig. 1. Modification of the anabolism/catabolism balance during OA. The OA process induces an increase in MMP and aggrecanase secretion and inhibits the expression of cartilage-specific markers, such as type II collagen and aggrecan.

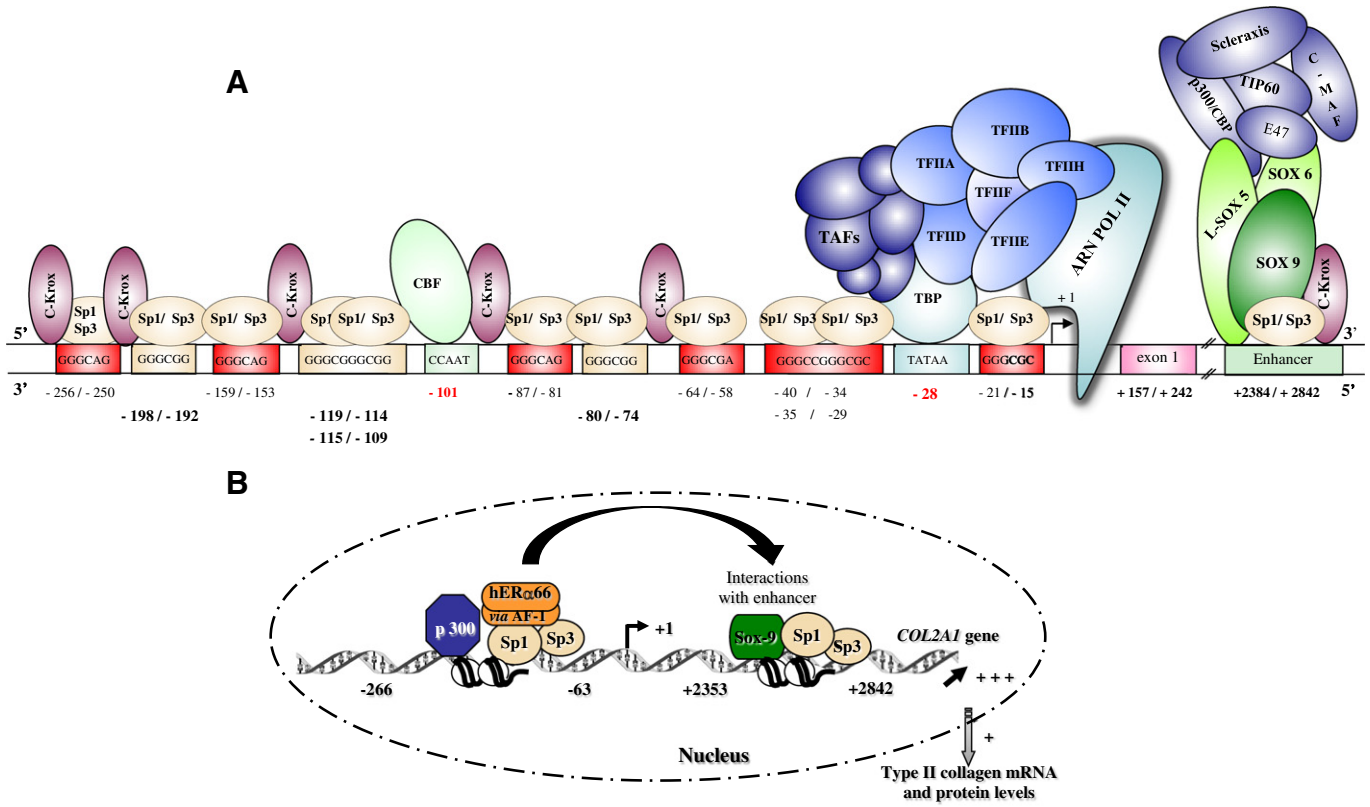

Fig. 2. Schematic diagram of cis-regulatory sequences and trans-factors binding to the promoter and/or the enhancer region of *COL2A1* gene. (A) The promoter of *COL2A1* gene contains several GC boxes (brown) and non-consensus (red) sequences that serve as binding sites for the transcription factors Sp1 and Sp3. The TATA box is located at −28 bp from the transcription initiation site and binds TBP (TATA Binding Protein) and general transcription factors (TAF: TATA Associating Factor). Intron 1 of the *COL2A1* gene contains a specific enhancer located between +2127 and +2842 bp, which binds many transcription factors such as SOX9, L-SOX5, SOX6, Sp1, Sp3 and c-Krox. Many transcriptional cofactors also bind in this region through protein–protein interactions (p300/CBP, TIP60, Scleraxis, E47). (B) Schematic representation of 17β-E_2 and hERα66 genomic pathways involved in the up-regulation of the type II collagen gene in articular chondrocytes. *COL2A1* does not contain ERE binding sites, so that hERα66 interacts with *COL2A1* promoter in a non-classical genomic pathway involving protein–protein interactions with transcription factors Sp1, Sp3 and SOX9. Some co-activators such as p300 are also involved in this transactivation process.

differentiation state of chondrocytes. We have demonstrated that the Sp1/Sp3 ratio decreases during chondrocyte dedifferentiation, which is correlated with a downregulation of type II collagen expression. In addition, and regardless of the differentiation state of chondrocytes, Sp1 stimulates the expression of the *COL2A1* gene whereas Sp3 inhibits it [27,28,31]. However, the expression and the DNA binding activity of both Sp1 and Sp3 are decreased in dedifferentiated chondrocytes compared to differentiated chondrocytes, but to a much lower extent for Sp3.

Both Sp factors are involved in the IL-6/sIL-6R- and IL-1β-induced downregulation of the expression of type II collagen via a −41/−33 bp region of the *COL2A1* promoter [31,32]. This sequence includes cis-regulatory elements that bind Sp1 and Sp3. Both cytokines enhance the Sp3/Sp1 ratio. Thus, during chondrocyte dedifferentiation, the change in the Sp1/Sp3 ratio leads to an increase in Sp3 binding activity on the promoter of *COL2A1*, preventing Sp1-induced transactivation [27,31]. In contrast, we recently demonstrated that 17β-E_2 stimulates, via the human estrogen receptor hERα66, type II collagen expression in differentiated and dedifferentiated articular chondrocytes. Transactivation of the type II collagen gene via the ligand-independent transactivation domain (AF-1) of hERα66 is mediated by the GC-binding sites of the −266/−63 bp promoter, through physical interactions between ERα, Sp1/Sp3, Sox9 and p300 [33]. Similarly, we have previously demonstrated the role of Sp1/Sp3, Sox9 and euchromatin-associated factors (p300, Tip60) in the IGF-I-induced upregulation of COL2A1 [28].

NF-kappa B belongs to a family of proteins involved in immune response and cellular stress. NF-κB is a heterodimer composed of two subunits: p65, which is responsible for its binding to DNA and transcriptional function, and p50 which modulates its activity. p65 is involved in the regulation of many genes and it behaves primarily as a transcriptional activator by binding to a GGGACTTTCC sequence. The expression of type II collagen is downregulated by NF-κB in response to IL-1β [32,34]. The DNA binding activity of NF-κB increases during the dedifferentiation of chondrocytes [27], whereas BMP-2 decreases the p65 DNA binding activity on *COL2A1* during the redifferentiation process [35].

Many transactivators and co-activators are also recruited in the enhancer and/or the promoter of *COL2A1* to stimulate its expression (e.g. p300/CBP, TIP60, Scleraxis, E47, c-MAF) [28,36–39].

Following the transcription of the *Col2a1* gene, a pre-messenger RNA is synthesized. During chondrogenesis, it undergoes alternative splicing, the two main variants being type IIA and type IIB collagen isoforms [40,41]. Type IIA collagen (exon 2 present) is an immature form of type II procollagen and its expression is found in undifferentiated mesenchymal cells and in chondrocyte precursors [41]. Type IIB collagen does not contain the exon 2 coding sequence, and its expression is restricted to mature differentiated chondrocytes. Thus, in addition to monitoring the expression of type II collagen, the assessment of a fully differentiated chondrocyte phenotype must include the analysis of the relative expression of IIB and IIA isoforms. Type IIA collagen contains an N-terminal propeptide consisting of 69 amino acids with many disulfide bonds. This short peptide has the ability to bind members of the TGF-β family, particularly TGF-β1 and BMP-2 [42]. During the early stages of chondrogenesis, the peptide encoded by exon 2 may then create a pool of growth factors necessary to maintain chondrocyte

differentiation. In addition, TGF-β1 and BMP-2 regulate the expression of the type IIA and IIB isoforms. For example, in murine chondrocytes, BMP-2 induces type IIB procollagen whereas TGF-β1 stimulates type IIA procollagen [43].

During the differentiation of MSCs into chondrocytes, two novel isoforms arising from alternative splicing of the *Col2a1* gene transcription were recently discovered, type IIC and IID isoforms [44]. The IIC isoform contains 34 nucleotides of exon 2, while the isoform IID includes all of exon 2 and three additional nucleotides. Type IID thus encodes an additional amino acid. However, it appears that the type IIC form is not translated, while the IID form is co-expressed with the IIA isoform during in vitro differentiation of human MSCs in chondrocytes.

Additionally, procollagens IIA/D can be re-expressed by dedifferentiating chondrocytes and in OA cartilage. Therefore, it is important to determine which isoform(s) is (are) synthesized in vivo in normal and pathological situations and, in vitro, to fully assess the phenotype of cells producing type II collagen. Antibodies directed against the cysteine-rich domain found in procollagens IIA and IID are already available, but antibodies detecting only the chondrogenic IIB form of type II procollagen are still lacking to date. We used a synthetic peptide encompassing the junction between exon 1 and exon 3 of the human sequence as an immunogen to produce rabbit polyclonal antibodies against procollagen IIB. These antibodies do not cross-react with procollagens IIA/D or with procollagens I, III and V, suggesting great potential for the analysis of the spatiotemporal distribution patterns of N-propeptides of procollagens IIA/D and IIB during normal development and in pathological situations [45].

2.2. Chondrogenesis and its regulation

2.2.1. Chondrogenesis

Cartilage formation begins in the mesenchyme by the condensation and differentiation of MSCs in pre-chondrocytes [46]. During this first phase of cell condensation, the cells express type I and type IIA collagens [47]. They also synthesize GAGs and adhesion molecules such as cadherin and neuronal cell adherence molecule (N-CAM) [48,49]. This is due to the presence in the cellular microenvironment of many soluble factors such as some members of the TGF-β superfamily. TGF-β1, TGF-β2 and TGF-β3 induce the synthesis of adhesion molecules such as syndecan, tenascin and fibronectin [50]. Simultaneously, most blood vessels disappear and only those stimulated by the vascular endothelial growth factor (VEGF) remain [46].

Chondrogenic differentiation continues, and pre-chondrocytes then express specific cartilage transcription factors such as SOX9, L-SOX5 and SOX6 [51,52]. At this stage of differentiation, pre-chondrocytes are transformed into mature chondrocytes and produce an ECM rich in PGs (aggrecan) and collagen fibers (collagen type II, IX and XI). In particular, they synthesize type IIB collagen which is the major marker of the differentiated state of chondrocytes.

Regarding chondrocytes found at the center of the condensation zone, they mature and proliferate extensively forming vertical columns of cells within the growth plate. Cells increase in size and become prehypertrophic, and then hypertrophic. The chondrocytes in this zone do not synthesize type II collagen, but mainly type X collagen, whose role is to facilitate the deposition of calcium into the matrix [53]. In this transition zone between bone and cartilage, alkaline phosphatase is also expressed. This enzyme releases inorganic phosphate which, when bound to calcium, allows the formation of hydroxyapatite crystals. Hypertrophic chondrocytes will also produce VEGF (a key angiogenic factor [54] for the appearance of blood vessels downstream of the growth plate).

Cells trapped in the calcified area (degenerative chondrocytes) eventually die by apoptosis and are removed by phagocytes. The empty space is invaded by osteoclasts that will ensure that the cartilage matrix is remodeled to prepare for the arrival and replacement of cartilage cells by osteoblasts. Part of the MSC population differentiates into osteoblasts expressing type I collagen, osteocalcin, osteonectin and osteopontin. Bone growth is closely related to the growth plate whose cellular architecture is well organized [49].

2.2.2. The key regulators of chondrogenesis

During the development of cartilage, several growth factors, differentiation and transcription factors are involved at different specific stages and levels. The differentiation of MSCs into mature chondrocytes and in hypertrophic chondrocytes requires a very precise combination of all these factors. The process of cell differentiation implies a constant change in the local microenvironment to guide cells to a differentiated state [55].

2.2.2.1. Growth factors. A summary of the general mechanism of action of the main growth factors is presented below and in Fig. 3. Some are described in the Supplementary data.

2.2.2.1.1. IGF (insulin-like growth factor). IGFs are proteins that can regulate growth, differentiation, migration and cell survival. There are two forms, IGF-I and IGF-II. The concentration of IGF-II in the blood and skeletal tissues is much greater than that of IGF-I. However, both isoforms exert their effects via the same receptor, the type I IGF receptor (IGF-IR). IGF-II also binds to the type II receptor (IGF-IIR) with a 100-fold greater affinity for IGF-I, leading to the degradation of IGF-II by lysosomes [56]. IGF-I has a major role in the growth plate. This factor is produced mainly by the liver, but also by the chondrocyte itself, ensuring autocrine and/or paracrine regulation [57]. IGF-I stimulates growth by inducing proliferation of growth plate chondrocytes, but also by stimulating the differentiation of MSCs in pre-chondrocytes [58].

In the blood, about 98% of the IGF is bound to one of the six classes of the insulin-like growth factor binding protein (IGFBP). In cartilage, IGF is bound to IGFBP-3 and IGF-I increases the mRNA and protein levels of type II collagen via the Akt signaling pathway [59]. IGF-I is also capable of signaling via the ERK–MAPK pathway, especially by decreasing the expression of Mmp -13 and that of the *Col2a1* gene, thereby inhibiting its chondrogenic effects [60]. In MSCs, IGF-I induces proliferation, regulates apoptosis and stimulates the expression of cartilage-specific markers such as type II collagen [61]. Finally, IGF-I is sometimes associated with TGF-β1 or BMP-2 to induce the differentiation of MSCs in chondrocytes [62].

Studies performed in the laboratory have demonstrated the chondrogenic action of IGF-I. For instance, in articular chondrocytes, IGF-I stimulates the synthesis of the type II collagen mRNA in particular by increasing the binding activity of the trans factors Sox9, L-Sox5 and Sox6 in the intron-specific enhancer *COL2A1* region (+2353/+2415 bp) [28,35]. In addition, when dedifferentiated human chondrocytes are treated with IGF-I, it induces the re-expression of type II collagen and SOX9 whereas it inhibits cyclooxygenase-2 (COX2) and MMP13 [63].

2.2.2.1.2. The TGF-β family and cartilage. The family of TGF-βs includes numerous ligands such as activin A, growth and differentiation factors (GDFs), TGF and BMPs. They are widely expressed in chondrocytes and constitute the class of growth factors almost exclusively involved in the process of chondrogenesis.

TGF-βs generally induce the differentiation of MSCs in chondrocytes, stimulate their proliferation, increase the production of the ECM and inhibit endochondral ossification [64]. Members of the BMP family induce the differentiation of MSCs in mature chondrocytes, but also the differentiation of proliferative chondrocytes to hypertrophic chondrocytes [65,66]. In chondrocytes, TGF-β increases the signaling induced by BMPs, whereas BMP-2 decreases that induced by TGF-β. They drive chondrogenesis only when TGF-β has induced the differentiation of MSCs in chondrocytes. Then, during the later stages of the differentiation process, BMP alone is able to exert its chondrogenic effects [67,68].

2.2.2.1.3. TGF-βs and the chondrocyte lineage. The condensation of MSCs in chondrocytes is initiated by TGF-β1. Of all the factors expressed during the early stages of chondrogenesis, TGF-β1 is one of the most

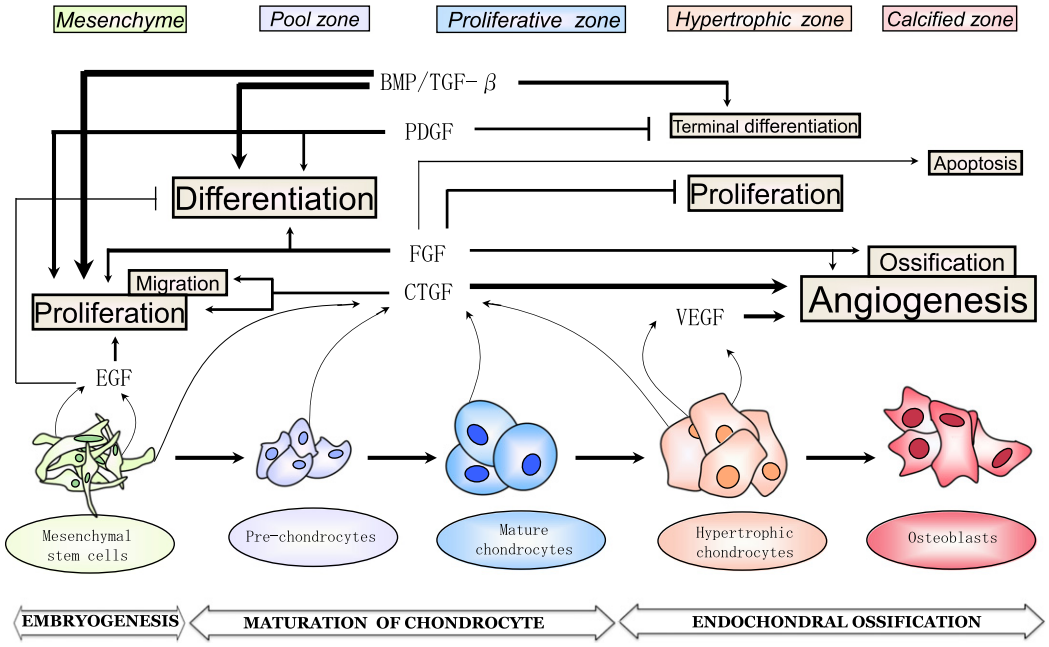

Fig. 3. Schematic overview of the role of growth factors at different stages of chondrogenesis. BMP (bone morphogenetic protein), TGF-β (transforming growth factor beta), PDGF (platelet derived growth factor), FGF (fibroblast growth factor), CTGF (connective tissue growth factor), and VEGF (vascular endothelial growth factor).

important. During this phase, it stimulates the synthesis of fibronectin [69] and N-cadherin to promote cell interactions [70].

In mice, TGF-β1 stimulates the synthesis of type IIA collagen and that of the α11 integrin subunit [71]. Increased expression of type II collagen by TGF-β is mediated by the association of Smad 3/4 and Sox9 and their recruitment in the *COL2A1* gene-specific enhancer [72]. In combination with BMP-2, TGF-β1 increases cartilage-specific markers (e.g. type II collagen, GAGs) [73,74], while the level of type I collagen appears to remain minimal [73]. The association of BMP-2 and TGF-β1 also prevents the stimulation of alkaline phosphatase by BMP-2 [74].

2.2.2.1.4. TGF-β1 and hypertrophy. TGF-β1 inhibits the terminal differentiation of chondrocytes by increasing the expression of PTHrP [75], inhibiting that of type X collagen, VEGF, osteocalcin and MMP13 [76]. Smad 1/5/8 is required to induce hypertrophy whereas Smad 2/3 blocks it. TGF-β1 decreases the expression of type X collagen [77] because Smad 2/3 binds to Runx2, inactivating the latter [78]. Differentiated MSCs express type X collagen after two weeks of culture in a chondrogenic medium. In human MSCs, TGF-β1 cooperates with the Wnt/β-catenin signaling pathway to induce chondrogenesis and it inhibits osteoblast differentiation by decreasing the expression of osteocalcin, alkaline phosphatase and Runx2 [79,80].

2.2.2.1.5. BMPs and the chondrocyte lineage. The diversity and number of BMP family members are so high that BMPs are expressed in each zone of the growth plate. Several BMPs, notably BMP-2, -4 and -7 are able to stimulate chondrogenesis and differentiation of MSCs. Since the effects of BMP-2 and -7 have already been reviewed (e.g., see [81,82]), we focus here only on the role of BMP-2 in cartilage development.

BMP-2 plays a major role in the condensation phase of MSCs in chondrocytes. In addition, MSCs expressing N-cadherin are even more receptive to BMP-2 stimulation to induce chondrogenesis [83]. BMP-2 has two direct and distinct effects on the orientation of the stem cell lineage: it appears to regulate differentiation, with the capacity to induce both chondrogenesis and osteogenesis [84].

In human embryonic stem cells cultured at high density, BMP-2 induces the formation of a rich ECM composed of collagens and PGs [85]. Similarly, after treatment with BMP-2, MSCs from adipose tissue can express phenotypic markers of mature cartilage (type II collagen/GAG) [86]. BMP-2 induces the expression of the transcription factor Sox9 in a dose-dependent manner [87] and increases the DNA binding activity of SOX9, L-SOX5 and SOX6 in the *COL2A1* gene enhancer [35,88,89]. BMP-2, unlike TGF-β1, stimulates the expression of type II collagen in its mature form (type IIB collagen) [71,90]. It has therefore the best chondrogenic effects compared, for example, to TGF-β1 on the induction of chondrocyte specific markers. In addition, BMP-2 stimulates type II collagen at low concentrations (50 ng/ml), whereas it inhibits the expression of this isoform at high doses (100 to 400 ng/ml) [91].

2.2.2.1.6. BMP-2: hypertrophy and osteogenesis. The differentiation of proliferating chondrocytes into hypertrophic chondrocytes is induced by BMP-2 at doses ranging from 100 to 1000 ng/ml, which leads to bone mineralization and the expression of type X collagen, alkaline phosphatase and osteocalcin [92,93]. At these concentrations, BMP-2 also inhibits the synthesis of PTHrP as well as its receptors [94]. In hypertrophic chondrocytes, BMP-2 stimulates the expression of type X collagen via the Smad 1/5/8 pathway in association with Runx2, whereas this pathway is not induced in non-hypertrophic chondrocytes [95]. In summary, BMP-2 has chondrogenic effects at low doses, osteogenic effects at high concentrations and positive effects on chondrocyte maturation.

2.2.2.2. Hormones and other peptides. Fig. 4 summarizes their mechanism of action during chondrogenesis. Only the major hormones and peptides are described in the following sections, some others are presented in the Supplementary data.

2.2.2.2.1. PTHrP (parathyroid hormone-related peptide). PTHrP belongs to the family of parathyroid hormones (PTHs). During the development of cartilage, PTHrP is produced mainly in the periarticular and proliferative area [96]. Even though most receptors of this hormone are present at the surface of pre-hypertrophic chondrocytes and osteoblasts, a small proportion of them are found at the surface of proliferating chondrocytes [97,98]. This peptide maintains chondrocytes in a proliferative state and inhibits their terminal differentiation into hypertrophic chondrocytes [99]. PTHrP blocks hypertrophy by stimulating the expression of the transcription factor Nkx3.2 and preventing Runx2 expression [100]. PTHrP acts as a negative regulator of the terminal differentiation of chondrocytes during chondrogenesis. This latter is also a physiological regulator of osteogenesis. During endochondral

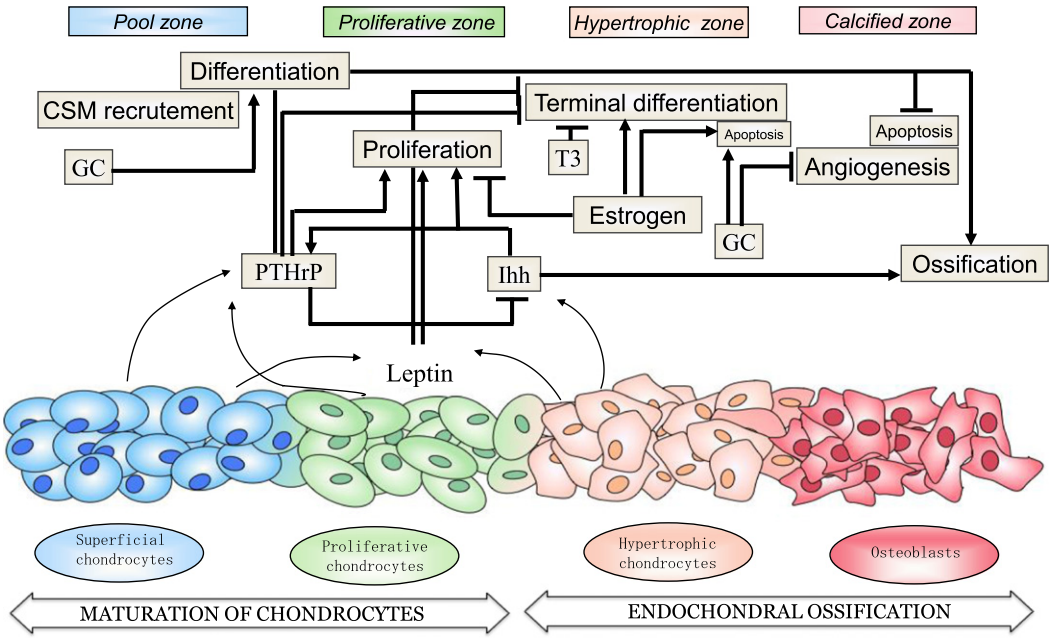

Fig. 4. Schematic overview of the role of hormones and other peptides at different stages of chondrogenesis. GC (glucocorticoids), T3 (triiodothyronine), Ihh (Indian hedgehog), and PTHrP (parathyroid hormone-related peptide).

ossification, the protein Indian hedgehog (Ihh) is associated with PTHrP in a regulation loop that influences chondrocyte differentiation [101] (see Supplementary data).

2.2.2.2.2. Triiodothyronine (T3). Triiodothyronine (T3) is a hormone synthesized by the thyroid gland. In target tissues, thyroxin (T4) is converted into active triiodothyronine (T3) by deiodination. In cartilage, this chemical reaction is ensured by the thyroid hormone deiodinase [102]. In rats, three of these four receptors are expressed in the reserve and proliferation areas, but not in hypertrophic zone. Triiodothyronine inhibits the proliferation of chondrocytes, stimulates the synthesis of type X collagen and increases the activity of alkaline phosphatase [103]. T3 also stimulates PG synthesis and activity of alkaline phosphatase [104]. Thus, T3 regulates chondrocyte proliferation, the organization of proliferative chondrocytes into columns and the terminal differentiation of hypertrophic chondrocytes.

2.2.2.3. The major transcription factors involved in chondrogenesis. Many transcription factors are involved at various levels during chondrogenesis and endochondral ossification. Depending on the location of chondrocytes within the articular cartilage, multiple transcription factors, specific to each zone, are involved in the process of chondrocyte differentiation.

Some of the transcription factors involved in chondrogenesis are described in the Supplementary data. Here, we focus only on the three key transcription factors involved in chondrogenesis.

2.2.2.3.1. Sox9, L-Sox5 and Sox6, the three key transcription factors for chondrogenesis. Sox9 is involved in the condensation phase of MSCs in pre-chondrocytes. Cells beginning to express Sox9 form aggregate and begin to differentiate. Sox9 plays a very important role in the phase following MSC condensation because it stimulates the expression of cartilage-specific markers and inhibits terminal differentiation of chondrocytes [51]. Sox9 also induces the expression of transcription factors L-Sox5 and Sox6, which definitively commit MSCs to developing into a chondrocyte lineage [51]. SOX9 binds to two responsive elements within the *SOX5* promoter, but none are present in the *SOX6* gene, suggesting a protein-protein type of interaction. In addition, introns 1 and 2 of the *SOX9* gene contain binding sites for SOX9 itself, revealing an auto-induction regulation in its tissue-specific expression [105].

The trio Sox9, L-Sox5 and Sox6 induces specific expression of type II collagen via its intron-specific enhancer (intron 1) and that of aggrecan [22,28,52,106]. In addition, the cofactor p300/CBP, Scleraxis/E47, Tip60 and c-Maf cooperate with Sox9 the enhancer, by directly binding to DNA or via protein–protein interactions to induce the transcriptional activity of the *COL2A1* gene [28,36–39] (Fig. 2). When it stimulates chondrogenesis, SOX9 acts as a dimer and is phosphorylated by PKA [107,108]. In addition to the *COL2A1* gene, the SOX9, L-SOX5 and SOX6 trio also induces the expression of type IX and XI collagens [52]. Similar to the *Col2a1* gene, *Col9a1* and *Col11a2* gene regulation are positively regulated with the trans-acting factors of the Sox family through their specific enhancer [108]. Thus, this trio coregulates the expression of the COL2A1, COL9A1, COL11A2 and ACAN (encoding aggrecan) genes in articular chondrocytes.

During endochondral ossification, SOX9 also behaves as an inhibitor of the expression of VEGF, OSX (Osterix) and RUNX2 which are respectively involved in angiogenesis, ossification and hypertrophy [109]. Thus, SOX9 inhibits the differentiation of proliferative chondrocytes in hypertrophic chondrocytes. However, during the dedifferentiation of chondrocytes in monolayer culture, SOX9 inhibits the transcriptional activity of the *COL2A1* gene via its recruitment to the 266 bp promoter through protein–protein interactions with other trans factors such as Sp1/Sp3 [28,110]. The action of SOX9 in chondrocytes depends therefore on the state of differentiation of these cells.

A recent, elegant study in 2010 analyzed the major sites of Sox9 interactions in the genome of rat chondrocytes [105]. The ChIP-on-chip experiments revealed that Sox9 has the ability to bind more than 30 genes involved in the regulation of the chondrocyte phenotype. The Sox9 transcription factor seems to regulate many cellular signaling proteins, but also matrix proteins or other transcription factors. In addition, it appears that all ECM genes contain at least two responsive elements for Sox9. It is now well-known that SOX9 interacts with a binding site located in the specific enhancer of intron 1 of the *Col2a1* gene; however, this study shows that SOX9 can also bind to intron 6 of the *Col2a1* gene [105]. The *COL1A1* gene, normally downregulated in chondrocytes, also contains two Sox9-binding sites, one in intron 1

and the other in its 3′ end. Furthermore, another study has shown that hypoxia inhibits the expression of type I collagen via the transcription factor HIF-1α and HIF-1α binds to the promoter of the *SOX9* gene and increases its expression [111]. Thus, taken together, it is plausible that the binding of SOX9 on the *COL1A1* gene acts as a negative regulator by inhibiting its expression. Lastly, the recruitment of SOX9 on the *RUNX2* and *OSX* genes may also involve transcriptional inhibition.

2.2.2.3.2. Nkx3.2 (NK3 homeobox 2). Also called bagpipe homeobox homolog 1 (Bapx1), Nkx3.2 is a transcriptional repressor that promotes somitic chondrogenesis. This transcription factor has a high binding affinity for the TAAGTG sequence in which the AGT motif appears to be essential for binding DNA [112]. Nkx3.2 expression is important for the recruitment of chondro-progenitors and it subsequently decreases throughout the differentiation process. It acts as a negative regulator of chondrocyte maturation by suppressing the expression of Runx2 [100]. Nkx3.2 may also induce axial chondrogenesis by inducing Sox9 expression in the mesoderm: during cell signaling induced by the BMP family and under the stimulation of Shh, Nkx3.2 and Sox9 mutually induce each other's expression. An autoregulatory loop between these two trans factors thus occurs and is maintained by BMPs [113]. For example, during the condensation of MSCs, BMP-2 induces the expression of Nkx3.2, which in turn suppresses that of Runx2 and inhibits terminal chondrocyte maturation into hypertrophic chondrocytes [114].

2.2.2.3.3. AP-2 (activator protein-2). Members of the AP-2 family are involved in various processes such as cell migration, differentiation, and apoptosis. They regulate their target genes by binding to GCCN3GGC palindromic sequences. Members of the AP-2 family form homodimers and heterotrimers to exert their activating effect on the transcription of target genes. This transcription factor is a regulator of chondrogenesis and is expressed in five isoforms AP-2α, AP-2β, AP-2γ, AP-2δ and AP-2ε [115]. These isoforms play an important role in chondrogenesis and the development of the spinal column during embryogenesis. AP-2α, AP-2β and AP-2ε isoforms are involved in the process of chondrocyte differentiation. AP-2α is expressed in the growth plate and in adult articular cartilage. This is a negative regulator of MSC differentiation in chondrocytes and its expression decreases during the endochondral ossification process. The patterns of expression for type II collagen and of AP-2α are inversely correlated [116]. A high concentration of AP-2α therefore maintains the chondrocyte in an early differentiation state and inhibits the transition of these cells into differentiated chondrocytes. AP-2ε is expressed during the late phase of chondrogenesis. Sox9 strongly stimulates the transcriptional activity of *AP-2ε* gene by binding to its promoter [117,118]. It is expressed in hypertrophic cartilage, where it stimulates the expression of integrin α10.

2.2.2.3.4. Zinc finger protein 219 (ZNF-219). This 77 kDa protein is composed of nine zinc fingers and many proline-rich regions [119]. Zinc finger domains 1–4 appear capable of acting as a nuclear localization sequence (NLS). ZNF-219 belongs to the family of Sp1 transcription factors. Sakai et al. revealed a consensus sequence for ZNF-219 which has high binding affinity [120]. This factor appears to bind to two copies of DNA whose sequence is CCCCCA and may act as a transcriptional repressor [120]. This conclusion is based on the fact that ZNF-219 decreases the expression of the *HMGN1* gene by binding to its promoter. In mice, this factor plays an important role in chondrogenesis [121]. It is expressed only in the proliferative zone of the growth plate and a knockdown or inhibition of Znf-219 suppresses MSC differentiation in mature chondrocytes. Znf-219 physically and functionally interacts with Sox9 to regulate chondrocyte differentiation and stimulates the transcription activity of Sox9, with which it also colocalizes. Thus, this zinc finger protein acts not only as a transcriptional repressor but also as an activator. Another study has shown that the HMGN1 protein binds to the SOX9 gene to inhibit its expression [122]. Znf-219 may thus promote chondrogenesis by inhibiting a Sox9 inhibitor. At the structural level, the last zinc finger of ZNF-219 seems to be essential for inducing chondrogenesis, since a mutant deficient in this domain is no longer able to bind to the promoter region of the *Col2a1* gene. Znf-219 may therefore act as a cofactor or as an adapter protein for the interaction between Sox9 and other proteins of the transcription initiation complex. In mice, ZNF-219 responsive elements are present in the promoter regions of chondrocyte-specific genes such as *Acan*, *Col2a1* and *Col11a2*. Lastly, ZNF-219 has no effect on the differentiation of MSCs into osteoblasts and does not colocalize with the transcription factor Runx2.

2.3. Pathological articular cartilage: osteoarthritis

There are many diseases associated with articular cartilage that can be categorized into several types: traumatic (chondral lesions), inflammatory (rheumatoid arthritis, chondrites), metabolic (chondrocalcinosis, gout) or degenerative (osteoarthritis). Below, we focus on the physiopathology of articular cartilage that requires osteochondral grafts, such as chondral lesions and osteoarthritis.

2.3.1. Osteochondral lesions

The chondral lesions or loss of chondral substances occur as a result of trauma to the joint, such as that caused by falls or sports injuries. The symptoms are highly variable. A survey of surgical interventions related to meniscal or ligamentous pathologies shows that chondral lesions of the knee appear to be the most common (63%) [123].

Articular cartilage has a very limited spontaneous repair capacity. However, studies performed on rabbits have shed light on a natural mechanism whereby injured cartilage tissue is replaced with fibrocartilage (rich in type I collagen fibers). In this mechanism, the injured area is repopulated by bone marrow stem cells that differentiate into chondrocytes and then gradually into osteoblasts and fibroblasts. ECM synthesis is also observed 10 days after the onset of the lesion. Chondrocytes around the lesion are not involved in the repair process, which is ensured by the proliferation and differentiation of stem cells from the bone marrow. However, 12 weeks after the onset of the injury, the newly synthesized cartilage matrix shows fissuring and gradually takes on the appearance of subchondral bone [124]. Most chondral lesions lead to the release of cartilage fragments into the synovial fluid, leading inevitably to early degenerative cartilage diseases such as osteoarthritis.

2.3.2. Osteoarthritis (OA)

OA, as the most common joint disease in the world, is one of the most important causes of pain and disability in adults, leading to significant socio-economic consequences. Its prevalence is further exacerbated by an aging population and increasing obesity. Thus, more than 80% of people over 75 years old develop OA. This disease is generally higher in women than in men, with 13% of women over the age of 60 developing OA compared to only 10% of men. In the United States, OA is the second leading cause of disability after cardiovascular diseases and each year there are more hospitalizations due to OA than to rheumatoid arthritis [125].

In Asia, it has been estimated that the percentage of the 65 and older population will more than double between 2008 and 2040, rising from 6.8 to 16.2% [126]. China and India are ranked as the top two countries with the most people aged over 65, 106 million and 60 million, respectively. The coming years will see millions of new cases of osteoarthritis in all populations around the world.

There are two types of OA, mechanical OA or structural OA. The first one, which will not be reviewed here, is observed when healthy cartilage undergoes excessive loads leading to its degradation. In contrast, structural OA involves the actual nature of cartilage, which once weakened and showing an abnormal appearance, will be rapidly degraded.

2.3.2.1. Risk factors of OA. Age is a major risk factor in the development of structural OA because the biomechanical properties of cartilage

gradually decrease with time. Aged chondrocytes are similar to terminally differentiated chondrocytes in the growth plate. Their differentiation status decreases with age and they are less able to respond to external anabolic stimuli [127]. The aging of cartilage and its relationship to the development of OA will be addressed more explicitly in the next chapter.

Sex or hormonal status is also a prime factor involved in the onset and development of structural OA. Thus, many studies have shown that the prevalence of OA affects women more than men. After menopause, the hormonal status of women changes, leading to a sharp decrease in estrogen concentrations correlated with an increase in the prevalence of OA in women over 50 [128,129]. Estrogen therefore seems to play an important role in preventing the development of OA. In addition, postmenopausal women who have taken estrogen replacement therapy develop OA less frequently than women who do not [130].

Genetic predisposition of some patients to OA is also a risk factor. According to family history, genetic mutations may be present in many genes involved in the ECM structure of cartilage and/or chondrocyte metabolism. For instance, many studies report that genetic mutations detected in the *COL2A1*, *COL11A1*, *COL9A1* and *COL10A1* genes result in structural changes in the cartilage matrix and provoke early OA [131]. Other mutated genes such as those of TGF-β or IGF-1 may cause similar changes. 50% of OA cases develop from inherited genetic mutations [132].

Diet, as in many diseases, is also a risk factor. A diet low in antioxidants such as vitamins C, D or E increases the probability of developing OA [133]. Similarly, frequent exposure to oxidants actively participates in the development of the disease.

Diseases that affect the osteoarticular system result, over time and indirectly, in structural OA. For example, gout, inflammation of the synovium, chondrocalcinosis, ochronosis or the epiphyseal osteonecrosis are all conditions that can weaken cartilage and induce its progressive degradation.

2.3.2.2. Aging of articular cartilage and OA. Age is an important risk factor for the onset and development of OA. However, aging of articular cartilage cannot be assimilated with a pathological process, because tissue aging is an inevitable mechanism. Numerous occurrences, from telomere shortening to reactive oxygen species (ROS), are involved in cellular aging without leading to disease. Thus, a recent study on 82 patients aged 90 showed that 60% of them have no OA, especially men [134].

However, in some special cases, aging and cartilage degradation mechanisms are correlated. ECM and chondrocyte metabolism change with age as well as cell death. Cell death is likely to be a very important risk factor because the degradation of articular cartilage is correlated with increased apoptosis of chondrocytes [135].

The ECM of aging cartilage show a higher frequency of small PGs rich in KS than long PGs rich in CS [136]. Other signs include microfibrillation on the surface of cartilage, decreased aggrecan aggregates and increased cross-linking of collagen [137]. The cartilage ECM is a reservoir for many growth factors and cytokines. Thus, the modification of the structural organization of the ECM during aging may decrease the bioavailability of these factors for chondrocytes, thereby leading to the deregulation of their cellular phenotype.

Aging articular cartilage shows an increased quantity of glycation end products (advanced glycation end products, AGEs) [138]. Glycation is a process that increases with age and leads to chronically elevated blood sugar levels. The glycation reaction generates glycated proteins that enter cells. Once inside cells, these proteins cannot be secreted by the cell or degraded, causing multiple mutations to DNA. Treatment of bovine articular cartilage with glycated protein increases its fragility and its rigidity [139]. On the other hand, the accumulation of AGEs in cartilage activates the glycation end product receptors (receptors for advanced glycation end products, RAGEs), which in turn induce the production of ROS and trigger catabolic cascades [140].

Other proteins may trigger catabolic signaling pathways such as small S100 calcium-binding protein A4 (S1004A) belonging to the S100 family of proteins. In response to IL-7 (interleukin 7), S1004A increases the expression of MMP13 via its RAGE receptor binding [141] and sequesters the tumor suppressor gene p53 [142].

ROS can be produced by chondrocytes or cells of the synovial membrane. Oxidant stress induces oxidation of proteins, lipids and causes DNA damage. Aging chondrocytes have more ROS and fewer active antioxidant enzymes [143]. In addition, in response to significant oxidative stress, chondrocyte death is greater when they originate from an older donor compared to a younger donor [144].

Although nitric oxide (NO) is present in adult articular cartilage, it increases significantly in aged tissues [145]. This intracellular increase in NO leads to chondrocyte apoptosis via the mitochondrial pathway and stimulates the production of pro-inflammatory cytokines that cause degradation of cartilage ECM [146].

2.3.2.3. Changes in cartilage homeostasis during OA. In OA, there is a change in cartilage homeostasis resulting in increased catabolism and decreased anabolism of chondrocytes. This mechanism is not the cause of the onset of OA, but rather its consequence. Older chondrocytes become much more sensitive to extracellular inflammatory signals and their response to growth factors decreases [3].

When the cartilage deteriorates, lesions begin to appear on the surface, destroying the orientation and integrity of the collagen network. Some chondrocytes are more exposed to growth factors present in the synovial fluid and this sudden bioavailability causes intensive proliferation, particularly in the superficial zone of cartilage. The chondrocytes then form a group of several proliferative clones, also called clusters. During the most advanced stages of OA, many empty lacunae also appear in the deep zones of cartilage. When the OA process is present, chondrocyte proliferation increases and the cells of the deep zone significantly re-express type X collagen before entering apoptosis [147,148].

In the early stages of OA, chondrocytes will first attempt to repair the damage by increasing its production of anabolic factors such as IGF-1 or TGF-β [149,150]. Upon exposure to these factors, chondrocytes dedifferentiate and produce mainly type I, IIA and III collagens, atypical forms for adult articular cartilage. Chondrocytes show more moderate synthesis of type II collagen and aggrecan and the small PGs (SLRPs) sometimes have an incomplete structure, e.g. lumican which lacks GAG chains of the KS type [4]. The phenomenon of cellular dedifferentiation reflects a loss of the chondrocyte phenotype that promotes the formation of non-functional cartilage, the fibrocartilage. This repair cartilage is more fragile than articular cartilage and is rapidly degraded under high mechanical stress. In parallel, chondrocytes and synoviocytes attempt to limit inflammation by producing certain anti-inflammatory cytokines such as IL-4, IL-10 and IL-13.

All the efforts mobilized to restore articular cartilage eventually fail. The lack of effective response to anabolic/catabolic signals and the increase of the sequestration of growth factors in the ECM of cartilage lead to the irreversible dedifferentiation of chondrocytes, apoptosis and degradation of the cartilage matrix.

Multiple proteolytic enzymes are produced and secreted by chondrocytes, osteoblasts or synoviocytes [151]. During OA, the pro-inflammatory cytokines such as IL-1β and TNF-α are responsible for the increased expression and/or activity of many MMPs and/or ADAMTS such as MMP1, 2, 3, 8, 9, 13 and 17 [1,2,152] and ADAMTS-4 and -5 [153,154] which will induce the degradation of cartilage-specific markers (type II collagen and aggrecan).

The release of cartilage fragments in the synovial fluid generates a secondary inflammatory reaction which is maintained by chondrocytes and synoviocytes. These cells produce pro-inflammatory cytokines or mediators or enzymes that act in a paracrine or autocrine manner such as IL-1, IL-6, TNF-α, prostaglandin E2 (PGE2), phospholipase A2 or cyclooxygenase A2 (COX-2) [155]. The synovial fluid of OA patients

also contains immune system cells such as macrophages and T-cells involved in cartilage turnover and which also participate in the stimulation of angiogenesis in osteophytes. Additionally, there is an increase in the serum concentration of C-reactive protein (CRP) caused by infiltration of pro-inflammatory cytokines in the blood such as IL-1β and TNF-α [156]. The gene expression of IL-1β is particularly stimulated by TNF-α via demethylation of two CpG islands in the IL1B promoter [157].

IL-1β and TNF-α have comparable effects because they both downregulate the expression of cartilage-specific markers. In addition, they induce the production of inflammatory mediators such as IL-6, IL-8 or NO. The transcriptional activities of the *IL1B* and *TNFA* genes are regulated by NF-κB [158]. This transcription factor also plays a very important role in the development of OA since it is involved in inflammation, cartilage degradation, cell proliferation and angiogenesis [159].

2.3.3. HtrA1, a particular protease involved in OA

High temperature requirement A (HtrA) was first identified in Escherichia coli as a heat shock protein that is resistant to high temperatures and then it was discovered also in plants and animals. Four proteins belong to the HtrA family (HtrA1-4). HtrA1 was isolated for the first time in fibroblast cell lines transformed by SV40 [160]. It is a serine protease enzyme, which is secreted in extracellular space and involved in cell proliferation. This protein is expressed in a variety of tissues such as skin, muscle, liver, pancreas or cartilage [161].

HtrA1 plays an important role in cancer development because its expression is decreased in melanoma as well as in ovarian cancer [162,163]. On the other hand, overexpression of this enzyme leads to a decrease in proliferation and cell migration, suggesting that it acts as a tumor suppressor gene in certain cancers. More recently, it has been shown that HtrA1 decreases bone mineralization by inhibiting the signaling pathway induced by BMP-2 through its SP and PDZ domains [164].

HtrA1 plays also an essential role in adult articular cartilage and during development of this tissue. Its expression is not found during the condensation phase of MSCs, but during the phase of terminal differentiation, when they differentiate into hypertrophic chondrocytes and osteoblasts [165]. This serine protease degrades components of the ECM such as aggrecan, type II collagen, decorin or fibromodulin, and also inhibits the signaling pathways induced by BMPs and TGF-βs. In pathological situations such as OA, the expression of HtrA1 is increased. It has been demonstrated that the levels of the HtrA1 protein are higher in OA cartilage compared to those of healthy cartilage [166]. Similarly, experimentally induced arthritis in mice increases the levels of HtrA1 [165]. Likewise, a high concentration of this enzyme is also found in the synovial fluid of patients with OA [167].

There is a large body of research that has highlighted the functions of HtrA1 in OA articular cartilage. HtrA1 induces aggrecan proteolysis in vivo by cleaving it in the IGD domain. Additionally, aggrecans containing the $VQTV^{356}$ epitope are found in greater amounts in OA cartilage and the accumulation of GAGs decreases [168]. HtrA1 also acts as an antagonist of the signaling pathways induced by the members of the TGF-β family. For example, HtrA1 inhibits the signaling pathways of TGF-β1 and BMP-2 in mice. HtrA1 is able to bind TGF-β1, TGF-β2, BMP-2 and BMP-4, but does not induce their degradation in vitro. The inhibition induced by HtrA1 is however directly related to its proteolytic activity. Thus, one hypothesis suggests that this enzyme can induce the degradation of the receptors of TGF-β family members [169]. Interestingly, TGF-β1 is able to stimulate the expression of HtrA1 in primary cultures of human chondrocytes [170]. Moreover, the activity of the HTRA1 gene is regulated by the transcription factor AP-2α which binds to its promoter [171].

Thus, the stimulation of the expression of HtrA1 during OA promotes the development of the disease by increasing catabolism and decreasing the anabolic response of chondrocytes. On the other hand, a recent study has shown that fibroblasts present in the synovial membrane can express HtrA1 which in turn degrade fibronectin. This increase in fibronectin degradation products enhances the synthesis of MMPs, thus favoring degradation of the ECM [167].

By preventing the beneficial effects of TGF-βs and BMPs in chondrocyte differentiation, HtrA1 physiologically and pathologically inhibits the differentiated state of mature chondrocytes.

2.3.4. Type I collagen, a marker of the dedifferentiated chondrocyte phenotype

Type I collagen is a heterotrimer consisting of two α1 chains and one α2 chain respectively encoded by the *COL1A1* and *COL1A2* genes. This isoform is found in many tissues such as skin, tendons or bone. Articular chondrocytes do not produce type I collagen, although adult mouse chondrocytes still express antisense *Col1a1* mRNA [172]. Furthermore, during the development of rat articular cartilage, protein expression of type I collagen is detected in the growth plate [173]. Thus, even if type I collagen protein is not found in adult articular cartilage, it may nevertheless play a role in the development and/or repair of cartilage.

The amplification of chondrocytes in monolayer culture is accompanied by cellular dedifferentiation, characterized by an increase in the expression of type I collagen and a decrease of cartilage-specific markers such as type II, XI, IX collagens and aggrecan [174,175]. There is also an increase in chondrocyte proliferation at the expense of their differentiation [176]. Comparison of the ECM produced by mature chondrocytes to that of dedifferentiated articular chondrocytes shows that the latter does not have the same biomechanical properties. This is because high expression of type I collagen and the concomitant decrease in type II collagen in dedifferentiated chondrocytes lead to a non-functional cartilage ECM, i.e. fibrocartilage.

Transcription of the *COL1A1* gene is regulated by multiple transcription factors such as c-Krox, Sp1, Sp3 or NF-κB. C-Krox stimulates the expression of *COL1A1* gene through the −112/−61 bp region via an interaction of the trans-acting factors Sp1 and Sp3 [26,177]. At high concentrations, Sp3 induces expression of *COL1A1* gene by binding to the region of its short proximal promoter from −174 to 42 bp [178]. Sp1 and Sp3 also stimulate the expression of type I collagen [179], but the Sp1/Sp3 ratio decreases as chondrocyte dedifferentiation occurs, with Sp3 competing with Sp1 leading to the transinhibition of *COL2A1* gene [180]. Finally, it has been shown that NF-κB decreases expression of *COL1A2* and *COL1A1* genes in fibroblasts [179,181].

3. Autologous chondrocyte transplantation/implantation (ACT/ACI) and cartilage engineering

There are many methods to relieve pain caused by cartilage degradation, but none restore functional hyaline cartilage. Drug management, employing a range of drugs from simple analgesics to non-steroidal anti-inflammatory drugs, is used to reduce pain, at least in part, or slow disease progression. When OA is well developed and there is no more hope for restoring a healthy cartilage surface, prosthetic joint replacement is the only solution for restoring the mobility of the affected joint. Today, there are many alternative surgical techniques, such as microfracture or autologous mosaicplasty, but neither produce functional cartilage. The tissue formed generally resembles fibrocartilage and surgeons must perform additional surgery on the affected joint. However, cartilage engineering based on ACI now offers new hope for restorative cartilage surgery.

3.1. The four generations of ACI

ACI was developed in 1987 by Lars Peterson and Matts Brittberg [5]. This technique, which aims at treating large chondral lesions by implantation of culture-expanded autologous chondrocytes within the cartilage defect, has already demonstrated its effectiveness (Fig. 5). It durably reduces pain and increases patient mobility [182] and clinical trials with 10 to 20 years of follow-up show satisfactory outcomes

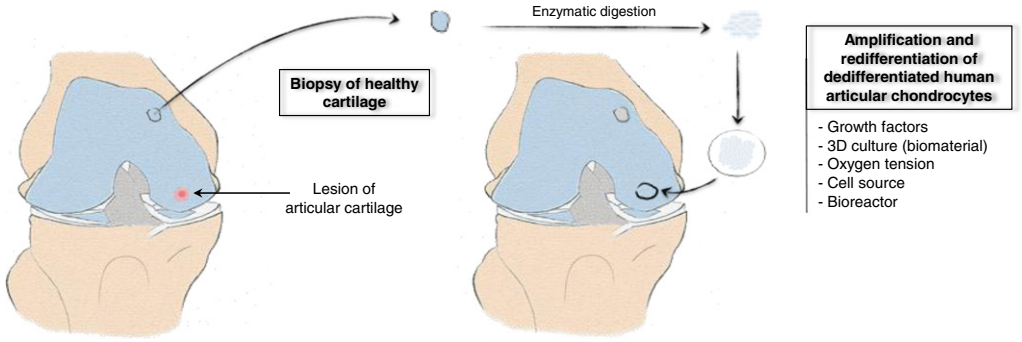

Fig. 5. Diagram of the fourth-generation of autologous chondrocyte implantation (ACI) technique.

[183]. Two years after implantation, the cartilage appears to be of hyaline nature and markers of the differentiated chondrocytes are expressed, such as type II collagen, aggrecan and COMPs. However, type I collagen is also detected, revealing a fibrous cartilage matrix [184,185]. Despite the success of this technique, there is still room for improvement. Successive enhancements have taken the technique four generations and have gradually led to cartilage tissue engineering [186]. The first improvements involved the procedure used for cell transplantation (e.g. progressing from a periosteum membrane towards a collagen membrane; the use of cell suspensions has been replaced by the use of cells embedded in three-dimensional matrices) (review in [187]). The processes currently under development for obtaining a biological substitute are now much more elaborate and employ molecular controls of chondrocyte differentiation. Thus, many components of ACI techniques have been tested, including chondrogenic molecules, various scaffolds, mechanical forces or oxygen pressure. Today, the objective is to find the combination that best enhances and stabilizes the characteristics of the chondrocyte phenotype in articular cartilage.

Therefore, and to overcome the problems related to the use of chondrocytes (e.g. the limited number of cells taken from biopsies, invasive techniques, dedifferentiation in culture, etc.), research has turned its attention to MSCs, which are an attractive alternative cell source for cartilage repair.

Thus, scientific studies aim to significantly improve the ACI technique in two ways: first by improving the chondrocyte phenotype and second by stabilizing the location of implanted cells within cartilage defects.

3.2. A major concern for all generations: phenotype control

Unlike the first three generations, which were developed in clinical trials, the fourth generation is in an experimental state. Many adaptations have attempted to improve the quality of the re-implanted tissue and their ultimate objective is to transplant mature differentiated hyaline cartilage (ECM rich in type II collagen and aggrecan without type I collagen). To reach this goal, many strategies have been explored such as the use of new biomaterials, new cell types (stem cells) or new cell culture techniques (chondrogenic factors, hypoxia, bioreactors, etc.). Thus, ACI is in the context of cartilage tissue engineering bringing to joint surface regeneration. Cartilage tissue engineering seeks to recreate new tissue and consists in seeding cells in a three-dimensional matrix (synthetic or biological) and whose cell lineage has been previously induced and/or maintained by biological mediators (cytokines, growth factors, etc.) [188]. The differentiated character of cartilage cells can be obtained by redifferentiation of dedifferentiated chondrocytes or by induction of specific differentiation of MSCs in chondrocytes.

3.2.1. Cell sources

Today many studies are exploring the use of chondrocytes to repair cartilage damage. The main research strategies first investigate the culture of chondrocytes in a biomaterial, which promotes the formation of a stable and functional ECM. Other more innovative strategies have developed co-culture systems to induce and/or stabilize the chondrocyte phenotype.

The use of autologous chondrocytes for transplantation offers the advantage of reducing the risk of immunological rejection and transmissible disease. However, the outcome has been generally disappointing due to the difficulties in maintaining the chondrocytes in a differentiated state during expansion. Moreover, this technique provokes morbidity at the donor site. To overcome the limitations of autologous chondrocytes (i.e. limited number of seed cells and dedifferentiation due to the OA phenotype or during the amplification of healthy cells), another strategy is to use autologous stem cells or allogeneic stem cells from umbilical cord blood (UCB) in undifferentiated state or differentiated state. Adult stem cells regulate tissue homeostasis by replacing dead cells with differentiated cells. However, they have a limited number of divisions when they are in an undifferentiated state.

The mesenchymal stem/stromal cells represent a promising alternative cellular model, but satisfactory protocols for converting them into chondrocytes and sufficient levels of cartilage matrix are still lacking. MSCs are found in a variety of tissues [189] and are already used to induce cell differentiation (see [190] for review), but with variable efficacy depending on their origin [191] and the lineage considered [192]. Moreover, from the literature, it is difficult to rank the origin of the MSCs according to their potential for proliferation and differentiation. Furthermore, studies vary greatly in the type and characteristics of donors and in cell culture conditions [193,194], preventing a clear picture of which subpopulations represent precursors of interest for a specific differentiation lineage.

Five years after the discovery of induced pluripotent stem cells (iPSCs) by Takahashi and Yamanaka [195], hyaline cartilage has been successfully obtained from mice dermal fibroblasts by introducing three transcription factors: c-myc, klf-4 and Sox9 [196]. The cells are therefore reprogrammed and differentiated at the same time, so they do not acquire the properties of embryonic stem cells (ESCs); this process is called transdifferentiation. The chondrocytes obtained by this process express cartilage-specific markers such as type II collagen and aggrecan, whereas type I collagen shows decreased expression due to the methylation of several sites found in the promoter of *Col1a1* and *Col1a2* genes [196]. Nevertheless, many of the cells form tumors when subcutaneously implanted in nude mice. These findings offer new strategies and new hope for the regeneration of diseased cartilage tissue.

3.2.2. Mesenchymal stem cells (MSCs) in cartilage engineering: which cells for which application?

Adult MSCs can differentiate into osteoblasts, adipocytes, and chondrocytes, but also into muscle cells or fibroblasts. Furthermore, they have a capacity for immunosuppression since they decrease T-cell proliferation [197]. The acquisition of this capacity requires prior activation by immune cells via the secretion of pro-inflammatory cytokines such as IFN-γ, IL-1β or TNF-α [198]. Thus, in addition to their

potential for repair, MSCs are distinguished by their immunomodulatory potency and their anti-proliferative and anti-inflammatory abilities. MSCs can also produce some inflammatory mediators such as prostaglandin E_2 and IL-6. This ability to inhibit inflammation and immune response in the host are probably due to the absence of HLA type II receptors on the surface of MSCs [199]. The HLA type II receptor is part of the major histocompatibility complex (MHC), a system of self-recognition, involved in the immune response of vertebrates.

In differentiation experiments on adult MSCs, many changes in the cellular microenvironment are observed. For example, genes not expressed in MSCs are expressed when MSCs differentiate into chondrocytes, such as glypicane 3, cadherin-11, MMP7 or type II collagen. Conversely, other genes show reduced expression such as CX3CR1, MMP1 and MMP9 [55]. A recent study has also shown that the morphology of MSCs plays an important role in guiding their differentiation. The flattened and adherent cells differentiate into osteoblasts whereas round and floating cells differentiate into adipocytes. As in the dedifferentiation of chondrocytes, MSCs adhering to plastic show an increase in the activity of the RhoA protein, which is responsible for the commitment of MSCs to the osteogenic pathway rather than the adipogenic pathway [200].

MSCs are mainly present in the bone marrow, but are also found in adipose tissue, the periosteum, muscle, synovial membranes, the umbilical cord and cartilage [201]. The source of MSCs is a very important consideration in regard to their application in regenerative medicine and sources vary with respect to the amount of stem cells they offer. When developing a protocol for therapeutic purposes, MSCs should be present in large quantities, collected with the least invasive procedure possible and have a highly reproducible protocol available. However, plasticity and differentiation potential vary according to the source [202]. Thus, MSCs from bone marrow (BM MSCs) and the synovium have greater chondrogenic potential than adipose and muscle MSCs [203]. However, primitive umbilical cord blood (UCB) MSCs have advantages compared to other adult sources, including non-invasive collection methods, hypo-immunogenicity, superior tropism and differentiation potential [204,205]. UCB MSCs can be guided towards chondrogenesis or used for their secretome as demonstrated for other sources [206–210].

The defining characteristics of MSCs are inconsistent among studies. Many methods have been developed to isolate and expand MSCs from a variety of tissues, which leads to diverse characterizations. Phenotypically, MSCs express a number of markers that are, unfortunately, not specific to them. However, it is generally agreed that adult human MSCs do not express the hematopoietic markers CD45, CD34, CD14, or CD11, but do express CD105 (SH2), CD73 (SH3/4), CD44, CD90 (Thy-1), CD146, CD71, CD271 as well as the adhesion molecules CD106, CD166, CD56, ICAM-1, and CD29 [211–220]. It is also generally accepted that all MSCs are devoid of the endothelial cell marker CD31. In a systematic review of the literature [221] Mafi et al. found that the expression of other cell surface markers — including STRO-1, HLA-A, HLA-B, HLA-C, HLA-DR, DP, DQ, HLA-I, EMA, Oct- 4, Oct- 4A, Nanog, Sox-2, TERT, Stat-3, fibroblast surface antigen, smooth muscle alpha-actin, vimentin and diverse integrin subunits — varies with the origin of the MSCs [222], their culture conditions and the chronology of the analysis [223].

In an effort to standardize MSC characterization, the International Society for Cellular Therapies (ISCT) determined in 2006 a set of minimal criteria to characterize MSCs [224]. The cells must be plastic-adherent when maintained in standard culture conditions and must express the specific cell-surface antigens CD105, CD73 and CD90, but not CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules. In addition, the MSCs must also be able to differentiate into osteoblasts, adipocytes and chondroblasts in vitro [211]. However, as stated earlier, these minimal criteria are not specific to MSCs and describe the properties shared by connective tissue cells [225,226]. The great discrepancy and inconsistency regarding the information available on the cell-surface profile of adult and perinatal MSCs and the lack of a comparative immunophenotype in standardized conditions make it impossible to compare different MSC sources. Further research is clearly needed in this field to overcome these problems. The ISCT thus encourages investigators to test for as many surface markers they judge important, using, if possible, a multicolor analysis to demonstrate that individual cells co-express MSC markers of interest for a specific scientific field, and lack hematopoietic antigens.

It is thus important to undertake a true comparative flow cytometry analysis of MSCs isolated from different tissues and expanded in the same culture conditions. With this goal, we tested an original panel of 27 surface markers selected from the literature. The different categories of MSCs (Wharton's Jelly, adipose tissue, dental pulp, and bone marrow) were cultured in medium without serum and immunophenotypic analysis was performed at an early passage after their isolation from the tissue. Detection of the co-expression of several markers should indicate cell subpopulations of interest. For instance, we looked for the combined detection of CD271, CD56 and Stro-1, all of which are surface markers that have been independently proposed as predictive markers for good proliferation, immunosuppression or ability to engage skeletogenesis [218,219,227–232]. Significant differences between the diverse categories of MSCs were observed (Fig. 6B). In particular, MSCs isolated from adipose tissue showed the shortest doubling time (Fig. 6A), but no combination of CD271, CD56 and Stro-1 was detected in this category of cells (Fig. 6B). It is now important to determine if this combination indeed reflects an enriched selection of skeletal precursors. Clearly, analyses using cell sorting techniques are of interest to evaluate the potential of chosen subpopulations for cell proliferation and for cartilage formation capacity.

3.2.3. Co-cultures

Co-cultures allow close paracrine communication between two cell types via the secretion of well-defined biological mediators. This type of culture is mostly used in experiments of cell differentiation and/or cell redifferentiation. Co-cultures can be indirect (separated by a porous membrane) or direct (physical contact between the two cell types).

Co-culture of dedifferentiated chondrocytes and healthy chondrocytes has been performed to test whether anabolic factors produced by differentiated chondrocytes favor the redifferentiation of dedifferentiated chondrocytes. Chondrocytes, harvested from a non-load-bearing area, were grown directly in an agarose hydrogel or dedifferentiated by monolayer passages before being co-cultured. The results show that this co-culture promoted the synthesis of PGs and collagens by the dedifferentiated chondrocytes [233]. However, immunohistochemical analysis revealed an increase in collagen type I concomitant with collagen type II [234].

Co-culture of chondrocytes and osteoblasts has also been explored. Jiang et al. tested whether the interaction of chondrocytes with osteoblasts is an essential step to ensure successful regeneration of the interface of an osteochondral defect [235]. Chondrocytes co-cultured with osteoblasts synthesize type II collagen but fewer GAG chains. Osteoblasts maintain their alkaline phosphatase activity, but show less bone mineralization compared to monocultures [235]. This type of interaction performed in vitro attempts to mimic the situation found in a healthy joint in vivo. Co-culturing of chondrocytes and osteoblasts affects both cell types, which regulate their own phenotype, and factors secreted by osteoblasts seem to induce chondrocyte proliferation [236].

Co-culture of chondrocytes and ESCs has been developed with human cells. The direct co-culture of these two cells types leads to the production of a cartilage matrix [237]. However, there was no analysis of the expression of specific and non-specific markers of cartilage, only a description of a fibroblast-like morphology for the chondrocytes used. The same year, another study demonstrated a fibro-cartilaginous

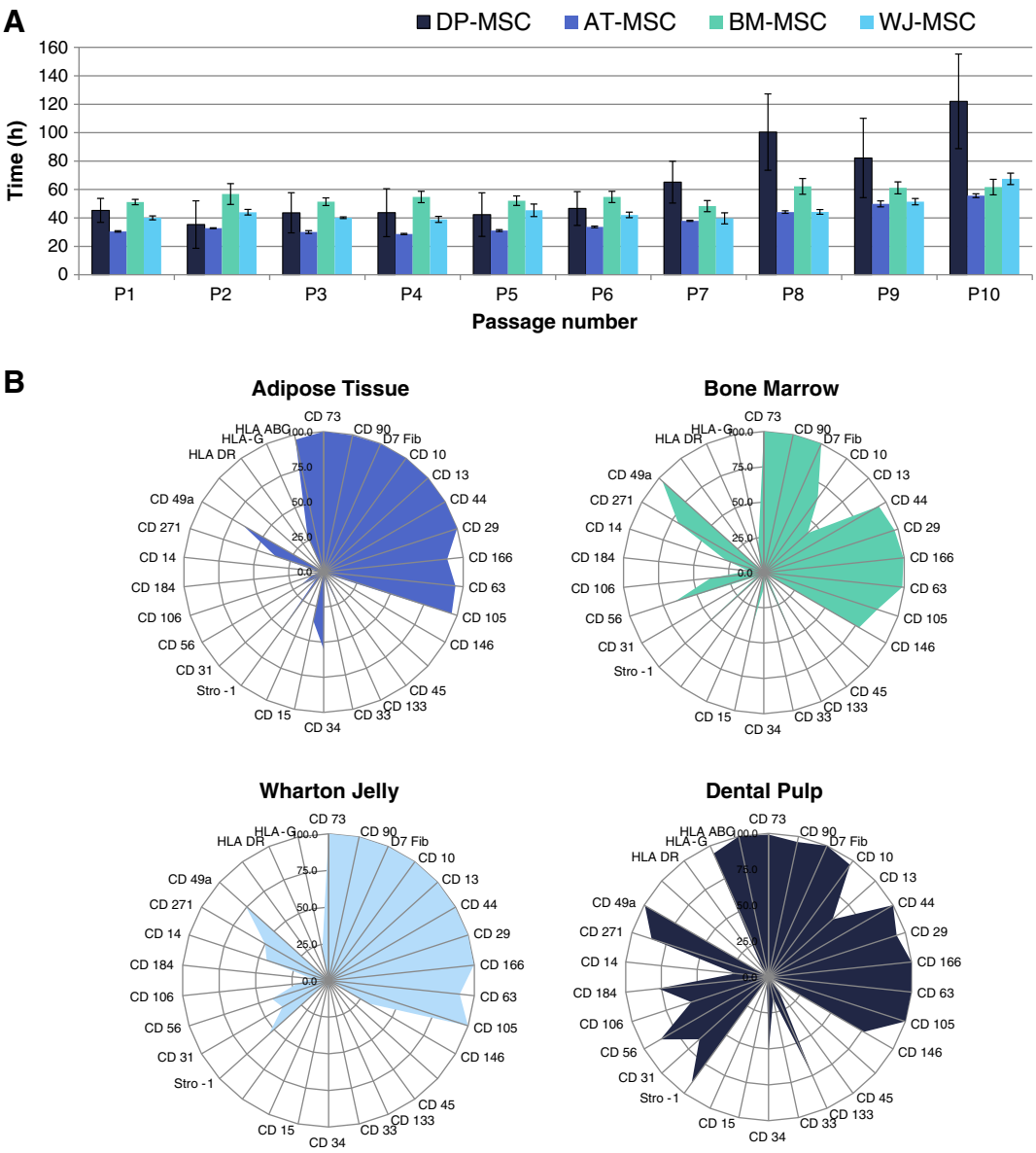

Fig. 6. Characterization of various MSCs. MSCs were isolated by adherence for cells from either adipose tissue (AT) or bone marrow (BM) or by explant culture for cells from Wharton's Jelly (WJ) or dental pulp (DP). The initial isolation phase was called P0 (passage 0). At the end of P0, the cells were detached using the xenofree dissociating reagent TrypLE®, plated at 5000 cells/cm^2 in T12.5 flasks for cell proliferation studies and in larger flasks for flow cytometry analysis at the end of passage 1 (P1). (A) Doubling times. Cell doubling times (DTs) and numbers (CDs) were calculated from Cellometer counts and cell culture time (CT) for 10 passages according to the following formulae: (1) CD = ln(nf / ni) / ln2 and (2) DT = CT / CD (nf: final number of cells at 80% confluence, ni: initial number of cells). (B) Comparative immunophenotypage. At the end of P1, an average of 5×10^6 cells was analyzed with a panel of 27 surface markers (CD: cluster of differentiation) by 8-color flow cytometry using a FACSCanto II cytometer equipped with three lasers (405, 488, and 633 nm). 300,000 events were recorded for each parameter to detect specific subpopulations. Unstained cells were used as negative controls and 7AAD stained cells as a viability control.

phenotype (expression of type I, II and VI collagens) during the differentiation of ESC cells in chondrocytes [238].

Co-culture of chondrocytes and MSCs is currently being investigated. This type of co-culture may promote MSC differentiation into chondrocytes and overcome the limitations of chondrocyte seed number. Some studies have explored human MSCs co-cultured directly with bovine chondrocytes in the presence of dexamethasone and TGF-β3. This interaction induces the expression of human type II collagen and produced a significant ECM after 1 to 4 weeks of co-culture [239]. Moreover, there is an increase in cell proliferation. A recent study provides new information regarding the direct co-culture of MSCs with chondrocytes. HACs were seeded in a three-dimensional matrix (three-dimensional micromass pellet) together with human MSCs and then treated with TGF-β1 and dexamethasone. Co-culture of MSCs and HACs in the absence of TGF-β1 and dexamethasone shows the expression of S100 proteins, type II collagen and GAG chains by HACs, whereas MSCs express no differentiated marker of chondrocytes. The MSCs increase matrix gene expression only in the presence of TGF-β1 and dexamethasone. However, the co-culture induces hypertrophy via increased expression of type X collagen and also stimulates cartilage mineralization by increasing the activity of alkaline phosphatase [240].

The objectives are to determine not only the optimal combination of chondrogenic factors but also the ratio of chondrocytes to MSCs that generates a hyaline cartilage-like matrix within a scaffold [241]. Another study demonstrated that MSCs co-cultured with chondrocytes stimulate cartilage matrix formation due to the trophic effect of MSCs on chondrocytes, rather than differentiating into chondrocytes, independently of MSC origin (bone marrow, synovial membrane or adipose tissue) [208]. More recently, Jeong et al. demonstrated that thrombospondin-2 secreted by human UCB MSCs promotes

chondrogenic differentiation of progenitor cells [209]. For this experiment, they used a co-culture system of chondrogenic progenitor cells derived from mouse limb buds and human UCB MSCs and validated paracrine action using micromass culture and an in vivo osteochondral defect model.

Finally, co-culture of chondrocytes and iPSCs has also been studied with, for example, iPSCs generated from osteoarthritic chondrocytes [242]. Increased cartilage markers, type II collagen, aggrecan and COMP were observed with osteoarthritic chondrocytes derived iPSCs, transfected with lentivirus carrying RFP and TGF-β1, co-cultured with chondrocytes in an alginate matrix. iPSCs are therefore probably a promising cell source for cartilage regenerative medicine; however, the methods for chondrocyte induction from iPSCs are not yet suitable for clinical applications.

3.2.4. Biomaterials for cartilage engineering

The use of matrix scaffolds is a key component in the success of cartilage engineering. The matrices used in the latter ACT generations are seeded with different sources of cells amplified in vitro (chondrocytes, MSCs; see above) allowing maintenance, recovery or procurement of a differentiated chondrocyte phenotype that can be transplanted in vivo. Matrices also serve as support for recruiting BM MSCs at the site of osteochondral defects to activate the subchondral repair process. In all cases, the nature of the scaffold, i.e. its physical and chemical characteristics, influence the quality of the neo-synthesized tissue [243–245]. Scaffolds should be (1) biocompatible to minimize, insofar as possible, any immunological responses and to integrate with the adjacent tissue, (2) biodegradable (gradual, controlled resorption should be simultaneous with cellular and tissue ingrowth), (3) porous to allow cell migration of loaded cells or native cells as well as diffusion of nutrients, gases and waste, (4) adhesive (for cell attachment as well as chondro-inductivity, and for delivery of bioactive molecules) and (5) structurally and mechanically compatible to support loading and deformation of the tissue without loss of volume [6,246]. Additionally, handling facilities, the manufacturing process and costs should be taken into account in the choice of the ideal scaffold. A considerable amount of biomaterials based on both natural and synthetic polymers have been manufactured and evaluated for cartilage engineering, including sponges, hydrogels, fibers, woven or non-woven meshes and microparticles, but few are commonly used in a clinical approach. Some examples include Cartipatch®, Atellocollagen®, Hyalograft®C, Tissucol®, Bio-Seed®-C [247,248]. Micro- and nano-polymeric particles as potential carriers of growth factors have also been developed to improve cartilage regeneration [249].

3.2.4.1. Natural biomaterials. Natural biomaterials derive from proteins (collagen, silk fibroin, gelatin), polysaccharides (chitosan, hyaluronic acid (HA), alginates, starch-based materials, cellulose, dextrans, agarose) and microbial-origin polyesters (polyhydroxyalkanoates (PHAs), poly-hydroxybutyrates (PHBs)). For an extensive review, see [246]. They show multiple bioactivities because many of them are body constituents or constituents of the extracellular matrix. They provide a natural, adhesive cell surface and carry the information necessary for cellular activity (cell growth and maturation). They are also biocompatible and biodegradable, but premature resorption may cause loss of shape and size of implanted biomaterials or cultured biomaterials. Moreover, natural scaffolds in the form of hydrogels have poor biomechanical properties making it very difficult for them to withstand compression in the articulation [244]. Collagen and hyaluronan-based matrices are the most popular natural scaffolds because they are normal constituents of articular cartilage.

Three-dimensional collagen matrices can be sponges, gels, membranes or foams. Collagen scaffolds are usually made of atelocollagen to remove the antigenic determinants and are chemically cross-linked. Type I collagen is the main isoform used to build three-dimensional matrices. Some matrices are composed of two layers, as is the case for the type I/III collagen membranes or sponges. Numerous studies in vitro and in vivo have shown that the membranes of type I/III collagens ensure chondrocyte anchoring, and maintain their phenotype for the MACI® procedure [250]. Type I/III collagen sponges have been successfully used for redifferentiation [35,251]. The use of type II sponges alone can maintain the differentiated state of primary rat chondrocytes for 3 weeks of culture, but does not induce redifferentiation of these dedifferentiated chondrocytes which become hypertrophic [252].

Matrices based on polysaccharides are currently employed in cartilage engineering [246]. For example, 18 months after allogeneic transplantation of rabbit chondrocytes cultured in agarose, there is a high production of type II collagen and GAGs in histological sections [253]. The same effects have been observed for alginate, which induces redifferentiation of dedifferentiated bovine articular chondrocytes in the presence of 5% oxygen [254]. Other biomaterials based on chitosan allow redifferentiation of chondrocytes [255,256], whereas polysaccharide matrices such as agarose and alginate, lead to the differentiation of MSCs in chondrocytes [244,257]. Human chondrocytes grown on a hyaluronan-based scaffold (HYAFF®11) re-express a differentiated phenotype [258].

Finally, some studies also combine different natural biomaterials to increase the chondrogenic potential of biomaterials. Some examples include the combination of alginate sponges with HA [259], HA with chitosan [260], silk fibroin with chitosan [261], and a ternary complex made of gelatin, chitosan and hyaluronan with heparin covalently immobilized onto the scaffold to bind bFGF [256]. However, these combinations often do not have adequate biomechanical strength and in vitro the newly synthesized ECM on these biomaterials is quickly degraded by the high pressure and friction that are exerted in the joint.

3.2.4.2. Synthetic biomaterials. There is a wide variety of biomaterials based on synthetic polymers, which can be classified in several groups: derivatives of poly(‹-hydroxyacids) are the most widely used synthetic degradable biopolymers: poly(glycolic acid) (PGA), poly(lactic acids) (PLA) and poly(lactic-co-glycocolic acid) PLGA; poly(ε-caprolactones) (PCL); poly(urethanes) (PUR); poly(propylene fumarates) (PPF); polyphosphazenes, poly(1,4-butylene succinates) (PBSu) and poly(ethylene glycols) (PEG). For an extensive review, see [246]. The synthetic polymer matrix currently used in clinical practice for cartilage repair is BioSeed®-C, consisting of a PGA/PLA copolymer and polydioxanone, in which autologous chondrocytes are seeded [262]. These synthetic biomaterials support chondrogenic differentiation [263,264] and are able to withstand the weight-bearing forces in the articulation, but they have certain disadvantages such as hydrophobicity and lack of informational structure for cell attachment. They undergo degradation mainly through hydrolysis and their degradation products are acidic or toxic, probably causing severe inflammation [248]. However, they are pathogen-free. They can be manufactured in a broad range of shapes and have low potential for immunological rejection.

3.2.4.3. Combinations of biomaterials. The current trend in cartilage engineering is to combine natural and synthetic biomaterials to exploit the advantages of each, since the composition, architecture (pore size, porosity and shape), and mechanical properties of the biomaterials affect chondrogenesis, cell behavior and cell fate [249]. Many groups combine a rigid scaffold with a hydrogel to obtain both good cell adhesion and differentiation and optimal mechanical properties of the implant, resulting in a great variety of hybrid scaffolds [247]. Grande et al. showed that bioabsorbable polymers such as PGA enhance PG synthesis, whereas collagen matrices stimulate synthesis of collagen in chondrocytes [243]. Many studies have associated a synthetic matrix with a matrix of type I collagen hydrogel or other hydrogels with cultured chondrocytes, or MSCs [265–267]. For example, bovine chondrocytes seeded in a hybrid biomaterial (natural type I collagen and synthetic PLGA knitted mesh) and transplanted in *nude* mice for 8 weeks result

in a significant synthesis of type II collagen, aggrecan and GAGs [268]. The mesh and the mechanical forces of the PLGA serve as the backbone while type I collagen is necessary for cell penetration and ensures the formation of new tissue. To promote chondrogenic differentiation, other studies try to mimic the natural ECM environment with RGD (Arg-Gly-Asp)-coated peptides on synthetic scaffolds [269].

Some strategies have tried to generate mature differentiated cartilage expressing specific cartilage markers; other strategies involve reproducing cartilage with different layers, such as found in vivo, by using multiphasic scaffolds [270]. Thus, a three-layer scaffold with PEG:CS:MMP-sensitive peptides incorporated in the top layer, PEG:CS in the middle layer, and PEG:HA in the bottom layer, creates three-dimensional niches that allow a single MSC population to differentiate into zone-specific chondrocytes and develop an organization similar to native cartilage [271]. A new generation of scaffolds has been designed to improve continuity between these distinct tissue layers by varying the percentage of polymers, pore size and distribution of bioactive molecules between the different layers [270].

Properties of scaffolds have been optimized to generate a hyaline-like cartilage, but because integration with adjacent cartilage was sometimes disappointing despite fixation of the grafted material with fibrin glue, integration with surrounding tissue must be taken into consideration [272]. Despite progress, current constructs also have inferior physico-chemical properties compared to native cartilage. One reason is that mechanical forces are neglected in cartilage culture. There has thus been an increase in the use of specific bioreactors for transmitting forces such as [fluid] shear, hydrostatic pressure and compression, under controlled conditions of pH, temperature, nutrient supply, oxygen tension and waste removal [273,274].

3.2.5. Why use hypoxia for cartilage engineering?

The effect of hypoxia on cartilage formation and remodeling has been neglected for long time, although chondrocytes are chronically exposed to low oxygen. The human body is exposed to oxygen concentrations equivalent to 160 mm Hg (20% O_2), but biological tissues are never exposed to more than 10–15% O_2 (i.e. in the lung, where pressure is at its highest: 100 mm Hg) [275]. Cartilage probably has the lowest oxygen concentration in the body because it is usually avascular. As a result, chondrocytes live in and are adapted to a hypoxic environment (compared to atmospheric concentration). Chondrocytes have the fascinating capacity to sense the level of oxygen with major biological consequences. Prolyl-hydroxylase (PHD) enzymes are key intracellular players under hypoxia, because their activity depends greatly on oxygen concentrations [276]. These enzymes specifically hydroxylate the hypoxia-inducible proteins (HIF-α), leading to the recognition of the specific hydroxylated prolyl by the protein pVHL (with E3 ubiquitin ligase activity) and then the degradation of HIF-α through the proteasome [277].

Under hypoxia, PHD activity decreases and HIF-α (HIF-1,-2 and-3) accumulates in the cytosol where it heterodimerizes with constitutive HIF-1β and translocates into the nucleus. The heterodimer binds to hypoxia-responsive elements (HRE) of target genes that help cells to switch to the glycolytic metabolism (Gapdh, Glut-1). Other genes contribute to the adaptation of cells to the lack of oxygen by promoting angiogenesis (e.g. VEGF) [278].

Studies on cartilage formation have shown that not only the cartilage but also the mesenchymal blastema are hypoxic. The expression of HIF-1α is required from the initial steps of chondrogenesis until joint formation, because abrogation of the HIF-1α gene in the mouse leads to the malformation of limbs and joints [279]. Hypoxia is also present in the growth plate, where it induces the secretion of VEGF via HIF-1α [280]. The production of VEGF is crucial for the survival of chondrocytes [281]. HIF-1α also drives matrix gene expression for genes such as type II collagen or aggrecan, the two major cartilage proteins [282]. In addition to its role in synthesis, HIF-1α is also a central regulator for collagen maturation [283], because it ensures collagen hydroxylation and induces collagen-prolyl-4-hydroxylase, the enzyme that enables the collagen cross-links in the developing growth plate [283,284].

Major advances have been made in deciphering the alternative role of HIF-2α in cartilage. The protein shares 48% of sequence identity with HIF-1α, including the very important transactivation domain TAD [285]. However, its expression is not redundant with HIF-1α and its role in response to hypoxia varies among tissues [286]. Some studies have specifically invalidated its expression in cartilage using Cre recombinase under the control of the Col2a1 promoter. When challenged by a destabilization of the medial meniscus that induces a phenotype similar to osteoarthritis (DMM), heterozygous mice show less cartilage degradation compared to their homozygous counterparts. Conversely, when HIF-2α is overexpressed after adenoviral expression, mice show more cartilage degradation after DMM [287]. Hypertrophic markers Col10a1, Mmp13 and Vegf have been identified as HIF-2α targets, suggesting that HIF-2α is a catabolic factor of cartilage in the mouse. Moreover, the aggrecanase ADAMTS-4 has also been identified as positively regulated by HIF-2α [288]. Surprisingly, the response of HACs to hypoxia is anabolic, since type II collagen and aggrecan synthesis is enhanced. In vitro, hypoxia has also an anti-catabolic effect because it inhibits cartilage degradation [289]. A microarray analysis has led to the identification of highly chondrocyte-specific genes, given the observation of increased matrix synthesis rather than cartilage degradation [290]. Strikingly, our studies have shown that the signaling pathway involves HIF-2α in the stimulatory cascade of Col2a1 [291]. We have also shown that HIF-1α is responsible for the decrease in Col1a1, thus limiting the fibroblast-like phenotype associated with monolayer culture [111]. Altogether, these data highlight the fact that HACs may behave differently from mouse chondrocytes: hypoxia has a positive effect on the differentiated phenotype in humans, but a negative effect in mouse cartilage. Thus, hypoxia promotes the anabolic response of HACs and decreases the catabolic response. In addition, the combination of a three-dimensional culture with low oxygen levels significantly increases type II collagen, aggrecan and Sox9 expression compared to monolayer culture [35,292]. Culturing the chondrocytes under a hypoxic environment could therefore be useful for improving current cell therapies for cartilage repair. We have optimized culture conditions with promising results for cartilage matrix synthesis [35]. Further studies on the underlying molecular mechanisms of hypoxia are required to fine-tune chondrocyte metabolism in vitro. As permanent stress exerted on these cells, hypoxia acts not only per se, but also in combination with growth factors. We tested the response of chondrocytes to BMP-2 under hypoxia and observed a much more pronounced effect on type II collagen deposition (Fig. 7).

The effect of hypoxia on the differentiation of MSCs in chondrocytes is, however, controversial. Malladi et al. [293] have demonstrated that a hypoxic environment (2% oxygen) inhibits chondrogenesis of MSCs derived from adipose tissue of mice, whereas Markway et al. [294] indicate that human BM MSCs in such oxic experimental conditions are able to differentiate into chondrocytes. Moreover, a recent study has demonstrated that hypoxia plays an important role in the migration of MSCs because the active form of RhoA is inhibited in the presence of low oxygen concentrations, such as the polymerization of actin fibers. Thus, although hypoxia appears to stimulate recruitment and cell differentiation, it also negatively regulates cell migration via HIF-1α [295]. Oxygen plays an important role in initiating the migration of MSCs and certain progenitors at lesion sites. However, NADPH oxidase activity increases under hypoxia, promoting the synthesis of ROS by MSCs and intensifying cell senescence [296].

The challenge in using MSCs as a cell source for articular cartilage tissue engineering is mainly to prevent the MSC-derived chondrocytes from undergoing hypertrophic maturation [297,298]. Some data indicate that hypoxia plays a pivotal role in this process. The potential role of hypoxia (2% oxygen tension) in the chondrogenesis of adipose stem

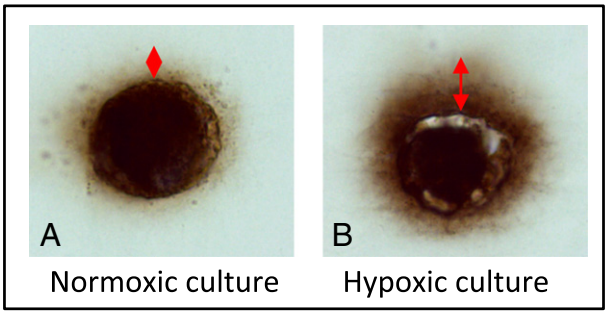

Fig. 7. Effects of oxic conditions on the expression of type II collagen in chondrocytes. Human articular chondrocytes were cultured in an agarose gel under various oxygen concentrations (20% O_2 vs. 1% O_2) with the same chondrogenic medium (ascorbate and BMP-2). Sections were labeled with a type II collagen antibody. As indicated by the red arrows, the most intense labeling around the chondrocyte was obtained under a hypoxic environment. This suggests that hypoxia enhances the chondrogenic response and leads to a greater accumulation of the neo-synthesized matrix.

cells has been examined in pellet culture in the absence or presence of BMP-2 and TGF-β3. The data suggest that hypoxia, at the gene level, favors the maintenance of a more chondrogenic phenotype, whereas the cells exhibit a hypertrophic phenotype under normoxia. The data reported by Ronzière et al. [299] suggest that in addition to defined culture conditions, hypoxia (2% O_2 tension) is another parameter that leads to a stable arrest of chondrogenesis before hypertrophy. Another challenge is to prevent or inhibit the synthesis of type I procollagen at the gene level as well as its deposition in the new extracellular matrix. Strategies other than hypoxia are required for cartilage tissue engineering, such as gene therapy (e.g. siRNA).

3.2.6. Mechanical stimulation

Adult chondrocytes live in an environment constantly subjected to mechanical compression and cartilage development also depends on these constraints. Thus, since the 2000s, other studies have considered culture of chondrocytes and/or MSCs in bioreactors to stimulate perfusion and mechanical forces. Four types of forces can be applied to the cells: hydrostatic pressure, direct compression, high and low fluid shear stress [300]. Some of these forces are capable of increasing the expression of specific genes in cartilage matrix such as type II collagen and GAGs [301,302]. Hydrostatic pressures exert chondroprotective effects and are also involved in the differentiation of MSCs into chondrocytes [303]. New bioreactors can develop bi-axial forces, and these bioreactors can receive neo-tissues or cells cultured in a three-dimensional matrix. Bioreactors foster a suitable culture medium by favoring the supply of oxygen and nutrients as well as adjusting the temperature, pH and mechanical stress. Thus, the use of bioreactors and mechanical conditioning can be useful for cartilage engineering to stimulate in vitro biosynthesis in chondrocytes within three-dimensional scaffolds before implantation. Moreover, this approach can also provide insight into mechanotransduction responses within cartilage-engineered constructs. For instance, agarose (or agarose/alginate) hydrogels constitute potentially clinically useful scaffolds for ACI [304]. These agarose hydrogels are excellent models for the analysis of mechanical response, because they are homogeneous and fully surround the cells embedded within, thereby allowing proper transmission of mechanical forces to the cells. Furthermore, the cell/agarose model has an advantage over cartilage explants in that the interactions of growth factors and mechanical stimuli on chondrogenic activity can be examined independently. We have studied chondrocyte/agarose constructs subjected to dynamic compression using the FX-4000C™ Flexercell® Compression Plus™ System (Flexcell) and have developed protocols to analyze steady-state levels of mRNA by RT-PCR, gene transcription by gene reporter assays and the phosphorylation state of signaling molecules by western blotting [305,306]. Very recently, we used this model cell model system for mouse chondrocytes and employed microarray analysis to investigate the overall changes in chondrocyte gene expression in response to dynamic compression [307]. We identified mechanosensitive genes that can serve as starting points for future investigations on the basic understanding of mechanotransduction in chondrocytes. Interestingly in regard to clinical applications, identification of mechanosensitive targets in cartilage-engineered constructs can help optimize mechanical conditioning for cartilage reconstruction.

3.2.7. Impact of chondrogenic factors in cartilage engineering

Soluble growth factors are widely used to enhance the bioactivity and regenerative capacity of biomaterials. They are added to the differentiation medium or conveyed by biomaterials [249,308]. Members of the TGF-β superfamily appear to be the most employed growth factors in cartilage engineering. They are used either to maintain or restore the chondrocyte phenotype, or to induce a chondrocyte lineage in an intrinsic or extrinsic way. Other growth factors such as bFGF, IGF-I, PDGF are usually used in combination with TGF-β members.

3.2.7.1. Maintaining or restoring the chondrocyte phenotype. It is well established that TGF-βs have the capacity to induce type II collagen and PG synthesis in primary chondrocytes [309,310], but TGF-β1 for example does not prevent the loss of the articular chondrocyte phenotype in rabbits and instead contributes to the dedifferentiation process [311]. Yaeger et al. also showed that TGF-β1, TGF-β2 and IGF-I alone are not able to induce aggrecan and type II collagen production by dedifferentiated chondrocytes [312]. This may explain why only some strategies use TGF-βs to restore the chondrocyte phenotype despite their great capacity to induce chondrogenic differentiation in MSCs. However, a few studies have overcome this drawback. For instance, oligo(poly(ethylene glycol) fumarate hydrogels with embedded chondrocytes and TGF-β1-loaded gelatin microparticles cultured for up to 28 days in vitro induce cellular proliferation and maintenance of the chondrocyte phenotype [313]. Synthetic hydrogels with heparin-binding TGF-β3 are also efficient in inducing differentiation of chondrocytes in vivo with a high specific ECM production [314].

In contrast, BMP-2, -4 and -7 are more widely employed in cartilage repair [81,315]. Among BMPs, here, we focus on BMP-2 effects in regard to its capacity to maintain or restore chondrocyte phenotype. BMP-2 not only prevents dedifferentiation of chondrocytes cultured in a monolayer [316], but it also restores their chondrocyte phenotype [89,317]. Moreover, BMP-2 promotes the synthesis of type IIB collagen (mature form) and the expression of the α10 integrin subunit, which is also a specific marker of cartilage, whereas TGF-β1 stimulates the expression of the non-chondrogenic form of type II procollagen, the type IIA isoform, and of a marker of mesenchymal tissues, i.e. the α11 integrin subunit [71]. However, some suggest that the effectiveness of BMP-2 is limited in time, with upregulation of MMPs, type I collagen and osteopontin [318]. BMP-2 use is limited by its capacity to induce chondrocyte hypertrophy. Thus, used at high concentrations, BMP-2 can induce markers of bone, whereas at low concentrations it induces markers of hyaline cartilage [91,93]. However, BMP-2 is largely employed in cartilage engineering with chondrocytes of various origins. BMP-2 has been employed with human auricular chondrocytes embedded in atelopeptide collagen, alginate, or PuraMatrix™ hydrogels [319] and with mouse chondrocytes isolated from the ventral parts of the rib cages seeded in agarose scaffolds [320]. BMP-2 and -7 significantly increase GAG content in septal chondrocytes suspended in alginate [321]. BMP-2 enhances the expression of type II collagen and aggrecan in HACs embedded in alginate beads or collagen sponges [35,322].

Other growth factors used alone such as IGF-I, PDGF and bFGF have been investigated less. Liu et al. showed that among 12 putative chondrogenic factors (including IGF-I, PTH, T3, FGF2), BMP-2, IGF-I and insulin were the most effective for inducing GAG accumulation, but the combination of BMP-2 and insulin was the most effective in

regard to redifferentiation after repeated passages [323]. In our studies, we have shown that BMP-2 is better than IGF-I in redifferentiation experiments on type I collagen sponges, because unlike IGF-I, it favors the type IIB form of collagen and does not stimulate type I collagen [35]. Moreover, the combination of BMP-2 and IGF-I improve the chondrocyte phenotype only at the mRNA level under hypoxia. Similarly, Jonitz et al. have demonstrated additive effects of TGF-β1 and IGF-I in the expression of cartilage-specific ECM components within pellet cultures only under hypoxia [324]. Several studies have used the properties of FGF to maintain the chondrogenic potential of expanded chondrocytes before their cultivation in three dimensions [325,326]. Our research group has also shown that chondrocyte expansion with FGF-2 and insulin increase chondrocyte responsiveness to a cocktail of BMP-2, insulin and triiodothyronine in cells cultured in collagen sponges [327]. Other studies have demonstrated that the BMP-2 effect can be enhanced by supplementing insulin and triiodothyronine in atellocollagen scaffolds [328]. However, a synergistic effect between FGF-2 and IGF-I has not been demonstrated when they are employed simultaneously in adult canine articular chondrocyte-seeded in type II collagen/GAG scaffolds in vitro [329]. Sequential application of growth factors has also been tested for promoting the chondrogenic capacity of chondrocytes with supplementation of, for example, TGF-β1/FGF-2 then IGF-I or alternating TGF-β1 or-β3 with IGF-I [330,331].

3.2.7.2. Induction of MSC chondrocyte lineages in vitro. For cartilage tissue engineering with isolated MSCs, MSCs must differentiate into chondrocytes in vitro for subsequent implantation in damaged cartilage. In most studies, chondrogenesis of MSCs is carried out in a standard medium (often high glucose DMEM) with supplements of dexamethasone, ascorbic acid, growth factors and ITS (insulin, transferrin and selenium) [332]. A large variety of experimental models are used to induce chondrogenesis in a three-dimensional matrix or pellets, with close contact between cells, where TGF-β1 and -β3 have the most important roles [298,333]. We discuss here only a few recent representative studies. TGF-β3 is able to induce the chondrogenesis of MSCs in alginate scaffolds, or immobilized in a PLGA-gelatin/CS/HA hybrid scaffold [334,335]. TGF-β1 alone is a potent inducer of MSC chondrogenesis in various scaffolds [244,266]. BMPs have shown relative effectiveness in inducing chondrogenesis of MSCs in pellets [336], or synovium-derived MSCs in alginate [337,338]. Together, BMPs and IGF-I, are used to extend the chondrogenic responses of TGF-βs, especially because IGF-I alone cannot induce MSC chondrogenesis [298]. Shen et al. have shown that BMP-2 enhances the TGF-β3 chondrogenic effects in BM MSCs cultured in alginate [257]. BMP-2 and TGF-β1 have also been combined to promote chondrogenic differentiation in type I collagen hydrogels [339]. In the presence of dexamethasone, IGF-I and TGF-β3 induce chondrogenic differentiation of BM MSCs embedded in type I collagen in a PLGA mesh [265].

To avoid hypertrophic maturation of MSCs, Gardner et al. suggest using PTHrP to stabilize the chondrocyte phenotype [298]. Thus, bFGF and PTHrP have been employed in MSCs induced by TGF-β3 in a pellet experiment, and, to avoid the inhibition of type II collagen synthesis, late incubation can be employed [340]. PDGF which has the capacity to prevent the progression of MSCs along the endochondral maturation pathway is another potential candidate [341].

3.2.7.3. Recruitment of BM MSCs to osteochondral defects. Another alternative cartilage engineering approach is to employ acellular repair techniques. We consider here only subchondral bone stimulation used to recruit BM MSCs to osteochondral defects, a strategy based on cell homing and on local delivery of growth factors by specialized biomaterials [308,342]. In this context, BMP-2 impregnated in collagen sponges or carried by PLA/PEG in a porous hydroxyapatite layer (IP-CHA) have been tested for their ability to repair cartilage defects and have given encouraging results [343,344]. Lee et al. regenerated the articular surface in rabbits by using a TGF-β3-adsorbed collagen hydrogel integrated in a scaffold of poly-ε-caprolactone/hydroxyapatite [345]. However, some suggest that TGF-β1 and -β3 should not be used in situ due to their numerous deleterious effects in joints (synovial proliferation and fibrosis, inflammation and osteophyte formation) [346]. The use of IGF-I and PDGF in acellular cartilage repair techniques has already been reviewed [347].

3.2.8. Gene and cellular therapy of cartilage

3.2.8.1. The use of transgenes in cartilage therapy. Gene therapy for cartilage repair involves introducing a vector, also called a therapeutic gene, in synovial cells and/or chondrocytes around the lesion or to perform ACI with chondrocytes genetically modified in vitro. The vectors introduced into cells may be genes encoding anti-inflammatory mediators or growth factors. The first researcher to consider gene therapy for cartilage was C. Evans, who in 1996 introduced a vector encoding an anti-arthritic cytokine in a joint affected by rheumatoid arthritis [348]. Since then, many genes have been introduced into chondrocytes to maintain their phenotype such as the genes from the TGF-β family, BMPs, IGF-I, FGF, Sox9, L-Sox5, and Sox6, but also gene constructs encoding type II collagen, aggrecan or COMPs [349]. The in vivo and ex vivo delivery of the therapeutic gene can be performed using adenoviruses, lentiviruses or non-viral vectors. The incorporation of transgenes, such as TGF-β1, BMP-2 or IGF-I, induce the expression of PGs and type II collagen in chondrocytes and decrease their dedifferentiation [350]. In addition, rendering chondrocytes resistant to external chondrocyte catabolic signals, such as those induced by the inflammatory cytokine IL-1 in chondrocytes, can be obtained by introducing a gene encoding a IL-1 receptor antagonist. This gene manipulation aims to make chondrocytes resistant to IL-1 in order to prevent them from inducing the secretion of proteolytic enzymes when they are located within the lesion [351].

Gene therapy of cartilage also seeks to incorporate vectors in MSCs to stimulate their chondrogenesis. For instance, MSCs that have integrated the gene encoding BMP-2 express high levels of type II collagen, and type I synthesis is decreased. The integration of this vector in this type of stem cell also stimulates the expression of proteins involved in cell-cycle inhibition such as p21 [352]. Other studies have used the TGF-β1 transgene to induce the differentiation of MSCs in chondrocytes [353].

Currently, the expression vector is driven by a viral promoter to ensure strong expression of the therapeutic gene. Although this system facilitates the study and analysis of biological effects, it is difficult to apply in clinical cartilage therapy. The use of such vectors requires optimization to control the level of expression of the target recombinant proteins, so as to avoid deleterious effects that can be obtained when certain genes are overexpressed.

An alternative to avoid integration of transgenes into the genome is to employ an RNA interference (RNAi) strategy for cartilage therapy, with miRNA or siRNA. miRNAs are endogenous and present major differences compared to siRNAs because they can inhibit several hundred mRNAs.

3.2.8.2. Emergence of the use of miRNAs for controlling chondrocyte genes. miRNAs are relatively new intracellular regulators of gene expression. They are encoded in the intronic sequences of genes and are present in long non-coding RNA [354]. They are first generated as primary RNA (named pri-miRNA), and matured by the enzyme Drosha in the nucleus and generate a 70 nucleotide precursor form (pre-miRNA). Once in the cytoplasm, a second enzyme complex, Dicer, cleaves the precursor into a small RNA duplex of around 20 nucleotides [355]. The mature miRNA then targets specific mRNA for degradation and/or inhibition of translation via a silencing complex (miRISC), where Argonaute (particularly Ago2) proteins are of particular importance [356]. Generally, the miRNA will bind specific 3′UTR sequences of mRNAs but can also target the 5′UTR [357].

In cartilage, miRNAs are expressed by chondrocytes of the growth plate with a specific pattern depending on their differentiation status

[358]. However, the exact miRNA-regulated process that forms chondrocytes in permanent articular cartilage is not known. To date, the most important demonstration of the crucial role of miRNA in cartilage physiology comes from the study of phenotype in Dicer knock-out mice. Since Dicer is crucial in the final step of miRNA generation, its invalidation prevents the normal production of miRNA. Abrogation of all miRNA specifically in the cartilage has been obtained using the elegant model whereby the Dicer gene is floxed and the Cre recombinase expression is under control of the Col2a1-promoter. The resulting mice show severe skeletal growth defects due to alterations in chondrocyte proliferation and hypertrophy [359]. Since then, some studies have focused on particular miRNA species in the context of cartilage tissue or in vitro studies of articular chondrocytes.

miR-140 is probably the most striking regulator of cartilage matrix. Its expression is very specific to cartilage tissues and is one of the most highly expressed miRNAs in developing human cartilage [358]. Miyaki et al. performed an extensive study of its role in cartilage using miR-140 knock-out mice and transgenic mice overexpressing miR-140 [360]. The loss of miR-140 contributes to cartilage degradation characteristic of OA, whereas its overexpression protects against OA progression. miR-140 is also under the regulation of Sox9 and directly targets ADAMTS-5 and MMP-13.

miR-675 is a novel example of cartilage-specific gene regulation. This mi-RNA was identified as being generated from a long non-coding RNA H19 whose expression is highly associated with the chondrocyte phenotype in culture. Its expression is also very high in mouse cartilage. In primary culture of chondrocytes, we have shown it participates in stimulating *Col2a1* gene expression independently of the presence or absence of Sox9, and that it targets a *Col2a1* transcriptional repressor [361]. Moreover, H19 and its product, miR-675, correlate strongly with the metabolic status of chondrocytes in cartilage and in culture, suggesting that H19 is an attractive marker for cell anabolism and potential target to stimulate cartilage repair [362]. miR-27b is another well-characterized miRNA: it regulates MMP-13 expression in human chondrocytes [363].

Other key cartilage miRNA have been identified by studying OA versus normal cartilage [364]. miR-146 is strongly expressed in the superficial layers of cartilage and low-grade OA cartilage [365]. Its expression is inversely correlated with that of MMP13, suggesting a role in OA progression. miR-140 expression has also been found to be modified, but either increases or decreases depending on the stage of disease [366,367].

In addition to miRNA profiling arrays which help identify signatures of miRNAs associated with chondrogenic culture conditions (e.g. BMP-2) or with the chondrogenic cell line ATDC5 undergoing chondrogenesis in vitro [368,369], a novel method consists in pulling down one protein of the RISC complex, the Ago protein followed by RNA sequencing. Martinez-Sanchez et al. did so in primary chondrocytes and identified miR-145 [370]. They further showed that miR-145 is a direct regulator of *Sox9* in normal articular chondrocytes, because it can bind specific non-conserved sites in its 3′UTR. Conversely, its overexpression in these cells reduces the expression of Sox9 as well as the levels of Col2a1 and aggrecan in combination with an increase of the hypertrophic markers Runx2 and Mmp13.

With more and more examples of the molecular basis of chondrogenesis involving miRNA, the use of miRNA-based tools to control chondrocytes in vitro or in vivo has become a viable strategy for cartilage repair. Extracellular RNAs are naturally secreted in particles such as exosomes. Thus, chondrocytes are physiologically influenced not only by growth factors and cytokines, but also by miRNA as paracrine molecules: variations in expression of specific miRNAs in biological fluids from patients with cartilage pathologies have been reported. This variation highlights the fact that miRNAs are produced and secreted in an attempt to counteract a pathological state or as a readout of the pathological fate itself, suggesting that they can be considered as pathological markers [371]. Overexpression of a given miRNA (miRNA mimics) or inhibition by a highly specific antagonist miRNA (LNA-antimiR) can be used during the in vitro culture of chondrocytes. This would ensure the proper expression of the chondrogenic gene, favoring an anabolic program and cartilage-specific matrix protein synthesis.

Thus, in addition to transcription factors or matrix genes, analysis of the expression of miRNAs appears to be a good approach to better understand the state of cell differentiation. These new phenotype markers can be used to determine more precisely the state of chondrocyte differentiation obtained from MSCs or from articular cartilage.

3.2.8.3. siRNA strategies in cartilage therapy. siRNAs are small RNAs that have the capacity to bind to a target messenger to degrade RNA and inhibit its translation to a transient manner. They can be expressed as short hairpin RNAs (shRNA) to mediate a long-term extinction of gene expression. However, the infection of cells with viral vectors encoding shRNA allows shRNA to integrate the genome. siRNA are used to reduce the damage of joint inflammation, but their use in structural OA damage remains restricted to in vitro studies. Three avenues of research will be developed corresponding to major challenges in cartilage therapy: to reduce ECM degradation, to reduce the appearance of type I collagen and to reduce cartilage hypertrophy.

Regarding the OA degradation process, expression plasmid vectors of shRNA targeting aggrecanase-1 and aggrecanase-2 transfected into cultured rat costochondral chondrocytes are effective in increasing the aggrecan and collagen II content of chondrocytes treated with IL-1® [372]. Thus, one recent study has shown that the intra-articular injection of lentivirus-mediated ADAMTS-5 siRNA prevents the degradation of articular cartilage in Sprague–Dawley rats [373]. The biological effects of MMP3 gene extinction has been studied in IL-1-treated chondrosarcoma as a model of OA chondrocytes and demonstrated an increase in cell viability, mitosis and in the HA and GAG content, together with a decrease in apoptosis [374].

Few groups have focused on the inhibition of type I collagen. To our knowledge, the first studies to report the use of siRNA to downregulate type I collagen in vitro were carried out with Cos-7 cells and human mesenchymal progenitor stem cells, or with human skin fibroblasts [375,376]. Others use adenoviral vectors encoding both TGF-β3 and type I collagen shRNA to control chondrogenesis of porcine chondrocytes and synovial MSCs [377]. The transition from differentiated chondrocyte to hypertrophic chondrocytes involves the transcription factor cbfa1/Runx2, which is also involved in type X collagen production and mineralization of cartilage tissue. To our knowledge, only one study reports the downregulation of cbfa in murine C3H10T1/2 MSCs, delaying osteoblastic differentiation, both in vitro and in vivo, while chondrogenesis seemed to be sustained [378].

Using siRNA as miRNA is hampered by the fact that they have a high molecular size and an anionic charge which opposes their entry into cells. Thus, they are either transfected by electroporation, or introduced by a transfecting agent. Other attempts have been made to improve their delivery through PTB-DRBD fusion protein with minimal side-effects [379].

3.2.9. Novel concepts in cartilage engineering: towards a fifth generation of ACI

Since its introduction in 1994 by Brittberg et al., ACT is constantly being improved [5]. Redifferentiation of chondrocytes has evolved substantially, and there is enhanced knowledge of the parameters that play an essential role in inducing the differentiated state of chondrocytes in culture such as hypoxia, culture within a biomaterial or use of chondrogenic factors. One of the remaining challenges in cartilage engineering that need to be taken into account is to prevent or inhibit the synthesis of type I procollagen at the gene level, as well as its deposition into the new ECM. However, the strategies using type I collagen RNAi are not widespread in cartilage engineering. Wang and collaborators has specialized in the use of dual adenoviral or lentiviral vectors encoding both TGF-β3 and type I collagen shRNA

to redifferentiate porcine chondrocytes or to differentiate synovium-derived MSCs in chondrocytes in an alginate hydrogel [377,380–382]. This strategy, in which cells are infected with viral vectors, leads to genetic modifications and is not suitable for clinical applications. However, the transitory action of siRNA allows the production of safe competent therapeutic cells. In this perspective, our group successfully transfected dedifferentiated chondrocytes with siRNA leading to prolonged COL1A1 knock-down in mouse chondrocytes embedded in an agarose hydrogel and in human chondrocytes seeded in a collagen sponge and cultured with BMP-2 [35,320]. In addition, we simultaneously explored the benefit of low oxygen tension, the contribution of BMP-2, the use of a collagen sponge and COL1A1 siRNA on the chondrocyte phenotype [35]. We also assessed the innovative combination of BMP-2, hypoxia, collagen sponge and siRNAs targeting COL1A1 and HTRA1 to enhance stabilization of human chondrocytes both in vitro and in vivo [383]. This approach has enabled us to significantly reduce the expression of the targeted mRNAs. Thus, the ratio of the major specific markers of cartilage are increased at the expense of type I collagen. The differentiation index is higher compared to dedifferentiated chondrocytes of OA cartilage, and a protease involved in matrix degradation is inhibited. We have also applied this protocol to define a culture system capable of inducing significant chondrogenesis in human BM MSCs. Thus, when MSCs are transfected with siRNA against COL1A1 and the protease HTRA1, seeded in type I collagen sponges and cultured in hypoxia with BMP-2 and TGF-β1, the mRNA and protein levels of cartilage-specific markers increase, and the expression of non-specific markers such as type I and III collagens decrease. In addition, extensive analysis of 16 genes involved in the regulation of cartilage homeostasis, together with specific and non-specific genes of cartilage ECM, also leads to the conclusion that the cell type obtained looks very much like chondrocytes of healthy cartilage [384] (Fig. 8). We made the same observations with UCB MSCs.

This work led us to describe the inhibition of the expression of type I collagen by siRNAs. Transient use of siRNA to induce and/or restore the chondrocyte phenotype may foster the emergence of a new generation of ACT. Many genes are induced during osteoarthritis and the use of siRNAs is an adequate tool for inhibiting the expression of a great number of genes. Thus, strategies other than those involving hypoxia to reduce hypertrophy are needed for cartilage tissue engineering, such as gene therapy (e.g. siRNA targeting cbfa1). There are likely other alternatives to induce chondrogenesis, such as inhibiting the trans-inhibitors of *COL2A1* (c-Krox, the p65 subunit of NF-κB and Sp3). This strategy could also be employed concomitantly with the BMP-2-induced increase in the DNA binding activity of *COL2A1* trans-activators (the Sox transcription factors and Sp1) and the BMP-2-induced decrease in the DNA binding activity of *COL2A1* trans-inhibitors (c-Krox, p65) with HACs seeded in collagen sponges under hypoxia [35].

To ensure non-negative, long-term effects of RNAi strategies in cartilage engineering, additional long-term studies are needed in an animal model closer to humans. These tests should also be performed in cropping systems standardized with respect to good manufacturing practices for human clinical use. As a result, we plan to perform pre-clinical trials in the horse to better understand the reaction of cartilage tissue after a few weeks of intra-articular implantation. This approach will allow us to explore the criteria necessary to begin to consider the first clinical trials and the development of cell therapy for cartilage repair in humans.

3.2.10. Clinical outcomes in clinical ACI

Effectiveness of ACI in cartilage repair of the knee have been studied in many clinical trials and analyzed in many reviews [183,385–387]. The literature reveals a large number of different knee scoring systems and methodology for clinical evaluation, making it difficult to directly compare different regenerative therapy approaches. Thus, it seems essential to standardize evaluation methods, what would imply, among others,

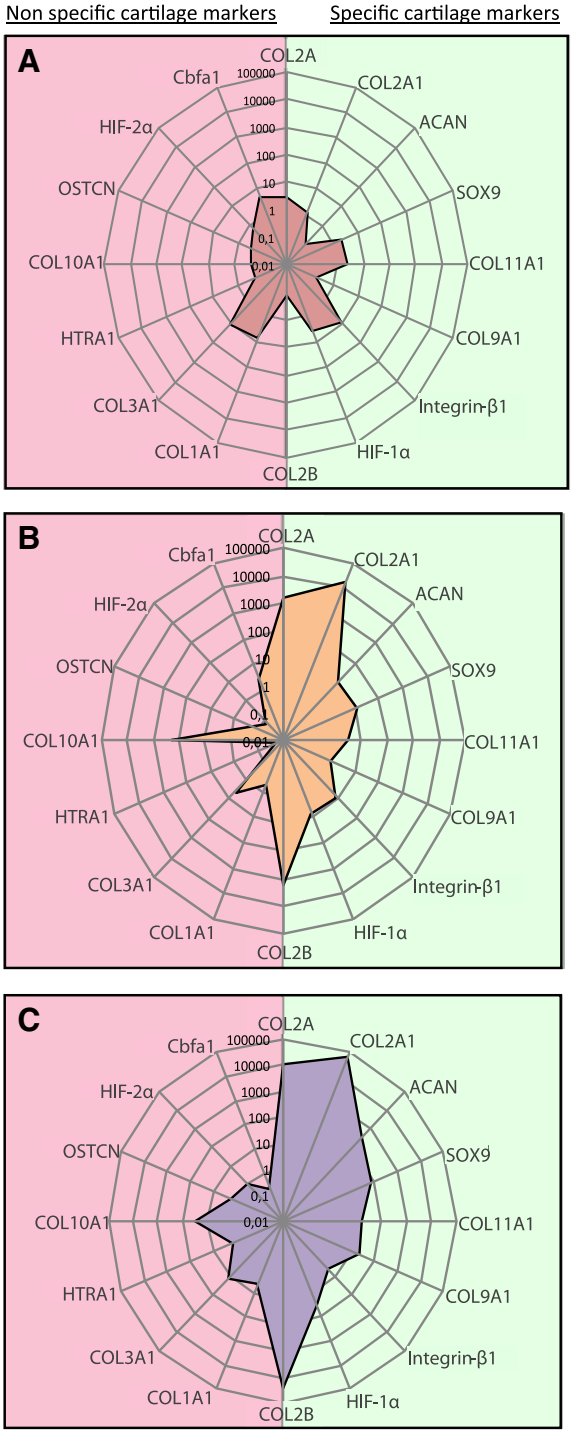

Fig. 8. Comparative analysis of the phenotype of native chondrocytes and differentiated hBM MSCs. mRNA was extracted either from sponge-embedded human bone marrow (BM) MSCs (A-B), or from native healthy cartilage after enzymatic digestion (C). (A) BM MSCs were amplified until P5 and seeded in type I/III collagen sponges, transfected with control siRNA (100 nM) at days 0 and 7. They were cultured for 14 days in hypoxia. (B) hBM MSCs were amplified until P5 and seeded in type I/III collagen sponges, transfected with *COL1A1* and *HtrA1* siRNAs (100 nM each) at days 0 and 7. They were cultured for 14 days in hypoxia with BMP-2 (50 ng/ml) and TGF-β1 (10 ng/ml). The mRNA levels encoding type II collagen (*COL2A1*), type IIA collagen (*COL2A*), type IIB collagen (*COL2B*), type I collagen (*COL1A1*), type X collagen (*COL10A1*), type XI collagen (*COL11A1*), type IX collagen (*COL9A1*), type III collagen (*COL3A1*), integrin-β1, hypoxia inducible factor 1 alpha (*HIF-1α*), hypoxia inducible factor 2 alpha (*HIF-2α*), osteocalcin (*OSTCN*), core binding factor 1 (*Cbfa1*), high temperature requirement A (*HtrA1*), aggrecan (*ACAN*), and SRY-type HMG box 9 (*SOX9*) were normalized to the levels of ribosomal protein L13a (*RPL13a*) mRNA and plotted in a radar chart.

some strict inclusion criteria at the risk of limiting the translation of the results from clinical trials to clinical practice.

However, two independent systematic reviews with distinctly methods arrived at coherent results suggesting favorable outcome after ACI [388,389]. Since the original clinical work by Brittberg and Peterson with periosteal cover describing good to excellent clinical outcomes at two years follow-up [5], clinical trials were also performed with the second and third generations of ACI [390–392]. The functional outcomes were estimated over several years by using various methods (IKDC, ICRS, Lylsholm, KOOS…) whereas histological outcomes remain incomplete.

What stands out from all this is that there is a considerable need for high quality studies with systematic, detailed reporting in the field of cartilage repair such as histologic evaluation of ACI effects. Preclinical animal studies represent valuable approaches for obtaining such data under the prerequisite of using appropriate animal models. In this field, the horse represents an excellent model for studying osteoarthritis pathophysiology as well as developing and testing new therapeutic strategies and biological markers for diagnosis and prognosis purposes. Several reasons may explain this relevance of the equine model. Horses are frequently affected by spontaneous osteoarthritis in both young equine athletes or later in older horses. Moreover experimental models of osteoarthritis and cartilage injuries have been developed and documented in details [393]. The size of equine joints permits also to test surgical procedures, particularly those used in ACI treatments, on an articular cartilage close to the human one in terms of thickness and chondrocytes volume density [394]. After treatments, horses can be evaluated accurately in terms of pain [395] and other clinical aspects. They can finally be submitted to exercise for evaluating the long-term outcome and functional recovery. Several studies have tested ACI in equine experimentally induced cartilage defects with positive results [396,397]. Frisbie et al. have also demonstrated the positive effect on arthroscopic, histological and immunohistochemical findings of ACI in the long-term healing (12 months) of clinically relevant defect (15-mm diameter) within equine femoral trochlea, after submitting horses to exercise [398].

4. Conclusion

Cartilage engineering has rapidly evolved and is entering a new, pivotal phase. The undeniable progress in biology and scaffolds holds promise for new treatments for cartilage defects. Scaffolds help determine cell phenotype and potential drug development, are essential for the development of cell therapy products. Various processes have been developed worldwide to produce scaffolds. However, the ideal scaffold remains to be determined, as well as the optimal cell source and stimulation protocol. Today, autologous chondrocytes are the current clinical option of choice but stem cells, especially from bone marrow, are a promising source of cells. MSC-based therapy holds great promise, because they have the potential to differentiate into functional cells and exert beneficial effects through their trophic and immunosuppressive factors. Considering the complexity of MSC properties, using MSCs in regenerative medicine requires further multidisciplinary knowledge at the fundamental and clinical level.

Cartilage engineering needs to transition away from classical in vitro processes towards elaborate bioreactors that can also handle hypoxia and/or RNAi and/or mechanical loadings. Above and beyond all the progress, the possibility of implementing devices using good manufacturing practices will undeniably shape the choices of strategies to follow. Thus, cost-effective ways of producing a safe biological substitute still need to be developed and tested in clinical trials. The scientific community now has auspicious tools that have not yet been fully tested for safe and effective long-term regenerative therapy. However, the knowledge and experience of scientists and clinicians will certainly overcome these technological barriers that govern the validation of new biological substitutes by public health authorities.

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Appendix A. Supplementary data

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