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BACHELOR THESIS

Osteoarthritis: Utilization of Chondrons in Articular Cartilage Repair

An Overview of the Developments and Applications During the Past Decade

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Abstract

Chondrocytes are cells that are responsible for the production and maintenance of articular cartilage in the knee. Chondron is the term used to denominate the chondrocyte and its surrounding pericellular matrix (PCM), which has a defined molecular composition and unique physical properties that support the chondrocyte. Predisposing risk factors such as age, trauma and obesity may be the underlying cause of articular cartilage damage, which is usually associated with osteoarthritis (OA). Despite the burden of over 500 million OA patients worldwide, treatment options are unsatisfactory. Techniques such as autologous chondrocyte implantation (ACI) use autologous chondrocyte isolates for *in vitro* monolayer expansion and re-implantation to repair articular cartilage defects. In these strategies, the PCM is often enzymatically removed, at the expense of hyaline cartilage quality and integrity. However, unequivocal evidence informs us to preserve the chondron to improve cell-induced cartilage formation. Some studies have therefore focused on the co-implantation of chondrons with cells such as mesenchymal stem cells (MSCs), adipose-derived stem cells (ADSCs) and chondrocytes to treat articular cartilage lesions in *in vivo* models. Instant MSC Product accompanying Autologous Chondron Transplantation (IMPACT) is a clinical trial in which the indispensable role of a chondron is warranted, thereby providing a refined alternative for ACI. This literature study reviews the attempts for and advances in the utilization of chondrons for articular cartilage repair in the past decade. It provides a comprehensive overview of both the scientific developments and clinical applications with a concluding value judgment and future prospective.

Samenvatting

Chondrocyten zijn cellen die verantwoordelijk zijn voor de productie en het onderhoud van articulair kraakbeen in de knie. Chondron is de term die gebruikt wordt om de chondrocyt en zijn omliggende pericellulaire matrix (PCM) aan te duiden, welke een gedefinieerde samenstelling en unieke fysieke eigenschappen heeft, die de chondrocyt ondersteunen. Ontvankelijk makende risicofactoren zoals leeftijd, letsel en overgewicht zijn mogelijk de onderliggende oorzaak van articulaire kraakbeenschade, wat meestal geassocieerd wordt met osteoartritis (OA). Ondanks de last van meer dan 500 miljoen OA patiënten wereldwijd, zijn behandelingsopties teleurstellend. Technieken zoals autologe chondrocytenimplantatie (ACI) maken gebruik van chondrocyt isolaten voor *in vitro* monolaag expansie en re-implantatie om articulair kraakbeenletsel te repareren. Bij deze strategieën wordt de PCM meestal enzymatisch verwijderd, ten koste van de kwaliteit en integriteit van het hyalien kraakbeen. Echter, overduidelijk bewijs informeert ons dat het intact laten van de chondron de cel-geïnduceerde kraakbeenformatie bevordert. Sommige studies hebben daarom de focus gelegd op de co-implantatie van chondronen met cellen zoals mesenchymale stamcellen (MSC's), vetweefsel afgeleide stamcellen (ADSC's) en chondrocyten om articulaire kraakbeen beschadigingen te behandelen in *in vivo* modellen. Instant MSC Product accompanying Autologous Chondron Transplantation (IMPACT) is een klinische proef waarin de onmisbare rol van een chondron wordt gewaarborgd om daarmee te voorzien in een verfijnd alternatief voor ACI. Deze literatuurstudie bespreekt de pogingen tot en de vooruitgangen in het gebruik van chondronen voor articulaire kraakbeenreparatie in het afgelopen decennium. Het biedt een verhelderend overzicht van de wetenschappelijke ontwikkelingen en klinische toepassingen met een concluderend waardeoordeel en toekomstperspectief.

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Introduction

Osteoarthritis (OA) is an age-related joint disease that involves the degeneration of cartilage. The most prevalent manifestations of OA include joint pain and disability in the elderly human population [1]. Numerous regenerative medicine strategies focus on the prevention of OA progression. Autologous chondrocyte implantation (ACI) is such a surgical procedure to treat deep focal articular cartilage damage. ACI is a well-established technique that isolates a patient's chondrocytes for *in vitro* monolayer expansion and subsequent re-implantation [2]. Chondrocytes are cells that are responsible for the maintenance of the structural composition of cartilage, which is referred to as the extracellular matrix (ECM). Furthermore, chondrocytes are embedded in a pericellular matrix (PCM), together forming a chondron. The PCM is of paramount importance for the chondrocyte, since it supports the anabolic and catabolic processes of the cell. Despite its pivotal role, the PCM is usually enzymatically removed in tissue engineering.

In 2010, Vonk *et al.* [3] reported that retention of the native PCM enhances the cartilage formation by chondrocytes. Interestingly, Vonk and colleagues elucidated an underlying molecular mechanism in a follow-up study [4]. The authors provide unambiguous evidence that PCM preservation prevents upregulation of collagenase-3 (MMP-13), responsible for collagen degradation, leading to weak and incompetent articular cartilage. Furthermore, it was revealed that discoidin domain receptor 2 (DDR2) modulates this expression of MMP-13 after direct contact with collagen in the ECM. These findings emphasize the irrefutable significance of the PCM for chondrocytic activity in healthy cartilage functioning.

Usually, medical procedures ameliorate over time, as new scientific insights arise or technologies advance. More than a decade has elapsed since Vonk *et al.* published their clarifying work that shed light on the procedure for ACI. What have the related scientific and clinical areas accomplished with these findings? This literature study aims to review the attempts for and advances in the application of chondrons in articular cartilage repair during the past decade. It provides a comprehensive overview of both the scientific developments and clinical applications with a concluding value judgment and future prospective.

Basic science of cartilage, chondrocytes and pericellular matrix

Articular cartilage

Articular, hyaluronic cartilage is a unique form of connective tissue that acts as a cellular cushion to withstand physical and mechanical forces in daily life activities. It lines the diarthrodial joints to provide a lubricated surface to minimize friction during articulation. Dissimilar to most other tissues, articular cartilage is an avascular, alymphatic and aneural connective tissue [5]. However, the synovial membrane, encapsulating the joints, provides a net of fenestrated blood vessels that nourishes the chondrocytes through diffusion [6]. Principally, articular cartilage comprises a dense ECM with a distribution of embedded chondrocytes. The abundant matrix of the ECM is predominantly composed of water, type II collagen, aggrecan and other non-collagenous proteins [7].

Zones

Various zones contribute to the composition of articular cartilage: the superficial (tangential) zone, the middle zone, the deep zone and the calcified zone (Figure 1). Each layer exerts an imperative role that leads to the interconnected dependency within the whole structure. The superficial zone protects and maintains the deeper layers, which is mediated by type II and type IX collagen fibers. Chondrocytes in this layer possess flattened, discoidal shapes [8]. This tangential zone makes up approximately 10% to 20% of the cartilage volume, whilst 40% to 60% is attributed to the middle zone. The middle zone is located immediately adjacent to the superficial zone and its proteoglycans and thicker collagen fibrils facilitate resistance to compressive forces. The deep zone comprises approximately 30% of the total cartilage volume. Of great importance in this zone are the high proteoglycan content and the perpendicular arrangement of collagen fibrils with respect to the articular surface. These factors account for the greatest compressive force resistance in articular cartilage. Finally, the calcified zone mediates the transition between the deep zone and the subchondral bone by anchoring collagen fibrils to the latter [5].

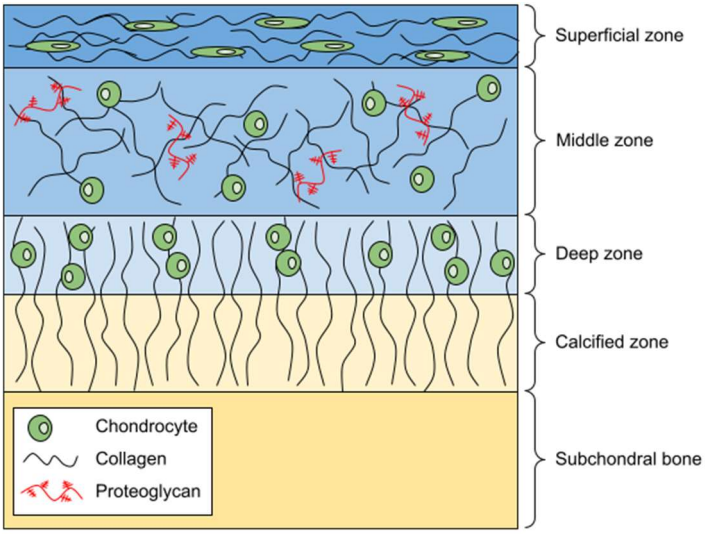

Figure 1. Cartoon representation of the zones and composition of articular cartilage. Articular cartilage can be subdivided into four distinct layers that contain components including chondrocytes, collagen and proteoglycan.

Regions

Even within each zone, a distinct division is present based on composition, proximity to chondrocytes

and collagen fibril diameter and organization. These discrete regions are identified as PCM, territorial and interterritorial region. The PCM constitutes the direct environment of the chondrocyte, immediately adjacent to the cell membrane. This matrix region will be extensively discussed in a following section. The territorial region surrounds the PCM and connects it to the interterritorial region. The territorial region is characterized by its fine collagen fibrils enclosing and thereby protecting the cells against mechanical stresses. Contrastingly, the interterritorial region expresses bundles of large collagen fibrils that are zone-dependently oriented. These fibers are arranged parallel to the surface of the superficial zone, obliquely in the middle zone and perpendicular to the articular surface in the deep zone [5, 8].

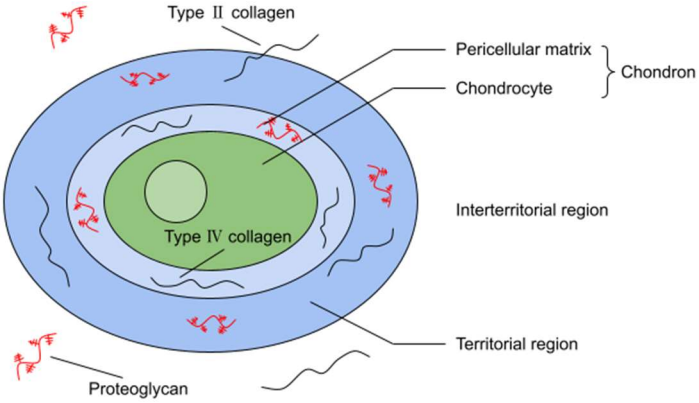

Figure 2. Illustrative representation of the surrounding regions of a chondrocyte and their components. Typically, the chondrocyte is immediately surrounded by a pericellular matrix, forming the chondron. Layers adjacent to the chondron are the territorial and interterritorial region, respectively.

Chondrocytes

Articular chondrocytes are typically quiescent, highly differentiated and metabolically active cells that originate from mesenchymal stem cells. These cells vary in size, shape and number, depending on the location within the cartilage and occupy approximately 2% of the total volume of articular cartilage. In response to several mechanical stimuli and growth factors, chondrocytes are responsible for both the synthesis and disintegration of the cartilaginous matrix. Although these highly specialized cells exert a fundamental role in the development, maintenance and repair of the resilient ECM, the intrinsic healing capacity in response to cartilage damage is limited. Due to their confined replication potential, chondrocytes invoke their optimal chemical and mechanical environment for optimal survival, predominantly mediated by the PCM [8].

Pericellular matrix

As mentioned previously, the PCM is a thin microenvironment directly surrounding the chondrocyte and facilitates its communication with the ECM. A unique structural element of the PCM is type VI collagen, that intracellularly assembles into tetramers and is exclusively located in the PCM in cartilage [9]. Therefore, type VI collagen is regularly used as a marker for chondrons.

The fibrillation (i.e. the process of forming fibers and fibrils) within the PCM is mediated by a complex of decorin, βig-h3 and biglycan, inducing the aggregation of type VI collagen. In this process, a molecular complex is constructed, in which the type VI collagen fibrils are connected to matrilins, which in turn, are linked to type II collagen and aggrecan [10]. This structure as a whole is considered as the building block of the PCM.

Unlike most tissues, the PCM is devoid of a distinct basement membrane that functions in the compartmentalization of a tissue. Although a typical basement membrane has not been identified in chondrocytic PCM, individual basement membrane proteins, including perlecan, nidogens, type IV collagen and laminins have been found [11]. Noteworthily, the basement membrane seems essential for the mechanical properties of the PCM. For instance, it was demonstrated that laminin and perlecan aid the tethering of the PCM to the chondrocyte [12]. In addition to being a structural network in the PCM of cartilage, collagen IV exerts a protective role on the chondrocyte by preventing apoptosis via stabilization of the chondrocyte environment [13]. Similarly, collagen VI was found to enhance cellular proliferation, inhibit MMP-13 production and protect chondrocytes against IL-1β-induced inflammation [14].

That the PCM is intrinsically native to its enclosed chondrocyte becomes apparent when both are regarded as one entity. In fact, a chondrocyte and its associated PCM are considered as one unit, which is called a chondron. The term was first coined by Benninghoff [15] in 1925, but its introduction into cartilage biology was delayed until Poole accomplished a series of studies in the 1980s and 1990s. A clarifying review by Poole [16] in 1997 paved the way for great advances in chondron and chondrocyte PCM research.

Osteoarthritis

Onset of osteoarthritis

Lesions in articular cartilage seldom heal and are frequently associated with joint pain and reduced joint function. Progression of such cartilage lesions generally foreshadows the onset of OA [17]. The prevalence of OA is positively correlated with both age and obesity, and is expected to increase as life-expectancy and youth overweight show a rising trend in Western countries [18]. In 2019, the burden of OA was estimated to involve over 500 million people worldwide and this number has risen by 48% between 1990 and 2019 [19].

A major feature and problem in the development of OA is the phenotypic instability of chondrocytes by means of morphological alterations. Chondrocytes

can undergo dedifferentiation, resulting in fibroblastic or hypertrophic forms that produce different extracellular proteins leading to a defective and impoverished matrix. For instance, the transition from a chondrocytic to a fibroblastic phenotype alternates cell shape and metabolism, leading to an elevated production of type I collagen and proteoglycans (*e.g.*, decorin). This disbalance in composition creates a mechanically incompetent fibrocartilaginous tissue. Alternatively, chondrocytes may produce alkaline phosphatase and collagen type X, indicative of hypertrophic chondrocytes, yet detrimental for cartilage integrity [7, 17].

Underlying mechanisms of osteoarthritis

In an early phase of cartilage damage, risk factors such as mechanical stresses create microcracks and natural pore deterioration in the subchondral bone of diarthrodial joints. This gives small molecules the excellent opportunity to cross talk via the channels through diffusion [20]. In an early response to injured cartilage, chondrocytes release transforming growth factor beta (TGF-β) via the SMAD2/3 signaling pathway. This initial reaction marks the resilience of chondrocytes, since it contributes to the maintenance of type II collagen, aggrecan and the chondrocyte itself. By incrementing the synthesis of TGF-β, integrin and collagen, chondrocytes attempt to mitigate the damage to the cartilage network [21]. Evidence [22] suggests that in an early response to mechanical forces on the articular cartilage, serine proteases (HtrA1) are activated to degrade the pericellular matrix. If repair mechanisms fail and are no longer able to cope with the persistent cartilage stress, degradation of aggrecan leads to exposure of type II collagen to cell surface receptors on chondrocytes, provoking a positive feedback loop that fortifies ECM destruction [23]. This exacerbating tailspin and its primary constituents will be dealt with in the following sections.

Matrix metalloproteinases

Biomarkers that are associated with articular cartilage degeneration and the early onset of osteoarthritis include several matrix-degrading enzyme families such as matrix metalloproteinases (MMPs), a disintegrin and metalloproteinase with thrombospondin type-1 motifs (ADAMTS) and aggrecanases [24]. MMPs are a family of calcium-dependent, zinc-containing endopeptidases, involved in tissue remodeling and degradation of the ECM, including collagen, glycoproteins and proteoglycans. MMPs are excreted by connective tissue and pro-inflammatory cells, including fibroblasts, endothelial cells, osteoblasts, macrophages, lymphocytes and neutrophils. Initially, these metalloproteinases are expressed as zymogens, inactive precursors, which are subsequently modified by other proteolytic enzymes to generate the active form. In normal physiological conditions, MMPs are minimally expressed. Besides regulation by growth factors, hormones and cytokines, the matrix metalloproteinases are endogenously modulated by tissue inhibitors of MMPs (TIMPs) and alpha-2-macroglobulins. An imbalance between MMP and TIMP activity can lead to various pathological conditions, including osteoarthritis. In pathological situations like osteoarthritis, a shift towards an overexpression of MMPs leads to cartilaginous tissue degradation [25, 26].

The first descriptions of MMPs emerged in 1949, as depolymerizing enzymes that promote tumor growth by producing connective tissue stroma [27]. In 1962, the first vertebrate MMP collagenase was isolated and identified as the enzyme responsible for tadpole tail resorption [28]. Over the course of 20 years, more mammalian enzymes were isolated and purified. It was not until 1985 that the field truly developed as several new members of the MMP family were identified through the use of molecular biology techniques [26, 29].

Collagenase-3

MMPs can be divided into several subgroups based on their preference for specific matrix macromolecules. One such subgroup comprises collagenases: MMPs that most efficiently degrade fibrillar collagen. In humans, three collagenases have been identified: collagenase-1 (MMP-1), collagenase-2 (MMP-8) and collagenase-3 (MMP-13) [25]. The general structure of collagenases consists of a propeptide domain, a metalloproteinase domain and a C-terminal hemopexin-like domain [30]. Of alleged importance is MMP-13, since it is pathologically over-expressed in the articular cartilage of patients with OA. Human MMP-13 was first discovered in 1994 by Freije *et al.* [31] and found to be produced in breast carcinomas. Besides collagen types I-IV, IX, X and XIV, MMP-13 also targets aggrecan, osteonectin, fibronectin, laminin, tenascin and perlecan in the ECM for degradation. In addition to MMP-13, the collagenase variants MMP-1, MMP-8, and MMP-18 are known to degrade collagen type II [32]. However, MMP-13 is found to cleave human type II collagen faster than MMP-1 with an enzyme-substrate efficacy (k_{cat}) at least 10-fold higher than MMP-1 [33]. For that reason, MMP-13 is so detrimental for the articular cartilage composition.

Although attention has been devoted to MMP-13, its expression and regulation remains highly complex, involving numerous intricate pathways and unelucidated mechanisms. However, MMP-13 expression induced by fibronectin fragments and type II collagen is best understood. On chondrocytes in articular cartilage, fibronectin fragments with the specific Arg-Gly-Asp sequence can bind to the α5β1 integrin that initiates a downstream signaling cascade, leading to increased gene expression of MMP-13 [4, 34]. MMP-13 and various other matrix metalloproteinases are released into the cartilage matrix destined for collagen degradation. After binding and locally unwinding of the triple-helical structure of collagen, the active

site of the proteinase can successfully hydrolyze the peptide bonds of collagen [35]. Another activation mechanism of MMP-13 involves the binding of type II collagen with a cell surface receptor of chondrocytes (Figure 3).

Discoidin domain receptor 2

In 1997, two independent groups discovered several collagen types as a ligand for discoidin domain receptors (DDRs) [36, 37]. One such receptor is DDR2, a cell surface tyrosine kinase receptor that is actively engaged in the communication with the ECM in articular cartilage. DDR2 is a transmembrane receptor that consists of an N-terminal discoidin (DS) domain, DS-like domain, extracellular juxtamembrane domain, transmembrane domain, intracellular juxtamembrane domain and a C-terminal kinase domain. The DS domain adopts a β-barrel structure with at the top, five protruding loops, creating a trench that forms the collagen-binding site. As a substrate, the DDR2 DS domain requires native, triple-helical fibrillar collagen with a GVMGFO motif, which are present on collagen types I-III [38].

The signaling function by DDR2 on the cell surface of chondrocytes in cartilage degradation plays a key role in the pathogenesis of osteoarthritis [23]. Degradation of the matrix enhances the exposure of chondrocytes to type II collagen, already at an earlier stage in OA development. The direct interaction of collagen with DDR2 on the cell surface of chondrocytes leads to an upregulation of MMP-13 through a protein kinase C-dependent pathway [4]. Furthermore, the Ras/Raf/MEK/ERK signaling pathways are reported to be involved in the increased expression of MMP-13 by DDR2 activation [39].

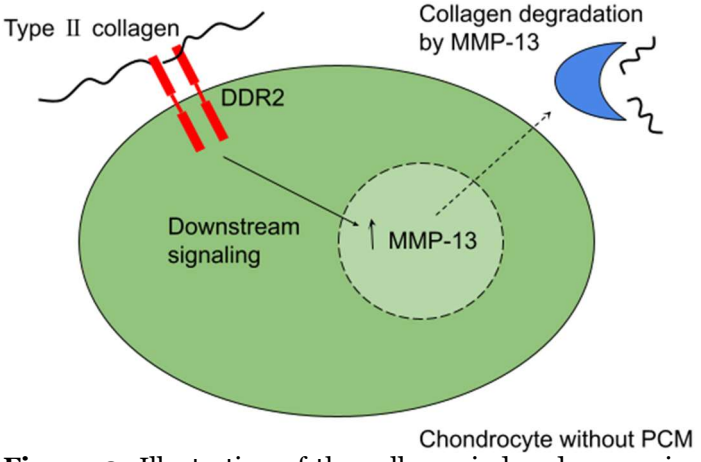

Figure 3. Illustration of the collagen-induced expression of MMP-13 via DDR2 on the surface of chondrocytes, directly exposed to the territorial region in articular cartilage. The intracellular downstream Ras/Raf/MEK/ERK signaling pathway leads to MMP-13 upregulation.

Articular cartilage repair

Classification

Despite intensive work in the area of cartilage regeneration, effective and satisfactory treatments for OA have proved challenging and remain unavailable [40]. Current clinical methods therefore predominantly focus on alleviating pain caused by articular cartilage damage. The practicability and efficacy of treatments for cartilage damage depend on several factors such as the location, area and depth of the lesion, as well as age, chronicity and physical activity of the patient. A widely used classification system to assess the lesion severity is the Outerbridge classification (Table 1), which adopts the depth and degree of cartilage damage as key factors for grade determination.

Table 1. Outerbridge classification

Grade	Description
Grade 0	Normal articular cartilage with smooth surface
Grade I	Soft and swollen cartilage with reduced proteoglycan content and increased water uptake
Grade II	The cartilage is blemished and the surface is cracked up until half the thickness of the cartilage. The area of the lesion does not exceed 1.25 cm^2 of the surface.
Grade III	The area of the lesion exceeds 1.25 cm^2 of the surface and damage is present deeper than 50% of the cartilage thickness. The subchondral bone may be exposed.
Grade IV	Defects are present over the full thickness of the cartilage. Destruction of the articular cartilage has completely exposed the subchondral bone.

Outerbridge classification for articular cartilage lesions [41]. Lesion severity is categorized into five grades (0-IV), from normal articular cartilage to most severe damage.

For grades I and II, conservative treatments are usually recommended, *e.g.*, body mass index (BMI) reduction, pharmacological treatment, patient education and rehabilitation. The best results for grades III and IV lesion severity demand surgical interventions such as physical stimulation by microfracturing or drilling, osteochondral transplantation, chondroplasty surgery or cell-based approaches like autologous chondrocyte implantation (Figure 4).

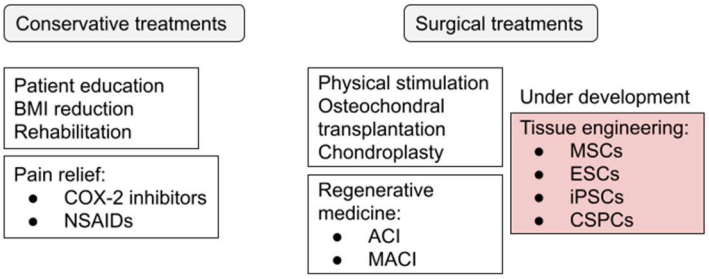

Figure 4. Illustrative overview of approaches to restore articular cartilage with a distinction between conservative and surgical treatments [42]. Abbreviations: body mass index (BMI); cyclooxygenase-2 (COX-2); nonselective nonsteroidal anti-inflammatory drugs (NSAIDs); autologous chondrocyte implantation (ACI); matrix-induced autologous chondrocyte implantation (MACI); mesenchymal stem cells (MSCs); embryonic stem cells (ESCs); induced pluripotent stem cells (iPSCs); chondrogenic stem/progenitor cells (CSPCs).

Physical stimulation

A minimally invasive and low-cost method for cartilage repair is microfracture. During microfracture, multiple perforations are created in the subchondral bone to allow pluripotent progenitor cells from the subchondral bone marrow cavity access the cartilage lesion [43]. Unfortunately, the produced cartilage is substandard fibrocartilaginous tissue, inferior to native hyaline cartilage. However, the regenerated fibrocartilage does succeed in filling the defects and preventing perifocal osteoarthritis [44], that may otherwise arise when left untreated [45].

Articular cartilage tissue engineering

To ameliorate tissue dysfunction and various devastating deficits in diseases, many surgical strategies have been developed to transplant artificial substituents such as joint prostheses, heart valves and complete organs. Unfortunately, non-biological components are often susceptible to serious infections, limited durability and inadequate biocompatibility. Likewise, organ transplantation provokes the problem of lifetime immunosuppression or even organ rejection. However, regenerative medicine including tissue engineering is a novel research domain that is promising for both tissue transplantation and disease treatment [41]. More specifically, tissue engineering is a multi-faceted discipline in biomedical engineering that uses the combination of engineering and biological science to restore the functions of damaged or malfunctioning tissues. The technology is based on a tripartite association, referred to as the "tissue engineering triad". This biological triad involves (i) the reparative cells that form the functional matrix, (ii) a supporting scaffold and (iii) bio-reactive molecules such as growth factors and cytokines. Recent advances in our understanding about the function of stem cells and growth factors in tissue regeneration have boosted cartilage tissue engineering [46]. Tissue engineering for articular cartilage repair is currently under development for the use of mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and chondrogenic stem/progenitor cells (CSPCs).

Mesenchymal stem cells

In the late 1960s, progenitor cells in the bone marrow were found to differentiate into osteoblasts [47]. In 1991, Caplain *et al.* [48] decided to name these cells "mesenchymal stem cells". It has been demonstrated that MCSs derived from bone marrow have the potential to differentiate into osteoblasts, adipocytes and chondrocytes [49]. In recent years, MSCs have therefore been of increasing interest for the field of regenerative medicine and tissue engineering. An advantage of MSCs is their abundant sources; they can be obtained from various tissues including bone marrow and adipose tissue [50]. Secondly, MSCs are strongly proliferative, self-renewing cells and possess a multidirectional differentiation potential for chondrogenesis [51-53]. For instance, adipose-tissue-derived mesenchymal stem cells (ADMSCs) in a collagen-based hydrogel can differentiate into chondrocytes and form tissue engineered cartilage [54]. Additionally, some studies indicate that bone-marrow-derived mesenchymal stem cells (BMSCs) can produce cytokines that stimulate chondrocyte proliferation and ECM synthesis [55]. Therefore, these aforementioned features make mesenchymal stem cells a suitable candidate for cartilage tissue engineering.

On the contrary, chondrogenic differentiation from MSCs also evokes several compromising difficulties in tissue engineering. Differentiation from human MSCs to chondrocytes is challenging due to the precise regulation through various growth factors in the chondrogenic medium, including TGF-β1 and bone morphogenetic protein-2 (BMP-2) [56]. A study by Gonzalez-Fernandez and colleagues [57] demonstrated that MSCs, complexed with plasmid DNA, encoding for TGF-β2 and BMP-2, significantly enhanced the production of hyaline cartilage ECM compared to the group without these growth factors. Another notable disadvantage of MSCs is the abundant formation of type I collagen in the neo-cartilage, indicative of aberrant fibrocartilage [58]. This results in merely a short-term joint mobility improvement, after the injection of autologous BMSCs into the knee joint of patients [59].

Embryonic stem cells

ESCs are pluripotent stem cells from the inner mass of a blastocyst, an early-phase embryo before the implantation into the uterus. ESCs have the advantageous ability for unlimited self-renewal in culture and to differentiate into nearly all somatic cells. Nevertheless, the utilization of human ESCs raises serious ethical concerns, since these cells are isolated from human embryos after *in vitro* fertilization. Moreover, a study [60] revealed that the direct injection of ECSs into mice can cause teratomas, tumors containing completely developed tissues, *e.g.*, teeth, hair, bone and muscle.

A defined chondrogenic medium for human ECSs containing dexamethasone and L-ascorbic acid 2-phosphate, drive these cells into differentiation. The greatest size of cartilage pellets is achieved through the addition of both TGF-β1 and morphogenetic protein-7 (BMP-7) into the medium [61]. Additionally, the production of type II collagen and glycosaminoglycan, specific for cartilage ECM, was found to be increased with TGF-β1 and BMP-7 addition in ECS culture [62]. Articular cartilage tissue engineering using ESCs is currently under development in clinical trials.

Autologous chondrocyte implantation

Since chondrocytes are naturally responsible for the secretion of the cartilage-specific extracellular matrix, they are regarded as the native candidate for

articular cartilage regeneration. These cells can be harvested from various sources such as articular, costal, auricular and nasal cartilage (derived from the joint, ribs, auricle and nasal septum, respectively) [63]. Hyaline cartilage is the preferred source of chondrocytes in the context of articular cartilage engineering.

In the pursuit of articular cartilage regeneration using chondrocytes, ACI has become an important strategy. ACI is a two-stage regenerative treatment that aims to restore articular cartilage defects with cultivated autologous chondrocytes in the second, open-knee surgery. After articular cartilage isolation during the first open-joint surgery, the matrix is usually enzymatically digested by collagenases *in vitro*, which leaves bare chondrocytes for monolayer expansion.

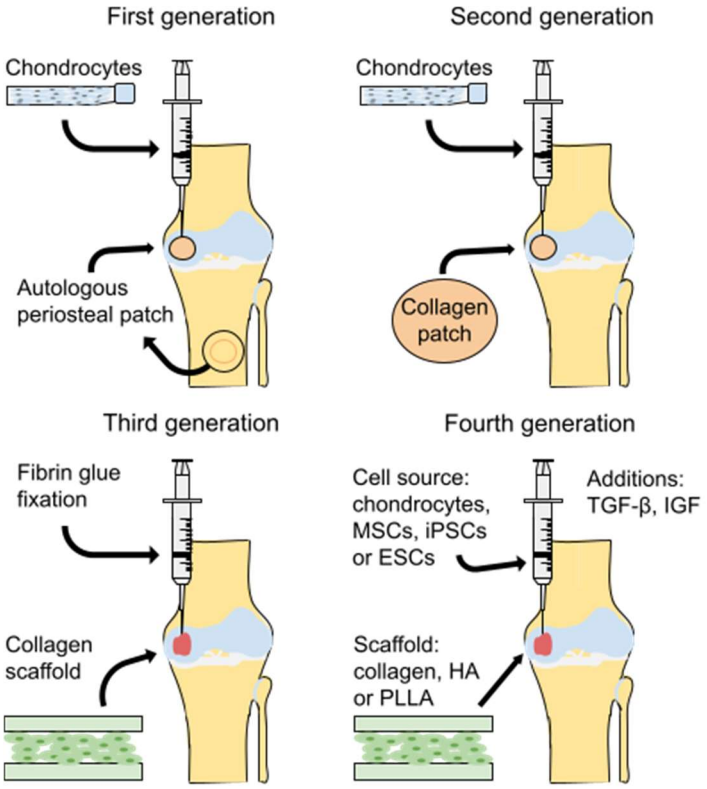

Figure 5. Overview of the four generations of ACI. In the first generation the cultured chondrocytes are injected underneath an autologous periosteal patch, which is replaced by a collagen patch in the second generation. The third generation involves a collagen scaffold that is fixated with fibrin glue. The fourth generation expands on the preliminary generations with the possibility of different cell sources, scaffolds and growth factors. Abbreviations: hyaluronic acid (HA); poly- L-lactic acid (PLLA); insulin-like growth factor (IGF).

The traditional first generation ACI technique involves the injection of a liquid suspension with chondrocytes underneath an autologous periosteal patch [64]. It has been reported that the procedure fails in about 15% of the people [65]. Adverse events have been reported with as many as 44% of the joints treated with ACI [66]. The disadvantages of the technique such as periosteal hypertrophy and delamination led to the development of a second generation ACI (ACI-C) which uses a collagen membrane to secure the chondrocytes inside the defect and to negate the need for a periosteal patch. A third generation approach is matrix-induced autologous chondrocyte implantation (MACI), which utilizes a porcine collagen type I/III scaffold to seed the autologous chondrocytes. This scaffold is simply secured into the lesion with a fibrin sealant [64]. Currently, the fourth generation of ACI expands on the possibilities for cell sources and scaffolds, and adds growth factors (Figure 5) [2].

Despite the efforts in refinement of the ACI techniques, chondrocyte dedifferentiation during *in vitro* monolayer expansion remains an important drawback of the treatment [2]. Dedifferentiation results in a decreased capacity of re-implanted chondrocytes for hyaline articular cartilage regeneration. This, together with the enzymatic matrix degradation step, questions the efficacy of ACI in contrast to the practice of chondrons in cartilage tissue regeneration.

Application of chondrons in cartilage repair

Reason for chondrons

Due to the limited intrinsic healing capacity of native articular cartilage, cartilage cell therapies including ACI are developed that use isolated chondrocytes and their expansion to obtain a sufficient amount of cells for *in situ* cartilage regeneration. As mentioned before, a major problem that impedes cartilage tissue engineering is the dedifferentiation of chondrocytes, which results in the loss of the specific chondrogenic phenotype. As a consequence, the production of type I collagen is elevated instead of type II collagen, leading to incompetent fibrocartilage.

The added value of using chondrons in cartilage repair rather than merely chondrocytes has been briefly touched upon in the introduction. Traditionally, chondrons are obtained from a rapid digestion of minced articular cartilage using 0.3% dispase (a protease that cleaves fibronectin and collagen I and IV) and 0.2% collagenase in phosphate-buffered saline. This procedure yields sterile, viable chondrons that resemble the composition and morphology of the *in situ* situation [67].

Evidence from studies by Vonk *et al.* [3, 4] leads to the conclusion that the preservation of the pericellular matrix of chondrocytes elevates the type II collagen production and reduces the MMP-13 and type I collagen levels. The authors show that the retention of the PCM of chondrocytes prevents collagen gaining access to DDR2 on the cell surface, thereby limiting the downstream kinase-C dependent pathway for MMP-13 production. The absence of MMP-13 prevents the breakdown of collagen in the matrix, which leaves the cartilage intact and functional. Additionally, another study [68] has found an enhanced production of glycosaminoglycan in chondron cultures compared to chondrocyte cultures, re-emphasizing the

beneficial effects of PCM preservation for cartilage production.

Considering the scientific evidence that reveals chondrons to outperform chondrocytes for healthy cartilage production, raises questions in the area of cell-based cartilage repair treatments. Recently developed therapeutic strategies involving chondrocytes for cartilage regeneration seem to have counterintuitively neglected the native PCM for its valuable properties. Fortunately, the applicability of chondrons in cell-therapy for the treatment of articular cartilage lesions has been assessed by a few groups, with one resulting clinical trial (Table 2).

Table 2. Studies involving chondrons in articular cartilage repair

Trial/authors (year)	Description	Model(s)
Bekkers *et al.* (2013) [69]	Evaluation of the combination of MSCs with chondrons in different ratios *in vitro* and animal models.	*In vitro,* mouse, goat
IMPACT (2013 - 2016) (NCT02037204)	First-in-man surgery: autologous chondrons are mixed with allogeneic MSCs and implanted in a fibrin glue carrier in a one-stage surgical procedure.	Human
de Windt *et al.* (2016) [70]	A study that demonstrates the safety and efficacy of allogeneic MSCs in stimulating articular cartilage regeneration in combination with autologous chondrons.	Human
de Windt *et al.* (2017) [71]	A study that demonstrates the safety and efficacy of the one-stage cartilage regeneration by IMPACT. Additionally, the authors suggest that MSCs function as stimulatory (trophic) factors.	Human
Korpershoek *et al.* (2020) [72]	To evaluate the safety and efficacy of IMPACT compared to nonsurgical treatment for large articular cartilage defects.	Human
Saris *et al.* (2021) [73]	A five-year follow-up report of the safety, clinical efficacy and durability after treatment with IMPACT.	Human
Jacer *et al.* (2018) [74]	To investigate the regenerative effects of intra-articular injection of ADSCs and chondrons for the treatment of induced osteoarthritis in the knee.	Rat
Duan et al. (2021) [75]	To explore the effects of a combination of chondrocytes and chondrons on matrix production and repair of defective knee cartilage.	Rabbit

A record of studies and a clinical trial utilizing chondrons in articular cartilage repair. IMPACT (with four accompanying papers) is the first clinical trial that involves autologous chondrons for cell therapy in articular cartilage lesions. Bekkers *et al.* provided a preclinical study in the context of IMPACT. Jacer *et al.* investigated the combination of ADSCs and chondrons in articular cartilage repair. Duan *et al.* combined chondrocytes and chondrons to repair articular cartilage defects.

Preclinical study

A preclinical study by Bekkers *et al.* [69] in 2013 evaluated whether the combination of MSCs and chondrons is suitable for a single-stage regenerative treatment for deep focal articular cartilage lesions. The authors' findings demonstrate that an *in vitro* combination of 10% to 20% chondrons with MSCs produces more cartilage matrix compared to chondrocyte/MSCs cocultures of different proportions. Interestingly, *in vivo* models with mice and goats show more matrix production with the 10%-20%/80%-90% combinations compared to scaffolds with only chondrons. Moreover, in cultures using MSCs and chondrocytes, the MSCs disappear over time. This finding suggests the importance of the trophic effects of MSCs in chondrocyte proliferation and matrix formation [76]. Namely, MSCs seem to possess a stimulating role for chondrocytes rather than differentiating into chondrocytes. In addition to their trophic effects, MSCs also possess anti-inflammatory and immunomodulatory properties [77]. Bekkers and colleagues report that the use of MSCs did not evoke clinically relevant graft rejection in their preclinical study, which may have been prevented by the low expression of major histocompatibility complex class I and II molecules on undifferentiated MSCs [78]. The authors conclude that the combination of chondrons and MSCs can be safely and successfully applied in a single-stage cell-based procedure to treat focal articular cartilage lesions in humans.

IMPACT

In 2013, a group from University Medical Center Utrecht embarked on a mission for the first-in-man surgery using chondrons and MSCs. The novel therapeutic approach that is based on this combination of autologous chondrons and allogeneic MSCs is called Instant MSC Product accompanying Autologous Chondron Transplantation (IMPACT) and is currently in clinical trials. IMPACT is a cartilage repair procedure that combines rapidly isolated autologous chondrons with allogeneic bone marrow-derived MSCs to treat defects on the femoral condyle or trochlear groove of the knee joint. To obtain chondrons, the IMPACT surgery involves a rapid digestion

protocol of debrided cartilage in Liberase MNP-S GMP Grade, a highly purified enzyme blend containing collagenase class I and class II from *Clostridium histolyticum* and thermolysin, a neutral protease isolated from *Bacillus thermoproteolyticus* [72, 79]. The autologous chondrons are recycled from the patient's defect site and supplemented with allogeneic MSCs in a one-stage surgical procedure, which, compared to two-stage ACI, decreases the patient's burden and significantly reduces the treatment costs [72, 73]. The cells are mixed with a fibrin cell carrier before injection into the cartilaginous defect.

Between 2013 and 2014, a total of 35 patients were subjected to the IMPACT procedure during the phase I/II stage. A potential consequence of stem cell transplantation is engraftment, a process that involves the migration of stem cells via the blood to the bone marrow, where the production of new blood cells and platelets is initiated. A DNA analysis [71] demonstrated that the MSCs do not engraft in the host tissue. Even eighteen months after surgery, no symptoms were reported that would indicate engraftment in the bone marrow, liver and lungs. After twelve months, structural evaluation of the hyaline cartilage using biochemical MRI scans and second-look arthroscopies revealed the proper integration with the native tissue. According to histological results, the quality of the repair tissue after IMPACT is noninferior or even superior to that after ACI [71]. In a mid-term follow-up [73] after 60 months, the clinical improvement was found to be sustained and no adverse effects suggested a foreign body reaction. Patient questionnaires including the Knee injury and Osteoarthritis Outcome score (KOOS) and the visual analog scale (VAS) for pain assessed the improvement after 60 months to be clinically relevant and statistically significant. However, of 35 patients, 5 (14%) required additional care due to osteochondral alterations after the IMPACT procedure. The IMPACT procedure was documented as a failure for these patients. In comparison, Knutsen *et al.* [80] determined 9 failures after ACI at five years (23%) and 17 at 15 years (42.5%), in a randomized controlled trial comparing ACI with microfracture.

Shortly, in a phase III randomized controlled trial, the efficacy of IMPACT compared to nonsurgical treatment will be explored in 60 patients with large (2-8 cm^2) articular cartilage defects. Patient recruitment is estimated to be completed around August 2021 [72].

The most relevant advantage of IMPACT compared to a two-stage procedure such as ACI, is the one-stage approach. The feasibility of the one-stage surgery is realized by off-the-shelf use of allogeneic MSCs and the recycling of chondrons from the rim of the patient's articular cartilage lesions. Recycling chondrons brings an additional advantage of donor-site morbidity prevention. After one surgical intervention, patients can immediately start convalescence instead of waiting for a cell expansion period of several weeks.

Nevertheless, although the therapeutic approach of IMPACT is primarily lauded, it poses an important challenge that merits the attention as well. The number of chondrons that can be obtained remains a limiting factor. In fact, it seems impossible to expand chondrons, since their PCM will impoverish upon cell expansion and they therefore become chondrocytes [81]. Since autologous chondron expansion is not applicable for IMPACT due to the one-stage nature of the procedure, occupation of only 10% to 20% chondrons is the best solution to the limited chondron availability.

In addition to the (pre)clinical studies considering the IMPACT trial, two independent groups have explored the utilization of chondrons for the repair of defective knee cartilage in combination with cells other than MSCs.

ADSCs and chondrons

In 2018, Jacer *et al.* [74] investigated the regenerative effects of intra-articular injection of adipose-derived stem cells and chondrons for the treatment of induced OA in rat knees. ADSCs from perirenal rat adipose were co-cultured with chondrons from primary newborn rat hyaline cartilage. In a rat model with induced OA, the group of researchers introduced the co-cultures into the intra-articular space. The effect of the combination of ADSCs and chondrons showed evidence of enhanced articular cartilage regeneration and increased type II collagen production compared to other groups. The results demonstrate that the production of healthy cartilaginous tissue by ADSCs and chondrons is feasible *in vivo*. The study implicates further research to elucidate pathways of repair and evaluate the efficacy and long-term safety of this system for treatment of OA in humans.

Chondrocytes and chondrons

A recent study by Duan *et al.* [75] explored the effects of a combination of chondrocytes and chondrons on matrix production *in vitro* and repair of defective rabbit knee cartilage. The authors found that combining chondrons and chondrocytes, particularly at a 1:1 ratio, significantly incremented the expression levels of aggrecan and type II collagen and production of glycosaminoglycan *in vitro*. Furthermore, the implantation of chondrons in association with chondrocytes substantially accelerated the repair of cartilage knee defects in rabbits. Duan and colleagues therefore postulate that this system may be a new promising strategy for intervention of defective knee cartilage.

Conclusion

This literature study reviewed the attempts for and advances in the application of chondrons for articular cartilage repair during the past decade. For this review, one clinical trial and three studies involv-

ing the utilization of chondrons in articular cartilage repair were taken into consideration. The finding of Vonk *et al.* in 2010, that the preservation of the PCM improves cartilage formation, was an auspicious starting point that gave the initial impetus to its future application in cartilage cell therapy. The fact that one clinical trial was initiated, in addition to a few ancillary studies is a positive development, although the expectations for a decade were higher.

Chondrons as a replacement for bare chondrocytes provide remarkable benefits in cell-based cartilage regeneration. Nevertheless, the application of chondrons in cartilage tissue regeneration alone may not be innovative enough to overcome the inertia of golden standards in the near future. However, the IMPACT trial may be part of a paradigm shift in which MSCs stimulate tissue repair rather than differentiate into the desired cell type.

Most scientists seem to either have little incentive to using chondrons in cell therapy or its benefits have eluded them. Concurrently, the *in vivo* studies utilizing chondrons in articular cartilage repair, augmented by auxiliary cells such as MSCs, ADSCs and chondrocytes do show the interest in the field of chondrons for regenerative medicine. Some of these initiatives require follow-up studies to elucidate pathways of repair, evaluate the efficacy and long-term safety of their system for treatment of OA in humans. Currently, great hopes are pinned to the clinical trials of IMPACT, which have, thus far, shown promising results to culminate in a future FDA approval.

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