

## Cartilage repair: past and future – lessons for regenerative medicine

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### Abstract

Since the first cell therapeutic study to repair articular cartilage defects in the knee in 1994, several clinical studies have been reported. An overview of the results of clinical studies did not conclusively show improvement over conventional methods, mainly because few studies reach level I of evidence for effects on middle or long term. However, these explorative trials have provided valuable information about study design, mechanisms of repair and clinical outcome and have revealed that much is still unknown and further improvements are required. Furthermore, cellular and molecular studies using new technologies such as cell tracking, gene arrays and proteomics have provided more insight in the cell biology and mechanisms of joint surface regeneration. Besides articular cartilage, cartilage of other anatomical locations as well as progenitor cells are now considered as alternative cell sources. Growth Factor research has revealed some information on optimal conditions to support cartilage repair. Thus, there is hope for improvement. In order to obtain more robust and reproducible results, more detailed information is needed on many aspects including the fate of the cells, choice of cell type and culture parameters. As for the clinical aspects, it becomes clear that careful selection of patient groups is an important input parameter that should be optimized for each application. In addition, the study outcome parameters should be improved. Although reduced pain and improved function are, from the patient's perspective, the most important outcomes, there is a need for more structure/tissue-related outcome measures. Ideally, criteria and/or markers to identify patients at risk and responders to treatment are the ultimate goal for these more sophisticated regenerative approaches in joint surface repair in particular, and regenerative medicine in general.

**Keywords:** cartilage repair • autologous chondrocyte implantation • clinical study • growth factors • cell types • inflammation

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## Introduction: the impaired repair capacity of cartilage and the beginning of cell therapy

'...from Hippocrates down to the present age, we shall find, that an ulcerating cartilage is found to be a very troublesome disease... ..and that, when destroyed, it is never recovered.' This statement by Hunter in 1743 is probably the most quoted sentence in the field of cartilage repair. But is it still true? Did developments in Tissue Engineering and Regenerative Medicine not change this paradigm?

Partial-thickness cartilage defects, even very small ones, do not heal spontaneously whereas full-thickness osteochondral lesions below a critical size do, albeit with fibrous cartilage tissue of inferior functional quality [1–3]. This very limited healing capacity of cartilage is partially due to its avascularity. Therefore, the classical wound healing response, where a set of complex biochemical events takes place to repair the damage, does not occur in cartilage. When cartilage is wounded, the tissue at the wound edge is damaged and cells die, resulting in debris similar to wounds in other tissues. However, this damaged and avital cartilage tissue will not be removed. Wound healing is prevented by avascularity in combination with an impaired migration capacity of cartilage cells through the dense extracellular matrix [3–5]. In full-thickness ICRS (International Cartilage Repair Society) grade IV (superficial osteochondral) defects, bleeding from the underlying bone marrow will, to some extent, trigger a natural wound healing reaction. Non-vital tissue will be partially resorbed and cells are attracted to the wound area, with proliferation, differentiation and new matrix production [3]. However, the resulting fibrocartilaginous repair tissue is of inferior quality, rather classified as scar tissue, a fact which is associated with an increased risk for gradual development of secondary osteoarthritis. Besides surgically punching or drilling holes through the subchondral plate, called marrow stimulation techniques, there was no other regenerative treatment option for symptomatic joint surface defects available until 1994 when the first cell therapeutic approach for cartilage repair, autologous chondrocyte implantation (ACI), found its way into the clinic [6].

### Autologous chondrocyte implantation

The original ACI procedure involves the isolation of chondrocytes from a cartilage biopsy harvested during an arthroscopic procedure from a less load-bearing area of the knee with the defect. After expansion in monolayer culture, the cells in solution are shipped back to the orthopaedic surgeon. The cell solution is injected during a surgical procedure under a flap of periosteum harvested from the tibia that has been fixed with sutures, fibrin glue (or both) to the edges of the cartilage lesions.

Since the first report by Brittberg *et al.* on the first 23 patients in 1994 [6], ACI has been performed in more than 30,000 patients

throughout the world (personal estimation by MB based on cases reported in literature and information from different companies using ACI). The clinical results have been reported from different centres worldwide. In a prospective clinical evaluation (evidence level II) of 244 patients with a 2–10 year follow-up [7], a high percentage of good to excellent clinical results (84–90%) was reported in patients with different types of single femoral condyle lesions, while other types of lesions had a lower degree of success (mean 74%). The reported histology mostly shows a mixed tissue repair of hyaline-fibrocartilaginous appearance. The total failure rate was 16% (10/61) at 7.4 years mean follow-up. All ACI failures occurred in the first 2 years and patients showing good to excellent improvement at 2 years had a high percentage of good results at long-term follow-up [7].

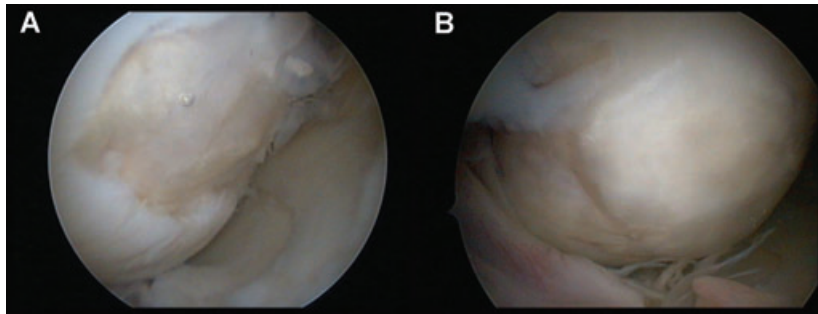
Reports on results with ACI from other centres [8–10] show similar figures with a high degree of success, however the evidence level of these reports is II or lower. In order to properly position this new treatment in an algorithm, and to establish its relevance for daily clinical practice, ACI needs to be evaluated in direct comparison to other cartilage repair techniques in prospective randomized trials [11]. The findings described above are related to what one defines as the first generation of ACI with cells in suspension covered with a periosteal flap. In a so-called second generation of an ACI procedure, the periosteum has been replaced with a collagen membrane. This approach was mainly developed to improve the surgical and patient friendliness, but it is still unclear how far this is affecting outcome. The third generation of cartilage repair products involves so-called combination products (CP) being either cells grown on a carrier membrane such as matrix-induced autologous chondrocyte implantation (MACI) or cells seeded and grown in a scaffold such as hyaluronic acid (Figure 1) or collagen.

### Combination products

Behrens *et al.* [12] studied 38 patients with localized cartilage defects treated with a cell seeded collagen membrane who were evaluated up to 5 years after the intervention. Eleven (73.3%) of the 15 patients that were followed for at least 5 years after surgery had significant improvement of clinical/functional results.

Zheng and coworkers [13] analysed histological samples from a cohort of 56 MACI patients to examine the phenotype of chondrocytes seeded on type I/III collagen scaffold over a period of 6 months. Biopsies showed the formation of cartilage-like tissue as early as 21 days, and in 75% of the biopsies taken from a group of 11 patients after 6 months, hyaline-like cartilage regeneration was seen.

Most of the ACI techniques are still performed as an open procedure. The use of a biological implant with cells seeded in a



**Fig. 1** Clinical view on femoral condylar cartilage lesion treated by autologous chondrocyte cultured in a hyaluronic scaffold (hyalograf-C). (A) The scaffold with cells has just been implanted and glued to the defect site transarthroscopically. (B) The same lesion at a second look arthroscopy 1 year post-surgery.

scaffold allows arthroscopic implantation. Marcacci *et al.* [14, 15] have worked with a biodegradable, hyaluronan-based biocompatible scaffold seeded with autologous chondrocytes. They presented a prospective study with 70 consecutive patients treated by arthroscopic ACI that were followed at least 24 months. Clinical improvement was statistically significant at 24 months and the second-look arthroscopy demonstrated a complete coverage of the grafted area with a hyaline cartilage-like tissue in 12 of the 15 analysed patients (evidence level II). A better clinical outcome was observed in young, well-trained patients and in the treatment of traumatic lesions.

### Randomized controlled studies

All the above-mentioned studies are limited in their scope as most of them do not reach the level of evidence needed to position these approaches in a treatment algorithm. Therefore, randomized prospective multi-centre comparative trials are required. To date, seven clinical randomized trials have been published in peer-reviewed papers, representing short-term and, at most, mid-term studies.

Horas *et al.* [16] performed a prospective clinical study to investigate the 2-year outcomes in 40 patients with an articular cartilage lesion of the femoral condyle who had been randomly allocated to be treated with either ACI or transplantation of an autologous osteochondral cylinder. Both treatments alleviated symptoms. However, the improvement provided by ACI lagged behind that obtained using osteochondral cylinder transplantation.

Bentley *et al.* [9] studied a total of 100 patients with symptomatic chondral and osteochondral lesions of the knee (mean age 31.3 years; mean defect size 4.66 cm<sup>2</sup>) randomized to undergo either ACI (58 patients) or mosaicplasty (42 patients) with a mean follow-up of 19 months (12 to 26). Functional assessment using the modified Cincinnati and Stanmore scores and objective clinical assessment showed that 88% had excellent or good results after ACI and 69% after mosaicplasty. Structural assessment by arthroscopy at 1 year demonstrated excellent or good repair in 82% after ACI and in 34% after mosaicplasty. This study has been criticized for a few reasons, including the lesions in this study were larger than the optimum size cartilage defect for mosaic arthroplasty is 1–4 cm<sup>2</sup> and the postoperative rehabilitation differed from that recommended for mosaic arthroplasty.

Dozin *et al.* [17] studied 47 patients that were randomly assigned to ACI or mosaicplasty and subjected to arthroscopic debridement of the lesion at the time of enrolment. Notable, and not unexpected, was the fact that 14 patients (31.8%) experienced substantial improvement following the initial debridement alone and, being clinically asymptomatic, received no further treatment. Among the 23 patients (52.3%) who could effectively be evaluated, a complete recovery was observed based on clinical examination in 88% of the mosaicplasty-treated patients and in 68% of the ACI-treated patients ( $P = 0.093$ ). Knutsen *et al.* [18] studied 80 patients who needed local cartilage repair because of symptomatic lesions on the femoral condyles between 2 and 10 cm<sup>2</sup>. Patients were randomized into ACI or microfracture treatment. At 2 and 5 years, both groups had significant clinical improvement compared with their preoperative status. At the 5-year follow-up, there were nine failures (23%) in both groups. Younger patients did better in both groups. The authors did not find a correlation between histological quality of the repair tissue and clinical outcome. However, none of the patients with the best-quality cartilage (predominantly hyaline) at the 2-year mark had subsequent failure. Saris *et al.* [19] conducted a prospective multi-centre trial to compare ACI with microfracture in patients with single ICRS grade III (>50% penetration to the cartilage, may be full thickness lesions, but not into the subchondral bone) to IV (penetrates subchondral bone, superficial osteochondral lesions) symptomatic cartilage defects of the femoral condyle. Patients aged 18–50 years were randomized to ACI ( $n = 57$ ) or microfracture ( $n = 61$ ). One year after treatment, ACI was associated with a tissue regenerate that was histologically superior to that after microfracture. Short-term clinical outcome was similar for both treatments. Saris *et al.* [19] used a well-characterized chondrocyte cell population (called ChondroCelect). In this expansion method of chondrocytes, care was taken to prevent as much as possible dedifferentiation, and was verified by the analysis of a gene marker profile that is predictive of the capacity to form hyaline-like cartilage *in vivo* and considered a potency assay. A specific gene marker cut-off score is used as the criterion for implantation. In the study by Saris *et al.* [19], 6 of the 57 patients did not receive ACI treatment because the gene expression profile was too low. So far, it is not known if low gene scores are indeed associated with less successful outcomes, and if high gene scores are potentially predictive of better clinical outcomes. Results of this analysis at 24 and

36 months have been partially reported (Saris *et al.*, AAOS February 2009, P422) and will be submitted soon (FP Luyten, personal communication).

Furthermore, there are two randomized studies comparing two types of ACI: ACI with collagen membrane (patch) *versus* MACI for osteochondral defects of the knee [20] and a prospective, randomized study comparing periosteum *versus* type I/III collagen membrane covered ACI [21]. There were no differences in the outcome of collagen covered ACI and MACI. A significant number of patients who had the periosteum covered ACI required shaving of a hypertrophied graft. It was concluded that there was no advantage in using periosteum.

Wasiak and Villaneuva published in 2006 [22] a review in the Cochrane database that included four randomized controlled trials (266 participants). They concluded that at that time there was no evidence of a significant difference in the outcomes between ACI and other cartilage repair interventions. They stated that additional good quality randomized controlled trials with long-term functional outcomes were required to provide clear guidelines for practice. The last randomized study from 2008 by Saris *et al.* [19] was not evaluated in this Cochrane review [22]. Future reports of the long-term results of that study and other ongoing studies will be of great interest.

Vavken *et al.* [23] studied clinical effectiveness and cost effectiveness of ACI in a review of six randomized controlled studies that assessed the effectiveness of ACI compared with microfracture or mosaicplasty. Four of these studies reported no or only insignificant differences between the procedures whereas two studies observed better results with ACI. The authors stated that the long-term results were good throughout, but the high quality of the regenerative tissue is a clear potential advantage of ACI. Cost-effectiveness models support ACI for the durability of its results and thus potentially lower costs in the long-term.

Taken together, these more recent randomized studies have given valuable information about what can and should be done in clinical cell therapy studies. They showed that the field is seriously exploring therapeutic options of earlier developed procedures. The clinical studies also demonstrate that much is still unknown and further improvement is required. Future cell therapeutic applications should give rise to a durable and well-integrated repair tissue, result in clinical improvement, eliminate or significantly postpone the need for subsequent arthroplasty, and be suited for both focal defects and early degenerative joint disease.

## Choice of cell types

The first critical issue in the development and improvement of a cell therapeutic strategy for cartilage repair is selection of the cell type. Articular cartilage was the first source of cells to be used in an FDA-approved method (ACI) to repair focal cartilage defects. There are now several tissue sources and cell types considered, each of which has certain advantages and disadvantages. Among

the several factors to be considered are ease of harvest, cell yield, purity, effective proliferative capacity, and the phenotype of the cells and the cartilage produced, also regarded by the regulators as potency assays.

One of the main drawbacks to the use of expanded articular chondrocytes is their dedifferentiation and loss of re-differentiation potential after expansion [24–26], as significant chondrocyte expansion appeared to be needed to make tissue engineering approaches feasible. Studies looking into the underlying molecular mechanism of this loss of chondrogenic potential have shown that stable *in vivo* chondrogenic potential correlates positively with a number of gene markers including Col2A1 and FGFR3 expression and its loss is marked by up-regulation of ALK-1 expression [11]. Furthermore, Sox5, Sox6 and Sox9 co-expression correlates with a stable chondrogenic phenotype in concert with a lack of osteogenic or hypertrophic chondrocyte markers [27].

In addition to articular cartilage, chondrocytes isolated from other cartilaginous structures (*i.e.* auricle, nasal septum, rib) have also been considered for cartilage repair procedures [28–30]. Chondrocytes from these different anatomic locations have distinctive growth and differentiation characteristics. Several studies have shown differences in growth and re-differentiation of chondrocytes of samples from different anatomical regions of rabbit [31, 32], bovine [33] and porcine origin [34, 35]. Similar demonstrations of site-specific differences were also noted in a study of human auricular, nasal and costal chondrocytes where auricular and nasal chondrocytes showed greater proliferative and re-differentiation capacity than costal chondrocytes [36], and in other studies which disclosed significant differences in cartilage-forming capacity between human articular- and auricular-derived chondrocytes [37, 38]. The overriding question for all of these studies using chondrocytes from different tissues and locations is whether or not the matrix elaborated by these chondrocytes will have the biomechanical properties necessary to withstand the compression and shear forces that will be experienced in the joint. However, this is also the question when articular chondrocytes are used for implantation. At the moment, we do not know the exact biomechanical properties needed and what the body can provide or compensate for. We also know little about tissue formation, organization (a tissue is more than an assembly of cells in a matrix) and integration into the environment after transplantation. These subtle aspects of tissue engineering biology are likely to be critical for long-term clinical outcome. Some hypothesize that to create a typical zonal structure, the cells from different cartilage zones should be seeded in a construct separately [39, 40]. Regardless of the correctness of this approach, it seems difficult to translate this into a robust manufacturing process, unless progenitor cells are used and principles of developmental tissue formation can be mimicked *in vitro* (Lenas P, Moos M Jr, and Luyten FP, manuscript in revision).

Cartilaginous structures, with the exception of articular cartilage, are covered by a perichondrium layer that contains cartilage progenitor cells. Perichondrial cells have been investigated for their utility as cell sources for tissue engineering as early as 1972, when whole perichondrial grafts were used to generate repair

cartilage [41]. The clinical results of perichondrium transplantations were initially reported to be good [42], however no differences were observed between debridement and drilling and perichondrium transplantation in the treatment of isolated cartilage defects after 10 years of follow-up, although slightly more problems were reported in the perichondrium transplantation group [43]. More recent studies report cartilage formation by cells isolated from perichondrium [44] and evidence for a subset of cells in the perichondrium with stem cell-like characteristics. This was based on long-term retention of BrdU label, extensive expansion in culture, and ability upon induction to develop to cells expressing either chondrogenic or adipogenic phenotypes *in vitro* [45]. It is an intriguing observation that perichondrium may contain a stem cell population that may be able to be expanded extensively in culture under the proper conditions.

Bone marrow is the best-known source of human adult mesenchymal progenitor cells (BMSC) that have been shown to have chondrogenic potential [46]. BMSCs have been tested for repair of articular cartilage in a rabbit model as early as 1994 [47], wherein it was shown that defects implanted with a type I collagen/MSC mixture showed slightly improved histologic scores for cartilage healing compared to a type I collagen/periosteal stem cell mixture, although variability in scores precluded reaching statistical significance. Since then, many experimental studies in rabbits have been performed where BMSCs have been combined with different biomaterials and/or growth factors [48–52] often with promising results. Some clinical studies using BMSCs to repair cartilage lesions have been reported. Most of these are case studies using bone marrow cells in an ACI-like procedure where cells are seeded in collagen and implanted in a cartilage defect covered with periosteum [42, 53, 54] or where the cells are percutaneously injected in the knee joint [55]. At this point, it is not known whether these procedures are able to reduce clinical symptoms, repair cartilage defects or slow down the progression of osteoarthritis.

One potential concern about the use of BMSCs is the question as to whether BMSCs produce stable hyaline cartilage. Several *in vitro* studies have shown that BMSCs produce type X collagen, a marker for hypertrophic cartilage [46, 56, 57] and chondrocytes from BMSCs implanted into SCID mice recapitulate endochondral bone formation, resulting in the loss of cartilage that is replaced by bone [58]. It may be that BMSCs are not destined to a hypertrophic chondrocyte fate based on a recent study by Liu *et al.* [59] who showed that a stable hyaline cartilage is formed when BMSCs are first allowed to mature *in vitro* for an extended time (12 weeks) prior to implantation. If true, this study makes bone marrow derived MSCs a viable source of cells for cartilage repair, with the caveat that the BMSCs will need to be pre-differentiated prior to use to differentiate into permanent cartilage after implantation. The molecular basis of stable hyaline cartilage is not yet completely understood. Therefore, it is unlikely that in the immediate future cartilage repair using BMSCs will be sufficiently robust to allow large-scale clinical use with predictable outcomes.

Next to production of matrix, BMSCs have been extensively documented to secrete trophic factors and in this way enhance wound healing or to act as immunomodulators [60–62]. By secre-

tion of these factors, they might influence the catabolic conditions in a joint with cartilage lesions and prevent progression of degeneration. This hypothesis is under investigation.

Another promising source of multi-potent progenitor cells is adipose tissue. The advantages of using adipose tissue as a source are the ease of collection and the large yield of cells that can be obtained [63]. Zuk *et al.* [63, 64] demonstrated that adipose-derived stromal cells (ADSCs) are multi-potent for several mesenchymal phenotypes, including chondrocytes, and thus are a potential source of cells for tissue engineering. The expression of hypertrophic chondrocyte markers, such as Runx2, Osterix and type X collagen, was observed in ADSCs [64] as was noted for BMSCs [57, 64]. In addition, ADSCs were reported to have a reduced potential for chondrogenic differentiation that may be a result of a lack of TGF $\beta$ 1-receptor expression and reduced expression of mRNAs for bone morphogenetic proteins (BMPs) [65]. This study also suggested that MSC of different anatomical origins may require different stimulant cocktails for their chondrogenic differentiation. ADSCs had a lot of attention in experimental studies the past years, however no studies in human beings have been reported yet.

Cells from the synovium have chondrogenic potential [66]. The study by Sakaguchi *et al.* [67] showed that human synovial-derived cells had greater chondrogenic potential than BMSCs, ADSCs, periosteal- or muscle-derived cells from the same patients, and a follow-up study showed that synovial-derived cells produced consistently larger cartilage aggregates than BMSCs from the same patients [68]. The synovial fluid of arthritic knees of patients has been reported to contain MSCs [69]. However, knowledge about these synovial fluid MSCs, their possible role in the aetiology of arthropathies and their potency for therapy is still limited. Additional support for the chondrogenic capacity of synovium-derived cell populations are developmental studies indicating that the precursor cells of the articular cartilage, marked by Gdf5, also end up in the postnatal synovium. Thus, the synovium appears to be a potential reservoir of cell populations developmentally destined to make articular cartilage and meniscal tissue [70]. This may be critical in the tissue organization and tissue integration aspects, important for long-term clinical durability.

Furthermore, multi-potent cells have been isolated from a variety of different sources like human amniotic fluid [71], umbilical cord [72] and tendon [73]. The potency of these cells for cartilage repair has not been demonstrated and no studies using these cells for human cartilage repair have been published until now.

Finally, pluripotent cells are also considered as a cell source for cartilage repair. Embryonic stem cells (ESCs) have potential as a source of cartilage due to their ability to be expanded almost indefinitely. Next to ethical concerns, the critical issues to overcome with any ESC application are to direct the cells along the differentiation pathway of interest to the exclusion of other pathways. Directing ESCs down the chondrogenic pathway in significant numbers has been a major challenge, but co-culture of human ESCs with adult-derived chondrocytes [74], hypoxic culture conditions and BMP-2 and BMP-4 have been shown to be chondro-inductive [75]. A hypertrophic phenotype and calcification of the

matrix have been described when ESCs are induced into the chondrogenic lineage [76, 77], indicating that ESCs may also be pre-programmed towards the hypertrophic phenotype. Genetic engineering is one option that could be used to direct ESCs towards hyaline cartilage, such as the transduction of ESCs with SOX5, SOX6 and SOX9 which induced stable production of hyaline cartilage in mouse ESCs [27]. It should be noted that this same combination of transcription factors was able to induce hyaline cartilage formation in human MSCs and dermal fibroblasts, which negates most advantages gained from using ESCs as a cell source. Perhaps the most promising result for ESCs to date is a study where 85% of the hESCs incubated with TGF $\beta$ 1 and  $\beta$ 1-integrin activating antibodies and subsequently co-cultured in transwells with bovine chondrocytes, differentiated towards chondrocytes, without signs of teratoma formation in nude mouse implants and no expression of type X collagen by RT-PCR [78].

Thus, although ESCs have chondrogenic potential and advances are being made in producing larger numbers of chondrogenic cells, the technology has not reached a level that is practical for tissue engineering purposes and ethical considerations still remain. Technological developments in stem cell biology may provide alternatives to ESCs. Successful cloning and reprogramming of adult animal cell nuclei by somatic cell nuclear transplantation (SCNT) or nuclear transfer (NT) provides stem cells tailored to the donor organism. In 2006, adult somatic cells were reprogrammed to regain pluripotency by transduction of four transcription factor to produce the so-called induced pluripotent stem cells (iPS) [79]. This breakthrough technique was further improved [80–82] and by the end of 2007 Yamanaka and his team managed to make the first human iPS [83]. Even though this is a great achievement, the random insertion of the dedifferentiation-initiating genes into the host cell's DNA remains a problem, and the method itself increases the risk of initiating cancer. Before these cells can be used as patient-specific cell therapy it will be crucial to characterize the cells exhaustively with respect to purity, potency and toxicity. Once implanted, cells cannot be removed anymore, and some may reside in the host lifelong.

## Regulating cellular activities

Immediately after the choice of cell source comes the question of cell regulation. How should we stimulate cells *in vitro* or *in vivo* or can we trust that the cells know what to do themselves such that they will be directed by the local environment?

There are several options to investigate and optimize these critical issues. First, the selection of specific cell populations characterized by preferably *in vivo* function relevant to the clinical application deserves more attention. For instance, cells that are able to produce stable hyaline-like cartilage can be selected from a pool of isolated cells by different approaches such as adapting cell culture conditions or using membrane markers. Dell'Accio *et al.* [24]

published that the capacity to form stable cartilage by articular chondrocytes was lost upon passaging and that this can be monitored using molecular markers such as FGF receptor 3. This approach to select a subset of cells from a more heterogeneous population to optimize the outcome deserves attention for other cell sources as well.

Furthermore, cells (either progenitor cells, or dedifferentiated chondrocytes) can be stimulated to differentiate towards the chondrogenic lineage and/or to produce cartilaginous matrix by addition of growth and differentiation factors or by application on or in biomaterial scaffolds. This approach can be taken *in vitro ex vivo* during expansion procedures or the manufacturing of the implant or directly *in vivo*. In the latter case, the combination implant is more complex in many aspects as it contains both cells and growth factors.

## Influence of growth factors on chondrogenic differentiation

Growth factors can be used to induce and enhance chondrogenic differentiation. Members of the TGF- $\beta$  superfamily have been demonstrated to be important in chondrogenic differentiation of expanded chondrocytes [84]. To avoid cartilage donor site morbidity, replicative chondrocyte dedifferentiation and aging (senescence) during *in vitro* expansion, attempts were made to stimulate mesenchymal stem cells (MSCs) to differentiate to cartilage during 3–4 weeks in the presence of cell culture medium containing transforming growth factor- $\beta$  (TGF- $\beta$ ) [85–88]. TGF- $\beta$  was also used to induce chondrogenic differentiation of progenitor cells derived from sources such as adipose tissue, synovial tissue and periosteum. BMPs, other members of the TGF- $\beta$  superfamily are also known to maintain the chondrogenic phenotype and induce chondrogenic differentiation of progenitor cells [88–90] and synergistic effects of BMPs and TGF- $\beta$  on chondrogenic differentiation were also reported [88]. TGF- $\beta$  has also been used together with FGF-2 [91], growth and differentiation factor-5 [92], IGF-I [93], or as part of platelet-rich plasma (PRP) [94], although IGF-I [95] and BMP-2 [96] and -7 [97] may also work alone.

Addition of growth factors during the expansion phase has been shown to be able to significantly improve the chondrogenic potential. Expansion of articular chondrocytes in the presence of various growth factors, such as a combination of TGF- $\beta$ 1 [90, 98], TGF- $\beta$ , FGF-2 and PDGF (BB) [99], TGF- $\beta$ 1 and FGF-2 [100], or 100 ng/ml of FGF-2 in defined medium [101] supported the preservation and re-expression of the cartilage phenotype better than culture conditions without these growth factors. A similar effect of FGF-2 on preservation of chondrogenic potential after culture expansion was demonstrated in BMSCs [102], while a similar combination of TGF- $\beta$ 1, FGF-2 and PDGF (BB) that was effective for increased expansion of human articular chondrocytes (above) was also useful for human auricular and nasal chondrocytes, but not for costal chondrocytes [36].

## Influence of growth factors on matrix deposition

Many studies have attempted to improve the quality of the extracellular matrix produced by chondrocytes using growth factors or other soluble compounds. Growth factors typically used in cartilage tissue engineering and matrix repair include IGF-I, FGF-2, TGF- $\beta$ , BMP-2, BMP-4, BMP-5 and BMP-7, the latter also known as OP1. Other less commonly used growth factors are FGF-9 and FGF-18.

IGF-I (or somatomedin-C) was described to have beneficial effects on cartilage matrix generation, it stimulated chondrocytes to deposit more collagen and proteoglycans [103, 104] and it is known to inhibit matrix degrading enzymes [105, 106]. IGF-I was even more potent in inducing matrix production when combined with BMP-7/OP-1 [107]. The use of IGF-I has been studied in *in vivo* models for cartilage repair, where it improved cartilage morphology, including increased collagen type II staining [95, 108].

FGF-2 inhibited the stimulatory effects of IGF-I [109] and stimulated production of matrix degrading enzymes [104, 109]. In *in vivo* models, FGF-2 appeared to have beneficial effects on cartilage repair [110, 111]. Even though cartilage morphology improved when FGF-2 was administered to cartilage lesions *in vivo*, analysis of matrix produced *in vitro* showed the opposite effect. This might be explained by the positive effect FGF-2 has on the phenotype of chondrocytes as mentioned earlier [112, 113] or by inhibition of aggrecanase activity as result of IL-1 [114]. Less is known about FGF-18 but evidence exists that it had beneficial effects on matrix production and cartilage repair, both *in vitro* and *in vivo* [115, 116].

TGF- $\beta$  is pleiotrophic and its effect strongly depends on the micro-environment. Addition of TGF- $\beta$  to chondrocytes in alginate did not affect matrix deposition but lowered the number of cross-links per collagen molecule [104]. When added to chondrocytes in monolayer, TGF $\beta$  stimulated the production of collagen that was heavily cross-linked [117]. In addition, it was shown to lower the production of cytokine-induced matrix degrading enzymes [48, 105, 118]. In general, intra-articular injection of TGF- $\beta$  led to an increase in proteoglycan content in articular cartilage although contrasting results also have been reported (see [119] for review). This raises the important issue discussed below that the effects of the growth factors could differ depending on the context and microenvironment, *e.g.* different repair results could ensue in traumatic and degenerative cartilage lesions.

BMP-2, -4, -5 and -7 influence matrix deposition by inducing collagen and proteoglycan production in cartilage explants [90] and in chondrocytes cultured in alginate [120–124]. Overexpression of BMP-2 *in vivo* induces matrix turnover and thereby contributes to the intrinsic repair capacity of damaged cartilage [125]. In addition to its strong pro-anabolic activity, BMP-7 has very prominent anti-catabolic properties [123]. Animal studies have demonstrated that this factor has the ability to repair cartilage *in vivo* in various models of articular cartilage degradation (see [107] for review). When BMP-7 and BMP-2 are combined in a chondrocyte alginate culture, they work synergistically [122].

Next to the effect of one or two defined growth factors, a pool of various growth factors in PRP demonstrated the potency to increase collagen production in chondrocyte cultures [126]. The possibility to use autologous blood to make PRP is one of the prominent advantages of this approach and it is currently investigated in clinics for a large range of disorders to stimulate tissue repair.

## Supportive effect of biomaterials

There is a tendency to move from ACI technologies to the use of CP using cell-seeded scaffolds, as they appear to be more 'user-friendly'. However, the characterization and manufacturing of a 3D implant is more complex. Also, it is very likely that the mechanisms and timed events of tissue repair are quite different when compared to the use of cell suspensions.

There is a large variety of both natural and synthetic scaffolds available. The variety of polymers and fabrication techniques available continue to expand. Pore size, porosity, interconnectivity, biocompatibility, shape specificity and degradation characteristics all influence the quality and/or amount of cartilage formed. Although many different scaffolds have been used for cartilage tissue engineering, not much is known about how matrices influence the quality of the matrix generated. Large differences in chondrogenic effects between matrices were described [127–131].

Hunziker and colleagues [132] reported that during normal development articular cartilage is physiologically reorganized by a process of tissue resorption and neof ormation, rather than by internal remodelling [132], suggesting that formation of cartilage matrix *in vitro* before implantation might not be the best approach for long-term durability. Testing of this hypothesis *in vivo* is important because it has important consequences for the direction of future applications. Therefore, it might be better to implant *ex vivo* expanded cells (either with or without scaffold) and to deliver a soluble factor or a mixture of factors, intra-articularly thereby stimulating the cells to create a piece of hopefully anisotropic cartilage on the site where it is needed. The factors already identified in the above-mentioned *in vitro* research might prove very useful here. To locally deliver the growth factors, controlled release systems may be necessary. Carriers that release factors range from nanoparticles to complex three-dimensional scaffolds, membranes for tissue-guided regeneration, biomimetic surfaces and smart thermosensitive hydrogels. This is an important and growing area of research in regenerative medicine.

Examples of such a controlled release systems are emulsion-coated scaffolds that release TGF- $\beta$ 1. By varying the copolymer composition used as coating, the release rate of TGF- $\beta$  could be precisely tailored from 12 to more than 50 days [133]. A poly lactic-co-glycolic acid (PLGA) microsphere system also seemed to be a functional device for the delivery of growth factors such as BMP-7 during the cultivation of articular chondrocytes [134]. Also systems have been designed for the controlled release of two factors [135, 136].

We have focused here on regulation of chondrogenic differentiation and matrix production. However, newer visions on cell

therapy aim at cells secreting modulating factors to stimulate the intrinsic repair capacity of the cartilage or to reduce the local inflammatory reactions to enable tissue repair indirectly [56, 60]. Results in cardiovascular research showing paracrine effects of factors secreted by MSCs are promising [137].

## **Interference of inflammation with cartilage repair**

In healthy joints, homeostasis exists. When this stable equilibrium of cartilage, bone, ligaments and synovium in a well-functioning biomechanical system is disturbed, a myriad of inflammatory factors and cellular responses come into play. The harmful effects on cartilage repair of biomechanical factors, such as malalignment, lack of menisci and ligamentous insufficiency, have been recognized and correction of these conditions at the time of cartilage repair procedure is recommended. Much less attention has been paid to inflammation and its eventual harmful effects on the repair process. Inflammation in the medical jargon is often considered as the source of evil, leading to classical symptoms and signs of inflammation, *rubor, tumour, dolor, calor and functio laesa*. Inflammatory pain and autoimmune inflammation in the medical setting often require treatment with non-steroidal, steroidal and biological anti-inflammatory drugs. In contrast to this pragmatic medical view, inflammation can be more broadly considered as an essential phase in tissue repair and regeneration and as an integral part of almost any host response to any noxious physical or chemical stimulus. The avascularity of the cartilage and, therefore, its inability to mount an effective inflammatory response, is considered to be the main reason for the poor repair capacity of the cartilage. This is in some cartilage repair methods overcome by drilling, abrasion or lately in particular microfracturing of the subchondral plate. These techniques result in a fibrin-rich clot of platelets (which from their alpha granules release repair factors like vascular endothelial growth factor VEGF, TGF- $\beta$ 1, PDGF) and recruitment of autologous BMSC or progenitors to fill smaller < 2.5–3 cm<sup>2</sup> cartilage defect. In a way, marrow stimulation is based on the provocation of inflammation, which leads to mobilization of endogenous bone marrow derived MSCs.

Inflammation is mediated mainly *via* pro-inflammatory cytokines, which may interfere with the chondrogenic and anabolic effects of growth factors used to stimulate chondrocytes and/or MSCs. Pro-inflammatory cytokines are considered to exert catabolic effects and to stimulate proteinases, which degrade extracellular cartilage matrix. Inflammation-induced *functio laesa* impairs cartilage tissue repair, maturation and vertical and lateral integration of the transplanted cells. Correspondingly, the most common complications of the procedure are symptomatic hypertrophy of the regenerated cartilage, disturbed fusion of the graft at the edges, partial graft failure as a result of too little cartilage production and delamination of already formed repair tissue. Several biomarkers have been described, which can be used to monitor inflammation and its consequences.

So far, ACI (ACI with a periosteal patch, ACI-P, or with a collagen cover, ACI-C) is perhaps optimally performed to repair critically located, relatively large >2 cm<sup>2</sup> (1–15 cm<sup>2</sup>), focal cartilage defects caused by trauma in otherwise healthy cartilage in young athletes. Treatment of such defects might have prognostic value in the prevention of secondary osteoarthritis later in life. In young patients, both the systemic and local milieu are relatively healthy, although the trauma and defect as well as implantation and implanted cells by themselves cause some inflammation that will affect repair [138]. Indeed, many of the clinical studies mentioned above reported that results were better in younger than older patients. High age and diabetes may compromise results of chondrocyte implantation and chondrogenesis also *via* increased production and accumulation of advanced glycation end-products (AGE). In immature cartilage under 20 years of age, the concentration of AGEs is low, but CML, CEL and pentosidine (different AGEs) increase 27-fold, 6-fold and 33-fold upon maturation, respectively [139]. This suggests that cartilage remodelling, *via* production of extracellular matrix, is much faster before the age of 20 years than later in life, when AGEs rapidly accumulate as a function of time. Acting through their receptor (RAGE), AGEs have immune-inflammation modulating properties but also impair expansion and trans-differentiation of MSCs to chondrocytes [140]. In the clinical setting, however, the effect of age *per se* on the outcome of various ACIs is not clear yet [141, 142].

In spite of the fact that pro-inflammatory cytokines are associated with enhanced remodelling, pro-inflammatory cytokines such as tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) or interleukin-1 $\beta$  (IL-1 $\beta$ ) have rarely been added to the chondrogenic inductive cell culture media, mainly because it is still a challenge to produce high-quality repair cartilage. Poor production of extracellular matrix and tissue destruction (catabolism) are locally present in patients even 1 year after ACI [143]. This local inflammation can be aggravated by the foreign body reaction caused by bioresorbable tissue engineering scaffolds [144] or by the iatrogenic inflammation eventually evoked by the vectors used for gene transfer to promote chondrogenesis. Pro-inflammatory cytokines are expected to be harmful for cartilage repair, therefore, in the clinical setting patients with cartilage damage due to arthritis are not considered to be eligible for (M)ACI although the progressive nature of the underlying disease is usually mentioned as the reason for a rather poor result in specific patients. To prove this paradigm, it would be necessary to show that, *e.g.* TNF antibodies or soluble TNF-receptors and IL-1 receptor antagonist exert synergistic effects together with the chondrogenic stimuli mentioned above. Attempts in this direction using *in vitro* co-culture systems suggest that the harmful effect paradigm holds [108, 145].

Pro-inflammatory cytokines and pro-apoptotic factors may be induced by local inflammation and also by the processing and storage of the cells and tissues prior to implantation [146]. It has been demonstrated that some chondrocytes used in chondrocyte transplantation undergo apoptosis [147], which may be due to a unique process known as chondroptosis. Therefore, the modulation of factors that interfere with this process may be of relevance to cartilage repair. Greater resistance to nitric oxide induced



apoptosis *in vitro* was seen after transfection of an anti-apoptotic Bcl-2 gene [148] and after chondrocyte transplantation *in vivo* after transduction of an anti-apoptotic FNK factor [149]. Chondrocytes and MSCs require matrix interaction to avoid anoikis, which is the form of apoptosis developing when mesenchymal cells lose their contact with extracellular matrix. Improved attachment to extracellular matrix reduces chondrocyte apoptosis [150]. Apoptosis of MSCs undergoing chondrogenesis in 3D alginate scaffolds was diminished with low-intensity ultrasound, which seemed to up-regulate several anti-apoptotic genes [151]. Second and third generation ACLI might help to avoid this form of chondrocyte apoptosis as the cells are seeded in or on a matrix.

TGF- $\beta$ 1 has recently been shown [152] also to protect osteoarthritic chondrocytes against apoptosis induced by a combination of TNF- $\alpha$  and mitogen-activated kinase phosphatase-1 inhibitor. Normal chondrocytes obtained from cadavers with no history of joint disease were not protected from apoptosis by TGF- $\beta$ 1 alone, but were protected from apoptosis if TGF- $\beta$ 1 treatment was done together with a phosphatase 2A inhibitor. These experiments show that TGF- $\beta$ 1 can be used to protect chondrocytes from apoptosis, but also that osteoarthritic chondrocytes behave differently from normal healthy chondrocytes [152].

What then are the markers that might indicate that chondrogenic differentiation of cells and cartilage repair tissue is jeopardized? Probably they overlap to a large extent with the mediators and biomarkers used to assess osteoarthritis, which is often referred to as primarily a disease of the hyaline articular cartilage. These markers not only include pro-inflammatory cytokines, proteolytic enzymes (MMPs and ADAMTSs) and connective tissue degradation products, but also anabolic growth factors, proteinase inhibitors (TIMPs) and markers indicating synthesis of extracellular matrix components. This may be due to the fact that the intricate feedback systems always lead to tissue response upon damage as an attempt to reinstall the body's homeostasis. Some of these markers, often used in osteoarthritis research, but probably suitable and relevant for cartilage regeneration studies as well, are given in Table 1.

## Tracking of transplanted cells *in vivo*

To further explore the therapeutic potential of regenerative treatment protocols for cartilage repair, appropriate animal models are necessary that allow tracing the fate of naive or manipulated donor cells in the host. In the field of cartilage regeneration, mainly two cell labelling approaches have been used thus far.

(1) One possibility is to use 'physicochemical labels' such as certain dyes or magnetic nanoparticles. An additional advantage of magnetic nanoparticles is that they can potentially be used in clinical settings because they are non-toxic and detectable by high-resolution MRI. Magnetic nanoparticles have been used both *in vivo* and *in vitro* in different cell types relevant for cartilage repair, where they have been shown not to affect cell behaviour [153–155]. A drawback of magnetic particles for histological

cell tracking is the fact that the specimens cannot be decalcified, because the decalcification process also removes the iron-containing particles. Another important disadvantage of cell labelling with particles or dyes is that the marker is diluted by cell divisions, and may be taken up by macrophages when transplanted cells are phagocytized [156]. For example, the membrane dye PKH26 was used to label chondrocytes in a goat study with ACLI and the data indicated that the labelled, thus implanted, cells contributed to the repair tissue [157]. However, although some labelled cells were still visible after 2–3 months, most of the labelling was gone by this time-point. It is unclear whether this observation was due to loss of labelled cells or due to marker dilution by cell proliferation.

(2) Because of the shortcomings of particles or dyes, the most widely used approach to label cells is to introduce marker genes into the genome of the cells under investigation by transducing cells *ex vivo* with a marker gene, or by using cells from transgenic donor animals with a stable genetic marker. The advantage of a stable genetic marker is that it will also be found in the progeny of the transplanted cells. Therefore, use of cells from transgenic donor animals is probably the most robust way of exploring the long-term fate of transplanted cells. The most widely used genetic markers in regenerative treatment protocols for cartilage repair are *Escherichia coli*  $\beta$ -galactosidase *lacZ*, and green fluorescent protein (GFP) from the jellyfish *Aequorea Victoria*. In a rabbit model of full thickness cartilage defects, muscle-derived cells and chondrocytes transduced to express *lacZ* were detected up to 4 weeks post-transplantation in the defects [158]. In GFP-transfected chondrocytes, using a retroviral vector, transgene expression and the number of implanted chondrocytes remained stable for at least 8 months *in vitro* and for 4 weeks *in vivo* [159]. In a goat model of osteoarthritis induced by ACL resection and total medial meniscectomy, MSC transduced to express GFP were found in the neomeniscal tissue, 6 weeks after intra-articular injection, but not at 20 weeks post-transplantation [160].

Although widely used, *lacZ* and GFP have several important disadvantages for histological cell tracking, especially in hard tissues. *LacZ* is heat labile, and requires pre-embedding staining [161]. Although GFP is an excellent marker for tracking of living cells [162], its fluorescent properties are greatly diminished during normal paraffin embedding, and sensitive detection of GFP in histological sections requires immunohistochemical detection methods [163]. Therefore, frozen sections are mostly used for histological analysis of GFP-labelled cells. However, preparation of frozen sections from hard tissues is technically extremely demanding. In addition, tissue autofluorescence is a major confounding factor in hard tissues. Recently, human placental alkaline phosphatase (hPLAP) has been described as highly suitable marker enzyme for studies involving genetically labelled cells in hard tissues [164]. This marker protein retains its enzymatic activity not only after paraffin embedding but also after a modified methylmethacrylate (MMA) embedding protocol [165], and can be detected by histochemistry and immunohistochemistry in a very sensitive fashion [164]. The availability of a genetic marker

**Table 1** Potentially suitable markers indicating that chondrogenic differentiation of cells or cartilage repair tissue is jeopardized

Name	Abbreviation	Specification
Soluble and intracellular factors		
Interleukin-1	IL-1	Pro-inflammatory molecules
Tumour necrosis factor- $\alpha$	TNF- $\alpha$	
Interleukin-6	IL-6	
Oncostatin M		
High mobility group box-1	HMGB-1	
Bone morphogenetic proteins	BMP	Anabolic factors
Calcitonin		
Fibroblast growth factor-2	FGF-2 or bFGF	
Growth and differentiation factor-5	GDF-5	
Insulin-like growth factor-I (= somatomedin C)	IGF-I (SmC)	
Transforming growth factor- $\beta$	TGF- $\beta$	
Inducible nitric oxide synthetase	iNOS	Enzyme producing nitric oxide
MAP kinases	MAPK	Intracellular signal transducing factors
Nuclear factor $\kappa$ B	NF- $\kappa$ B	
Prostaglandins	PGE <sub>2</sub> etc.	Cause vasodilation and increase vascular permeability leading to swelling and sensitization and stimulation of the nociceptors
Caspases and various pro- and anti-apoptotic factors		Factors involved in the internal and external effector apoptosis pathways and its regulation
Proteinases		
Metalloproteinases able to degrade extracellular matrix	MMP-1, -2, -3, -9 and -13, MT1-MMP	Degrade collagen and/or gelatine, in particular in neutral pH, but can also degrade other components of the cartilage matrix
Tissue inhibitors of MMPs	TIMP-1 etc.	Inhibit, stabilize or target the action of the MMPs
Cathepsin K	Cat K	Degrades effectively collagen, in particular under acidic conditions
A disintegrin-, metalloproteinase- and trombospondin-containing molecules	ADAMTS-4 and -5 etc.	Degrade in particular the proteoglycans (aggrecans, the ground substance of the cartilage) in the cartilage extracellular matrix
Collagen and other structural components of the cartilage		
Type II <sup>1</sup> , III, IX, XI collagens		Cartilage supporting fibres
Cartilage oligomeric protein COMP and other collagen decorating molecules <sup>2</sup>		Regulate the thickness of the collagen fibres and cross-link them to other molecules, store differentiation and growth factors
Hyaluronan and hyaluronic acid binding protein HABP		Act as the core molecule in proteoglycans in the cartilage and form water binding hydrogel in the synovial fluid
Aggrecans		Water binding molecules in the ground substance of the cartilage
Degradation products		
IICTP, IINTP, CTx-II		Degradation products of type II collagen
Depolymerization products of hyaluronan		Depolymerization is also reflected in a diminished viscosity of the synovial fluid

<sup>1</sup> Production is measured by measuring the concentration of the amino- and carboxyterminal globular propeptides released from the procollagen molecules before they can organize into the near quarter-stagger.

<sup>2</sup> In cartilage decorin/PGII, biglycan/PG I, fibromodulin, lumican, PRELP (Proline arginine-rich end leucine-rich repeat protein), mimecan (osteoglycin, osteoinductive factor) and chondroadherin.

suitable for the detection of labelled cells in hard tissue sections may significantly advance the field of cell therapy for cartilage repair in the future.

Another important problem that has hampered the preclinical development of regenerative treatment protocols for cartilage repair is the issue of immune-mediated rejection of labelled cells in immunocompetent hosts. During recent years, it has become quite clear that membrane or even intracellular expression of any foreign protein, and, thus, of any marker protein, will elicit immune-mediated rejection of transplanted cells carrying the marker gene in the recipients [166–170]. Even though joints may be immune-privileged, immune-rejection might seriously interfere with the validity of animal models aimed at testing the usefulness of cell therapy, especially in long-term studies. Most investigators could not detect a large number of labelled cells in cartilage defects after a few months in immunocompetent hosts [157, 158, 160]. It is currently unknown whether this is due to loss of the marker gene, slow immune-mediated rejection of labelled cells, or replacement of transplanted cells by cells of host origin. Because inflammatory processes are crucial in the progression of osteoarthritis, immunocompetent animal models are highly desirable.

The problem of immune-mediated rejection of labelled cells can be circumvented by the use of genetic DNA markers such as the non-expressed TK-tsA cassette [171]. Also, cells from male donors can be tracked in female recipients by the presence of the Y chromosome. However, there is accumulating evidence that male cells can be rejected in immunocompetent females under certain circumstances. For example, skin grafts from male donors are rejected in female recipients, at least in certain mouse strains [172]. In any case, the detection of genetic DNA markers in histological sections requires *in situ* hybridization or *in situ* PCR. Although methods for *in situ* hybridization in plastic-embedded soft tissues have been described [173], these methods are very difficult to apply to plastic sections of joints in a routine and reliable fashion. Recently, a novel *in vivo* technology for long-term studying of labelled cells in the complete absence of immune-mediated rejection in immunocompetent hosts was developed by inducing specific tolerance to the marker gene hPLAP by neonatal exposure of wild-type Fischer 344 rats to cells from hPLAP transgenic Fischer 344 rats of the same inbred strain [174]. This immunocompetent marker tolerant model is almost fully equivalent to the situation in human patients in whom autologous adult stem cells or autologous chondrocytes are used for cell therapy and holds great promise to provide answers to important questions in the further development of regenerative therapeutic protocols for cartilage repair: What is the long-term fate of transplanted chondrocytes? Do MSC engraft in cartilage defects? Do cells from different sources behave differently in terms of therapeutic potential for cartilage repair? Can scaffolds improve the engraftment of transplanted cells and the therapeutic outcome? How do inflammatory conditions in a traumatic or osteoarthritic joint influence engraftment, long-term survival and differentiation of transplanted cells?

## Conclusion and future directions

In order to obtain more robust and more reproducible results in cartilage repair procedures, more basic information is needed on the processes of tissue formation and integration including the fate of the cells, cell viability and quality of the extracellular matrix. Also, variables that may affect the outcome of the clinical studies, such as patient characteristics and joint environment, should be taken into account. The role and use of factors that stimulate extracellular matrix formation (like growth factors, drugs but also biomechanical factors) as well as the vulnerability of the newly formed tissue to inflammation and degradation need more basic research.

Different applications will very likely have different optimal procedures. Cell type, culture parameters and scaffolds should be carefully selected and tested for each application. There are multiple potential sources for chondrogenic cells to engineer replacement tissue, none of which has yet been shown to be ideal. Articular cartilage has been extensively studied as a source for chondrogenic cells and autologous chondrocytes have been used in ACI procedures, but the results have shown limitations in expandability and ability to differentiate into hyaline cartilage. However, more recent modifications in growth conditions and the potential for using chondrocytes from alternative anatomic locations may overcome some of these limitations. Another promising source are the progenitor cell populations derived from bone marrow. Bone marrow stromal cells have the advantage of being very expandable but suffer from their propensity to form hypertrophic chondrocytes, which invites replacement by bone. Other sources of chondrogenic progenitor cells also show promise, such as cells from perichondrium, synovium and adipose tissue, but these sources have not been studied extensively and some might bear the same risk of hypertrophy as BMSCs. The use of long-term cultures of these cells may overcome this limitation, but carries along with it the necessity to pre-form the cartilage *in vitro* prior to implantation, as opposed to seeding cells *in vivo* prior to differentiation.

One of the most challenging aspects in this field and the field of tissue engineering in general relates to the manufacturing processes. As was seen in the past with protein therapies, it may take quite some years to come to design and implement fully automated highly robust processes for the production of biological implants. Cell suspensions are still relatively 'easy', the manufacturing of 3D biological implants will require an additional level of monitoring involving sophisticated bioreactors and biosensors. These technology developments are required to make the production of these implants affordable. Quality controlled production processes are also a prerequisite if we want to gain more insight through clinical studies.

Stringent and challenging regulatory requirements have stimulated industry to find one stage surgical procedures that operate without cell culture. An example of such is the cartilage autograft implantation system (CAIS, dePuy) where cartilage is minced and mixed with a synthetic scaffold and fixed in the defect with

resorbable staples. Animal experiments showed signs of hyaline cartilage formation [175] and clinical development is ongoing. Another development is the combination of commonly used bone marrow stimulation techniques with interactive biomaterials. Although initially the results of animal experiments appeared not very successful [176, 177], more promising results have been reported lately [178, 179].

The proof-of-the-principle study of ACI was first discovered in New York and verified in rabbits in 1989 [180]. Brittberg and colleagues [6] started with the first study in patients in the late 1980s and reported them in 1994. Since then, many patients have been treated with the ACI cell-based therapy. Although there are quite a few clinical studies at the moment, there is a need for more, well-designed clinical studies. The most used outcome parameters in cartilage repair studies are pain and function. Although from the patient's perspective these are the most important outcomes in the short term, there is a need for more objective and quantifiable outcome measures. These outcome measures should address structure/tissue-related aspects that are important for long-term outcome since studies have shown that there is no direct correlation between pain/function scores and radiographic scores [181]. Attention is now directed to expand imaging modalities such as MRI [182, 183], and to find biomarkers that predict progression or disease status. The search for the most optimal (panel of) biomarker(s) is still in progress [184], and the incorporation of biomarkers in cartilage repair studies might be instrumental in helping to identify patients at risk, responders to treatment and uncover the mechanism(s) and processes taking place over time, after cartilage repair procedures.

However, these types of clinical studies are expensive and together with support from academic, national and other non-profit resources such as the European Union, additional industrial support will be required to take this approach. In view of this, transparent regulatory requirements adapted to this field and the field of regenerative medicine in general are a prerequisite to achieve this goal.

We have started with investigating the possibility to repair traumatic defects in knee joint cartilage. However, the patient groups are often heterogeneous which complicates interpretations of the outcome of the studies. More information on the patient profile (including age, gender, weight, joint status, location of the defect, underlying cause) as well as the identification of responders to treatment, proper patient tailored rehabilitation and better information on the patients' expectations will help improving treatment outcome. In addition, other groups of patients with cartilage lesions at other anatomic sites are waiting for repair too, such as patients with cartilage defects in ankle, hip and shoulder joints. Today, it is not known whether cartilage repair procedures can be used for these joints too and if or how the outcome will differ. Also patients with (early) osteoarthritis could in the future be eligible for cartilage repair treatment. This will be more complex because of the catabolic, inflammatory status of the joints and possibly the genetic basis of the disease. At the moment, we do not know

enough about the vulnerability of the constructs when used in an inflamed joint.

Lastly, patients with non-joint cartilage defects such as nasal septum perforations, malformed auricle or defects in larynx or trachea after (repeated) airway stenosis or tumour resection are also potential patients for cartilage engineering [185]. If cartilage in the airway is to be repaired, cell injection is not an option. For these patients, we need to form a construct that has sufficient structure to keep the airway open from the moment it is implanted [186]. Because biomaterials in the airway almost always lead to inflammatory responses, the construct should be scaffold-free (at least upon implantation).

Finally, we have gained knowledge on cellular responses to stimuli as well as the experience with the clinical studies on cartilage repair that teach us more *about* the healing responses and what can be done in patients. The developments in the areas of biomaterials, cell culture, growth factors, imaging and the development of promising cell sources bodes well for the future for cartilage engineering. The challenge for the cell biologists is to develop the cell isolation and culture conditions necessary to expand and differentiate different cell sources efficiently and then, with the use of advances in the fields of biomaterials and bioreactors, to fabricate a provisional tissue or tissue intermediate into the appropriate size, shape and tissue organization. With the advent of advanced imaging capabilities, the challenge for the imaging engineers is to devise more and more effective means of assessing cartilage *in vivo*, so that all of these developing treatment modalities can be assessed accurately, and at different time-points. As these different technologies come together, the prospects of producing functional cartilage tissue for clinical applications with good long-term results become more and more likely. After the initial hype, it is time for hope. The knowledge gained in this area has raised many new questions on mechanisms, and there is a renewed and more realistic hope for improvements in technical and clinical outcomes in the years to come.

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