

Review

Major biological obstacles for persistent cell-based regeneration of articular cartilageAndre F Steinert¹, Steven C Ghivizzani², Axel Rethwilm³, Rocky S Tuan⁴, Christopher H Evans⁵ and Ulrich Nöth¹¹Orthopaedic Center for Musculoskeletal Research, König-Ludwig-Haus, Julius-Maximilians-University, Würzburg, Germany²Department of Orthopaedics and Rehabilitation, University of Florida, Gainesville, FL, USA³Institut für Virologie und Immunbiologie, Julius-Maximilians-University, Würzburg, Germany⁴Cartilage Biology and Orthopaedics Branch, National Institute of Arthritis, and Musculoskeletal and Skin Diseases, National Institutes of Health, Department of Health and Human Services, Bethesda, MD, USA⁵Center for Molecular Orthopaedics, Harvard Medical School, Boston, MA, USACorresponding author: Andre F Steinert, a-steinert.klh@mail.uni-wuerzburg.de

Published: 5 June 2007

This article is online at <http://arthritis-research.com/content/9/3/213>

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Arthritis Research & Therapy 2007, **9**:213 (doi:10.1186/ar2195)**Abstract**

Hyaline articular cartilage, the load-bearing tissue of the joint, has very limited repair and regeneration capacities. The lack of efficient treatment modalities for large chondral defects has motivated attempts to engineer cartilage constructs *in vitro* by combining cells, scaffold materials and environmental factors, including growth factors, signaling molecules, and physical influences. Despite promising experimental approaches, however, none of the current cartilage repair strategies has generated long lasting hyaline cartilage replacement tissue that meets the functional demands placed upon this tissue *in vivo*. The reasons for this are diverse and can ultimately result in matrix degradation, differentiation or integration insufficiencies, or loss of the transplanted cells and tissues. This article aims to systematically review the different causes that lead to these impairments, including the lack of appropriate differentiation factors, hypertrophy, senescence, apoptosis, necrosis, inflammation, and mechanical stress. The current conceptual basis of the major biological obstacles for persistent cell-based regeneration of articular cartilage is discussed, as well as future trends to overcome these limitations.

Introduction**Structure and function of articular cartilage**

Articular cartilage is a highly specialized tissue that protects the bones of diarthrodial joints from forces associated with load bearing and impact, and allows nearly frictionless motion between the articulating surfaces [1,2]. The extracellular matrix (ECM) of articular cartilage is distinct from that of other connective tissues, consisting of an intricate network containing predominantly fibrillar collagens and proteoglycans. The collagens, types II, IX and XI, form a fibrous framework that gives the tissue its shape, strength and tensile

stiffness [3]. Collagen type VI is found pericellularly around chondrocytes [4], and collagen type X is found in calcifying cartilage [5]. Although collagen type I is the most prevalent collagen throughout the body, the primary constituent of the articular cartilage matrix is type II, comprising 80% to 90% of the collagen content [3]. The proteoglycans in articular cartilage in their most abundant form exist as large hydrophilic aggregates, which contain the fluid component and control its movement. The level of compaction of the proteoglycans within the collagen lattice will determine their level of hydration and, in turn, the stiffness of the articular cartilage. The synthesis, incorporation and degradation of ECM proteins are orchestrated by chondrocytes that populate the matrix at low density [3]. Because articular cartilage is avascular, nutrients for the chondrocytes are supplied from the capillaries of the synovium and must diffuse into the synovial fluid and then into the cartilage matrix. Co-ordinated synthesis and proteolytic breakdown of certain ECM components by chondrocytes enables certain components of the cartilage matrix to undergo turnover and maintenance [3]. Factors that impair chondrocyte function can disrupt the equilibrium of synthesis and catabolism in favor of cartilage degradation, which over time can lead to osteoarthritis (OA) [6,7].

Capacity of articular cartilage for repair

As a result of injury or disease, articular cartilage frequently incurs damage, but has very limited ability to regenerate. In chondral defects, where a lesion is contained within the articular cartilage, there is no involvement of the vasculature.

ACT = autologous chondrocyte transplantation; BMP = bone morphogenetic protein; ECM = extracellular matrix; FGF = fibroblast growth factor; IGF = insulin-like growth factor; IHH = indian hedgehog; IL = interleukin; IL-1Ra = IL-1 receptor antagonist; MMP = matrix metalloproteinase; MSC = mesenchymal stem cell; NO = nitric oxide; OA = osteoarthritis; PTHrP = parathyroid hormone related peptide; RA = rheumatoid arthritis; SOX = SRY (sex determining region Y)-box; TGF = transforming growth factor; TNF = tumor necrosis factor.

Consequently, progenitor cells in blood and marrow cannot enter the damaged region to influence or contribute to the reparative process. Resident articular chondrocytes do not migrate to the lesion, and no production of a reparative matrix occurs. Thus, the defect is not filled or repaired and essentially remains permanently [1,2].

Osteochondral cartilage defects have suffered damage to both the chondral layer and bone plate, causing the rupture of blood vessels and ingress into the bone marrow. In this case, a repair response is initiated and generally begins with a hematoma that forms when blood escapes the damaged vasculature or marrow and enters the lesion [1,2]. The fibrin network within the hematoma traps platelets that, in turn, release various bioactive factors, including platelet derived growth factor (PDGF) and transforming growth factor (TGF)- β , that stimulate vascular invasion and migration of undifferentiated mesenchymal progenitor cells into the fibrin clot [1,8]. Within several days of injury, the progenitor cells proliferate and may or may not differentiate into chondrocytic cells that synthesize cartilaginous matrix. The resulting repair tissue, however, is poorly organized and contains significant amounts of collagen type I. This fibrocartilage tissue is mechanically inferior and breaks down with time and loading, becoming fragmented or disintegrating altogether [1,2,8].

Present status of cartilage repair

The specialized architecture and limited repair capacity of articular cartilage, coupled with the high physical demands placed upon this tissue, make it exceedingly difficult to treat cartilage injury medically. Currently, there exists no pharmacological agent that promotes the healing of articular cartilage lesions, whether chondral or osteochondral. Thus, physicians have attempted various surgical methods to restore articular surfaces, which fall into three broad classifications.

The first type of approach entails the use of mechanical penetration of the subchondral bone by abrasion arthroplasty, Pridie drilling, or microfracture to disrupt the vasculature and marrow. This results in a large clot that fills the defect and enables the natural repair response to form fibrocartilage repair tissue, which is subordinate to normal cartilage in terms of mechanical properties [1,2,8,9]. However, these procedures are cost effective and clinically useful, as patients often have reduced pain and improved joint function, and are, therefore, generally used as first-line treatment for focal cartilage defects [9-12].

The second approach involves attempts to regenerate hyaline cartilage repair tissue through transplantation of tissues such as periosteum, perichondrium or osteochondral grafts [2,8,13,14]. Although short term results have been positive for a number of patients, the long term clinical results are uncertain, with tissue availability for transplant, especially in large cartilage defects, being a major limitation [1,2,8]. Therefore, the autologous chondrocyte transplantation (ACT)

procedure has been developed and used clinically since 1987, in combination with a periosteal cover, to treat chondral or osteochondral defects of the knee with good clinical results [12,15-17]. However, for the first generation of ACT, various adverse events have been reported, including graft failure, delamination, tissue hypertrophy and chondromalacia [18,19]. In order to overcome some of these impairments, modern modifications of this procedure involve embedding chondrocytes in a three-dimensional matrix before transplantation into cartilage defects [19-21]. To date, only a few clinical studies have been conducted comparing ACT with other repair procedures for large defects, and these have shown no major differences in the respective outcomes [22-26]. Despite these advances, with most surgical interventions resulting in improvement of clinical symptoms such as pain relief, none of the current treatment options have regenerated long-lasting hyaline cartilage tissue to replace damaged cartilage [1,2,12,16].

Tissue engineering approaches that deliver a matrix seeded with chondrogenic cells (chondrocytes or progenitor cells) and chondrogenic factors have also been evaluated experimentally, with basically the same long term overall results *in vivo* thus far [27,28]. Despite good *in vitro* data with different approaches, the reasons for the failure of cell-based cartilage repair approaches to form hyaline repair tissue *in vivo* remain largely unclear to date [2,27-29]. Although proof of principle has been provided that implanted chondrocytes can contribute to structural cartilage repair, the origin of cells persisting at the repair site, whether these are the originally transplanted cells or migrated ones from the subchondral marrow or the adjacent synovium, has not been entirely clarified yet [2,30,31]. Emerging data are elucidating not only the fate but also the function of the transplanted cells at the repair site, for example, suggesting that transplanted mesenchymal cells not only function as repair cells but also as trophic mediators that stimulate the surrounding tissues toward repair [32].

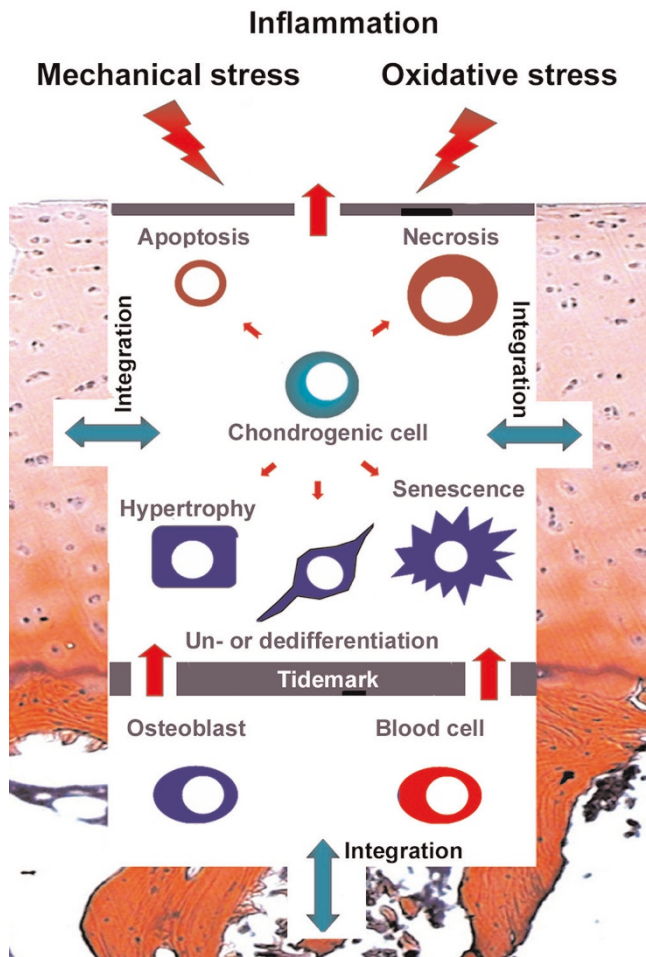
Biological obstacles to hyaline neocartilage formation comprise differentiation insufficiencies, loss of transplanted cells or tissues, matrix destruction and integration failures (Figure 1), which all can occur due to various reasons. Insights into these mostly interrelated mechanisms might help us to systematically direct therapeutic approaches toward the formation of a more hyaline cartilage repair tissue.

Differentiation cues

Cartilage development and chondrogenesis

During development, the process of chondrogenesis gives rise to the formation of cartilage and bony skeletal tissues. Chondrogenesis is typically initiated at sites of skeletal element generation by localized proliferation of mesenchymal cells guided by multiple growth factors and morphogens such as Wnts, TGF- β s and fibroblast growth factors (FGFs) [33,34]. Subsequent aggregation and condensation of these

Figure 1



Biological obstacles associated with cell-based approaches to cartilage tissue engineering. Formation of hyaline neocartilage can be hindered due to loss of transplanted chondrogenic cells by cellular efflux, apoptosis or necrosis, differentiation insufficiencies, including fibroblastic, hypertrophic or osteogenic differentiation (red arrows), matrix destruction by mechanical, oxidative and/or inflammatory stressors (red flashes), as well as integration failures within the cartilage and/or bone compartment (green arrows) of the defect.

cells is mediated by both cell-cell (neural cadherin and neural cell adhesion molecule) and cell-matrix (hyaluronan and CD44, fibronectin, proteoglycans and collagens) adhesion, as well as numerous intracellular signaling pathways transduced by integrins, mitogen activated protein kinases, protein kinase C, lipid metabolites and cyclic AMP and so on [35-40]. At this stage, the undifferentiated mesenchymal cells produce an ECM enriched with collagen type I, hyaluronan, tenascin and fibronectin. Mesenchymal cells in the precartilage aggregate and begin to synthesize and express molecules associated with chondrogenic differentiation, followed by their differentiation into chondroblasts and synthesis of extensive ECM containing proteoglycan and cartilage-specific collagen types II, IX, and XI [33,34].

Synthesis of collagen type I is reduced, as is expression of the cellular adhesion molecules. During development of the limbs and other bony tissues, chondrogenesis is gradually replaced by endochondral ossification. The chondrocytes within the cartilaginous tissue mature, hypertrophy and express collagen type X with reduced production of type II. The cartilage becomes vascularized and is infiltrated by osteoprogenitor cells. The chondrocytes undergo apoptosis, while the osteoprogenitor cells differentiate into osteoblasts and replace the cartilage with mineralized bone tissue [33,34]. During the developmental process a portion of the chondrocytes remains nonhypertrophic and expands to form the permanent cartilaginous tissues [33,34]. In the adult cartilage tissue, however, many of these developmental mechanisms are lost or only partially restored once the tissue is injured and repaired. Although adult cells, such as chondrocytes and mesenchymal stem cells, show fundamental biological differences compared to embryonic stem cells, including their limited potential to self-renew, they have been shown to be capable of greater plasticity than previously expected and to retain their potential to undergo chondrogenesis following considerable expansion *in vitro* [27,41-44]. A further understanding of the differentiation capacity of these cells, especially under aged and arthritic conditions, might be key to the successful application of adult chondrogenic cells in cartilage repair without evoking the ethical and legal issues associated with the use of embryonic stem cells [28,41,45-47].

Un- or dedifferentiated chondrogenic cells

Still a matter of debate is whether to transplant a fully *in vitro* differentiated construct or a graft containing a homogeneous population of rather undifferentiated cells and signaling molecules, to ensure that the desired differentiation process takes place *in vivo* under physiological conditions of mechanical loading [2]. Each approach has its advantages and disadvantages. The former allows chondrogenic differentiation under controlled *in vitro* conditions, but is often associated with biocompatibility and integration problems [48,49]. The latter promotes integration well, but presents the risk of uncontrolled and undesired differentiation processes to occur [48,50]. Various cell types have been used to repair cartilage lesions, including chondrocytes, perichondrial or periosteal cells, and mesenchymal progenitor cells from bone marrow and other sources.

Chondrocytes

As mentioned above, only autologous chondrocytes are used in clinical practice. Human articular chondrocytes lose their chondrogenic phenotype upon monolayer expansion and change their morphology to a fibroblastic appearance [51]. However, dedifferentiated chondrocytes can re-differentiate toward the chondrogenic lineage in three-dimensional culture, particularly after conditioning by growth factors such as FGF-2, epidermal growth factor (EGF), TGF- β , or PDGF-BB during monolayer expansion [52-54]. The same is

observed for constructs generated from OA chondrocytes, except with reduced rates of collagen production compared to constructs made from non-OA chondrocytes [55]. Next to age and underlying disease, a limited number of cell doublings is also important to sustain proper commitment in chondrocytes [42,45], while extensive *in vitro* expansion of articular chondrocytes resulted in loss of *in vivo* cartilage formation [56].

Chondrocytes have been employed in conjunction with different matrix materials and growth factors *in vitro* and cartilage repair approaches *in vivo* (for reviews, see [2,29,57,58]). In each instance, improved repair was reported for the respective treatments, with the lesion being filled to a greater volume of tissue compared with untreated controls; however, the repair tissue generated was mainly fibrocartilaginous in nature. The transplanted chondrocytes or chondroblasts were concluded to have a beneficial effect on the spontaneous repair response, over and above that elicited by the matrix itself [2,27-29].

Perichondrial/periosteal cells

Cells derived from periosteum and perichondrium have been shown to have considerable chondrogenic potential [59]. These cells reside within the proliferative stratum of the cambial layer of the perichondrium and periosteum, respectively, and have been isolated for tissue engineering purposes [59]. The chondrogenic potential of perichondrial and periosteal cells for their use in cartilage repair has been tested extensively *in vitro* and *in vivo* following seeding into various matrices [2]. However, the need for two surgical interventions, the highly variable results achieved in experimental studies thus far, as well as the long-term instability of the repair tissue formed, may have prevented this approach from entering clinical practice to date.

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multilineage progenitor cells responsible for the turnover and repair of mesenchymal tissues, such as bone, cartilage, ligament, muscle, and fat [41,44,60,61]. Although no clear definitive phenotype of MSCs has been described, through the use of the proper culture conditions, expanded MSCs can be stimulated to differentiate along specific pathways, such as chondrogenesis, adipogenesis, and osteogenesis [41,43,60,61]. With regard to cell-based cartilage repair approaches, MSCs provide an attractive alternative to chondrocytes. Unlike mature chondrocytes, which must be surgically harvested from a very limited supply of non-weight-bearing articular cartilage, MSCs are relatively easy to obtain from bone marrow, and will maintain their multilineage potential with passage, enabling considerable expansion in culture [8,29,32,44,46,62,63]. For the purposes of cartilage repair, extensive analyses of the appropriate microenvironment to stimulate MSCs toward chondrogenesis *in vitro* have been performed. MSCs are commonly isolated by adherence to

cell culture plastic or density-gradient fractionation, and, therefore, represent a heterogeneous population of cells [41,44,46]. Positive selection for chondroprogenitor cells has been achieved by conditioning media with growth factors such as FGF-2 or TGF- β during monolayer expansion [64]. Although no definitive MSC marker has been identified, selection protocols for the same immunophenotype within the MSC population might also be employed to improve chondrogenic phenotype stability, with STRO-1, CD73 and CD106 among the most promising candidates for positive selection and CD11b, CD45, CD34, CD31 and CD117 among those for negative selection [44,46].

Key steps in using MSCs for articular cartilage repair include the development of effective methods to stimulate MSCs toward chondrogenesis, maintenance of an articular cartilage phenotype without ossification or fibrinogenesis, and a delivery system to localize the cells within a lesion without inhibiting chondrogenic differentiation or the integrity of the repair tissue [2,8,27-29,44]. Building from these results, transplantation of chondrogenic grafts or chondrogenesis in osteochondral defects *in vivo* have been attempted with varying levels of success [2,8,27-29,44,65,66]. Such systems have been crucial to the elucidation of cell signaling pathways during chondrogenesis and the contributions of specific factors to this process, which include growth and adhesion factors, as well as mechanical stimuli [2,27-29,44]. However, although short term success in generating hyaline-like cartilage repair tissue after weeks *in vivo* has been demonstrated, the long-term stability of the tissue formed has been questioned, which is not surprising given the inferior fibrocartilaginous nature of the retrieved tissue after longer time points [2,27-29,44]. For the treatment of bone defects, bone-marrow stromal cells within three-dimensional constructs have already been applied to humans [67]. The first clinical results of the transplantation of autologous bone marrow stromal cells for the repair of full-thickness articular cartilage defects have also been reported [68,69]. Three patients were treated with collagen gel MSC-constructs, which were covered with a periosteal flap, and fibrocartilaginous defect filling was found after one year, as well as a significantly improved patient outcome in the respective follow-ups after one, four and five years [68,69].

Chondrocyte hypertrophy and osteogenesis

During development of the limbs and other bony tissues, chondrogenesis is gradually replaced by endochondral ossification as described above, with indian hedgehog (IHH), parathyroid hormone related peptide (PTHrP) and Wnt/ β -catenin pathways among the main regulators of these processes [33,34,70]. These regulatory mechanisms can be partially recapitulated *in vitro* by using chondrocytes or chondroprogenitor cells under the appropriate high-density three-dimensional culture conditions [27,28,42,44]. Although it was shown that *in vitro* chondrogenesis without significant levels of end stage hypertrophy is possible by using normal

articular chondrocytes [71,72], data from *in vitro* chondrogenesis using MSCs [72-81] or OA chondrocytes [47,82,83] together with members of the TGF- β superfamily reveal a significant level of chondrocyte hypertrophy, indicated by high levels of collagen type X expression. Although the use of collagen type X as a marker of chondrocyte hypertrophy in MSC-based systems has been questioned [84], it correlates well thus far with the existing *in vivo* data. For example, MSCs genetically modified to express bone morphogenetic protein (BMP)-2 show a significant level of tissue hypertrophy and osteophyte formation when transplanted orthotopically to osteochondral defects [85] or ectopically [79,86] in small animal models. Correspondingly, TGF- β 1 has been shown to induce fibrosis in joints of nude rats when it was directly delivered by first generation adenovirus [87]. Furthermore, implantation of chondrocytes genetically modified to express BMP-7 has been shown to generate good hyaline cartilage repair tissue after 6 weeks *in vivo*, but only bad results have been reported after one year, with loss of 72% to 100% of the transplanted cells [88]. We argue that this might be attributed to mechanisms of hypertrophic differentiation and subsequent apoptosis, although experimental proof is not yet available.

Possible strategies to avoid hypertrophic differentiation of neocartilage constructs might be based on the use of cells that are not subjected to high doses of TGF- β superfamily members, but are to their inhibitors, such as noggin, chordin [89,90] or that are modified using small interfering RNA-mediated knock down approaches, while chondrogenesis may be achieved via different pathways (for instance SOX9). Other potent molecules that might be considered for the facilitation of cartilage repair include IHH, a member of the hedgehog family of cell surface-associated ligands. IHH is expressed in prehypertrophic chondrocytes of the growth plate and functions to inhibit chondrocyte hypertrophy by maintaining expression of PTHrP through a negative feedback loop [91,92]. Altering the expression of such proteins during chondrogenesis may serve to delay the onset of hypertrophy and formation of bone, while increasing the pool of proliferating chondrocytes. Indeed, addition of PTHrP has been shown to inhibit chondrocyte hypertrophy during *in vitro* chondrogenesis of primary, adult MSCs [81,91,93-100]. Hypertrophic differentiation of chondrocytes, as well as bone formation, is also regulated by the Wnt/ β -catenin pathway [101,102]. Specifically, recent developmental biology studies indicate that the transition from a nonhypertrophic to a hypertrophic chondrocyte appears to be under the control of the canonical Wnt signaling pathway (Wnt-5a) [34,70,102-104]. These molecules have also been shown to be involved in *in vitro* chondrogenesis of adult MSCs, making these molecules attractive targets for a possible therapeutic use [101,102,105].

With regard to osteogenic infiltration from the underlying subchondral bone, adequate sealing structures in replace-

ment of the tidemark (for example tissue sealants or semi-permeable polymers) could be employed when treating osteochondral defects in order to avoid the entry of osteoblasts and red blood cells within the cartilage part of the lesion (Figure 1; see also 'Integration failures' below) [2,20].

Senescence

Another mechanism limiting the function of transplanted cells for cartilage repair is aging or senescence. Articular chondrocytes and MSCs, like all human cells, can only undergo a finite number of replications *in vitro*, a phenomenon known as Hayflick's limit or replicative senescence [106-109]. Cellular senescence is associated with many molecular mechanisms, including telomere erosion and oxidative damage caused by O₂, H₂O₂ and tert-butylhydroperoxide, leading to activation of p53, retinoblastoma gene and insulin-like growth factor (IGF)/Akt pathways [106-109]. In articular cartilage tissue, the number of chondrocytes decreases with increasing age, chondrocyte function deteriorates, and the ability of the cells to maintain or restore the tissue declines [110,111]. In parallel, senescent cells accumulate with age, and produce degradative enzymes and pro-inflammatory cytokines, which can disrupt the tissue structure and consecutively decrease tissue function [110,111]. The cells synthesize smaller aggrecans and less functional link protein, leading to the formation of smaller, more irregular proteoglycan aggregates [110,111]. Chondrocyte mitotic and synthetic activities decline with age, as well as age related responses to anabolic cytokines [110,112]. In parallel, chondrocyte and MSC telomere length declines and senescence-associated β -galactosidase expression increases with advancing age, suggesting that senescence contributes to the age-related deterioration of chondrocyte function, limiting their use for cartilage repair approaches [45,109,112-114]. Mediators that also promote premature stress-induced senescence of resident and transplanted cells include ethanol, ionizing radiations, mitomycin C, or excessive mechanical stress, and have been associated with age-related pathologies such as OA, which also involves pro-inflammatory cytokines such as IL-1 or tumor necrosis factor (TNF)- α [110,112,115,116]. Thus, senescent cells might contribute to aging and age-related pathology by stimulating tissue remodeling and/or local inflammation, which would compromise tissue structure and function. Telomerized cells have been used for tissue engineering applications [117], and other measures to inhibit replicative senescence have also been proposed [118]. It remains to be seen if such measures can be used to improve the outcome of cartilage repair approaches, especially when dealing with aged cells and patients [112].

Delivery of chondrogenic factors and gene therapy

With regard to cell-based therapies for articular cartilage regeneration, it is widely thought that exposure of chondrogenic cells (chondrocytes, periosteal/perichondrial cells,

MSCs) to specific stimuli that promote chondrogenic differentiation and maintenance of the chondrocyte phenotype could significantly enhance the repair potential of this type of procedure and improve the clinical outcome. Potentially useful in this respect are members of the TGF- β superfamily, including TGF- β 1, 2, 3, and several BMPs, IGF-1, FGFs, and EGF, among others (reviewed in [29,66]). Another class of biologics that may be useful in cartilage differentiation repair are transcription factors that promote chondrogenesis or the maintenance of the chondrocyte phenotype. SRY (sex determining region Y)-box 9 (SOX9) and related transcription factors L-SOX5 and SOX6 have been identified as essential for chondrocyte differentiation and cartilage formation [99,100,119]. Signal transduction molecules, such as SMADs, which are mediators of TGF- β and BMP signals, are also known to be important regulators of chondrogenesis [120,121]. However, the short half-lives of recombinant proteins and a lack of effective delivery methods for intracellular acting molecules hinder the clinical application of these factors.

Gene transfer offers an alternative approach to protein delivery that may satisfactorily overcome the limitations of conventional methods [66,95,122,123]. By delivering cDNAs that code for therapeutic proteins to specific target cells, the genetically modified cell is converted to a biofactory for protein production [66,122,123]. Through the localized delivery of gene transfer vectors or genetically modified cells to specific sites of cartilage damage, sustained protein synthesis can be concentrated at the site of injury or disease with minimal collateral exposure of non-target tissues [66]. Gene transfer approaches *in vivo* using various transgenes, including IGF-1, BMP-2, BMP-7, FGF-2, and SOX9, accelerated the healing of cartilage defects in various repair models [66,85,88,119,122-133]. Although short-term success in generating hyaline cartilage repair tissue after six weeks *in vivo* has been demonstrated, long-term *in vivo* analyses indicate only unsatisfactory results [66,88,123]. The possible causes are reviewed in this article. For a successful gene therapy approach for cartilage repair, the mode of delivery, level and duration of transgene expression, as well as the type and dosage of vectors used, have to be well considered [66,123]. For example, ubiquitous intra-articular expression of strong anabolic transgenes such as TGF- β 1 can cause severe fibrosis in joints [87], and excessive adenoviral loads are inhibitory to chondrogenesis of primary MSCs [134].

Loss of transplanted cells or tissues

As adult human articular cartilage is avascular and thought to be a post-mitotic tissue, with virtually no cell turnover and resident cells being encased within the dense extracellular matrix, there is no compensatory mechanism for loss of native and transplanted chondrogenic cells. In cartilage defects, cells can be lost due to leakage of cell suspension, apoptosis and necrosis.

Delivery, surgical and biomaterial issues

Surgical procedures such as tissue harvest for the ACT procedure or lesion-edge conditioning (performed to improve the fitting of an implant) inevitably affect healthy cartilage tissue surrounding the lesion site. These procedures are associated with an extensive margin of apoptosis and/or necrosis along the cut surfaces that is likely to create an unfavorable biological environment for graft integration, with scalpel margins being less affected than trephine wounds [2,48]. When periosteal flaps are used in first generation ACT procedures, significant problems were reported, including delamination, loss of flaps and cell suspension [18,135].

Because of this and other reasons, chondrocytes have been incorporated in various matrix materials before transplantation in newer generations of ACT, including collagen and hyaluronic acid [19,136-138]. Various other scaffold materials have also been tested *in vivo* and *in vitro* for cartilage tissue engineering purposes (extensively reviewed elsewhere [2,19,20,27,29,139-142]). From a biological perspective, beneficial material features would include not only a three-dimensional environment for differentiation and biocompatibility (no signs of cytotoxicity, apoptosis, senescence), but also would promote the release of factors supporting one or more biological aspects of repair, as outlined in other chapters of this review. Such so called third generation biomaterials have been manufactured for tissue engineering purposes [143-145]. For example, polylactic acid scaffolds have been engineered to release recombinant BMP-2 in a sustained fashion, which was sufficient to induce a favorable chondrogenic response *in vitro* [146]. However, the ideal material properties for a successful cartilage regeneration approach *in vivo* remain to be defined, including the biomechanical stability of the matrix, kinetics of resorption, selection of bioactive factor, cellular target(s) and mechanism of stimulation [142,146-148]. Given these imperfections, a guided biomaterial development focusing on cell and cartilage tissue specific requirements seems desirable.

Apoptosis and necrosis

As pointed out earlier, various stressors can affect neo-cartilage formation and integration, including pro-inflammatory cytokines such as IL-1 or TNF- α , nitric oxide (NO), free radicals and oxidants, mechanical stress, glucocorticoids and others [53,149]. Depending on the cell type, as well as dose and type of stressor, the transplanted cells will react in different ways. For instance, a high, cytotoxic dosage of a stressor could cause a level of damage such that cellular biochemical activities decrease, leading to cellular death by necrosis. The level of damage sustained by cells determines whether programmed cell death (apoptosis) can unfold or, if the damage is lower, senescence can occur [150,151]. Since even after *in vitro* selection, transplanted cell populations are not homogeneous, the dose of the stressor will shift the percentage of cells executing each of the possible programs - cellular proliferation or inflammation, senescence (see above), apoptosis, and necrosis [152].

As outlined above, apoptosis naturally occurs during limb development following end stage terminal hypertrophic differentiation of chondrocytes, before osteoprogenitor cells infiltrate the cartilage anlagen and chondrogenesis is gradually replaced by endochondral ossification. Furthermore, it has been suggested that apoptosis of chondrocytes during development may act to control chondrocyte number in cartilage tissue, and current evidence indicates that chondrocyte apoptosis is involved in OA pathogenesis [153-155]. However, in cartilage repair, apoptotic cell death is obviously an undesired phenomenon, either when it occurs within the transplant or along with necrosis at the cut lesion edges of cartilage [48,156]. Apoptosis is characterized by specific hallmarks, such as loss of cellular membrane asymmetry, cell shrinkage, release of cytochrome c, chromatin condensation and degradation of nuclear DNA to 180 base-pair oligomers, with caspases 3, 7, 9 and others mediating the apoptotic signaling program [109,153,157,158]. *In vitro*, chondrogenic cells respond to the same apoptosis-inducing signals as other cells, including FasL/CD95, TNF- α , TRAIL (TNF-related apoptosis inducing ligand), IL-1, matrix metalloproteinase (MMP)-9, calcium and phosphate ions, NO, ceramide, retinoic acid, or serum deprivation and mechanical forces [150,154,159]. For example, TNF receptor mediated signaling simultaneously stimulates pathways for apoptosis induction via caspases and pathways for suppression of apoptosis via nuclear factor (NF)- κ B [109,150,154]. Thus, the balance of these two signaling pathways determines the fate of cells after TNF- α stimulation. Interestingly, BMPs, specifically BMP-2, -4 and -7, have been identified as key regulators inducing apoptosis during digit development, which was reversed by the BMP antagonist noggin [160]. Next to the avoidance of stressors named above, the use of apoptosis inhibitors that directly bind and inhibit caspases, such as Bcl-2 or Bcl-XL, might be of therapeutic value in order to expand the lifespan of transplanted and resident cells [160-162]. Remarkably, it has also been shown that the dedifferentiation of chondrocytes, as well as the inhibition of apoptosis, is regulated by the Wnt/ β -catenin pathway [34,163]. Under arthritic conditions, Wnt-7a induces dedifferentiation of articular chondrocytes by stimulating transcriptional activity of β -catenin, whereas NO-induced apoptosis is inhibited via the activation of cell survival signaling, such as phosphatidylinositol 3-kinase and Akt, which block the apoptotic signaling cascade. Whether this mechanism can be harnessed therapeutically for the maintenance of chondrocyte populations, capable of building hyaline neocartilage, remains to be elucidated [163].

Besides induction of apoptosis, where cells can be eliminated without inflammation, cell death can be mediated by necrosis following a significant inflammatory response, due to the leakage of cell contents. Whereas the term 'necrosis' in the strict sense refers to changes secondary to cell death by any mechanism, including apoptosis, it is used here to describe a caspase-independent mode of cell death,

as used previously by other groups [164-166]. Necrotic cell death is characterized by swelling of the cells and organelles, uncontrolled release of lysosomal enzymes and nuclear condensation (pycnosis) [164-166]. Thus, necrosis for cartilage repair is especially detrimental because of the associated inflammatory response, which interferes with chondrogenesis and the integrity of the cartilage matrix. The avoidance of cytotoxic stimuli during cartilage repair procedures, such as trephine wounds, needle stitches, and others (see above), is of ample importance for a successful repair, as well as anti-inflammatory measures where necrosis cannot be completely avoided.

Given the lack of cell supply in cartilage, major cell loss in cartilage and neo-cartilage will inevitably lead to tissue failure, as chondrogenic cells are the only source of matrix synthesis in these tissues. Furthermore, recent studies are beginning to indicate a central role of the collagen framework in the maintenance of cartilage cells. However, whether cell loss is primary or secondary to cartilage matrix destruction is still a matter of debate [154,167].

Matrix degradation

Damage to the cartilage ECM can arise focally after acute cartilage injury in an otherwise healthy joint, or from an underlying disease process, such as in OA or rheumatoid arthritis (RA).

Mechanical factors

As mentioned above, mechanical factors are integrally involved in cartilage matrix integrity, as well as survival and death of chondrogenic cells in native cartilage as well as repair tissues [157,168,169]. Quantitative analyses revealed that cartilage repair tissue after ACT has inferior mechanical properties compared to healthy cartilage in a large animal model [170,171]. *In vitro* studies using bovine cartilage explants showed that compressive stress, as low as 4.5 MPa, was able to induce apoptosis, while other parameters of matrix degradation, such as disruption of the collagen fibril network, tissue swelling, release of glycosaminoglycans, and increased nitrite levels, were apparent only at higher stress levels [172]. Beyond a threshold of 6 MPa, cell viability is inversely proportional to the applied stress [173]. In contrast, physical parameters have also been identified that aid the cartilage tissue quality *in vitro* [174-176] and *in vivo* after cell-based cartilage repair, comprising intermittent active or continuous active/passive motion and other techniques (reviewed in [2]). Therefore, post-operative care and physical therapy also play important roles in determining the healing outcome.

Inflammation and degeneration

Joint trauma, cartilage repair responses with or without different matrix materials, and diseases such as OA or RA can cause joint inflammation of varying intensities. The cytokines involved, including IL-1 and TNF- α , have direct and indirect pro-inflammatory effects to regulate the degradation

of ECM components by stimulating secretion of proteolytic enzymes and other mediators, such as MMPs, aggrecanases and other members of the 'a disintegrin and metalloproteinase with thrombospondin motifs' (ADAMTS) gene family, NO, and prostaglandin E₂ [149,177,178]. The importance of IL-1 is reinforced by the fact that it has been shown to inhibit chondrogenesis in chondrogenic cells *in vitro* by downregulation of chondrocyte differentiation state marker genes, such as those encoding SOX9, collagen type II and others, while upregulating NO, prostaglandin E₂ and MMP-3, and the finding that the effects could be inhibited by the addition of the IL-1 receptor antagonist (IL-1Ra) [179-181]. However, much of this work was derived from research studying OA and RA [179,182-184]. Cell-based cartilage repair in OA knees has been attempted, with equal results compared to cartilage repair procedures in non-arthritic knees [50]. Inhibition of pro-inflammatory cytokines such as IL-1 may offer a useful supportive approach to the management of cartilage injury by reducing gene expression of genes involved in cartilage matrix degradation, and thus favoring a beneficial repair environment for transplanted chondrogenic cells [132,185-187]. The application of anti-inflammatory cytokines is hindered by delivery problems. For example IL-1Ra is available as the drug 'Kineret' for the treatment of RA, and is self-administered at a daily dose of 100 mg by subcutaneous injection, because it is neither feasible nor safe to deliver IL-1Ra by repeated intra-articular injection [179,184]. This treatment leads to a peak IL-1Ra serum concentration of approximately 1 µg/ml, which is transient; the concentrations of IL-1Ra achieved in synovial fluid are unknown but they are likely to be low and also transient, explaining the limited success thus far using this drug in RA [179,184]. Gene delivery strategies to overcome these delivery issues have been devised [188-193], with a phase I clinical study having been completed for the treatment of RA [194,195] and an ongoing study for the treatment of OA [196]. Should these technologies prove to be safe and effective in the future, their use could be beneficially extended to the area of cartilage repair.

For treatment of local defects the use of growth factors, anti-inflammatory cytokines or matrix molecules offers much promise as an approach to promote hyaline cartilage repair [29,66,122,197]. Since local defects are contained within healthy cartilage, it is likely that expression of certain growth factor and chondroprotective genes will be required for the length of time necessary to promote cartilage healing. The potential for gene therapy to promote cartilage repair in chronic diseases like RA and OA is more limited, due to more extensive cartilage loss, and altered metabolic responses of OA and RA chondrocytes.

Integration failures

One of the main biological challenges associated with cell-based cartilage repair procedures is integration of the graft tissue with the host. Depending on the nature of the cartilage

injury, whether it is a chondral or osteochondral lesion, different aspects of transplant integration with cartilage and bone have to be appreciated.

With respect to integration of cartilage with cartilage, the nature and status of the tissue that comprises the wound lesion edge is central to tissue integration. As mentioned above, controlling aspects of cell differentiation, apoptotic or necrotic cell death together with matrix synthesis is the main factor influencing successful integration. Despite some cell migration potential from cartilage tissue in young experimental animals [49,198], chondrocytes appear to have only a limited ability to infiltrate existing cartilage matrices and even to occupy empty chondrocyte lacunae [48]. However, integrative repair and chondrogenesis is not only a function of gross matrix synthesis, but is also affected by adjacent tissue structure and composition [199].

Another aspect is that blunt trauma to cartilage induces a greater proliferative response that extends to a greater distance from the lesion edge compared to burst and sharp trauma. However, in the case of sharp trauma, the basal cells enter proliferation before surface zone chondrocytes, which is not the case in blunt wounds [48]. Several measures have been used to promote this process by establishing good contact between cartilage transplants and native articular cartilage, including the use of collagen cross-linkers, brief enzymatic digestion and biological glues (tissue transglutaminase and other adhesives; reviewed in [2]).

With respect to the repair of osteochondral defects, two distinct types of tissues, articular cartilage and subchondral bone, are involved. In designing a multiphase implant, the healing of the underlying subchondral region of the defect site is critical as it supports the overlying neocartilage regeneration [49,200-202]. In order to protect the cartilage graft from stromal cell invasion from the underlying bone and dedifferentiation in osteochondral lesions, there have been attempts to mimic the tidemark with the use of biological sealants [20]. However, there have not been any controlled studies to date to validate this procedure. Initial studies have been performed to generate biphasic transplants in order to promote graft integration with the underlying bone *in vitro*, with a tidemark being already integrated [49,200-202]. Also, initial *in vivo* studies regenerating the subchondral layer by using MSC-seeded poly-ε-caprolactone scaffolds have been performed with successful results [203]. Although the fabrication of neocartilage with an appropriate zonal organization has been attempted [204,205], exact reproduction of the different zones of native articular cartilage, including the tidemark and underlying bone, has not been achieved to date. A fruitful interplay between cells, morphogens and scaffold technology will be crucial for successful cartilage-like patterning of transplants for successful integration. Optimization and development of these approaches will determine whether constructs are applicable for partial- or full-thickness defects.

Table 1**Therapeutic strategies to augment cell-based cartilage repair**

Impairment	Pathomechanism	Therapeutic strategy	References	
Differentiation insufficiencies	Un- or dedifferentiated cells	Timed growth factor release systems/gene delivery		
		TGF- β superfamily members	[76,85,88,134]	
		FGFs	[131]	
		SOX/Smads	[119-121,124-126]	
	Hypertrophic differentiation	Cell selection		
		Growth factor selection	[52-54,64]	
		Immunophenotype selection	[41,46]	
		Inhibition of hypertrophy		
		BMP inhibitors: noggin, chordin, siRNAs	[89,90,160]	
		PTHrP/IHH	[81,91-95]	
Osteogenesis	Wnt5a	[29,103,163]		
	No dexamethasone	[75]		
	Inhibition of osteogenesis			
Senescence	BMP inhibitors (noggin, chordin), siRNAs	[89,90,160]		
	Establishment of a barrier/tidemark to bone	[49,198]		
	Senescence protection			
	Age	[107,110]		
	Telomere erosion	[117]		
	Oxidative damage	[115,117,118,151,187]		
Chemical stress	Anti-oxidative selenoproteins, superoxide dismutase	[115,117,118,151,187]		
	Anti-inflammatory agents (IL-1Ra, sIL-1R, sTNFR)	[132,185-188,190,191,193-195]		
Mechanical stress	Mechanoprotection	[157,168,169]		
Cell loss	Inefficient cell delivery	Guided, homogeneous cell delivery	[2,48]	
		Apoptosis		
		(NO induced, stress)		
	Necrosis	Anti-apoptotic measures		
		Bcl-2, Bcl-XL, anti-FasL	[151,161,162,164]	
		Anti-inflammatory agents (see also above)	[132,185-188,190,191,193-195]	
		Anti-oxidative agents	[115,117,118,151,187]	
		Age	[2,48]	
		Surgical protection (no needle stitches, no unnecessary harm to cartilage lesion borders)		
		Mechanical stress	[2,48;168,169]	
Chemical stress	[132,185-188,190,191,193-195]			
Oxidative stress	[115,117,118,151,187]			
Matrix degradation	Matrix degradation	Delivery of matrix components	[197]	
		Inflammation	[132,185-188,190,191,193-195]	
	Mechanical stress (shear stresses, compressive forces)	Anti-inflammatory agents (IL-1Ra, sIL-1R, ICE inhibitor, sTNFR, anti-TNF-antibodies, TACE inhibitor, TIMP-1, -2, MMP inhibitors, IL-4, -10, -11, -13, GFAT)		
		Mechanoprotection		
		No trauma	[2,48;168,169]	
		Avoidance of non-physiological loads	[1,2,8,9,12,20]	
Establishment of correct knee axis and stability	[1,2,8,9,12,20]			
Antioxidants	[115,117,118,151,187]			
Integration	Cartilage to cartilage	Cartilage matrix crosslinks	[20,49,200-202]	
		Tidemark formation	[49,198]	
	Cartilage to bone	Stimulation of cell migration	[27,29,66]	
		Chondroblasts above tidemark Osteoblasts below tidemark		

BMP, bone morphogenetic protein; FasL, Fas-Ligand; FGF, fibroblast growth factor; GFAT, fructose-6-phosphatase amido transferase; ICE, IL-1 converting enzyme; IHH, indian hedgehog; IL, interleukin; IL-1Ra, IL-1 receptor antagonist; MMP, matrix metalloproteinase; NO, nitric oxide; PTHrP, parathyroid hormone related peptide; sIL-1R, soluble IL-1 receptor; siRNA, small interfering RNA; SOX, SRY (sex determining region Y)-box; sTNFR, soluble TNF receptor; TACE, TNF-alpha converting enzyme; TGF, transforming growth factor; TIMP, tissue inhibitor of matrix metalloproteinases; TNF, tumor necrosis factor.

Conclusions and future perspectives

Cell-based approaches to cartilage repair have only been introduced to the clinic by means of the ACT procedure, with good clinical results, but are still far from generating a repair tissue that is comparable to native cartilage in terms of tissue

quality and stability. Although current treatments have limited effectiveness, an improved clinically useful repair approach does not necessarily have to demonstrate complete regeneration of normal tissue. The aim of this review is to critically analyze the current clinical and experimental

cartilage repair approaches from a cell and molecular biological perspective in order to encourage the development of more rational approaches to repair. Considering the main biological obstacles to cartilage repair as outlined in this review (Figure 1), an ideal construct would contain hyaline differentiated tissue, and become fully integrated with the adjacent cartilage and bone, without the induction of an inflammatory response, senescence, apoptosis or necrosis. This aim may be overly optimistic to achieve in the near future, but the enabling technologies exist to address these issues. Efficient delivery of chondrogenic, anti-inflammatory and anti-oxidative factors seem to be of key importance (Table 1). As most of these factors are recombinant proteins, which have short half-lives, a repeated local administration is likely to be necessary to achieve the desired result, creating delivery problems. Gene transfer techniques could be adopted that might help us to overcome the limitations of the current treatments for damaged articular cartilage. Nonetheless, what remains to be determined are the exact factors needed for hyaline repair, including their bioactive level and duration of expression to meet the complexities of treating this tissue.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

This work is supported by grants from DFG (STE1051/2-1 to AFS and UN), IZKF (D-23/1 to AFS and AR), and NIH NIAMS (Intramural Research Program Z01 AR41131 to RST, and grants AR48566 and AR50249 to SCG and CHE). We apologize to investigators whose work could not be cited due to space limitations.

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