TITLE: Cartilage Tissue Engineering for Degenerative Joint Disease

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Abstract

Pain in the joint is often due to cartilage degeneration and represents a serious medical problem affecting people of all ages. Although many, mostly surgical techniques, are currently employed to treat cartilage lesions, none has given satisfactory results in the long term. Recent advances in biology and material science have brought tissue engineering to the forefront of new cartilage repair techniques. The combination of autologous cells, specifically designed scaffolds, bioreactors, mechanical stimulations and growth factors together with the knowledge that underlies the principles of cell biology offers promising avenues for cartilage tissue regeneration. The present review explores basic biology mechanisms for cartilage reconstruction and summarizes the advances in the tissue engineering approaches. Furthermore, the limits of the new methods and their potential application in the osteoarthritic conditions are discussed.

1. Introduction

The damage and loss of organs and tissues leads to metabolic and structural changes that can cause significant morbidity, and decrease the quality of life. Currently employed therapies for the treatments of joint tissues loss or disease are unsatisfactory as they rely on metal joints prosthesis which offer structural replacement albeit limited functionality. Furthermore, artificial implants lack tissue's physiological activities and often do not provide the lifelong solution for the patient. The field of tissue engineering (TE) has emerged over the past decades to improve the treatments for tissue and organ failure, [1-3].

The goal of TE is to provide living biological/physiological substitutes that could replace tissue loss due to disease, congenital abnormalities, or trauma. Ideally, the biological substitute should structurally and morphologically resemble native tissue and be able to perform similar biological functions. In comparison to artificial implants biologically engineered tissue may offer a better long term performance due to the enhanced biocompatibility, integration into surrounding tissues, and the ability to remodel according to the body requirements. TE can be broadly defined as the structural and functional reconstitution of mammalian tissues where the cells, biomaterials and biological cues are combined. TE is a highly multidisciplinary field that combines the knowledge from materials science, cell and molecular biology, engineering and medicine. The possibility to

apply the TE approach as a treatment for osteoarthritis is even more challenging given that the disease affects the entire joint. Therefore, the TE package should combine the restoration of normal composition and function of the damaged articular cartilage while avoiding further degeneration of cartilage and the surrounding tissue.

2. Hyaline cartilage and chondrogenic pathways

Articular cartilage is a highly specialized tissue that provides low friction and allows for efficient load bearing and distribution. The major constituents comprise specialized cells – chondrocytes, embedded in highly hydrated and organized extracellular matrix (ECM) consisting of collagens fibers and proteoglycans. The mixture of fluid and matrix provides viscoelastic and mechanical properties necessary for efficient function of cartilage tissue. Chondrocytes are the single cellular component of hyaline cartilage.

Under physiological conditions a balance between anabolic and catabolic cell activities maintains the structural and functional integrity of the ECM. This constant process is dependent of several factors, including growth factors, cytokines, mechanical loading, aging and injury. Articular cartilage has no blood vessels, it is not innervated and normal mechanisms of tissue repair perform poorly to form only fibrocartilagenous tissue. The existing therapies for cartilage repair are limited and physicians are often obliged to wait until the cartilage degeneration reaches the point where a partial or total joint replacement can be applied as a treatment. Nevertheless, depending on the age, activity level, and degree of cartilage damage, several methods to decrease pain and attempt cartilage repair have been applied. They include lavage, shaving, debridement, laser abrasion, Pridie drilling, microfracture techniques and mosaicplasty [4-6]. Novel treatment relying on cell therapies and TE has gained substantial importance in the orthopaedic field; however, the complexity of interactions between cells, matrix and other factors makes the reproduction of articular cartilage *in*

vitro extremely challenging. Therefore, it is crucial to understand the processes that regulate chondrogenesis in order to attempt regeneration of the adult tissue through a defined stimulation of specific signaling pathways.

During limb bud development in the embryo, chondrogenesis of mesenchymal cells is regulated through cell-cell adhesion (condensation), cell-cell matrix interaction, biomechanical signals and a diversity of growth factors including TGFβ superfamily members- bone morphogenic proteins (BMPs), fibroblasts growth factor (FGF), epidermal growth factor (EGF), insulin-like growth factor (IGF)-1, parathyroid hormone-related peptide (PTHrP) and Indian hedgehog (Ihh) [7, 8]. BMPs are involved in almost every aspect of chondrogenesis, from commitment to terminal differentiation via regulation of PTHrP/Ihh and FGF pathways in the growth plate [9, 10]. BMPs promote Ihh expression triggering the Ihh/PTHrH negative feed-back loop that regulates the onset of chondrocyte hypertrophy. FGFs are another group of molecules that cross-talk with BMPs during chondrogenesis. FGF antagonizes BMP-4 induced chondrocyte differentiation leading to reduced bone size [11]. Additionally, FGF-2 inhibits Ihh expression, promotes hypertrophic differentiation and suppresses chondrocyte proliferation [12]. IGF-1 is expressed in the condensing region of the developing cartilage as well as in mature cartilage and synovial fluid. IGF-1 also enhances matrix synthesis *in vitro* and *in vivo* [13-15]. Furthermore, *in vitro* administration of exogenous IGF-1 blocks interleukin-1 induced degradation pathways of proteoglycans in chondrocytes [16, 17]. The complex interconnected pathways involved in chondrogenesis, cartilage maintenance, and the progression of cartilage degeneration have yet to be unraveled. Further understanding is necessary to selectively interfere or control these pathways in order to enhance the long-term stability and function of implanted TE cartilage in treating not only injured, but also diseased tissue.

3. Osteoarthritis

3.1. Etiology of OA

Osteoarthritis (OA) is a debilitating, progressive joint disease often associated with the aging process. It represents a combination of several disorders in which biomechanical properties of cartilage are altered leading to tissue softening and ultimately degradation [18]. The main characteristic of OA is an imbalance between chondrocyte anabolic (synthesis) and catabolic (resorptive) activities. The degenerative process may be initiated with the loss of proteoglycans from the ECM followed by disruption of collagenous fibrillar network leading to cell apoptosis/necrosis and deterioration of the functional tissue. Given that OA is a pathology encompassing articular cartilage, subchondral bone, ligaments, capsule, and synovial membrane, before any thought can be given to the TE approach, it is necessary to understand the pathways that underline the degenerative processes.

The etiology of OA, although not fully understood, is comprised of several interconnected factors: age, programmed cell death (apoptosis), local inflammatory processes and mechanical stress.

3.2. Age related changes – chondrocytes senescence and apoptosis

Articular cartilage undergoes age-related changes that increase the risk of joint degeneration leading to the development of OA [19, 20]. These changes include structural and biochemical matrix reorganization, surface fibrillation, alteration in proteoglycan composition, increased collagen linking and decreased tensile strength and stiffness [21]. There are well described cellular changes associated with aging in different tissues that could also explain the decline of cartilage function [22]. The ability of chondrocytes to maintain metabolic homeostasis is shown to decline with age [20] leading to alterations in proteoglycan and collagen composition and organization [21,

23]. A decrease in cell number and/or biosynthesis could account for these observations. Indeed, decreased cell numbers have been reported in OA cartilage, although synthesis of matrix macromolecules was not altered in isolated OA chondrocytes grown in monolayer cultures [24].

Interdependent mechanisms that decrease functionality of cells with age and lead to cell senescence include cumulative oxidative damage, accumulation of mutations and genetic instability, and telomere shortening [25-27]. In chondrocytes, characteristics of aging include synthesis of smaller, more irregular aggrecans accompanied with decreased synthesis of proteoglycans, increased expression of senescence-associated β-galactosidase activity, telomere erosion, and decreased response to IGF [28-30]. The aging of chondrocytes isolated from healthy cartilage has been demonstrated by slower proliferation rates in culture of cells from healthy individuals older than 30 years [31]. In addition, glycosaminoglycan content of corresponding micromass pellet cultures was lower despite the exposure to growth factors indicating that aging also affects chondrocyte ability to respond to growth factors and re-differentiate. Furthermore, chondrocytes from normal but aged patients had a secreted protein pattern resembeling that of chondrocytes from OA patients and not young individuals [32]. Other studies have also shown that the responsiveness of aged chondrocytes to growth factors TGFβ, IGF-1 and EGF and cytokine interleukin -1α (IL-1α□□□) is altered [33-35]. In OA cartilage, expression of β-galactosidase was increased close to but not away from the OA damage sites suggesting that cell senescence plays a role in the progression of aging cartilage towards disease [36]. In the post-traumatic OA chondrocyte senescence was also accelerated [37] and freshly isolated OA chondrocytes were less responsive to IL-1β [38] further implicating cell senescence in the development of arthritis.

Programmed cell death (apoptosis) is an essential mechanism for homeostasis of all tissues. A variety of experimental models have demonstrated that chondrocyte apoptosis occurs after injurious impact, release of cytokines and nitric oxide (NO), and is related to aging [39, 40].

Although several studies have demonstrated a high rate of apoptosis upon mechanical trauma [41], the role of apoptosis in OA remains controversial [42, 43]. While some studies indicated increased rates of apoptosis in OA, linked to proteoglycan depletion from the ECM [44, 45], others have shown increased apoptosis only in the calcified cartilage layer [46]. Furthermore, apoptosis in chondrocytes may be as unique as the tissue itself; a novel term "chondroptosis" has been suggested to differentiate classical from chondrocyte-specific apoptotic pathways [47]. Whether as a result of injury or aging process, cell death and the consequent inability to repair and maintain cartilage play an important role in the development of OA.

3.3. Cytokines, oxidative damage and chemokines

Local inflammatory processes within the cartilage itself accompanied with deregulated cytokine activities, namely interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) have been shown to contribute to pathological development of OA [48, 49]. Cytokines are soluble or cell-surface molecules that play an essential role in mediating cell-cell interactions. In the normal adult cartilage their presence is low as the chondrocytes' activity is limited to tissue maintenance. In the development of OA the delicate balance between matrix synthesis and degradation is perturbed. The initial inducers of cartilage catabolism in OA have not been identified. Potential stimuli include mechanical stress [50, 51], and degradation products of ECM components including fibronectin fragments which stimulate production of matrix-degrading proteases [52-55]. IL-1 is a prototypical proinflammatory cytokine implicated in OA cartilage degradation which stimulates production of matrix metalloproteinases (MMPs). MMPs are a family of enzymes that degrade collagen, elastins and other ECM components [56]. In OA cartilage IL-1 co-localized with MMP1, 3, 8 and 13 and other proinflammatory catabolic enzymes [57]. In animal models intra-articular injection of IL-1 resulted in proteoglycan loss while the inhibition of IL-1 via IL-1 receptor antagonist (IL-1ra)

slowed the progression of cartilage loss [49]. TNF-α effects also include stimulation of degrading proteinases and suppression of matrix synthesis. Importantly, TNF-α and IL-1 exert synergistic effects in enhancing cartilage damage and inhibition of proteoglycan synthesis [58, 59]. Finally, IL-1 and TNF-α increase nitric oxide synthase leading to an increase of NO [60]. NO radicals have deleterious effect in joint cartilage including downregulation of matrix synthesis and upregulation matrix degradation via activation of MMPs [61-63]. Furthermore, NO increases chondrocyte susceptibility to oxidants while free radicals produced by NO were also shown to induce chondrocyte apoptosis [44, 64, 65]. The degradation of aggrecan, the second most abundant component of ECM is mediated through another family of metalloproteinases, aggrecanases [66, 67]. IL-17 was also demonstrated to contribute to the pathology of OA. IL-17 induces collagen degradation and NO production in human chondrocytes [68-70]. Recently, IL-17 was found to activate not only collagenases MMP 3, and MMP13 but also aggrecanase 1 and thus has been proposed as a target for reducing cartilage degradation [71]. Control of MMP activity is achieved through tissue inhibitors of metalloproteinases (TIMPs) [72]. Imbalance between MMP activity and TIMP inhibition is a characteristic of OA cartilage leading to collagen type II degradation [73].

A role for chemokines and their receptors in cartilage degradation in OA has recently been reported [74]. Chemoattractive cytokines (chemokines) are a large family of mediators of inflammation and immunity closely resembling cytokines [75]. From the major chemokines subfamilies (CXC,CC,C, CX3C) human chondrocytes can produce CC and CXC chemokines and express their corresponding receptors. Engagement of these receptors induces the release of matrix degrading enzymes such as MMP 1, 3, and 13, and N-acetyl-beta-D-glucosaminidase. Furthermore GROalpha, a CXC chemokine acting on CXCR2, can activate an apoptotic pathway in chondrocytes that leads to chondrocyte cell death. These findings suggest that chemokines can act

as an autocrine or paracrine loop on chondrocytes and can contribute to the pathological patterns of OA.

3.4. Post-traumatic causes of OA

Injurious and excessive mechanical stress can also result in depletion of proteoglycans and damage the collagen network [50, 51]. Mechanical factors leading to joint damage can be viewed as factors that either compromise joint protection or excessively load the joint [76, 77]. The first category comprises factors increasing joint vulnerability, including malalignment, muscle weakness, genetic predisposition, and aging, while the second implicates obesity, certain physical activities, and acute trauma. Posttraumatic arthritis can result from irreversible cartilage damage sustained at the time of injury, and chronic overloading resulting from articular incongruity and instability [78]. Joint injuries shown to invoke posttraumatic OA include direct and indirect joint impact loading, meniscal, ligament and joint capsule tears, and intra-articular fractures [79, 80]. The chondrocytes' response to mechanical loading is recognized as an integral component in the maintenance of articular matrix homeostasis. The eventual result of inappropriate mechanical loading is degradation of ECM although the mechanisms by which the degradation progresses initiates are not completely understood. Studies examining the contribution of chondrocyte apoptosis to matrix degradation in bovine and human cartilage explants subjected to mechanical loads representative of traumatic mechanical injury demonstrated an increase in cell death [81, 82]. It has been postulated that chondrocyte apoptosis caused by impact injury could initiate a pathogenesis similar to that observed in OA [41]. Several loading regimes have been investigated with regards to chondrocyte apoptosis and proteoglycan loss. Cyclic loading of bovine cartilage caused cell death, loss of proteoglycans and increase in matrix degradation enzyme MMP-3 [83]

while mechanical shear stress applied on human articular cartilage increased the production of oxidants leading to pre-mature senescence [28, 37].

4. Cartilage TE in OA? The need for "Tissue Re-engineering"

Joint replacement with the metal joint prosthesis represents the main treatment for the OA affected joints. An important drawback of the materials currently used in this treatment is that they do not withstand patients' physical activities and are prone to wear out, loosen and occasionally break. Thus, promoting repair very early in the degenerative process is the logical attempt to avoid joints replacement. Ideally, the biological signals should be provided in a TE package to diminish inflammatory process, initiating the reparative processes and prompting the patient's own tissue to complete the regeneration. The key constituents for successful TE are cells, a carrier such as matrix scaffold, signaling molecules and correct mechanical stimuli [84]. Scaffolds support cell infiltration, proliferation and subsequent differentiation in response to signaling molecules and mechanical stimulation, and can provide initial mechanical strength to a TE construct. Two possible approaches for tissue regeneration have been developed – preparation of cells which are subsequently injected into the lesion (with or without scaffold) allowing regeneration to occur *in vivo*, and tissue reconstruction *in vitro* whereby a ready-to-use graft is transplanted into the defect.

The first approach for cartilage TE has been termed autologous chondrocyte implantation (ACI), and has become the dominant clinical cell based therapy for the repair of cartilage lesions over the past decade. In this technique, expanded articular chondrocytes are implanted under a periosteal flap after surgical debridement of the lesion. ACI has demonstrated excellent short to mid term repair [85-88] although the evaluation of longterm repair remains somewhat controversial [4]. The second approach aims to produce neocartilaginous tissue combining cells with various biomaterials, bioreactor systems and growth factor cocktails [89-94]. Both type of cell-based

approaches comprise isolation of cells from a low-load bearing area and subsequent expansion *in vitro*. The enzymatic degradation of extracellular matrix (ECM) during the isolation procedure affects cells as demonstrated by their change in gene expression profile [95]. After initial attachment, cells start to proliferate, adopt a polygonal morphology and become fibroblast-like. Phenotypic changed are accompanied with switches in gene expression, including loss of collagens type II, IX and XI and aggrecan and concomitant upregulation of collagen type I, III and V [96, 97]. Furthermore, their surface marker profile dramatically changes towards a more mesenchymal progenitor-like cell upon prolonged *in vitro* culturing [98]. The sum of these changes has been termed de-differentiation. Several attempts to grow chondrocytes in suspension and overcome de-differentiation process have been made, including chondrocyte growth on non-adherent plastic surfaces [99], in agarose [100, 101] or alginate gels [102]. Although chondrocytes retained their capacity to produce ECM, proliferation rates were considerably impaired.

Various strategies employed to promote re-differentiation of passaged chondrocytes include cell growth in three-dimensional (3D)-like cultures such as micromass pellet cultures and natural or artificial scaffolds, media supplements including anabolic cytokines and growth factors [103], variation in oxygen tension [104] and mechanical stimulation [105].

Treatment of cartilage lesions via a TE approach has mostly been employed in small lesions resulting from traumatic injuries, and in a younger population. The situation is very different in OA. The translation of this form of therapy under degenerative conditions has not yet been successful. This can be probably explained by the extent of the disease that affects the OA joint. Thus, the TE approach should address several phenomena including blocking the production of pro-inflammatory factors and suppressing the progression of the degenerative process affecting both, cartilage and bone compartment.

At present only small and contained degenerative lesions could be tentatively treated using available TE methods. In the case of extended degenerative conditions, a treatment with currently available clinical TE procedure is insufficient and needs further development. The requirements for the treatment of extended OA lesion include resetting the entire joint local environment to the physiological baseline. To this end a "Tissue Re-engineering approach" that addresses the joint resurfacing, inflammation and mechanical issues may offer a successful tissue regeneration.

5. Cell sources for cartilage TE

To date several cell sources have been investigated as potential candidates for the cell therapy based approach for cartilage TE, including normal and OA chondrocytes and mesenchymal stem cells (MSC) derived from a variety of tissues. Embryonic stem cells represent a promising cell source but many ethical issues need to be resolved prior to their clinical application. All cell type candidates are similar in that they have lost their intrinsic "knowledge" of which tissue they need to produce, thus identification of growth factors to trigger correct signaling cascades is essential. Additionally, several parameters need to be assessed when choosing the cell type including cell availability, cellular phenotype comprising gene and protein expression, and cellular capacity to re-differentiate and produce appropriate cartilaginous ECM.

Table 1. Cell types for tissue engineering

Chondrocytes	articular
	auricular
	septal/nasal
	costal
Mesenchymal stem cells	bone marrow

	adipose tissue
	muscle tissue
	peristeum
	synovial membrane

Table 1. Cells with chondrogenic capacities to be employed for TE.

5.1. Chondrocytes

Chondrocytes are the cells of choice for all current ACI procedures. Adult chondrocytes with matrix forming capabilities have been isolated from several sources, including low load-bearing area of articular joint cartilage, as well as septal, auricular and costal cartilage [106-111]. However, due to the process of de-differentiation, growth factors are currently required to activate re-differentiation pathways leading to chondrogenesis. The best candidates that could provide appropriate signaling are molecules involved in embryonic chondrogenesis, namely members of TFGβ family, BMP-2 and BMP-7, and IGF.

Combination of basic FGF with TGF has been employed to stimulate the process of de-differentiation in rabbit chondrocytes with the idea to obtain secondary chondroprogenitor cells [112]. These chondroprogenitor cells were subsequently able to re-express chondrocyte phenotype *in vitro* and form hyaline cartilage in an *in vivo* assay. Basic FGF has been demonstrated to increase accumulation of proteoglycans of adult canine articular chondrocytes embedded in type II collagen-glycosaminoglycan scaffold [113]. Studies of the role of TGFβ-1 and BMP-2 in rat periosteal chondrocytes cultured in aggregates indicated that while cell treatment with BMP-2 alone results in hypertrophy, combined treatment lead to formation of abundant ECM [114], suggesting a role of BMP-2 in neochondrogenesis followed by terminal differentiation by TGFβ-1. The hypertrophic effect of BMP-2 was confirmed in bovine articular chondrocytes embedded in polyglycolic acid

(PGA) scaffold, while BMP-12 and BMP-13 increased growth rate and modulated the composition of engineered cartilage. The role of BMP-2, 3, 5, 6, and 7 was assessed in bovine articular chondrocytes embedded in alginate *in vitro* and in a nude mouse model [115]. BMP-7 proved the most efficient in stimulating matrix synthesis and in suppressing the infiltrative response of mouse fibroblastic cells thereby preventing transplant destruction. In human articular chondrocytes a combination of FGF and TGFβ-1 increased cell proliferation rates and also allowed for more efficient chondrocytes re-differentiation in pellet cultures [103]. Recently, the role of several prostaglandins was evaluated for chondrogenesis of human de-differentiated articular chondrocytes [116]. While PGE(2) reduced the expression of collagen type I in pellet cultures, PGD(2) and PGF(2) alpha enhanced chondrogenic differentiation and ECM production.

5.2. OA chondrocytes

Several limiting factors are associated with the use of chondrocytes from OA joints, including the number of cells that can be obtained from a diseased tissue, capacity of cells to proliferate *in vitro*, and responsiveness to growth factors necessary to trigger re-differentiation process. Chondrocyte numbers are decreased by 38% in OA cartilage as assessed histomorphometrically and via the number of isolated cells [24]. While proliferative capacity of chondrocytes in OA cartilage is increased *in vitro* and may account for chondrocyte clustering observed *in vivo*, results *in vitro* are still inconclusive given that both lower and higher proliferative rates have been reported [32, 117]. Finally, mechanical insult, joint instability and imbalance between anabolic and catabolic cytokines lead to altered cellular responses in OA chondrocytes and could make them inappropriate for reparative or regenerative therapy [43, 118].

The gene expression profiles from both healthy and OA chondrocytes indicate an increased expression of collagens without changes in their ratios [119]. In contrast, OA chondrocytes express

significantly higher levels of matrix degrading enzymes MMP1, MMP2, MMP3, MMP13 and aggrecanase-1 [38]. The most striking observation in OA chondrocytes is the synthesis of molecular markers characteristic of de-differentiated chondrocytes, namely collagen type I in the chondrocyte clusters, collagen type X in the upper zone, and re-expression of collagen type IIA [120].

Despite all the identified differences, recent data indicate that OA chondrocytes retain their differentiation potential upon isolation and proliferation *in vitro* [117]. In the micromass pellet cultures OA chondrocytes continued to proliferate for 14 days thus increasing the pellet size in contrast to normal chondrocytes. The proteoglycan production was comparable to normal chondrocytes, and the collagen-rich matrix was present, although the total collagen was significantly lower. Additionally, in a 3D-scaffold based on hyaluronic acid, OA chondrocytes were also able to produce cartilage-specific matrix proteins. These results raise hope that despite their differences in comparison to normal chondrocytes, OA chondrocytes could be employed as a cell source for TE treatment providing that the disease can be controlled. Recent data using human chondrocytes from patients with the history of trauma, demonstrated that cells exposed to a hyaluronan based scaffold reduced apoptosis and decreased gene expression as well as secretion of degradation cytokines, namely, MMP-1, and MMP-13, and NO [121]. At the same time, the expression of cartilage specific genes SOX9, collagen type II and aggrecan indicated differentiation towards chondrogenesis.

5.3. Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are self-renewing progenitor cells that have the potential to differentiate into chondrocytes, osteoblasts, adipocytes, fibroblasts, and other tissue of mesenchymal origin [122, 123]. They reside in many tissues within the adult organism and display the capacity to regenerate the cell pool of a given tissue [124-126]. MSC discovery opened new

avenues for therapeutic approaches because of their inherent accessibility and repair capacities. Several aspects need to be taken into consideration for cell therapies using MSCs: maintenance of undifferentiated status during the expansion *in vitro,* homing mechanisms that guide delivered cells to a site of injury and factors that induce and most importantly maintain cell differentiation status *in vivo*.

Although pluripotency has been demonstrated for MSC derived from bone marrow, adipose and muscle tissues as well as synovial membrane [122, 127-130], a growing body of evidence indicates that pluripotency decreases during MSC proliferation *in vitro* [131, 132]. A large number of signaling molecules that coordinate differentiation of MSCs into chondrocytes have been extensively investigated. Basic FGF was demonstrated to increase proliferation rate and life span in rabbit, dog and human MSC while maintaining their potential to differentiate towards fat, cartilage and bone [133, 134]. The family of BMPs has a pivotal role in prechondrogenic condensations and the transition of chondroprogenitor cells into chondrocytes [135-137]. Specifically, BMP-2 is expressed in the condensing mesenchyme of the developing limb [138], and regulates chondrogenic development of mesenchymal progenitors [139-141]. BMPs were demonstrated to induce chondrogenesis of MSCs *in vitro* [142, 143]. An important observation in some of these studies is further development towards hyperproliferative state indicating potential final differentiation to bone [114, 144]. The exclusive development towards chondrogenesis was observed only upon administration of recombinant BMP2 in pellet cultures of human MSC [142]. The exposure of equine MSCs to TGFβ-1 resulted in higher collagen II expression in monolayer cultures [145], and was superior to the treatment with hyaluronic acid and synovial fluid for chondrogenesis in pellet cultures [146]. TGFβ-1 was also demonstrated to induce chondrogenesis in bovine MSCs in pellet culture [147]. Insulin growth factors (IGFs) play a central role in chondrogenesis, and IGF-I has a potent chondrogenic effect in MSCs [148]. The effects of TGFβ-3, BMP-6 and IGF-1 analysed with

human MSC in pellet cultures demonstrated that a combination of TGFβ-3 and BMP-6 or TGFβ-3 and IGF-1 were more effective for chondrogenic induction [149].

The first application of MSCs in cartilage repair was conducted in rabbit where full thickness defects were filled with collagen scaffold seeded with MSC and mechanically loaded [150]. The shortterm results indicated regeneration of cartilage and bone. In a rabbit model MSC transplanted into collagen scaffold impregnated with recombinant BMP-2 enabled cartilage regeneration [151]. In a rat model implantation of MSC expressing BMP-2 or IGF-1 efficiently filled cartilage defect. Recently, in a murine model, only MSC expressing BMP-2 were shown to produce cartilage [152]. Unfortunately, none of the studies could demonstrate long lasting tissue formation suggesting that more investigation is needed before MSCs can be used in cartilage regeneration.

Potential use of MSCs has been also investigated in a goat meniscectomy OA model. Local injection of MSCs isolated from caprine bone marrow and labelled with green fluorescent protein (GFP), together with hyaluronic acid, stimulated regeneration of meniscal tissue and retarded the progressive cartilage destruction normally seen in this model of OA [153]. The presence of fluorescent cells in the newly formed menisci testified the contribution of MSC to the repair process. The results suggest a therapeutical benefit from injection of MSC for the traumatic type of injury to the meniscus that could thereby prevent further degeneration toward OA.

To assess possible application of MSCs in humans, proliferative and differentiation capacity of bone derived MSCs obtained from late stage OA patient was compared to bone marrow derived MSCs from healthy donors [154]. Cell proliferative potential as well as chondrogenic and adipogenic differentiation of MSCs from OA patients *in vitro* were significantly reduced compared with that of the healthy donors. These results suggest a change of bone marrow MSC capabilities in OA patients. The mostly osteogenic capacities of OA MSCs could explain the increase in bone

density and cartilage loss that are observed in OA patients. Importantly these data incite carefully designed and extensive further studies of bone marrow derived MSC from OA patients.

Previous studies have demonstrated the capacity of isolated, single-cell derived human articular chondrocyte clones to differentiate into cartilage, fat and bone [155]. However, these cells were not characterized for their surface molecules and not defined as MSCs. A more recent study has confirmed that de-differentiated adult human chondrocytes represent a population of multipotent cells capable to differentiate into adipogenic, osteogenic, chondrogenic, myogenic and neurogenic lineages [156]. Finally, the presence and characteristics of MSCs isolated from the cartilage of OA patients were examined [157]. While both MSCs isolated from healthy donors and OA patients had a surface marker profile characteristic of MSC, greater number of MSC was obtained from OA patients irrespective of whether the donor site was affected by the disease. The increased frequency of progenitor cells in OA cartilage could result from increased proliferation of resident progenitor cells, de-differentiation of chondrocytes (as demonstrated by their changed genetic profile) or from recruitment of MSC from synovial membrane or synovial fluid [130, 158].

MSCs have been tested directly for the repair of OA knees in humans [159]. Bone marrow derived MSC were expanded in culture, embedded in collagen matrix and transplanted. The follow-up after 6 months indicated similar clinical aspects in control cell-free and MSC treated lesions, but better arthroscopic and histological outcomes. Although promising data were obtained, results from long-term studies are still required.

As outlined above, the major hurdle in the OA treatment is the presence of inflammatory cytokines whose action needs to be controlled prior to or in parallel with the reparative attempts. Several studies have recently dealt with this issue. Dual expression of IGF-I and interleukin-1 receptor antagonist (IL-1Ra) were studied in the horse OA model [160]. Cartilage explants were exposed to the milieu of monolayer synovial membrane-derived cells expressing IGF-1 and IL-1Ra.

The data confirmed that combining the anabolic action of IGF-1 and the catabolic blocking of IL-1Ra protected and partially restored cartilage matrix. Another study employed gene transfer of TIMP-1 into bovine chondrocytes and demonstrated resistance to the catabolic effects of IL-1, including reduced MMP activity and a decreased loss of collagen type II [161].

6. Scaffolds and Bioreactors

6.1. Scaffolds

Scaffolds represent one of the key components for the TE approach. Their application ranges from a substitution of periosteal flap in the ACI treatment to a drug delivery device that could enhance tissue regeneration and reduce the OA related inflammatory processes.

As an alternative to tissue flaps in the ACI treatment, highly porous scaffolds may be used to maintain differentiated cells in a given area, or to encourage proliferation of chondrocytes as a technique for regeneration enhancement by encouraging cell migration [162]. Beyond being a simple mechanical substrate, the scaffold interacts with cells, bioactive molecules and mechanical signals in a dynamic and synergistic manner to contribute to the process of regeneration [163]. The main characteristics of an ideal scaffold include sterility, biocompatibility, biodegradability and sufficient mechanical properties to support cell differentiation and matrix production. Furthermore, the nature and type of defect determining the size and shape of the tissue to be regenerated together with the joint conditions of the patient must be taken into consideration when selecting the appropriate scaffold [163]. Finally, in the case of OA degeneration the choice of scaffold is particularly challenging due to the involvement of other joint tissues, namely synovium and subchondral bone, which are also affected by the disease.

The unique scaffold properties suited to regenerate bone and cartilage are very different. While there are many materials that may be applied for the problem of resurfacing OA joints, we will focus on those that have been extensively used, or have shown promising results in the TE application. These materials, in form of matrices, can be broadly categorized according to their chemical structure into natural, protein-based polymers, carbohydrate-based polymers, artificial materials and combinations thereof (see Table 2).

Table 2. Chemical classes of matrix

1.	Protein-based or natural polymers
	Fibrin
	Collagen
	Laminin (Matrigel)
	Gelatin
2.	Carbohydrate-based polymers
	Polylactic acid
	Polyglycolic acid
	Hyaluronan
	Agarose
	Alginate
	Chitosan
3.	Artificial polymers
	Dacron (polyethylene terphtalates)
	Teflon (Polytetrafluoethylene)
	Carbon fibers
	Polyestherurethane
	Polybutyric acid
	Polyethymethacrylate
	Hydroxyapatite
4.	Within/between classes
	Cross Linkage
	Chemical modification
	Geometrical modifications
	Matrix combinations

Table 1. Classes of scaffolds used for joint resurfacing.

Protein-based, natural polymers may contain ligands that can be recognized by cell-surface receptors and have the advantage of known biocompatibility and fewer regulatory constraints.

Potential drawbacks of these materials include lack of large quantities for clinical application, difficulty of processing into scaffolds, concerns of immunogenicity, disease transfer for allografts, and varied degradation rate from the patient.

The natural polymer that has received the most attention is collagen. In 1983, it was found that chondrocytes seeded on collagen gels maintain differentiated phenotype and GAG production for six weeks [164]. Wakitani et al. have reported that when MSCs were seeded into collagen gels implanted in osteochondral defects in rabbits, embryogenesis was recapitulated and both bone and hyaline cartilage were formed [150]. However, mechanical properties of the regenerated tissue were significantly lower compared to normal tissue, and evidence of degeneration was detected after 24 weeks ([3, 150]). Collagen matrices have also been found to stimulate new collagen production by transplanted cells as compared to other scaffolds [165]. In a recently published study, porcine collagen membrane was combined with microfracture to repair osteochondral defects in a sheep model with good results. Further improvement was achieved by seeding autologous chondrocytes onto the collagen membrane [166]. Methods that can demonstrate substantial repair of lesions involving both cartilage and subchondral bone may provide future promise to the possibility of repairing at least localized OA lesions. Collagenous matrices or collagen-imitating scaffolds are increasingly emerging as highly suitable vehicles for cell and growth factor transport into cartilage lesions. Collagens represent not only major constituents of connective tissues in terms of integrity and function, but are also major targets of tissue destruction and regeneration and thus might become major tools to achieve tissue repair [167].

Another protein-based candidate for osteochondral repair is fibrin glue, produced by polymerization of fibrinogen with thrombin [168]. Fibrin matrix was used as support for chondrocytes in full-thickness articular cartilage defects in horses, and regeneration of cartilage with a surface of hyaline-like tissue containing high percentage of type II collagen and sulphate

GAGs was achieved [169]. However, fibrin is proinflammatory and induces its own degradation further leading to its substitution by cellular components within the extra-vascular tissue. Its degradation products are however physiological and thus non-toxic although there are reports questioning its immunogenecity [170]. Further, the application of fibrin glue in replacement of structural tissues is limited by its lack of mechanical stability.

Synthetic polymers are available in unlimited supply and are easily processed into desired shapes and sizes. These materials are versatile because their physical, chemical, and degradation properties may be modified to meet the specific requirements of a given application. Copolymers, polymers blends and composites with other materials may also be manufactured to impart desired properties for certain applications.

Polylactic/polyglycolic acids, both individually and in combination have been investigated as scaffold material to repair cartilage defects for more than two decades [3, 94, 168, 171, 172]. Structural modifications to these polymers have yielded different matrix properties ranging from fine fibrillar meshworks to foam [173]. Compared to fibrin, collagen, Poly(L-lactic acid) (PLLA), and poly (DL-lactic-co-glycolic acid) (PLGA), PGA was shown to provide a better scaffold for *in vitro* cartilage regeneration, as demonstrated by cell densities equivalent to those found in natural tissues, and by continuous cellular production of type II collagen [174]. Although such engineered constructs have also been tested for articular cartilage repair in animal models, mainly in rabbits [94, 171, 175-177]), they have not been applied in human patients. The possible reasons include the graft induction of foreign body giant cell reaction [100] and the hydrolytic activity of the polymer substrate, which yields both toxic and partially cytotoxic degradation products. These potentially deleterious effects have, as yet, not been thoroughly investigated. A recent efficacy study for drug delivery using PLA microspheres in the 35-105 micron size range, loaded with 20% Paclitaxel, a chemotherapeutical agent resulted in Paclitaxel release in a controlled manner over several weeks in

a rabbit model [178]. Thus local drug delivery approach using PLA scaffold may be a potential formulation for the intra-articular treatment of inflammation in arthritic conditions. Triptolide, an immunosuppressive drug was also studied in a collagen induced arthritis rat model and demonstrated positive curative effect [179].

Hyaluronan is a physiological component of the articular cartilage matrix. It forms macromolecules of remarkable length and molecular weight which are biocompatible and biodegradable [180]. In theory, hyaluronan would be an ideal matrix to support cartilage repair if it could be implanted in an unmodified form. However, in order to achieve the required matrix physiochemical properties and structural organization, hyaluronan is in practice cross-linked by esterification or other means [180] resulting in compromised biocompatability [181]. Matrices composed of hyaluronan have not been applied alone to enhance spontaneous repair responses, but were frequently used as carriers for chondrocytes or bone marrow derived *MSC* in the treatment of cartilage defects of the knee [182]. In a recent review Marcacci has reported results from a 3 year clinical study of a cohort of 141 patients suffering from acute cartilage defects treated with an implant composed of a esterified derivative of hyaluronic acid (HYAFF 11) seeded with chondrocytes [183]. At the follow-up 91.5% of patients improved their condition according the International Knee Documentation Committee subjective evaluation; 76% and 88% of patients had no pain or mobility problems as assessed by the EuroQol-EQ5D. Hyaluronic acid based biomaterials have also been shown to create an environment in which the cells downregulated the expression of catabolic factors. Decreased levels of MMPs and NO were observed in the supernatants of chondrocytes grown on hyaluronan-based scaffolds, and cell apoptosis decreased during the culture period [121]. The results demonstrated a potential ability of hyaluronan scaffold to reduce the production of molecules involved in cartilage degenerative diseases and may indicate its beneficial effect in the treatment of early OA lesions. Modulation of the inflammatory cascade

by the intrinsic scaffold physical/chemical properties may prove to be of major importance in OA treatment strategies. An alternative concept is based on gene-activated matrices, where a specific gene is locally delivered in order to enhance healing or block degenerative/inflammatory processes [184].

Medicine today is continually adopting less invasive procedures that reduce morbidity and length of hospital tenure, while increasing the pace of recovery and return to normal activity. This trend towards minimally invasive procedures has also reached the field of TE and is a source of motivation for development of injectable cartilage engineering systems [185]. As a part of such system the scaffold must have physical properties that would allow an injection via a syringe or catheter. Once implanted, the scaffold material should solidify, acquire necessary mechanical properties and maintain a desired form and shape in a specific location without diffusion or movement[186]. Hydrogels are a class of materials that satisfy the requirements for a successful injectable TE system. Examples of injectable hydrogel systems that have been employed in TE include Pluoronics®, a block copolymer of polyethylene glycopolypropylene glycol [187], collagen [166], Matrigel TM [188], an ECM extract derived from a solubilized basement membrane preparation extracted from EHS mouse sarcoma, and fibrin glue [189]. The injectable polymer systems that crosslink via physical interactions are simple to apply as no external initiator or cross linking agent is required for the hydrogel formation. Unfortunately, hydrogels created from physically cross linked polymer often suffer from weak mechanical properties that limit their application [190]. A summary of injectable material is listed in Table 3. Given that OA is a generalized degenerative disease, an additional role of injectable hydrogels would be to deliver regulatory factors to the joint while providing resurfacing capabilities.

Table 3. Injectable scaffold biomaterials for cartilage tissue engineering

Natural Materials
Alginate

	Collagen
	Fibrin glue
	Hyaluronic acid (crosslinked)
	Chondroitin sulfate
Synthetic materials	
	PLG speheres
	PEG
	Polyvinyl alcohol
	Polypropylene fumrate

Table 3. Natural and synthetic materials applied as a liquid for injectable TE system. PEG:polyethylene glycol; PLG: Poly(lactide-coglycolide)

6.2. Bioreactors for cartilage TE

The term "bioreactor" in the context of cell and tissue culture indicates a device where specific physicochemical culture parameters can be reproducibly maintained at defined levels. By providing inherent control over the required bioprocesses, the use of bioreactor systems has the potential to improve the quality of engineered cartilage tissues and to streamline their manufacture. Moreover, bioreactors are expected to play a key role in the establishment of advanced model systems to investigate mechanisms of cartilage degeneration and repair, and possibly to predict the behaviour of engineered grafts upon implantation into an OA joint.

6.2.1. Bioreactors to establish and maintain 3D cultures

The initial step in the *ex vivo* generation of a cartilage graft, namely the seeding of chondrogenic cells onto a porous scaffold, establishes the three-dimensional environment and likely has a strong influence in determining the uniformity of successive tissue formation. Simply pipetting a highly dense cell suspension into the porous scaffold is the most commonly used seeding technique, but the manual and user-dependent process lacks control and reproducibility. Stirred-flask "bioreactors" can increase the control and reproducibility of the process when seeding cells into thin and highly porous scaffolds [191]. However, due to insufficient penetration of cells into

the interior region of thick or less porous scaffolds, stirred-flask systems can also yield low seeding efficiencies and non-uniform cell distributions, with a higher density of cells lining the scaffold surface [192]. Perfusing a cell suspension directly through the pores of a 3D scaffold using a direct perfusion bioreactor was shown to be more effective and reproducible in generating uniformly seeded constructs than the above techniques, in particular when seeding thick and low porosity scaffolds [192].

After distributing the chondrocytes throughout the porous scaffold, a key challenge is to maintain this distribution and the cell viability within the internal region of the construct during prolonged culture. Due to mass transfer limitations, cartilaginous constructs cultured under conventional static conditions (i.e., with unmixed culture media) are frequently inhomogeneous in structure and composition, containing a hypoxic necrotic central region and dense layers of viable cells encapsulating the construct periphery. Stirred-flask and rotating vessel bioreactor systems have been shown to enhance mass transport to/from chondrocyte-scaffold constructs, thus resulting in tissues with increased fractions of cartilage-specific molecules in the inner core [193]. Interestingly, as compared to the turbulent flow within stirred flasks, the dynamic laminar flow in rotating wall vessels, associated to reduced levels of shear, supported the formation of cartilaginous tissues containing higher amounts of more uniformly distributed GAG and collagen [191]. Bioreactor systems applying a direct perfusion of culture medium through the scaffold pores can also mitigate mass transfer limitations throughout the engineered constructs. Perfusion of chondrocyte-seeded scaffolds was shown to support elevated GAG synthesis and retention within the ECM, [194, 195] as well as a uniform distribution of viable human chondrocytes as indicated in Figure 1[196]. In conjunction with computational fluid dynamics modeling of the fluid-induced shear stresses and mass transport within the porous architecture of the 3D scaffold [197, 198], a perfusion system can

provide a well-defined physicochemical culture environment which has great potential to generate cartilage grafts of clinically relevant size.

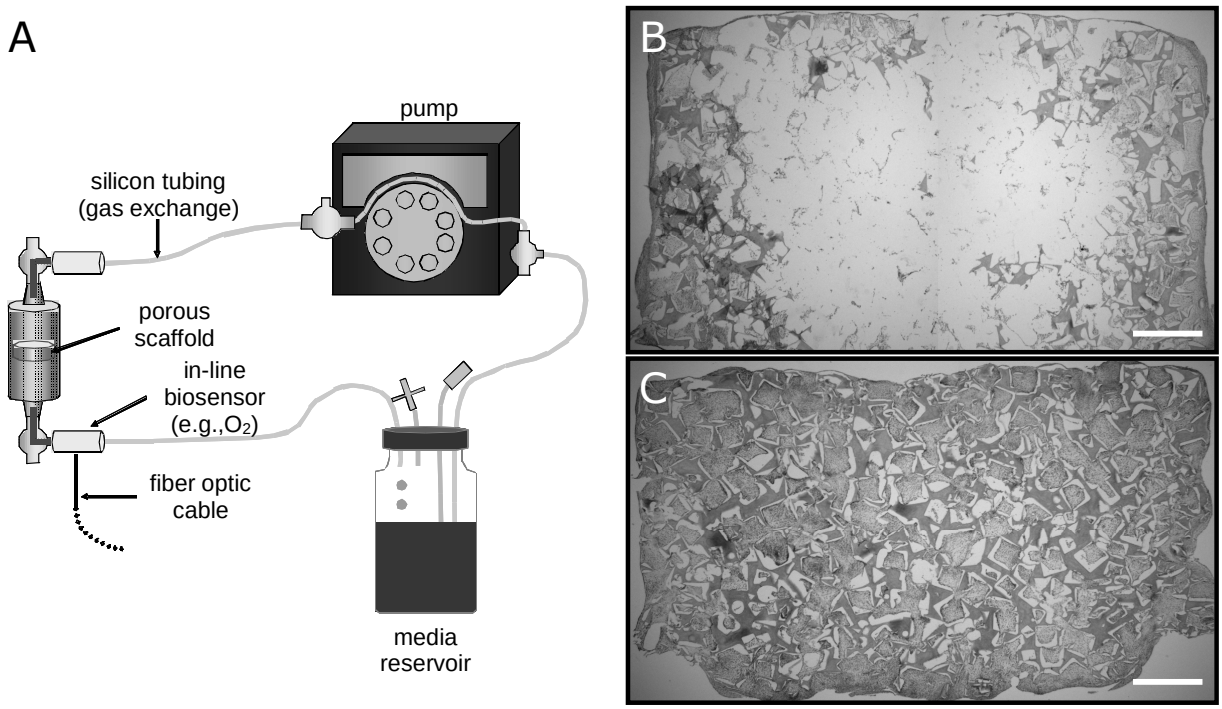

Figure 1. (a) Perfusion bioreactor system for the engineering of clinically relevant-sized cartilage grafts. Culture media is perfused through the pores of the cell-seeded 3D scaffold to reduce mass transfer limitations throughout the engineered construct. (b) Cartilage constructs generated under static culture conditions are frequently inhomogenous in structure, with cells and matrix concentrated at the construct surface and a necrotic interior region. (c) In contrast, cartilage engineered using the direct perfusion bioreactor was shown to be highly uniform, with cells and matrix distributed throughout the entire graft. Scalebar is 1mm. [196]

6.2.2. Bioreactors for mechanical conditioning

Physiological joint loading plays a critical role in differentiating *MSC* during cartilage development, and in developing and maintaining the structural and functional properties of articular structures in youth and adulthood [199, 200]. Thus, it is not surprising that mechanical loading,

applied using a variety of devices [201] has been included as a key element in cartilage TE strategies, in order to enhance cell differentiation and/or tissue development *in vitro*. Indeed, a number of studies have reported stimulated chondrocyte metabolism and/or enhanced cartilage matrix production in response to dynamic loading, although these responses were greatly dependent upon the specific magnitude and/or frequency applied [105, 202-207]. Despite these numerous proofs of principle that mechanical conditioning can upregulate gene expression and tissue development, little is currently known about which specific mechanical forces, or regimes of application (i.e. magnitude, frequency, continuous or intermittent, duty cycle), are most stimulatory. The field is further complicated by the fact that the effects of mechanical stimuli on tissue engineered cartilage may vary substantially using different scaffold systems [208] or constructs at different stages of development [209]. Moreover, a crucial issue that still remains to be demonstrated is whether *in vitro* mechanical preconditioning of engineered cartilage would result in grafts with increased chances of long-term *in vivo* success [210].

6.2.3. Bioreactors for controlled model systems

The role of bioreactors for mechanical conditioning of cartilaginous constructs could be broadened beyond the conventional one of enhancing cell differentiation and/or *ECM* deposition in engineered tissues. They could also serve as controlled *in vitro* models to study pathophysiological interactions between physical forces and soluble factors on engineered cartilage development. For example, a bioreactor applying controlled regimes of loading and specific inflammatory cytokines (e.g., IL-1β or TNF-α) might be used to investigate the response of an engineered tissue to an environment simulating an OA joint, before more complicated and costly large size animal models are introduced.

In the context of the identification and validation of effective drugs for OA, the bioreactor-based reproducible generation of cartilaginous tissues under standardized conditions would offer the possibility to use engineered cartilage as a 3D model system for drug screening. Exposure of the engineered constructs to defined stimuli (biochemical or biomechanical) could also lead to the identification of anabolic targets, which represents a key step in drug discovery.

6.3.4. Bioreactors for cartilage tissue manufacture

One of the major challenges to bring an autologous cell-based engineered cartilage product into routine clinical practice including the treatment of OA would be to translate research-scale production models into clinically applicable manufacturing designs that are reproducible, clinically effective, and economically acceptable while complying with Good Manufacturing Practice (GMP) requirements [211]. Bioreactors have the potential to meet this challenge by automating and standardizing the manufacture of engineered grafts in controlled closed systems [212, 213].

7. Mechanical stimulation

Physiological joint loading is essential for the development and maintenance of normal articular cartilage. During development, both movement and mechanical load play a critical role in differentiating embryonic MSC into chondrocytes leading to development of the articular surface [199, 214]. In young humans, morphological properties of articular structures are further defined (or developed) by mechanical loading of the joints [199, 200]. Even in adulthood, physiological joint loading is necessary and responsible for the maintenance of articular structures and leads to varying mechanical properties between different joints and within each joint [200, 214]. Variations in loading have been shown to alter gene expression, chondrocyte density, and biosynthetic response thereby resulting in different organization schemes of the constituents within the ECM

[199, 200, 214]. For example, regions such as the patellofemoral articulation that are subjected to high shear loads possess a high concentration of collagen fibrils in the superficial layer of the cartilage that are aligned in the direction of shear loading [199].

Given the importance of mechanical loading in the development and maintenance of native articular cartilage, it is not surprising that mechanical stimulus can have a significant impact on the behavior of isolated chondrocytes (or chondrocytes cultured *in vitro*), cartilage explants, and tissue-engineered cartilage constructs *in vitro* [202, 206, 215]. Functions of mechanical stimulus for *in vitro* tissue systems include control of cell phenotype, delivery of nutrition and removal of waste products, and mediation of the synthesis and organization of matrix molecules. For a synopsis of methods used to apply mechanical load to cells *in vitro*, see the review by [201]. Increases in the synthesis of type II collagen, proteoglycan, and other important matrix molecules have been accomplished by mechanical preconditioning tissue-engineered constructs [206, 215, 216]. Further, mechanical properties of scaffold-based tissue-engineered constructs such as aggregate modulus [206], tensile modulus [217], dynamic stiffness and oscillatory streaming potential [216] have been shown to increase significantly with mechanical preconditioning. The efficacy of increasing the mechanical properties of repair cartilage tissue with respect to its long-term success *in vivo* is not well understood [210, 218] and remains to be demonstrated.

8. Outlook of combined TE parameters in the treatment of OA

The key element in TE approach is to employ biologically based mechanisms in order to achieve repair and healing of damaged and diseased tissues. Chondrocytes from normal as well as OA cartilage may be suitable candidates as a cell source while pluripotent mesenchymal cells isolated from different tissues have also demonstrated the capacity to produce cartilaginous tissue. A wide range of natural and synthetic scaffolds have been demonstrated to support cells

proliferation and subsequent differentiation. Different types of bioreactors were successfully employed to establish and maintain 3D culture systems, provide mechanical conditioning and enhance tissue regeneration. Finally, knowledge of action of growth factors, cytokines, chemokines, protease inhibitors, and kinases has shed light for the crucial signaling cues in articular cartilage regeneration. Can this knowledge be applied for the treatment of OA?

The first goal in treating OA is to arrest and if possible reverse its progress regionally or globally. The TE approach should thus be designed as a “Tissue-Reengineering” to block the ongoing inflammatory process while stimulating the regenerative process (Figure 2). For this purpose application of a combination of growth factors is essential. Selected growth factors could be integrated as a part of the treatment in several ways: i) as recombinant proteins supplied together with cells (for example during ACI procedure), ii) as recombinant proteins impregnated in the scaffold, or iii) as genes expressed by genetically modified cells. Scaffolds, such as HIAFF11, that support reduction of inflammatory cytokines will certainly prove beneficial for the OA treatment. Importantly, due to the disease spreading the subchondral bone, that compartment should also be included in the treatment. Finally, proper timing for the treatment, in the case of OA as early as possible, may prove crucial for successful TE application in treatment of OA lesions.

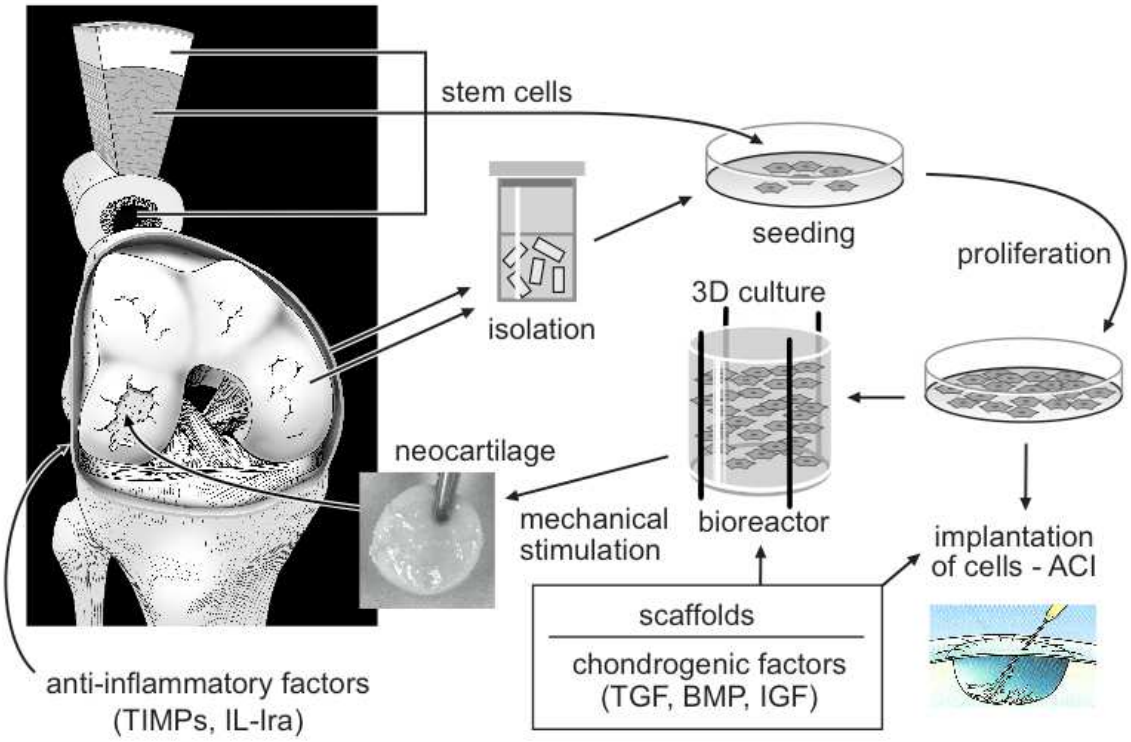

Figure 2. Cartilage tissue re-engineering. Schematic presentation of a potential treatment for OA lesions. Stem cells with the potential to regenerate cartilaginous tissue can be obtained from bone marrow, muscle, fat, synovial membrane and cartilage, and expanded in monolayer cultures until sufficient cell numbers are obtained. Subsequently, cell could be combined with scaffolds and growth factors, and either directly injected in the lesion (ACI) or further grown in bioreactors *in vitro* to form new tissue. Neocartilage would finally be implanted in the lesion. All implantation procedures would include anti-inflammatory factors in order to decrease further degeneration of the joint.

Although TE approach has demonstrated promising results *in vitro*, new challenges will emerge with its translation into the clinical setting. To increase the chances for success, further research must address immunological issues (depending on the cell source – autologous or

heterologous), integration of the engineered cartilage into the patient's own tissue and the variability of tissue development depending of potentially diseased surrounding tissue, age and physical activity.

9. References

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