

# Tissue engineering of functional articular cartilage: the current status

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**Abstract** Osteoarthritis is a degenerative joint disease characterized by pain and disability. It involves all ages and 70% of people aged >65 have some degree of osteoarthritis. Natural cartilage repair is limited because chondrocyte density and metabolism are low and cartilage has no blood supply. The results of joint-preserving treatment protocols such as debridement, mosaicplasty, perichondrium transplantation and autologous chondrocyte implantation vary largely and the average long-term result is unsatisfactory. One reason for limited clinical success is that most treatments require new cartilage to be formed at the site of a defect. However, the mechanical conditions at such sites are unfavorable for repair of the original damaged cartilage. Therefore, it is unlikely that healthy cartilage would form at these locations. The most promising method to circumvent this problem is to engineer mechanically stable cartilage *ex vivo* and to implant that into the damaged tissue area. This review outlines the issues related to the composition and functionality of tissue-engineered cartilage. In particular, the focus will be on the parameters cell source, signaling molecules, scaffolds and mechanical stimulation. In addition, the current status of tissue engineering of cartilage will be discussed, with the focus on extracellular matrix content, structure and its functionality.

**Keywords** Articular cartilage · Tissue engineering · Chondrocyte · Signaling molecules · Mechanical stimulation

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## Introduction

Osteoarthritis is a degenerative joint disease characterized by pain and disability (Temenoff and Mikos 2000). It involves all ages and 70% of people aged >65 have some degree of osteoarthritis (Engel 1968). Natural cartilage repair is limited because the intrinsic regenerative ability of the tissue is low and cartilage lesions in case of trauma or diseases tend to progressively degrade (Hunziker 2002; Buckwalter and Mankin 1998). Current clinical treatment strategies like mosaicplasty, autologous chondrocytes injection and microfracture have varying success rates, but average long-term results are unsatisfactory (Kreuz et al. 2006; Redman et al. 2005; Bentley et al. 2003; Hunziker 2002; Buckwalter and Mankin 1998). A general drawback of these therapeutic strategies is that the newly formed tissue lacks the structural organization of cartilage and has inferior mechanical properties compared to native tissue, and is therefore prone to failure (Hunziker 2009). The contribution that *in vitro* cartilage tissue engineering can make is to create a more durable and functional replacement of the degenerated tissue, which is therefore more likely to survive the mechanical conditions in a joint after implantation. One ultimate goal in this field of research is to develop a replacement that has a structure and composition resembling native cartilage, yielding similar mechanical behavior and which fully restores joint functionality.

This review will focus on issues related to functionality of tissue-engineered cartilage. First, we discuss the most important parameters for cartilage tissue engineering studies, including cell source, signaling molecules, scaffolds and mechanical stimulation. Second, we will discuss the current status of tissue engineering of cartilage, focusing on ECM content, structure and its functionality. Finally, we identify common limitations and provide further recom-

mendations for future approaches to engineer a cartilage matrix *in vitro* that can provide a functional replacement of damaged articular cartilage *in vivo*.

### Important parameters for cartilage tissue engineering studies: cell source, signaling molecules, scaffolds and mechanical stimulation

#### Cell source

The ideal cell source for cartilage tissue engineering is one that can easily be isolated and expanded, and which synthesizes abundant cartilage-specific extra-cellular matrix components, e.g., aggrecan and type II collagen. The most investigated cell sources for their potential in cartilage tissue engineering are chondrocytes and stem cells (for a detailed overview, we refer to Table 1 in Chung and Burdick 2008).

#### *Chondrocytes*

Chondrocytes are the most obvious cell source. They are able to produce, maintain and remodel the cartilage ECM *in vitro*. However, only a small number of autologous chondrocytes are available, and cells harvested from diseased joints are relatively inactive. Unfortunately, chondrocyte expansion in monolayer causes dedifferentiation, characterized by decreased proteoglycan synthesis and type II collagen expression and increased type I collagen expression (Darling and Athanasiou 2005b; Goessler et al. 2004; Goessler et al. 2005). The age of chondrocytes is also an issue that needs to be considered. In most cartilage tissue engineering studies, chondrocytes from immature animals are used, which proliferate faster and have increased chondrogenic potential compared to chondrocytes from older human donors (Hidaka et al. 2006; Pestka et al. 2011). Unfortunately, chondrocytes from older (OA) patients are metabolically less active *in vitro* (Wenger et al. 2006; Dehne et al. 2009). Even though these limitations can be partly counteracted with altered culture conditions, such as rotating bioreactor cultures (Marlovits et al. 2003), culture in serum-free media (Giannoni et al. 2005), culture with reduced oxygen tension (Foldager et al. 2011; Strobel et al. 2010) and the addition of growth factors (Barbero et al. 2004; Terada et al. 2005), the use of these cells for cartilage repair is not favorable. Another disadvantage of the use of isolated articular chondrocytes is morbidity at the donor site and loss of joint function.

#### *Stem cells*

A possible solution for overcoming the limited supply of primary chondrocytes is the use of multipotent stem cells,

mainly from bone marrow and adipose tissue. Bone marrow-derived stem cells (BMSCs) can be easily obtained and can be induced to differentiate into cartilage, even after expansion (Song et al. 2004; Boeuf and Richter 2010). Chondrogenic differentiation of BMSCs for cartilage tissue engineering purposes is facilitated by the application of TGF- $\beta$  in various 3D culture environments (Worster et al. 2001; Mauck et al. 2006; Angele et al. 1999; Li et al. 2005; Coleman et al. 2007; Williams et al. 2003; Meinel et al. 2004; Wang et al. 2005; Chen et al. 2004; Buxton et al. 2011; Alves da Silva et al. 2010). The main limitation of the use of BMSCs for cartilage tissue engineering is that matrix accumulation and the subsequent mechanical properties of BMSC-laden constructs are lower than those of chondrocyte-seeded constructs (Erickson et al. 2009; Mauck et al. 2007; Thorpe et al. 2010; Vinardell et al. 2010). A possible explanation could be that during culture *in vitro* MSCs increase expression of collagen type X, which is a hypertrophic chondrocyte marker (Barry et al. 2001; Koga et al. 2009). Some reports have shown that the expression of hypertrophic-related genes could lead to cell death or calcification followed by vascularization when implanted (De Bari et al. 2004). Furthermore, MSCs continue to express collagen type I (Steck et al. 2005). Recently, several promising results have been published that show the feasibility of inhibiting collagen type I and X expression and thereby controlling the chondrogenic differentiation pathway of MSCs (Rampersad et al. 2011; Petit et al. 2011; Bian et al. 2011; Fischer et al. 2010).

Adipose-derived stem cells (ADSCs) have been shown to be capable of differentiating into chondrocytes in 3D culture systems in the presence of ascorbate, dexamethasone and TGF- $\beta$  (Estes and Guilak 2011; Ronziere et al. 2010; Puetzer et al. 2010; Buckley et al. 2010; Diekman et al. 2010). In these studies, production of cartilage-specific matrix components was shown as well as increased mechanical properties. Even though ADSCs are able to differentiate into chondrocytes, their chondrogenic potential is lower compared to BMSCs, which suggests that more research needs to be done to improve the chondrogenic potential of these cells.

Besides bone marrow and adipose tissue, other sources such as muscle, synovium and periosteum are also being investigated for cartilage tissue engineering purposes, all of which have been shown to have chondrogenic potential, but which is still lower compared to BMSCs and/or ADSCs (Salgado et al. 2006; Li et al. 2011; O'Driscoll 1999).

#### Signaling molecules

Several cytokines, hormones and growth factors are known to influence the anabolic and catabolic processes by chondrocytes. Therefore, a number of growth factors,

including transforming growth factor (TGF- $\beta$ ), insulin-like growth factor (IGF-1), bone morphogenetic proteins (BMPs), and to a lesser extent fibroblast growth factors (FGFs) and epidermal growth factor (EGF), have been used in cartilage tissue engineering studies *in vitro* to promote the chondrogenic phenotype, to stimulate ECM production and to promote chondrogenesis of MSCs (for a detailed overview, we refer to Table 2 in Ahmed and Hincke 2010). Members of the TGF- $\beta$  superfamily play a major role in cartilage development and repair. Mainly, the isoforms TGF- $\beta$ 1, 2 and 3 enhance chondrocyte proliferation and increase ECM synthesis by chondrocytes (Morales 1991; Bujia et al. 1996; van der Kraan et al. 1992). Further, TGF- $\beta$ 1 and 3 promote chondrogenesis of MSCs (Grimaud et al. 2002; Li et al. 2005; Schulz et al. 2008; Puetzer et al. 2010; Xu et al. 2008). IGF-1 can stimulate the anabolic activity of chondrocytes and can induce chondrogenesis of MSC cell types (Yoon and Fisher 2008; Veilleux and Spector 2005; Kurth et al. 2007; Indrawattana et al. 2004; Fukumoto et al. 2003; Gooch et al. 2001; Seifarth et al. 2009). BMPs, especially BMP-2 and BMP-7, promote chondrogenesis of MSCs and increase matrix production by chondrocytes and MSCs (Kurth et al. 2007; Park et al. 2005; Hicks et al. 2007; Kaps et al. 2002).

#### *Combinations of signaling molecules*

Administration of a combination of growth factors to chondrocyte and MSC cultures *in vitro* may increase their impact. For example, combinations of IGF-1/TGF- $\beta$ 1, IGF-1/TGF- $\beta$ 2, IGF-1/BMP-2 and IGF-1/bFGF/TGF- $\beta$ 2 exerted additive anabolic effects on chondrocytes and stimulated ECM synthesis (Chua et al. 2004; Seifarth et al. 2009; Wiegandt et al. 2007; Elder and Athanasiou 2009; Yasuda et al. 2006). However, other studies have reported that combinations of IGF-1/TGF- $\beta$ , bFGF/ TGF- $\beta$  and FGF-2/IGF-1 did not further improve histological features or mechanical performance of the engineered cartilage (Arevalo-Silva et al. 2001; Veilleux and Spector 2005).

Combinations of growth factors have also been used to induce chondrogenic differentiation of MSCs. For example, a combination of IGF-1 and TGF- $\beta$ 1 induced chondrogenic differentiation of MSCs (Xiang et al. 2007) and combinations of TGF- $\beta$ 2/BMP-7, TGF $\beta$ 2/BMP-6, TGF- $\beta$ 2/BMP-2 and TGF- $\beta$ 2/IGF-1 promoted chondrogenesis of MSCs, with TGF- $\beta$ 2/BMP-7 being most effective (Kim and Im 2009; Im et al. 2006). Also, combinations of TGF- $\beta$ 3 with BMP-2, BMP-4, BMP-6 and IGF-1 have been shown effective, both in monolayer and 3D cultures (Sekiya et al. 2005; Hennig et al. 2007; Indrawattana et al. 2004; Takagi et al. 2007).

*Dose and timing of administration* It has become clear that the effect of application of signaling molecules is not only

dependent on the type of factor that is applied but other parameters are also involved, such as dose and timing of administration and the cell type on which they act. For example, transient application of TGF- $\beta$ 3 resulted in higher compressive properties and GAG content of chondrocyte-laden hydrogels (Lima et al. 2007; Byers et al. 2008) and MSC-laden constructs (Huang et al. 2009; Mehlhorn et al. 2006; Caterson et al. 2001), compared to continuous application of TGF- $\beta$ 3. It has been suggested that TGF- $\beta$  may act to ‘prime the pump’, which makes continuous application superfluous. Other studies have employed sequential growth factor addition with the goal of first increasing proliferation within the constructs with a combination of FGF-2/TGF- $\beta$ 1 followed by enhancing matrix production with IGF-1 (Pei et al. 2002). In most cartilage tissue engineering studies, the commonly used concentration of growth factors such as TGF- $\beta$ , FGF-2 and BMPs is 10 ng/ml (Ahmed and Hincke 2010). However, continuous treatment of chondrocytes in agarose with 1, 2.5, 5 and 10 ng/ml TGF- $\beta$  resulted in comparable enhancement of both physical and biochemical properties (Byers et al. 2008).

#### *Mechanical stimulation*

A well-established cue for improving the mechanical properties of tissue-engineered cartilage is mechanical stimulation. Bioreactors have been developed to apply mechanical loading regimes to cell-seeded constructs (for a detailed overview, we refer to tables and figures in Schulz and Bader 2007). Direct confined or unconfined compression and hydrostatic pressure are the two most investigated loading regimes in cartilage tissue engineering studies. Direct dynamic compression applied to chondrocyte-seeded constructs generally induces increased ECM production and/or proliferation and has been shown to improve compressive properties of the engineered tissue (Bian et al. 2010; Kock et al. 2009; Kelly et al. 2006; Kisiday et al. 2004; Mauck et al. 2002). More recently, dynamic compression has been applied to MSC-seeded constructs, where it stimulated the accretion of cartilage-like extracellular matrix (ECM) components relative to unloaded controls (Mauck et al. 2007; Kisiday et al. 2009; Park et al. 2006; Thorpe et al. 2010). Application of hydrostatic pressure *in vitro* has improved the properties of tissue-engineered cartilage (Hu and Athanasiou 2006b; Miyanishi et al. 2006a, b). However, as with direct compression, the outcomes of these studies depend largely on the loading parameters used. Besides the effect on metabolic activity of the cells, hydrostatic pressure also stimulates the chondrocytic phenotype of chondrocytes *in vitro* (Candiani et al. 2008; Heyland et al. 2006; Kawanishi et al. 2007). Furthermore, hydrostatic pressure has been used to stimu-

late chondrogenic differentiation of bone marrow-derived (Luo and Seedhom 2007; Miyanishi et al. 2006a, b; Wagner et al. 2008), adipose-derived (Ogawa et al. 2009), and synovium-derived stem cells (Sakao et al. 2008) with promising results. Other loading regimes that have been investigated are shear loading, sliding/rolling indentation loading, tensile loading, centrifugal force, and gravity (Darling and Athanasiou 2003a, b; Schulz and Bader 2007; Khoshgoftar et al. 2011; Sun et al. 2010; Wimmer et al. 2009) with mixed results. In conclusion, it is necessary to investigate which specific (combinations of) mechanical stimuli, as well as their parameters, result in optimal response of the cells in cultured constructs.

### Scaffolds

The goal of the use of biomaterial scaffolds in cartilage tissue engineering is to provide the cells with a comfortable niche which stimulates cells to synthesize cartilage matrix, and to (temporarily) replace the function of the native matrix until new cartilage has formed. To fulfill that function, the scaffold should preferably (1) be biodegradable in a controlled way without toxic byproducts, (2) have a porosity that allows diffusion of nutrients and waste products, (3) support cell viability, proliferation, differentiation and ECM production, (4) be able to fix to and integrate with the tissue at the defect site, and (5) give mechanical support to the engineered tissue. Many natural and synthetic polymers have been used as scaffold material in cartilage tissue engineering (for a detailed overview, we refer to Table 1 in Ahmed and Hincke 2010).

#### *Types of scaffold*

Natural polymers can be subdivided into protein-based, such as silk, fibrin and collagen, and carbohydrate based, such as agarose, alginate, hyaluronan and chitosan. Many of these are hydrogels, which makes them appropriate for engineering tissues such as cartilage, which have high water content. These can be designed as injectable in liquid form, which mixes well with chondrogenic cells. The most attractive feature of hydrogels is that cells encapsulated in the scaffold maintain their spherical chondrocyte phenotype and do not (de)differentiate. Hydrogels are interesting for studies in which mechanical loading is used, because they are able to transduce mechanical loads such that forces can be exerted on the cells (Spiller et al. 2011). Finally, natural scaffold materials, particularly fabricated by biologics, are believed to permit natural ECM remodeling with construct maturation (Chung et al. 2006; Burdick et al. 2005; Li et al. 2005; Welsch et al. 2010).

The most widely used synthetic polymeric scaffolds in cartilage tissue engineering are the poly- $\alpha$ -hydroxy esters,

especially polylactic acid (PLA) and polyglycolic acid (PGA), because of their biodegradability and US Food and Drug Administration (FDA) approval for clinical use (Yoon and Fisher 2006). Scaffolds made of these polymers have better mechanical strength than hydrogels, which makes it easier to fix them in a defect and improves their load-bearing properties (Munirah et al. 2008). In addition, it is easier to modify the properties of these scaffolds, which makes it easier to tune, for example, their degradation characteristics, structure and mechanical strength. A disadvantage of synthetic polymers is that cells often do not maintain their chondrocytic phenotype and produce ECM with inferior properties (Chen et al. 2003).

#### *Scaffold architecture, porosity and stiffness*

Porosity, pore size and interconnectivity of scaffold materials are important since these properties influence cell migration and diffusion of oxygen, nutrients, waste products and signaling molecules (Nuernberger et al. 2011). For example, inhomogeneous oxygen delivery from the periphery towards the center of cell-seeded constructs may lead to cell death in the central regions but not in the periphery (Volkmer et al. 2008; Malda et al. 2004; Sengers et al. 2005a, b).

In addition, a porous material improves mechanical interlocking between the implant and the surrounding natural cartilage, providing a greater mechanical stability at the interface. Porosity and permeability have a remarkable effect on proliferation and phenotype of chondrocytes (Lien et al. 2009; Stenhamre et al. 2010; Jeong and Hollister 2010). The pore size for scaffolds to promote proliferation is optimal between 100 and 500  $\mu\text{m}$  (Ikada 2006; Lien et al. 2009). Porosity and architecture can also be used to induce topographical organization. Woodfield et al. (2005) produced a 100% interconnected pores scaffold with pore size gradients, which promoted an inhomogeneous cell distribution and zonal distribution of GAGs and collagen type II.

Stiffness of scaffolds also influences the mechanical environment of the seeded cells which in turn can influence cell differentiation and tissue growth in culture (Kelly and Prendergast 2006). Increasing substrate stiffness influences chondrocyte morphology which changed from a rounded shape with nebulous actin on weaker substrates to a predominantly flat morphology with actin stress fibers on stiffer substrates (Genes et al. 2004). Further, the load on cartilage is a stress and not a strain, hence the strain applied to the cells at first is a function of the scaffold stiffness and then a combination of scaffold and ECM properties as the tissue is produced. For example, high agarose concentrations (3%) yield initially stiffer tissue constructs, presumably due to more efficient retention of matrix products, but



long-term tissue properties become significantly inferior to those with 2% agarose (Ng et al. 2005).

### Biodegradability

Spatially and temporally controlled degradation of the scaffold can affect production and deposition of new tissue. Optimal degradation kinetics ensures initial stability and shape of the scaffold, but do not hinder new cartilaginous ECM deposition. Several degradable scaffolds have been adopted for cartilage tissue engineering (Freed et al. 1994). It has been shown that scaffolds that degrade slowly lead to increased and more homogeneous ECM deposition compared to fast degrading scaffolds (Meinel et al. 2004; Solchaga et al. 2005; Bryant and Anseth 2002). Further, degradation of the scaffold allows for integration and remodeling of the new tissue into the surrounding cartilage after implantation (Bryant and Anseth 2003). To direct temporal degradation of scaffolds, hydrolytically degradable components (Bryant and Anseth 2003), matrix metalloproteinase (MMP)-sensitive peptides (Lutolf et al. 2003; Park et al. 2004), and exogenous enzymes (Ng et al. 2009a; Rice and Anseth 2007) have been introduced. For example, Ng et al. (2009a) has shown that controlled degradation of agarose scaffold with the enzyme agarase resulted in increased collagen content and dynamic mechanical properties relative to control over time in culture, which they hypothesize to be the result of enhanced nutrient transport and increased space for collagen fibril development with time of culture. In addition, it has been shown that, in evolving MSC-laden hydrogels with mesh sizes that change over time due to crosslink degradation, GAG and collagen II content were increased, and mechanical properties were superior to non-evolving hydrogels (Chung et al. 2009).

### Tissue-engineered cartilage: content, structure and functionality

The joint is mechanically a very demanding environment. For a tissue-engineered cartilage implant to survive those conditions, it needs to have sufficient material properties to withstand or respond to normal joint loading. This does not necessarily mean that the engineered tissue is an exact copy of the natural tissue; the tissue may further develop and adapt properties post-implantation. The questions arise, what exactly are these ‘sufficient material properties’, and how much do we need to improve our current tissue-engineered cartilage.

#### Proteoglycan content

Several studies have been able to engineer cartilage constructs in vitro with native sGAG content and equilib-

rium compressive properties (Lima et al. 2006, 2007; Elder and Athanasiou 2008; Bastiaansen-Jenniskens et al. 2008; Waldman et al. 2006). sGAG content and compressive properties improved with increasing culture duration and cell seeding density (Chang et al. 2001; Mauck et al. 2002; Puelacher et al. 1994), and with addition of anabolic growth factors and/or increased serum supplementation (Pei et al. 2002; Gooch et al. 2001; Mauck et al. 2003). Interestingly, the deposition of sGAG was significantly enhanced when dynamic loading was applied to chondrocytes-seeded constructs (Chowdhury et al. 2003; Mauck et al. 2000).

#### Collagen content

The major shortcoming of tissue-engineered cartilage is believed to be the lack of collagen content and consequently its poor tensile properties. Collagen reaches only 15–35% of the native content after 5–12 weeks (Hu and Athanasiou 2006a; Miot et al. 2006; Eyrich et al. 2007). Culture conditions that have a significant impact on collagen synthesis in vitro include cell source (Waldman et al. 2003), cell seeding density (Williams et al. 2005; Revell et al. 2008), scaffold properties (Woodfield et al. 2005), growth factors (Darling and Athanasiou 2005a; Jenniskens et al. 2006; Blunk et al. 2002) and mechanical stimulation (Mauck et al. 2000; Waldman et al. 2006; Hu and Athanasiou 2006b; Elder et al. 2006; Elder and Athanasiou 2008; Kock et al. 2010). A possible hypothesis that may explain low collagen contents in constructs is that GAGs, which are initially rapidly synthesized, impede increased collagen content. Altered transport pathways of nutrients (Asanbaeva et al. 2007), or reduction of cell straining by environmental loading may cause decreased collagen synthesis. Altered transport of synthesised products (Asanbaeva et al. 2007) or altered extracellular biochemical environment may modulate collagen self-assembly (Saeidi et al. 2009). Finally, altered cellular mechanical stimulation may induce MMP expression, resulting in collagen degradation. Also, collagen type I in vitro degradation has been demonstrated to be strain-dependent (Huang and Yannas 1977), and this likely also holds for collagen type II (Flynn et al. 2010). If so, then it is worthwhile to explore strains in cartilage tissue engineering constructs, to evaluate whether these strain conditions would either prevent or induce enzymatic collagen degradation. In the latter case, we may proceed to seek loading conditions that would prevent collagen degradation from occurring.

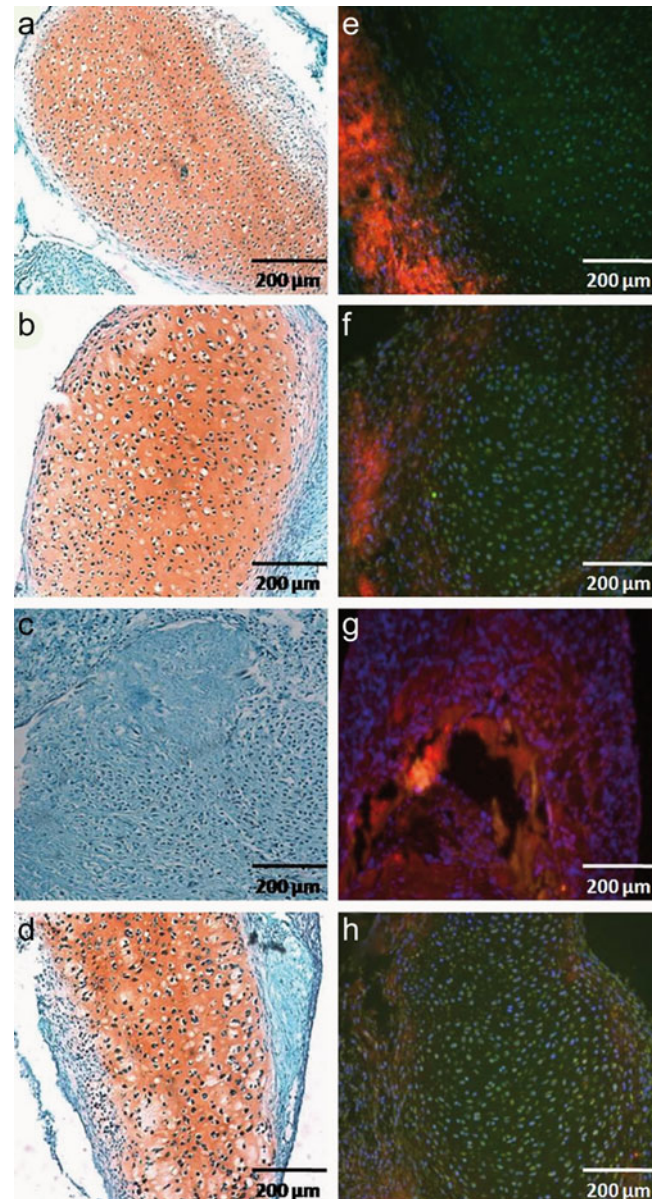
Furthermore, the excessive amounts of GAGs compared to collagens in tissue-engineered cartilage are believed to negatively influence tensile properties of the tissue (Responde et al. 2007). Studies involving the application of the enzyme chondroitinase-ABC, which degrades GAGs (Prabhakar et al. 2006) and thus reduces stress on the collagen network, have demonstrated increased tensile

properties of cartilage explants (Asanbaeva et al. 2007) and self-assembled tissue-engineered cartilage (Natoli et al. 2009; Bian et al. 2009). This effect is likely due to more or altered crosslinking, larger fibril size or altered fibril orientation (Responde et al. 2007).

### Collagen orientation

The importance of the arcade-like collagen structure for the load-bearing properties of native cartilage is well-emphasized in literature (Korhonen and Herzog 2008; Owen and Wayne 2006; Wilson et al. 2007; Shirazi and Shirazi-Adl 2008; Shirazi et al. 2008; Bevill et al. 2010). It is logical to assume that this collagen architecture reproduced in engineered cartilage tissue would lead to superior mechanical properties. However, only a few studies have focused on the importance of depth-dependent material properties in engineered cartilage. However, using depth-dependent scaffold properties or cell sources did not lead to an arcade-like collagen structure (Kim et al. 2003; Malda et al. 2005; Ng et al. 2005, 2006; Klein et al. 2007; Moutos et al. 2007). Mechanical loading could be another stimulus for obtaining an anisotropic distribution of collagen in engineered cartilage. The rationale is that, at birth, cartilage contains a random collagen structure. However, a few months after animals start to walk, cartilage develops its arcade-like structure (van Turnhout et al. 2010). Radial confinement of self-assembled constructs increased collagen organization in the direction perpendicular to the articular surface, with no change in collagen or GAG content (Elder and Athanasiou 2008). Furthermore, using polarized light microscopy, it has been shown that unconfined compression aligns collagen fibers perpendicular to the compressive loading direction (Kelly et al. 2006), i.e. aligned with the direction in which it cyclically elongates due to Poissons effects and incompressibility of the tissue. The strain field generated by applying unconfined compression may be useful to generate a superficial zone with collagen fibers parallel to the surface or higher modulus near the surface (Kelly et al. 2006; Khoshgoftar et al. 2011). However, a physiological collagen network with additional vertical fibers in the deep zone may not be produced by this loading regime, since vertical strains are absent. A numerical study by Khoshgoftar et al. (2011) suggests that a loading regime involving indentation with subsequent sliding of the indenter can stimulate the formation of an appropriate superficial zone with parallel collagen fibers. Adding lateral compression to this loading regime may stimulate the formation of a deep zone with perpendicularly aligned fibers, creating an arcade-like collagen architecture. Currently, in our group, experiments are pending in which this loading regime is applied to chondrocyte-seeded agarose constructs in order to create a

physiological collagen network in the engineered cartilage. This particular sliding indentation setup has already been shown to stimulate collagen synthesis in periosteum tissue (Fig. 1) (Kock et al. 2010). In that study, periosteal explants were embedded in between agarose layers, which induced cartilage formation, confirmed by synthesis of sGAG and



**Fig. 1** Sections of cultured periosteal explants, stained with Safranin-O (red, proteoglycans)/Fast Green (blue, collagen) (a–d, magnification  $\times 40$ ) and with antibodies for collagen types I and II (e–h, magnification  $\times 40$ ). Cartilage was produced by the explants between agarose layers, with and without addition of TGF- $\beta 1$  (a, b) and collagen type II was synthesized in this cartilage (e–f). Only collagen type I was visible in explants that were cultured under tension by dynamic loading and no cartilage was formed (c, g). When dynamic loading was combined with TGF- $\beta 1$  supplementation, cartilage formation was visible (d) and collagen type II could be seen in the chondrogenic area (h)

collagen type II (Fig. 1a, e). Addition of TGF-β1 to the culture medium did not further enhance this chondrogenic response (Fig. 1b, f). Applying sliding indentation only to the periosteum inbetween agarose layers enhanced the production of collagen type I, leading to the formation of fibrous tissue without any evidence of cartilage formation (Fig. 1c, g). However, when stimulated by both TGF-β1 and sliding indentation, collagen production was still enhanced, but now it was collagen type II, while sGAG was found to be similar to TGF-β1 or unloaded samples (Fig. 1d, h).

Zonal organization

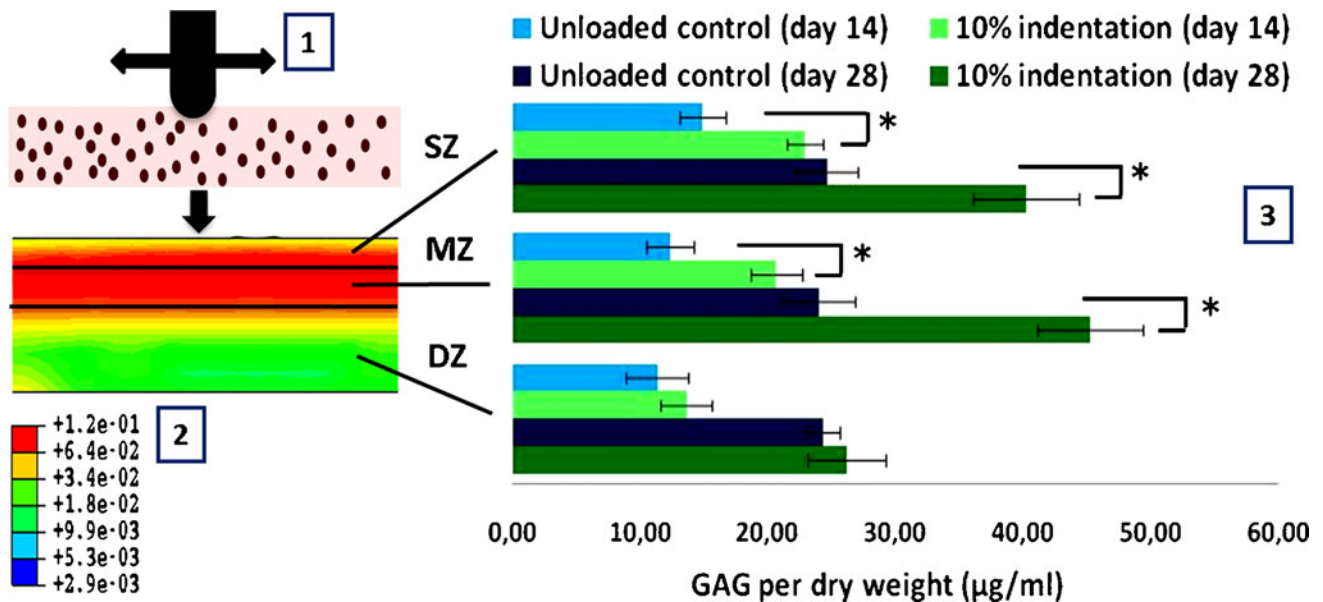
Articular cartilage engineering studies typically use homogeneous cell mixtures from juvenile animals that produce cartilage tissue with large amounts of ECM, but lack zonal organization and structure. Considering the prevalence and importance of zonal variations in normal articular cartilage, recent studies have aimed at engineering cartilage with zonal structure, function, or both. Approaches to mimic the zonal structure and function include cell-based, scaffold-based, a combination of cells and scaffold (hybrid), and methods based on application of depth-dependent strain fields.

Cell-based methods typically replicate the native distribution of chondrocyte populations by isolation of zonal chondrocytes, which are employed in specific regions of a construct and are shown to preserve their zone-specific phenotype and to secrete specific zonal markers (Kim et al.

2003; Klein et al. 2003; Waldman et al. 2003; Schuurman et al. 2009; Malda et al. 2010). However, in those studies, the depth-dependent material properties of the engineered cartilage were generally not comparable to native cartilage (Klein et al. 2007).

Scaffold-based methods include porous gradient scaffolds and multilayer hydrogels. An anisotropic pore architecture within 3D PEGT/PBT copolymer scaffolds developed using a 3D fiber deposition technique promoted anisotropic cell distribution, and GAGs and collagen type II distribution, like that in the superficial, middle, and lower zones of immature bovine articular cartilage (Woodfield et al. 2005). Other studies have used bi- or multilayered hydrogels to support the cartilage production by the different zonal subpopulations. Using zonal populations of chondrocytes seeded into layers of 2 and 3% agarose, bilayered cartilage constructs were produced with zonal chondrocyte organization and depth-dependent biochemical content, qualitatively similar to native cartilage (Ng et al. 2009b). Interestingly, this depth-dependent effect was not seen when full-depth chondrocytes were used in the same culture set-up, emphasizing the need for cells with typical zonal characteristics (Ng et al. 2005). Very recently, hydrogel-based bio-printing approaches have become available which provide organization via both scaffold architecture and controlled deposition of cells at predefined locations (Klein et al. 2009a, b; Cohen et al. 2006).

Recently, researchers have combined cell- and scaffold-based methods to induce spatially-varying properties into tissue-engineered cartilage constructs. Nguyen et al.



**Fig. 2** Sliding with an indenter over an chondrocyte-seeded agarose construct (1). The sliding indentation protocol led to a depth-dependent strain field (maximal principal strains) (2), with highest strains in the superficial zone (SZ) and the middle zone (MZ) and

lowest strains in the deep zone (DZ). The sliding indentation protocol induced depth-dependent ECM deposition (3), leading to the highest GAG content in the top half of the construct (SZ and MZ), which receives high strains according to numerical simulations. \* $p < 0.0017$



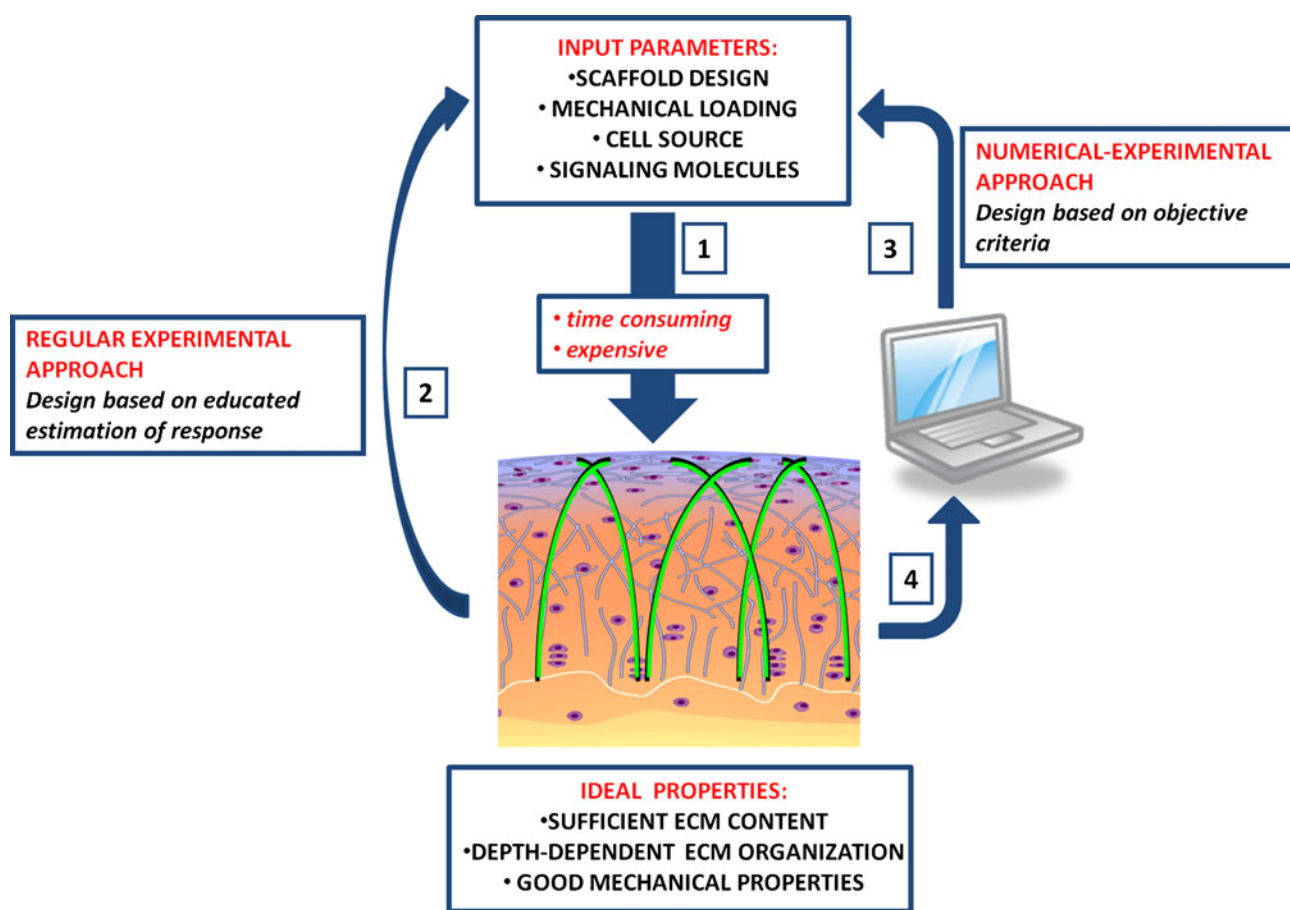
(2011a, b) demonstrated that layer-by-layer organization of specific combinations of natural and synthetic biomaterials can direct MSCs to differentiate into zone-specific chondrocytes and creates a native-like articular cartilage with mechanical and biochemical properties varying with depth.

A different approach to induce depth-varying inhomogeneity within chondrocyte-seeded agarose constructs is based on application of mechanical loading. We hypothesized that by applying depth-dependent mechanical cues to the chondrocytes, the tissue would be stimulated to form depth-dependent material properties. For this, we developed a custom-built bioreactor that indents constructs with a bar, which moves over the construct without relieving the indentation strain, a loading regime we refer to as sliding indentation (Kock et al. 2010). The sliding indentation protocol induced depth-dependent ECM deposition, leading to the highest GAG content in the top half of the construct (Fig. 2), which receives high strains according to numerical

simulations (Khoshgoftar et al. 2011). This confirms the hypothesis that depth-dependent mechanical cues give rise to depth-dependent matrix content. Currently, experiments are running to further investigate the effect of depth-dependent strain magnitudes and orientations on collagen production and orientation, since this is the major depth-varying component in articular cartilage, which is known to significantly contribute to the mechanical properties of the tissue.

### Conclusions and future directions

Current treatments for in vivo repair of articular cartilage damage, including mosaicplasty, microfracture, and autologous chondrocytes injection, have successfully been shown to relieve pain and improve joint function, but long-term results are unsatisfactory. The major drawback of these methods is that these mostly result in the formation of



**Fig. 3** Outlook on the approaches for tissue engineering of cartilage with sufficient ECM amounts, ECM organization and mechanical properties. The traditional approach relies on experimentally exploring the effect of (a combination of) different input parameters (1, 2). These experiments are very time consuming, labor intensive and therefore expensive. We propose a computer-aided approach which includes theoretical and computational evaluation of the influence of

different input parameters in a modeling approach (3). With such models, it is possible to discriminate promising protocols from those with poor potential via in silico experiments. In addition, the outcome of experiments could be used for optimization and validation of the theoretical and computational models (4). This approach is less based on trial and error, less time consuming and therefore cheaper



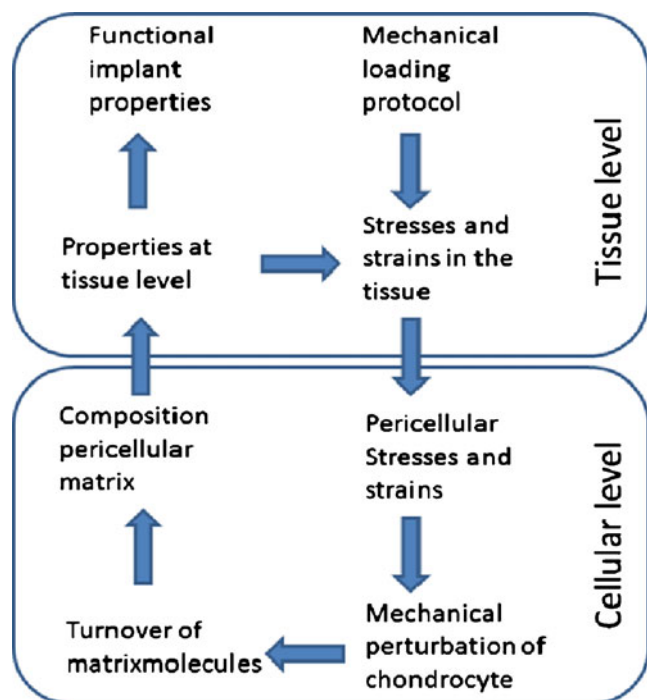
fibrocartilage with inferior mechanical properties, which is likely to degrade over time because of its insufficient load-bearing capacity. Tissue engineering has been proposed as a promising solution to circumvent this problem. The major advantages of engineering cartilage *in vitro* are that culture conditions can be precisely controlled and that its material properties can be evaluated during culture, in contrast to *in vivo* approaches which greatly depend on the conditions at the donor site. Implantation of a construct with properties that enable it to withstand *in vivo* loads will have a higher probability for success.

In the past decades, enormous progress has been made in the optimization of strategies for tissue engineering of functional articular cartilage. However, there are still many issues to be addressed before engineered cartilage can be used as a clinical therapy. Finding an optimal cell source is the first critical issue. Although primary native chondrocytes perform best, their limited availability makes their use unrealistic. Preventing loss of phenotype when chondrocytes are expanded is a major challenge. Stem cells seem to be a promising alternative, but they produce cartilage tissue with inferior properties compared to chondrocytes. In the next years, it will become clear whether, and if so which, stem cells could be the optimal cell source for cartilage tissue engineering studies. The second issue involves the choice for scaffold material. Natural and synthetic materials have been investigated, but until now none of these fulfill all the necessary requirements. Third, appropriate biochemical and/or mechanical triggers for matrix production and tissue organization are needed. It remains challenging to derive optimal stimuli that can promote proliferation and differentiation of cells and stimulate the synthesis of proper and sufficient ECM components and the secretion of enzymes that can remodel the produced ECM.

In this respect, the most important questions that remain are: which characteristics should the engineered cartilage possess in order to function as well as the healthy tissue, and how do we get there? It is clear that ECM content is important, but it is unclear to what extent we need to reproduce the native matrix components in engineered cartilage implants pre-implantation. It is possible to obtain native amounts of GAG in engineered cartilage, but collagen content is still far below native. In our opinion, future research should particularly focus on approaches to increase collagen content, which is essential for proper mechanical functioning of the tissue. Further, in order for tissue-engineered cartilage to be mechanically functional, we think that it is essential that the depth-dependent matrix organization, especially the arcade-like collagen architecture, should be reproduced to some extent. But how can this be best achieved? Some attempts have been made, but the native structural ECM organization has not yet been reproduced. Finally, for successful repair, complete integra-

tion of the neo-cartilage with the surrounding tissue is required, which is an aspect that demands opposite properties from those required for mechanical load bearing.

Exploring all these different aspects experimentally will be challenging, costly and time-consuming. We would progress faster if we could reduce the number of experimental conditions to explore. This may be achieved if we could refine or enhance the interpretation of experimental results, or if we were able to predict the outcome of particular experimental conditions and thereby discriminate promising protocols from those with poor potential. One way to achieve this is through theoretical modeling (Fig. 3). Models may provide insight into aspects that are difficult to assess during the experiment. For example, the profiles of glucose, lactate and oxygen throughout a tissue engineering construct in time are difficult to measure, but can be computed based on nutrient utilization data. These computed profiles allowed extended interpretation of measurements related to compromised nutrition in the core of engineered cartilage (Sengers et al. 2005b), and explained why mixing of culture medium in rotating wall vessel bioreactors partly compensates for this compromised nutrition (Sengers et al. 2005a). Numerical studies have



**Fig. 4** Determining optimized mechanical loading regimes for engineering functional cartilage involves understanding how mechanical loading at the macroscopic levels perturbs cells at the microscopic level, how that perturbation stimulates the chondrocyte to adjust its pericellular matrix by matrix turnover, and how that microscopic tissue development modulates the functional properties at the macroscopic scale. Ultimately, modeling will need to cross these scales to predict how mechanical perturbation would modulate tissue properties with time of culture

also been dedicated to understanding how mechanical loading applied at the macroscopic level would perturb chondrocytes at the microscopic level. These perturbations depend on the properties of the scaffold (Appelman et al. 2011) and the pericellular matrix (Guilak and Mow 2000). The premise is that such insight could be used to optimize scaffold properties, or the mechanical stimulation protocols for tissue engineering. However, application of these insights is difficult, because the cellular microenvironment changes with time during tissue development. To incorporate cartilage matrix development is a major challenge that modelers are currently exploring (Sengers et al. 2004; Klisch et al. 2008; van Donkelaar et al. 2011). Models that take into account the actual, measurable tissue composition (Wilson et al. 2006; Klisch et al. 2008) are of particular interest, because these allow direct translation between predicted proteoglycan and collagen contents and biochemical data, or between predicted matrix distributions and histology. The next step in these developments is to add effects of mechanical perturbation to these growth and development models, in order to predict tissue content, distribution, and collagen orientation depending on the applied loading protocol (Khoshgoftar et al. 2011). Once this has been established, it will be possible to predict the effects of loading protocols on functional tissue development. Such predictions may lead to the design of promising tissue engineering protocols, and reduce the number of experiments with poor potential (Fig. 4).

In summary, cell source, scaffolds, signaling molecules and mechanical loading are considered to be the most important parameters to optimize for improved tissue engineering cartilage. Ultimately, the combination of these factors should result in mechanically functional tissue-engineered cartilage with sufficient collagen content and depth-dependent matrix organization, which can be implanted and which will withstand the mechanically demanding in vivo environment. Cell source and signaling molecules may be essential to enhance total matrix contents. However, these are not likely to trigger tissue orientation. Therefore, we postulate that only by controlling the mechanical cues will we be able to engineer a cartilage with its particular collagen fiber orientation and inhomogeneous matrix distribution.

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