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REVIEW

Cartilage tissue engineering and bioreactor systems for the cultivation and stimulation of chondrocytes

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Abstract Damage to and degeneration of articular cartilage is a major health issue in industrialized nations. Articular cartilage has a particularly limited capacity for auto regeneration. At present, there is no established therapy for a sufficiently reliable and durable replacement of damaged articular cartilage. In this, as well as in other areas of regenerative medicine, tissue engineering methods are considered to be a promising therapeutic component. Nevertheless, there remain obstacles to the establishment of tissue-engineered cartilage as a part of the routine therapy for cartilage defects. One necessary aspect of potential tissue engineering-based therapies for cartilage damage that requires both elucidation and progress toward practical solutions is the reliable, cost effective cultivation of suitable tissue. Bioreactors and associated methods and equipment are the tools with which it is hoped that such a supply of tissue-engineered cartilage can be provided. The fact that in vivo adaptive physical stimulation influences chondrocyte function by affecting mechanotransduction leads to the development of specifically designed bioreactor devices that transmit forces like shear, hydrostatic pressure, compression, and combinations thereof to articular and artificial cartilage in vitro. This review summarizes the basic knowledge of chondrocyte biology and cartilage dynamics together with the exploration of the various biophysical principles of cause and effect that have been integrated into bioreactor systems for the cultivation and stimulation of chondrocytes.

Keywords Bioreactor · Tissue engineering · Biomedical engineering · Cartilage · Chondrocytes · Physical stimulation

Introduction

The main interest in cartilage research emerges from joint ailments. Adult articular cartilage has a limited ability to recover after degenerative and rheumatic diseases as well as traumatic injuries. Diseases of hyaline cartilage represent one of the major health problems especially in industrialized countries with high life expectancy (Buckwalter 2002; Flugge et al. 1999). Currently, more than 40 million US American citizens (approximately 15% of the overall population of the USA) suffer from arthritis. It is estimated that nearly 60 million US American citizens will be affected by the year 2020. Arthritis is a term for a group of different disorders that are characterized by inflammation of one or more joints including the knee, the shoulder, elbow, hip, and ankle joint (Ayad et al. 1998; Leavitt et al. 2005). Arthritis actually comprises more than 100 different disease patterns, which collectively affect virtually all parts of the body. Pain and swelling as typical consequences of joint inflammation reduce considerably the quality of the affected patients' lives (Lawrence et al. 1998). The socio-economic consequences of arthritic diseases (e.g., inability to work, early retirement) and costs for health care are

Dedicated to Prof. K. Arnold on the occasion of his 65th birthday.

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immense. Of the many diagnoses characterized as rheumatic disorders (e.g., gout, rheumatic fever, and lyme arthritis), rheumatoid arthritis (RA) and osteoarthritis (OA) account for the very most of the diseases. The erosion of the articulating surfaces of joints, known as osteoarthritis, currently affects more than 200 million citizens worldwide and in particularly about 21 million people in the USA. More than 25% of the over 50 year olds suffer from OA and more than 80% of those over 65 suffer from some form of degenerative joint disease. In Germany 150,000 to 180,000 arthroscopic operations are carried out annually, while there are 1,500,000 such operations in the US and 350,000 in the EU. Currently, no reliable long-term curative strategies have experienced wide clinical acceptance. The conservative surgical treatment (in more than 50% of patients) by total joint replacement leads to societal costs of approximately US §$15 billion per annum in total. This situation is the driving force behind the numerous ongoing efforts to develop new—tissue engineering—concepts and—regenerative—technologies for the treatment of degenerative and traumatic joint surface defects. It is necessary, as it is with all artificial organ and tissue engineering endeavors, to consider the in vivo situation of articular cartilage with regard to its structure and composition and the biochemical and mechanical environments (see "Introduction", "Dynamics of articular cartilage", "Conditions in vivo") in order to identify suitable in vitro culture conditions for the stimulation of chondrocytes in bioreactor apparatuses (see "Bioreactors") and for the production of autologous cartilage grafts in GMP (good manufacturing practice)—capable manufacturing devices (see "From chondrocyte loaded laboratory devices to stem cell based bed-side manufacture?"). Focus on these last two sections will be on those bioreactors in which cultivated chondrocytes or intact excised cartilage tissue are intentionally exposed to mechanical stresses.

Types of cartilage

It is possible to distinguish three major types of cartilage that differ in the biochemical composition and structure of their extracellular matrix (ECM), the resulting mechanical properties and therefore their occurrence in the human body. One type is the yellowish elastic cartilage characterized by chondrons with only a few cells, a small concentration of proteoglycans (PGs), but much elastin. Elastin is interwoven into the collagen mesh and the more this protein is present, the more flexibility is provided to the tissue. Elastic cartilage is surrounded by perichondrium and appositional growth is guaranteed through cell differentiation of the connective tissue. This tissue type is less vulnerable to degenerative changes. Elastic cartilage is suitable to resist bending and so it can be found in the epiglottic cartilage, the smaller laryngeal cartilage, the external ear and auditory tube, or the small bronchi (Buckwalter 1997; Montes 1996; Möricke 1997). A second type of cartilage is the fibrocartilage, providing other physical characteristics. Like the elastic type it contains a small concentration of PGs, but by contrast far less elastin. The meniscus is a fibrocartilaginous tissue composed primarily of an interlacing network of collagen fibers with a longitudinal, circumferential orientation which gives it unique functional properties. The ECM of fibrous cartilage consists of approximately 60–70% collagen, 8–13% non-collagenous proteins, and 1% PGs in dry weight. The water content in native meniscal tissue is around 70–75% (Flugge et al. 1999). Meniscal cells in the superficial layers depict oval or fusiform morphology, a little cytoplasm and rough endoplasmic reticulum whereas the rounded or polygonal cells in deep zones show morphological similarities to articular chondrocytes. However, these cells predominantly synthesize thick collagen type I fibers to resist compression or tensile stress. In conclusion, fibrocartilage is an ideal material for disci and menisci (Buckwalter 1997; DeHaven and Arnoczky 1994; Leeson et al. 1985; McDevitt and Webber 1990; McNicol and Roughley 1980; Möricke 1997). The third and most widespread cartilage in the human body is the hyaline type. As the name already implies, it appears as a white and slightly bluish tissue with a macroscopically smooth surface. Its resistance to compression or tensile forces is due to the net-like organized structure of the collagen type II fibers combined with a high concentration of PGs. Hyaline cartilage can be found in the nose, the trachea, bronchi, and most joints—in synarthroidal as well as in diarthroidal joints (Buckwalter 1997; Buckwalter and Mankin 1998b; Meachim and Stockwell 1973; Möricke 1997). In synchondroidal joints, e.g., between the ribs and sternum, in the intervertebral discs, or in the epiphyseal junction of the femur, it builds up a continuous connection between bones. In diarthroidal joints hyaline cartilage covers the contact zones of two interlocked bones and is called articular cartilage (Fig. 1). Articular cartilage is a unique type of connective tissue. Its outward appearance as a simple thin layer covering the articulating joint surfaces belies a specific structure and unique functional and mechanical properties. The two layers are separated by the viscous synovial fluid. A capsule encloses the entire joint and retains the synovial fluid (Schünke 2000).

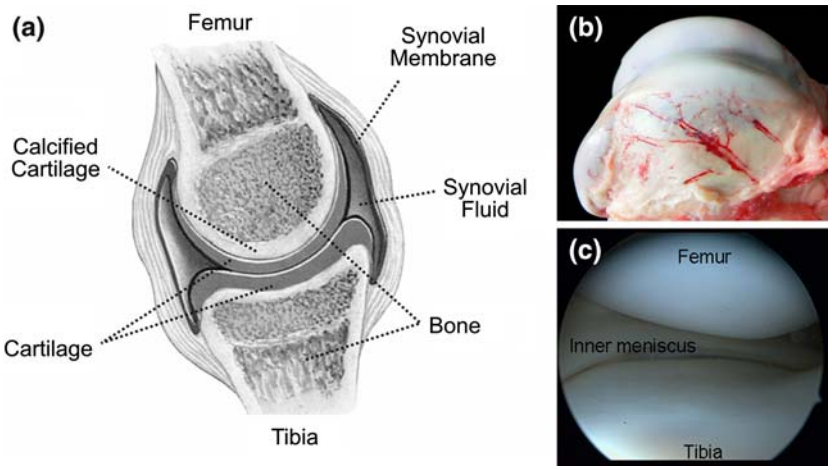

Fig. 1 Sketch of a knee joint (**a**), *side view* on femoral condyle of an open joint (**b**) , and *arthroscopic view* of a healthy human knee joint (**c**)

Structure of articular cartilage

Articular cartilage is a well-characterized tissue. It has the lowest volumetric cellular density of any tissue in the human body. Chondrocytes are the exclusive cell type. In humans, chondrocytes contribute only about 1% of the tissue volume. The remaining 99% is made up of a complex ECM (Buckwalter et al. 1988; Hamerman et al. 1970; Heath and Magari 1996; Stockwell 1967). The chondrocytes are situated in small cavities (lacunae) within the cartilage tissue. The spherical cells are found as single, isolated cells, or in a chondron, an aggregate of several chondrocytes. Even in chondrons there is no direct cel–cell contact. Cilia extend into the surrounding ECM (Buckwalter and Mankin 1998b; Leeson et al. 1985; Meachim and Stockwell 1973; Palfrey and Davies 1966; Stockwell 1979). The cells sense the structure and composition of the ECM and carry out their primary function that is to maintain it. The chondrocytes themselves synthesize all necessary ECM components (Buckwalter and Mankin 1998b; Cohen et al. 1998; Meachim and Stockwell 1973). A prominent golgi complex and a very large granular endoplasmatic reticulum enable synthetic activities. Although several typical ultrastructural features may be identified, none of these is sufficient to uniquely identify chondrocytes. Instead, they are identified by the surrounding ECM (Buckwalter 1997; Leeson et al. 1985; Palfrey and Davies 1966; Stockwell 1979). The unique viscoelastic properties of articular cartilage are a consequence of the molecular nanoarchitecture and zone-specific organization of the matrix components in this tissue, which are synthesized by the chondrocytes (Fig. 2). The ECM of articular cartilage consists (as wet mass) of about 60–85% water and dissolved electrolytes. The complex solid framework is composed of collagens (10–30%), PGs (3–10%) and non-collagenous proteins and glycoproteins (Buckwalter 1997; Buckwalter et al. 1990; Mankin and Thrasher 1975; Muir 1973; Ratcliffe and Mow 1996; Scott 1999). In the ECM surrounding chondrocytes, three regions can be classified according to biochemical composition, function, and appearance. The pericellular matrix covers the surface of every chondrocyte as a thin layer. The territorial matrix envelops the pericellular regions of a single cell or one chondron. The interterritorial matrix makes up the bulk of the cartilage volume and provides the tissue with its characteristic functional properties. The pericellular and territorial matrix regions serve to bind the cells to the interterritorial matrix, to protect them from damage under tissue load and to transmit mechanical signals into the cells. (Buckwalter et al. 1988, 1990). An additional characteristic of cartilage which has a strong bearing on its biochemistry and biomechanical behavior is the

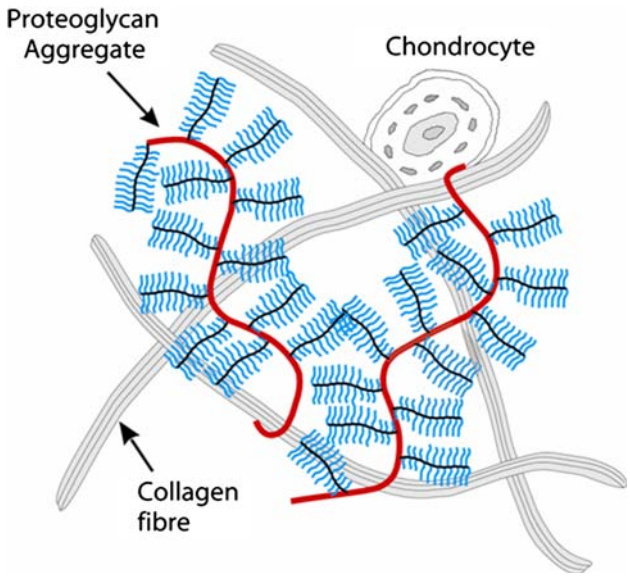

Fig. 2 Illustration of the extracellular matrix (*ECM*) organization of articular cartilage

complete lack of blood vessels, lymphatic vessels, and neurons. All intra-tissue transport is therefore completely reliant on avascular processes and is consequently slow. The supply of nutrients and oxygen and the removal of waste products are affected by an overlying layer of fibrillar vascular tissue, the perichondrium (Buckwalter et al. 1988; Buckwalter and Mankin 1998b; Leeson et al. 1985; Möricke 1997).

Composition of articular cartilage

Currently, the collagen superfamily contains 27 different types which function as structural components of the peri- and ECM in vertebrate tissues (Eyre 2004). Articular cartilage contains seven collagen types wherein a heteromer of types II, IX, and XI forms the characteristic basic architecture and types III, VI, XII, and XIV was found in small fractions in this tissue (Eyre 2002). In articular cartilage 90–95% of collagen in the ECM is formed by collagen type II fibrils, which provides tensile strength of the tissue (Fig. 3). In association with type XI it forms a mesh wherein type IX—member of the collagen subgroup "FACIT" (Fibril Associated Collagens with Interrupted Triple helices) is covalently linked to the surfaces of the type II fibrils and further enables a cross-linked framework to aggrecan (Eyre 1995; Smith and Brandt 1992). The non-fibrillating type VI forms elastic fibers and can be found around cells of the middle zone and throughout the ECM in small amounts of up to 1% of overall collagen (Wu and Eyre 1989). Young and coworkers localized small amounts of collagen type III on the surface of the collagen type II fibril network of adult human cartilage (Young et al. 2000). Collagen type X can be observed in a mineralized form in the calcified zone of articular cartilage. It is predominantly associated with hypertrophic cartilage and has often been seen as unique to that tissue. In adult articular cartilage positive immunostaining for collagen type X indicates chondrocytic hypertrophy during the development of arthrosis (Aigner et al. 1993; Gibson and Flint 1985; Schmid et al. 1990). The highly negatively charged glycosaminoglycans (GAGs) of the PGs provide the cartilage its swelling capacity (Comper and Laurent 1978; Knudson and Knudson 2001). In articular cartilage two main PG families could be observed. The majority (50–85%) of the overall PG content in this tissue type were presented by large aggregating PGs like versican and the major biomolecule aggrecan, which consist of a protein backbone, the core protein, to which unbranched GAGs side chains of chondroitin sulfate (CS) and keratan sulfate (KS) are covalently attached (Fig. 4) (Schwartz et al. 1999; Watanabe et al. 1998). In cartilaginous tissues from other sources, heparan sulfate in the temporomandibular joint disc (Axelsson et al. 1992) and dermatan sulfate in the knee mensicus (Herwig et al. 1984) may also be present. In the most abundant PG, the aggrecan, KS chains are located primarily near the hyaluronan strand, whereas on the other hand, the CS chains are more distant from the hyaluronic acid (HA) strand. The distribution of both GAGs depends on the zone of the cartilage source and varies amongst individuals due to genetic differences (Maroudas and Venn 1977; Venn and Maroudas 1977).

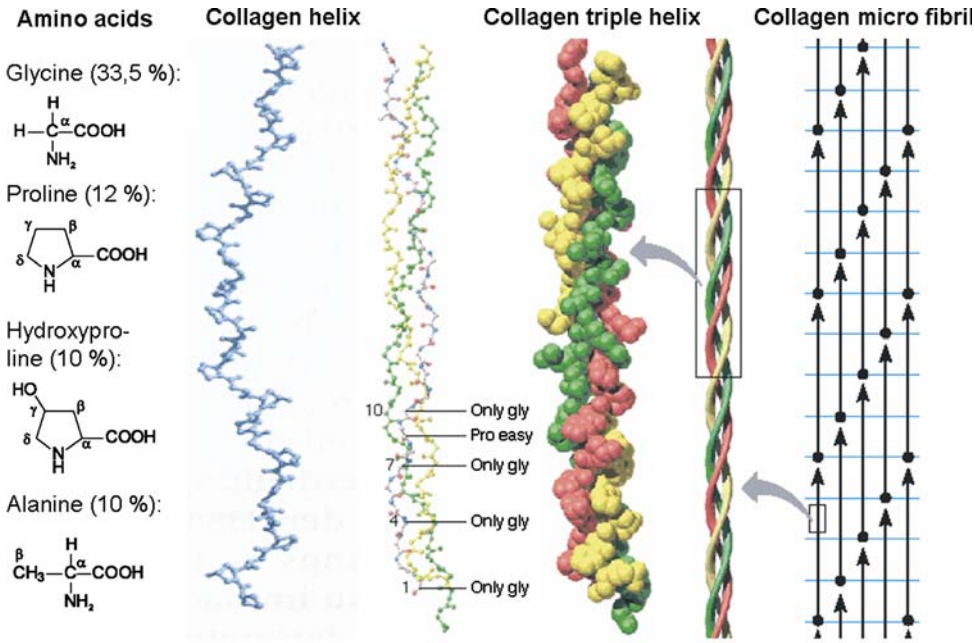

Fig. 3 Diagram of collagen structure from the isolated amino acids to collagen fibrils. The collagen right-handed triple helix is formed from three individual polypeptide chains resulting in a (Gly-*X*–*Y*), repeat structure which characterizes all collagen types. The *X* and *Y* position is often occupied by proline and hydroxyproline. Each of the three α-chains of articular cartilage specific type II collagen fibrils forms an extended left-handed helix with a pitch of 18 amino acids per turn. The three chains, staggered by one residue relative to each other, are supercoiled around a central axis in a right-handed manner to form the triple helix

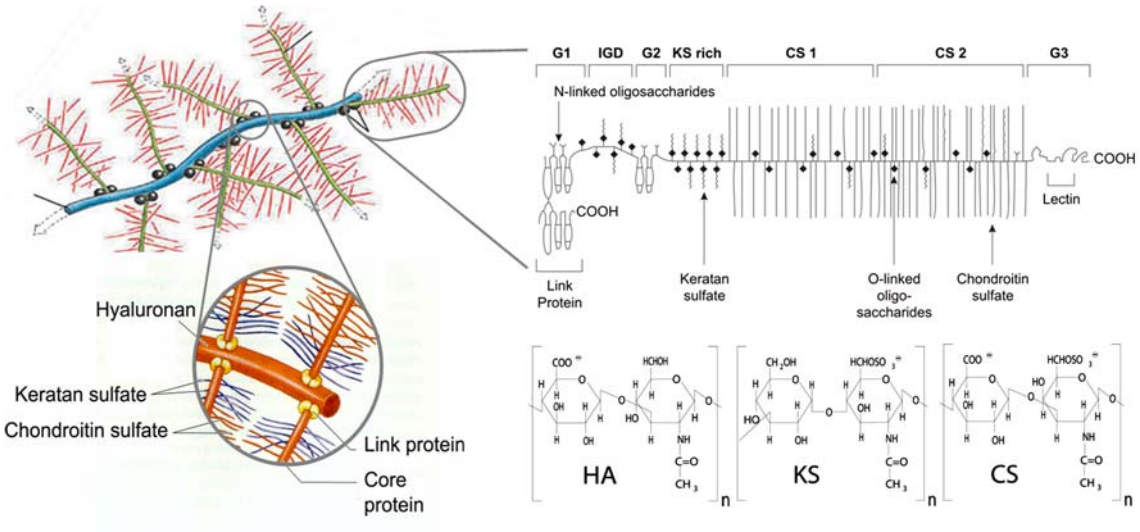

Fig. 4 Schematic sketches (*left*) and chemical structures (*right*) of the most relevant polysaccharides of proteoglycans (PGs) in articular cartilage. The PGs consist of a strand of hyaluronic acid (*HA*), to which a core protein is non-covalently attached. On the core protein, glycosaminoglycans (*GAG*s) such as keratan sulfate (*KS*) and chondroitin sulfate (*CS*) are covalently bound in a bottle brush fashion. The resulting architecture of the large aggregating proteoglycan aggrecan depicts the stabilization of this core protein to hyaluronan by the link protein

The isomers of CS, 4- (C4S) and 6-sulfate (C6S) are produced in vivo by chondrocytes in various proportions, also in dependence of the species and donor site reaching from equal relations to an increased ratio of C6S:C4S in human articular cartilage during the development from birth to skeletal maturity as reported by Bayliss et al. (Bayliss et al. 1999). Due to the enormous negative charge of GAGs side chains, aggrecan provides the compressive stiffness of cartilage by fulfilling the Donnan ion distribution law. The Donnan osmotic pressure effect in cartilaginous tissues leads to the swelling of the matrix with interstitial water, which becomes initially pressurized and forced out of the aggrecan network under enormous absorption of the applied forces (Ateshian et al. 1997; Lai et al. 1993; Mow et al. 1992). In mature articular cartilage the family of small interstitial or rather small leucin-rich PGs (SLRP) contributes around 10% to the total PG content (Table 1). For example, biglycan is found in the pericellular matrix of chondrocytes from load bearing zones were it binds to non-fibrillating type VI collagen and TGF-β (Bianco et al. 1990). Decorin occurs mostly in collagenous matrices, where it binds with types I and II collagen, TGF-β and fibronectin (Hildebrand et al. 1994; Schmidt et al. 1991). Besides collagen variants and PGs as major components of cartilage, a couple of non-collagenous and glycoproteins (Table 1) are present in cartilage tissue. The function of most of them is not well described and is still under investigation. It seems that these proteins were responsible for the cartilage-specific aggregation and integration of the ECM network and they also influence chondrocytes by promoting cell attachment to different substrates (Heinegard and Oldberg 1989). The cartilage oligomeric matrix

Table 1 Structural macromolecules in articular cartilage

Component	Elements	Weight
Water		60–80%
Collagen type II		10–20%
Aggregating PGs	HA, KS, C4S, C6S	5–7%
Aggrecan		
Versican		
Non-aggregating PGs		<5%
SLRP class I	Biglycan, Decorin	
SLRP class II	Fibromodulin, Lumican, PRELP; Perlecan, Epiphycan	
Syndekan family	Amphiglycan, TGF-β3 Receptor β3 glycan, Glypican	
Other collagen types	Collagen types III, V, VI, VII, IX, X, XI, XII, XIV	
Non-collagenous and glycoproteins	COMP, Link protein, Anchorin, Fibronectin, Tenascin, Thrombospondin, Chondroadherin, Fibrillin	
Lipids	Phosphatidylserine	

SLRP small leucine-rich proteoglycan, *PRELP* prolin arginine-rich and leucine-rich repeat protein, *COMP* cartilage oligomeric matrix protein (=Thrombospondin-5)

protein (COMP, Thrombospondin-5) was prominent in territorial and interterritorial zones of ECM (Hedbom et al. 1992; Shen et al. 1995). An in vitro study has shown the interaction of COMP with fibrils of types I and IX collagen. During arthrosis the detected amount of COMP in blood and synovial fluid was found at increased levels (Saxne and Heinegard 1992).

Properties of articular cartilage

Since articular cartilage is present in most joints it has to resist great loadings of several times the body weight in the hip or the knee, for example. This requirement demands a highly organized tissue structure that itself implies the source for new problems. In a cross-section of every articular cartilage at least four different depth zones can be defined varying in their biochemical composition, the structure of the matrix components and even the number and shape of the embedded chondrocytes. From the superficial to the deep zone, the number of chondrocytes is decreased, whereas the dimension and metabolic activity are increased. Further on zone specific variations of collagen fiber orientation can be observed (Fig. 5). The cartilage surface displays an acellular dense layer of horizontally orientated and highly ordered collagen fibers. It is still a matter of discussion whether this lamina splendens might be an optical artifact. Right below this layer next to the synovial fluid, single spindular-shaped chondrocytes and highly concentrated collagen—86% of dry weight—are orientated in parallel to the surface. Both together are called the superficial zone that resists shear stress and reduces friction. This superficial zone makes up about 20% of the thickness of articular cartilage. The deeper transitional zone is thicker and incorporates metabolically very active cells of spherical or rounded shape. The chondrocytes there maintain the highest concentration of PGs of all zones and collagen fibers organized in a mesh form. These properties render the transitional zone ideal to withstand compression. The middle zone is the thickest one—60% of tissue depth—with the longest collagen fibers and columns of cells arranged perpendicular to the surface. The thin deep zone offers chondrocytes with ellipsoid morphology and is composed of 67% collagen content by dry weight. The thin and rigid calcified zone is where the long collagen fibers finally originate and the cartilage is anchored to the subchondral bone underneath; only a few, small chondrocytes appear here (Aydelotte and Kuettner 1988; Aydelotte et al. 1992; Buckwalter et al. 1990; Cohen et al. 1998; Weiss 1978). Among cartilage tissues the hyaline articular type has an additional quality that results in severe biological consequences. As the only exception, articular cartilage completely lacks the perichondrium and is covered by synovial fluid instead. Without the vascularized perichondrium the supply to the cartilage tissue is limited to slow processes like diffusion and convection taking place in the synovial fluid (Hodge and McKibbin 1969; Honner and Thompson 1971; Schünke 2000). The process of diffusion might be sufficient to feed the upper zones of the cartilage tissue. But in order to reach the deeper zones joint movements and the accompanied fluid convection throughout the tissue are essential to supply the chondrocytes. Nutrients and oxygen have to cross a longer distance from the blood to the cartilage anyway, leading to a low and hypoxic metabolism with a lower capacity for regeneration. Movements of the synovial fluid caused by joint motion are essential for keeping the tissue metabolically active and functional. Besides this important nourishing function, synovial fluid is of biomechanical importance since it distributes loads across the joint surface and decreases friction within the joint (Buckwalter 1997;

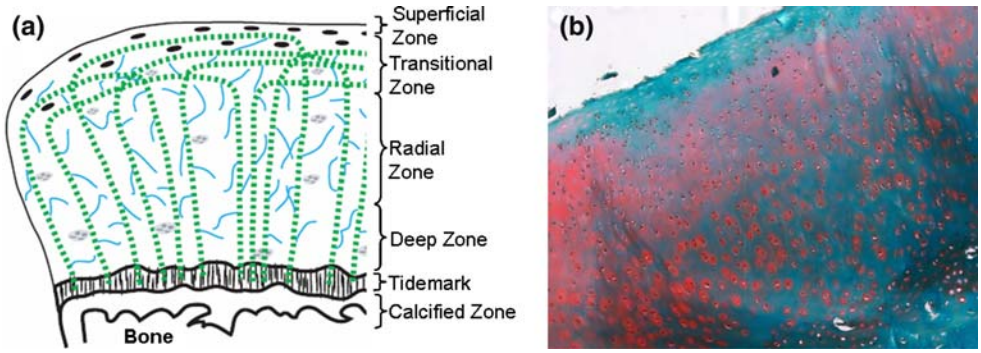

Fig. 5 Schematic drawing (**a**) of articular cartilage demonstrates the zonal arrangement and macromolecular organization by the illustration of PGs (*blue*) and collagen fibrils (*green*). Histological staining with *Safranin O* and *fast green* (**b**) of full-depth articular cartilage harvested from ovine medial femoral condyle depicting columnar formation. Subchondral bone is stained *green*, articular cartilage is *red* because of its proteoglycan (*PG*) content and calcified cartilage as a mixture of both results in a *pink* staining

Bursac et al. 1996; Leeson et al. 1985; McKibbin 1973; Möricke 1997). The lack of a perichondrium results in a weaker ability for self-regeneration. The perichondrium layer contains fibroblastic precursor cells capable of migrating into damaged cartilage where they surround themselves with matrix and replace the tissue in a process similar to appositional (exogenous) growth occurring during embryogenesis. Without a perichondrium this mode of cartilage regeneration is not possible. In conclusion the regeneration of damaged articular cartilage is only possible by the processes of interstitial (endogenous) growth that means proliferation of inherent cells and swelling of the tissue (Buckwalter 1997; Leeson et al. 1985; Möricke 1997). Considering this information articular cartilage actually has a very low, if any, capacity for self-repair (Buckwalter and Mankin 1998b; Heath and Magari 1996; Mankin 1982; Meachim and Stockwell 1973).

Healing-damaged articular cartilage

Due to the unique structural and functional characteristics articular cartilage tolerates a remarkable amount of intensive, cyclic, and static physical stress. Under normal conditions, the mechanical strength of articular cartilage remains constant despite structural alteration during ageing processes. The degeneration or loss of this impressive specific load-bearing capacity of articular cartilage caused by traumatic events (microtrauma, osteochondritis dissecans) or chronic and progressive degenerative joint diseases like OA and RA has immense patho-physiological, biophysical, clinical, and social consequences. The exact mechanism of the cartilage disintegration in arthritic joints, however, is not known. Once articular cartilage is degenerated or damaged by extensive overuse, injury, inflammation, or ageing, intrinsic repair mechanisms might replace the tissue. In the case of microtraumata and distinct chondral or partial thickness injuries [Grade II according to Outerbridge (1961) and types I to IV according to the Jackson and Bauer classification (Bauer and Jackson 1988)] such as flaps and fissures which do not penetrate the subchondral bone, cells initially undergo necrosis. After around 3 days adjacent and surviving chondrocytes begin to proliferate and to produce ECM, which is indicated by the morphological prominence of chondrocyte clusters with temporary increased type II collagen synthesis for a few days. Long-term examination offers almost macroscopically unchanged chondral lesions, and even a few cases proceed to OA (Hunziker 1999). Besides the limited regenerative abilities caused by the perichondrium and described above, another intrinsic mechanism is well known when deep cartilage defects occur and reach down to the subchondral bone (Grade III and IV according to Outerbridge). Damage of this kind is repaired by precursor or stem cells of mesenchymal origin from the subchondral bone marrow invading into the damaged site and replacing the hyaline cartilage with inferior fibrocartilaginous tissue (Caplan et al. 1997; Shapiro et al. 1993). In detail, osteochondral or full-thickness lesions gain access to vascular supplies and therefore bleeding occurs and the defect site is filled wth a fibrin clot. During the first week after injury multipotent mesenchymal stem cells (MSCs) from bone marrow migrate into this clot and start their resorption. In the following weeks stem cells differentiate into chondrocyte (-like cells) as indicated by the enormous synthesis of PGs which leads to the complete filling of the former defect site by repair tissue which offers some similarities to hyaline cartilage. After a couple of weeks the repair tissue visually resembles hyaline cartilage, but a decreased ECM content lacks the strength and properties of normal articular cartilage. Long-term observation with functional and histological examinations of the repair tissues in full-thickness defect sites demonstrate fibrillated fibrocartilage with mechanical inadequate properties instead of hyaline (-like) tissue formation (Caplan et al. 1997; Mitchell and Shepard 1980; Shapiro et al. 1993). Beyond the natural mechanisms a lot of different man-made methods to heal-damaged or degenerated articular cartilage already exist. Only some of them are used regularly in clinical reality. The technique most used is the resection of damaged cartilage and the implantation of a prosthesis made of synthetic material. But there are other therapeutic methods that can be combined if necessary (Buckwalter and Mankin 1998a; Goldberg and Caplan 1999; Menche et al. 1998; Minas 1999; O'Driscoll 1998). A couple of surgery techniques utilize the intrinsic cartilage self-repairing mechanisms originating in the subchondral bone. Mostly in the case of partial thickness defects, chondral shaving, the precise generation of deep microfractures or the (Pridie)-drilling into subchondral bone at the damaged site allows precursor cells from the bone marrow to migrate into the fracture and to differentiate into chondrocytes building up new fibrocartilaginous tissue (Kim et al. 1991; Pridie 1959; Steadman et al. 1997). Further techniques use autogenic or allogenic cellular material. These techniques involve the injection of chondrocytes, autologous chondrocyte transplantation (ACT) or autologous chondrocyte implantation (ACI) (Brittberg et al. 1996; Gillogly et al. 1998; Minas and Peterson 1999; Peterson et al. 2000), or similar cell solutions into the damaged site, the transplantation of perichondrial (Bouwmeester

et al. 1997) or periosteal tissue (Angermann et al. 1998; Hoikka et al. 1990; O'Driscoll 1999; von Schroeder et al. 1991) to cover the damaged site or a combination of both. It is also possible to replace the degenerated tissue by osteochondral plugs in a one-step operative technique called the osteoarticular transfer system (OATS) or mosaicplasty (Hangody et al. 1998). The decision which cell type is supposed to be used to heal-damaged tissue is likewise important. The choice depends on the actual medical indication and might vary between adult mature chondrocytes, MSCs from various sources, and progenitor cells from the periosteum, perichondrium or genetically modified cells (Brown et al. 2000; Caplan et al. 1997; Goomer et al. 2000; Hunziker 2002; Lee et al. 2003; Mandelbaum et al. 1998; Mason et al. 2000; O'Driscoll 1998). In most approaches one of these cell types is cultivated in vitro on some form of scaffold to create a preformed stable construct which is suitable for the implantation as a matrix-coupled autologous chondrocyte transplant (MACT) (Behrens et al. 2006). The function of scaffold materials is primarily to maintain structure and enable cells to attach, but they can also be linked with pharmacological agents in order to enhance cellular functions like attachment, proliferation or differentiation as well. Corticosteroids, HA or various growth factors have been used for that purpose, but have also been injected alone in liquid form into the remaining cartilage to promote healing. They can even be synthesized and overexpressed by the cells themselves after genetic modification (Boyan et al. 1999; Hunziker and Kapfinger 1998; Iwata 1993; van Beuningen et al. 1994). In most (pre)clinical approaches scaffold materials are transplanted either directly into defect cartilage or they have been combined with other techniques. The scaffold materials can be synthetics like teflon, carbon fibers, or polymers like f (PGA) or polylactic (PLA) acid and their copolymer PGLA, for example (Freed et al. 1994; Sittinger et al. 1994). But they can also consist of biological materials like fibrin clots or collagen (Ochi et al. 2001; Ting et al. 1998). During the past few years the clinical application of chondrocyte seeded approved MACT matrices based upon collagen type I hydrogels (e.g., CaReS®, Atelocollagen), and HA derivates (Hyalograft C) has been one of the main surgical procedures for repairing full-thickness cartilage defects (Andereya et al. 2006; Behrens et al. 2006; Marcacci et al. 2005; Marlovits et al. 2006). Advantages of these three-dimensional (3D) grafts include increased morphological, biochemical and biomechanical properties, better intra-operative handling, and a shorter convalescence (Fig. 6). However, limitation of chondrocyte availability and their decreased mitotic and metabolic activity have been an issue.

Dynamics of articular cartilage

Articular cartilage is affected in vivo and in vitro by several biomechanical forces like direct compression, tensile and shear forces, or the generation of hydrostatic pressure and electric gradients as well as changes in the pH. The dynamic processes that occur in cartilage are necessary to maintain its structure and function and have to be applied in the tissue engineering of cartilage as well. In order to understand these processes different mechanical stimulations and their dynamic effects are outlined here.

The biphasic model

To understand and describe the processes taking place within cartilage, this tissue is at minimum considered to have a biphasic system. One phase is represented by the solid components of the ECM that form a porous-permeable composite material. The molecules that participate in this composite material are a mesh of collagens attached to each other by non-collagenous proteins and the non-covalently bound PGs. The second phase represents the interstitial water together with the ions

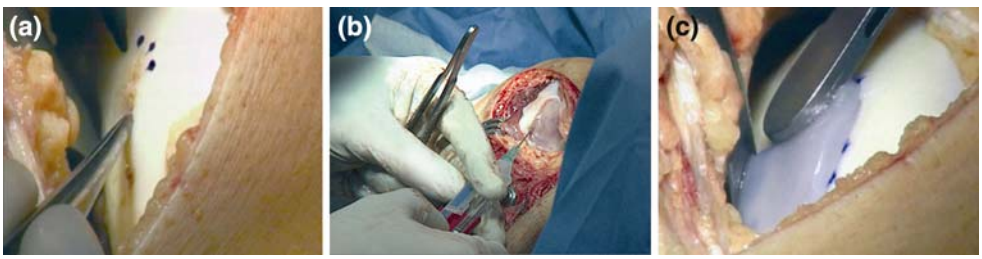

Fig. 6 Surgical procedure for the implantation of a matrix-coupled autologous chondrocyte transplant (*MACT*) into a full-thickness defect of femoral condyle. The *left image* (**a**) depicts the debridement of the cartilage defect site. The *middle* and *right pictures* show the glueing with fibrin (**b**) and the fixation (**c**) of the adjusted cell-seeded collagen type I matrix (CaReS®) into the former defect side

solubilized in it. It is likewise possible to describe cartilage as a system with three phases, separating the fluid phase into a water and an ion phase. Either model is useful in the mathematical description of the viscoelastic properties of cartilage (Cohen et al. 1998; Mow et al. 1992; Mow and Wang 1999). The dynamics within articular cartilage result from the structural and the chemical properties of its components. PGs predominantly consist of highly sulfated and therefore negatively charged disaccharides (Lindahl and Hook 1978; Muir 1973; Roughley and Lee 1994). These GAGs are responsible for the capacity to bind positive charged ions and with that the entire fluid phase. The osmotic potential caused by this fixed high local concentration of negative charges results in a tendency for water inflow. But the PGs ability to swell is limited by compressive forces from the outside and by the collagen network inside the ECM that resists the tensile forces caused by swelling. An equilibrium is reached when the swelling pressure on one side and the coherent and compressive forces—that form the matrix stress—on the other side are even (Cohen et al. 1998; Mow et al. 1992; Mow and Wang 1999; Schinagl et al. 1996; Setton et al. 1999).

Applying compression

Uniaxial compression has been extensively used to apply load since it was considered to be the most important form of loading to act on cartilage in vivo. But what kinds of processes occur during compression? Soon after loading the cartilage with a uniaxial compressive force, an increasing internal hydrostatic pressure arises in the tissue. This is because the negative charges of the solid phase provide frictional resistance to a shifting of the fluid phase through the tissue. This slow movement together with the inability of aqueous solutions to be compressed is the reason for the increasing hydrostatic pressure. This high hydrostatic pressure prevents a quick deformation of the tissue. The hydraulic permeability inversely correlates with the fixed charged density of the PGs, while the collagen at this stage does not offer much resistance to compression (Cohen et al. 1998; Heath and Magari 1996; Setton et al. 1999; Soltz and Ateshian 1998). Despite the low hydraulic permeability the fluid phase is squeezed out of the tissue, if the compression is maintained. The more fluid is squeezed out, the more load has to be borne by the collagen network of the solid phase. Like in other viscoelastic materials deformations of the solid phase occur relatively quickly in the beginning and slow down afterward. There are two reasons for this. The first reason is the change in porosity. The more the solid network is compressed, the smaller the remaining pores are. In conclusion, a lower permeability results in a decrease in fluid flow and a more compact and rigid solid phase. The second reason for the deformation process slowing down may not be realised at first sight. With increasing deformation of the solid phase, the tensile stress for the collagen network increases as well (Cohen et al. 1998; Eckstein et al. 2001; Grodzinsky and Urban 1995; Setton et al. 1999; Soltz and Ateshian 1998). Theoretically, this tensile stress lasts until all collagen fibers are aligned in parallel along the axis of loading. If the tensile force passes this point, the collagen network gives way and will be torn. The ability to resist tensile forces caused by a long lasting compression is mainly dependent on the concentration of collagen and the orientation and number of network crosslinks, but is not influenced by the PGs. The gradual deformation of the cartilage as a result of the compression described here is an effective way to protect the rigid parts of the ECM from high load peaks (Cohen et al. 1998; Mow et al. 1992; Schinagl et al. 1996; Setton et al. 1999; Soltz and Ateshian 1998).

Applying hydrostatic pressure

There are a number of investigations where hydrostatic pressure has been used as the only applied load. Hydrostatic pressure does not need to harm the tissue if a free and direct exchange between the surrounding aqueous liquid and the liquid phase inside the tissue is possible. A slowly increasing hydrostatic pressure will advance into the liquid phase and reach the same level as outside. In this case equal forces act from every side on the network of collagens and PGs and will not damage it. There are also no internal fluid flows. But hydrostatic pressure that increases quickly does not advance into the liquid phase and will put stress on the solid phase with a long-lasting impact—similar to the processes during compression (Iwata 1993; Setton et al. 1999). It is very interesting to note that both under in vivo and in vitro conditions the pressurization of the interstitial fluid supports the majority of applied compressive loads and only less than 10% of the load remains for a direct compression of the solid phase (Eckstein et al. 2001; Mow and Wang 1999; Soltz and Ateshian 1998). This means that not compression but the indirect application of hydrostatic pressure is the most important load occurring in joints.

Applying shear stress

Shear stress is another force that cartilage has to resist when the synovial fluid is pressed alongside the smooth

surface of the tissue as a consequence of joint movement. Cartilage responds on pure shear by deforming but with no change in the volume, no pressure gradient and no fluid flow. The upper zones of cartilage react flexibly and are able to shift vertically up to 15° (Cohen et al. 1998; Heath and Magari 1996; Mow et al. 1992; Setton et al. 1999; Zhu et al. 1993). Even the embedded chondrocytes in the superficial zone seem to react on this permanent shear stress in the way that they shape their form following the force vector. Since the deeper and calcified zones of cartilage are not able to react that flexibly, the shear stress is actually greatest in this zone close to the subchondral bone. Responsible for the resistance against shear stress are not the PGs, but the collagens directly proportional to their concentration (Cohen et al. 1998; Schinagl et al. 1996; Setton et al. 1999; Smith et al. 1995; Zhu et al. 1993).

Transduction of biomechanical stimuli

Without doubt chondrocytes are capable of reacting on biomechanical stimulations and converting them into intracellular signals. In fact this capability is not limited to chondrocytes; other cell types are influenced in their normal cellular functions, too. Hydrostatic pressure within a physiological range is for example known to have effects on exocytosis, transcription, and translation rates and it alters the activity of Na^+/K^+-ATPase. It is therefore not surprising that mechanical stimuli induce biochemical changes at all, but chondrocytes are a cell type where the effects are essential for the maintenance of the entire tissue (Browning et al. 1999; Grodzinsky and Urban 1995). How do chondrocytes sense mechanical impacts? There is evidence that they react on the shifting of currents and the resulting electrical fields or changing osmolarity induced by mechanical forces. Even an artificial change in the local pH or the generation of an electrical field across cultivated chondrocytes yields biochemical reactions similar to those observed after usual mechanical loadings. These facts indicate the close interaction between all factors influencing the tissue. It is known for example that hydrostatic pressure shifts the equilibrium of the HCO_3/CO_2 buffer system and as a result changes the pH (Browning et al. 1999; Grodzinsky and Urban 1995). These explanations do not exclude the existence of cellular mechanosensors. Integrins and other surface proteins providing direct contact with the ECM act as mechanosensors. They assure the adherence of their cells to extracellular components, span through the cellular membrane, and start intracellular signal transduction pathways (Fukuda et al. 1997; Millward-Sadler et al. 2000; Wright et al. 1997). Additionally, ion channels are known that can be activated electromechanically; they react on mechanical stimulation and the resulting changes in the membrane potential. Finally, it is possible that the deformation of chondrocytes itself caused by compression may participate in the mechanical signal transduction pathway (Guilak et al. 1995; Millward-Sadler et al. 2000; Wright et al. 1996).

Influencing cartilage biochemistry

Numerous observations in clinical reality have provided evidence that mechanical stress even if applied in the form of passive joint motion is necessary to maintain healthy or regenerate-damaged articular cartilage. A lack of mechanical stress on the other hand results in cartilage degeneration. Despite this clear evidence it is turning out to be very difficult to determine detailed causal relationships between the kind of mechanical stimulation, changes in structures and concentrations of ECM, and the resulting mechanical properties of the tissue. This is true for the in vivo situation and for the in vitro situation generated in bioreactors as well. Most investigations in which cartilage material has been cultivated primarily focussed on and quantified anabolic or catabolic biosynthetic responses to different loading patterns. Nevertheless, general statements are still especially difficult since differences emerge when the cartilage of various species, ages, or joints are compared. At least it is possible to state that there are stress limits in the application of mechanical stimuli that lead to pathological changes in the cartilage explants or the tissue-engineered constructs when crossed (Grodzinsky and Urban 1995; Heath 2000; Sah et al. 1992, 1989). In detail different regulatory systems seem to exist, influencing the metabolism of collagen and GAGs by mechanical stimuli. Concerning the collagen present in cartilage tissue or tissue substitutes it might be possible to state that shear forces or the application of high hydrostatic pressure result in an increasing synthesis, while static loadings, tensile forces or the lack of shear forces result in a decreasing synthesis of collagen. The concentration of collagen together with its organization and orientation within the sample influence its mechanical behavior. It is especially difficult to state general rules about how the biochemistry of PGs can be influenced. This is not only because of the concentration of PGs but also their size and form of linkage which can be influenced by mechanical culture conditions and all these factors are essential for the tissues' functional behavior. At least it can be reliably stated that the content of GAGs can be influenced positively by shear or tensile forces, high hydrostatic pressure and by direct compression if applied intermittently

and at high frequencies. If on the other hand static compression or low hydrostatic pressure is applied, the concentration of GAGs decreases. It has further been determined that chondrocyte proliferation is stimulated by shear forces or perfusion, probably because it enables better supply with nutrients and oxygen. It is true for chondrocytes as well as for other cell types that a better differentiation can be achieved at higher initial cell densities (Grodzinsky and Urban 1995; Heath 2000; Sah et al. 1989, 1992).

Conditions in vivo

The construction idea behind the design of a bioreactor is always to be able to create a well-defined system in which culture conditions are regulated. If a certain tissue is supposed to be cultivated, the bioreactor system should enable the environment to mimic the physiological conditions that appear in vivo. Since articular cartilage is kept functional under unique mechanical and metabolic conditions in vivo, precise knowledge about these conditions is essential and should be considered when constructing a bioreactor.

Loading

Under physiological conditions large forces are applied to the thin layers of articular cartilage. These forces are the result of normal joint movements in which the body weight or a multiple of the same cause the loading. To describe these loads generated within a joint simplified models have been used in the past. The assumption in order to create such mathematical models was that homogenous layers of cartilage fit perfectly into each other. Likewise the mechanical loads were assumed to follow simple time courses like the form of a sinusoidal wave (Heath and Magari 1996; von Eisenhart et al. 1999). More recent investigations were able to provide evidence and quantify the much more complex situation in a joint. Experimental setups have been based on the behavior of cadaverous tissue in testing devices or thin pressure sensors fitting into small notches of cadaverous tissue or artificial hips that were actually transplanted. It soon became clear that pressure is not homogeneously distributed across the cartilage surface and forms irregular isobars instead. For the articular cartilage of the major weight bearing joints in the hip and the knee average loadings of about 0.5–7.7 MPa and average compression amplitudes of more than 13% have been measured during normal movements like walking. When the same joints are subjected to activities like repeated knee bending or stair climbing, peak pressure rates with maximum values of 18 MPa are possible. Even during resting the surrounding muscles and tendons of a joint participate in the generation a small load (Afoke et al. 1987, 1990 Grodzinsky and Urban 1995; Heath and Magari 1996; Hodge et al. 1986; Mow and Wang 1999; von Eisenhart et al. 1999; Weightmann and Kempson 1973). Not only the local pressure distribution but also the pressure variation within time has also been shown to be very complex. In some experiments techniques with a high resolution in time were used and helped to characterize the pressure courses arising in a joint as irregular and abrupt. A maximum pressure increase of 6.5 MPa in only 0.2 s could be measured in one investigation, for example (Hodge et al. 1986). As already mentioned earlier, different types of forces affect articular cartilage during joint movement. A great number of studies quantify only the predominant contact and hydrostatic pressure. They do so because the major effect of all loading events is the generation of hydrostatic pressure (Eckstein et al. 2001; Mow and Wang 1999). Only a few studies have been performed trying to quantify other forms of loading. In one of these studies the shear forces on human articular cartilage have been measured in terms of the equilibrium shear modulus named with the Greek letter "μ" and quantified to have a size of about 2.6 MPa. Likewise the vertical shift of articular cartilage named with the Greek letter "δ" as a result of shear forces has been quantified to reach a ranging from 9° to 15° (Hayes and Mockros 1971; Simon et al. 1990; Spirt et al. 1989; Zhu et al. 1993). Probably the best way to imagine the typical loading processes affecting articular cartilage is to understand them as a rolling movement of direct compression together with a generation of shear and tensile forces and high hydrostatic pressure (Heath and Magari 1996).

Structural variations

If tissue-engineered cartilage is supposed to replace damaged tissue within a joint, it is essential to understand the relationship between function and structural characteristics. Both function and structure vary within a single joint, between joints in the same species and between species. These variations include chemical, physical, and morphological properties (Meachim and Stockwell 1973). Human articular cartilage of the knee or hip joints reaches a thickness of up to several millimetres. It has long been observed that cartilage thickness varies to a great extent probably as a result of the prerequisite of its load bearing function. Using techniques like ultrasound investigation or magnetic resonance imaging it could be shown that within an

individual joint the thickness of the cartilage layer is inhomogeneous and probably varies dependent on the load it has to resist. Additionally, it could be disproved that the differences in the cartilage thickness between individuals are a result of their individual load history. People exercising sport on a regular basis over many years did not display different cartilage thicknesses compared with those doing no exercise. Likewise gender did not affect cartilage thickness but the surface size of the cartilage layer. A similar effect is known in animals where the surface area but not the thickness increases with the size of the animals (Eckstein et al. 2001; Grodzinsky and Urban 1995; von Eisenhart et al. 1999). This stiffness of articular cartilage belongs to the heterogeneous morphological variants as well. It could be found that those cartilage areas subjected regularly to high loads are stiffer compared to those areas subjected to less stress. Additionally, big variations of cartilage stiffness between individuals have been observed, although the reasons for that remain unknown (Lyyra et al. 1999; Swann and Seedhom 1993). Several investigations tried to measure and determine the factors responsible for the thickness of the space between cartilage layers that is filled with synovial fluid. The distance between cartilage layers with a thickness of up to 3 mm each has been shown to be dependent on the applied loading and varies between 1.5 mm down to a direct and nearly complete surface contact even at physiological loadings (Grodzinsky and Urban 1995; von Eisenhart et al. 1999).

Oxygen supply

As already explained one special feature of articular cartilage is its limited nourishing supply by the synovial fluid which slows down metabolism. It is also true that the oxygen supply is affected and only allows hypoxic metabolism. Microelectrodes were able to quantify an oxygen gradient between the cartilage surface and deeper zones beginning with 7.5% oxygen tension at the superficial zone down to 1% oxygen tension close to the calcified zone. The extent of this gradient is probably dependent on the investigated species. There is some evidence that the oxygen gradient is the reason for different types of PGs occurring in different zones since some of their biosynthetic pathways are oxygen-dependent (Brighton and Heppenstall 1971; Kim and Han 2000; Rajpurohit et al. 1996; Scott 1992; Silver 1975). In conclusion the standard cell culture conditions with 21% oxygen tension do not mimic the physiological situation within cartilage. Cultivations under cell culture standard conditions result in different biochemical characteristics and promote a less differentiated cell population. Although the availability of oxygen may possibly limit the chondrocytes metabolism, they seem to be well adapted to the hypoxic situation. Nevertheless it is known that normoxic in comparison to hypoxic conditions enhance at least the proliferation and stimulate collagen type II secretion in cultured chondrocytes (Hansen et al. 2001; Rajpurohit et al. 1996; Scott 1992; Scott and Haigh 1988; Ysart and Mason 1994).

During embryogenesis

Although during embryogenesis some cartilage tissues develop from ectoderm, cartilage is mostly a tissue of mesodermal origin like bone or other connective tissues. In the ontogenetic development, mesodermal cells give rise to a loosely organized embryonic connective tissue—the mesenchyme. Round-shaped cells of this loosely organized tissue condense and develop in the process of chondrification the primordial skeleton by the fifth week of human pregnancy. The primordial skeleton is an intermediate of growing cartilage from which most bones derive during a process called endochondral ossification. Seven weeks later the formation of bones and joints is complete and only thin layers of hyaline cartilage at the articular surfaces are left (Buckwalter 1997; Hall 1983; Stockwell 1979; Wheater et al. 1979). During the rest of the pregnancy and beyond significant changes in the biochemical composition of cartilage still occur. In the prenatal and postnatal growth process cartilage displays a high chondrocyte density with proliferating and metabolically very active cells. In general, the concentration of total protein decreases, the concentration of collagen remains constant, but those of total PGs and linker protein increase. One example for biochemical modifications taking place is the changing ratio of C4S to C6S in favor of the first. This changing ratio is of such importance that it is used as a marker for chondrogenic differentiation. Another example is the decreasing size and number of CS chains, while size and number of KS chains increase (Bayliss and Ali 1978; Elliott and Gardner 1979; Lash et al. 1974; Roughley et al. 1987). The question remains whether and which mechanical loadings are applied during embryogenesis. At least in the mice model such loadings have been observed to be caused by the first muscle contractions and it is reasonable to expect them in the human embryo too (Burger et al. 1991; Klein-Nulend et al. 1986). Whether an approach of tissue engineering should mimic the conditions during embryogenesis or those in the adult is probably dependent on the cell type used for the cultivation.

Bioreactors

With few exceptions bioreactors used for cultivating cartilage tissue try to mimic the mechanical loading experienced by cartilage in vivo which results from the motion of and forces on joints (Figs. 7, 8). There are also a few systems in which other forms of stimulation including electrical fields, ultrasound, or centrifugal forces are applied to the cultures (Lee et al. 1982; Maeda et al. 2001; Parvizi et al. 1999). Cartilage explants are normally used in such systems to study the consequences of the application of these forces. Cell–polymer constructs are cultivated in these systems for subsequent use as functional tissue replacements. The mechanical stress that is generated in the various bioreactors is also applied to mechanical test systems and there are a number of similarities in construction (Brown et al. 2000; Mauck et al. 2000; Palmoski and Brandt 1984; Wong et al. 1997). The various systems are classified here according to the principal type of stress being applied. Unless otherwise indicated, the culture environment consists of a temperature of 37°C, 90–100% relative humidity and a gas phase mixture comprising 20% O_2 and 5% CO_2.

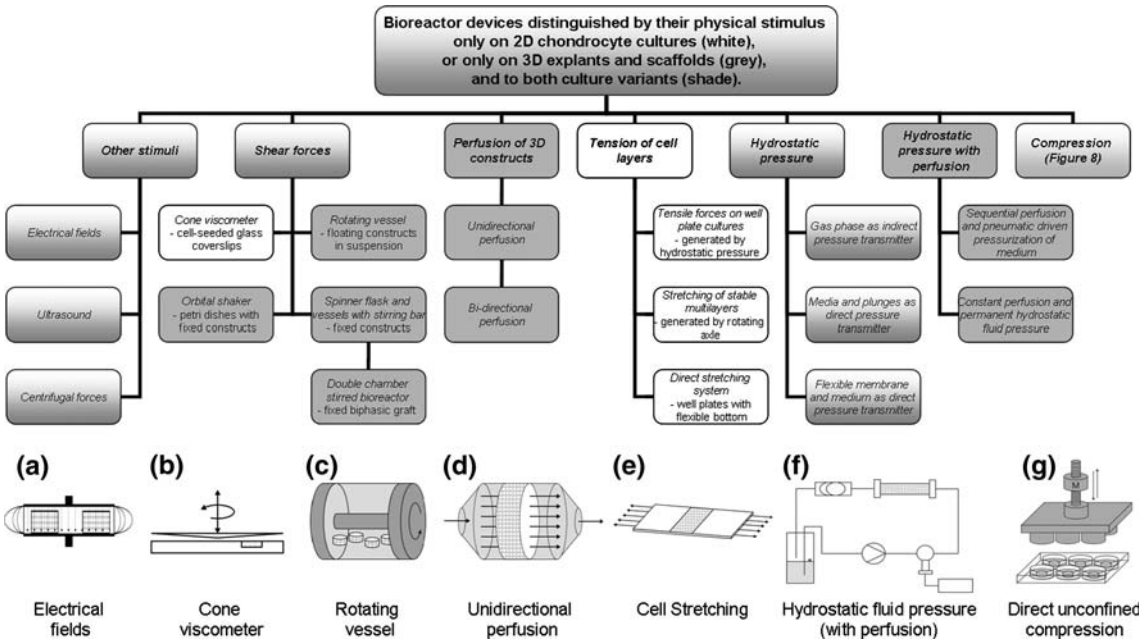

Fig. 7 Schematic overview of the principles of cause and effect in common bioreactor systems for the cultivation and stimulation of chondrocyte cultures. An exemplary sketch of the bioreactor apparatus is given for each physical stimulus

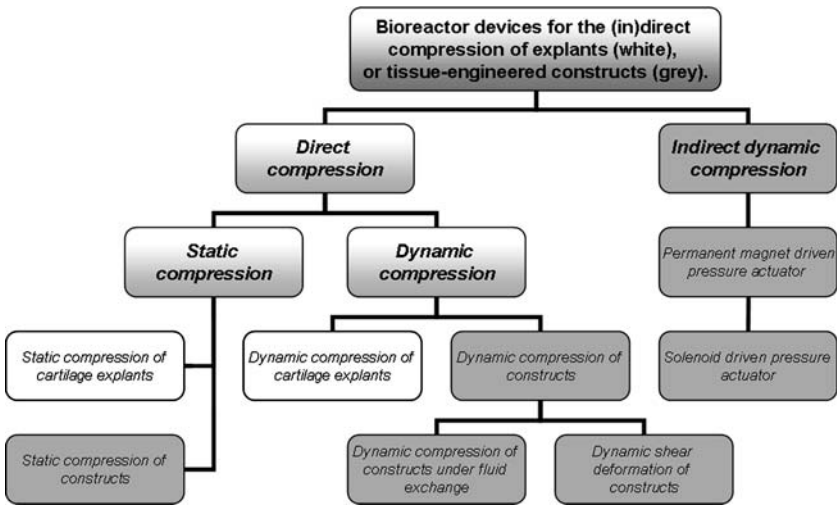

Fig. 8 Overview of operation methods for the compression of articular cartilage explants or artificial cell-based constructs in bioreactor systems classified by their applied loading protocols and further implemented stimuli

Systems applying shear forces

Shear forces can be applied very easily by various methods. The easiest method for generating shear forces is to use simple petri dishes with fixed constructs inside that are cultivated either statically or under shear stress on an orbital shaker (Freed 1993). A second and major approach to study the effects of shear forces is the cultivation in a spinner flask or a vessel with a stirring bar. For that purpose cell–polymer constructs can be fixed inside these vessels or chondrocytes can be cultivated on microcarriers. Depending on whether solid or porous microcarriers are used, shear forces reach the entire cell population or only cells growing on the surface. The generation of shear force in orbital-shacked petri dishes or in spinner flasks has been used by many groups and is very common (Bouchet et al. 2000; Brown et al. 2000; Freed 1993; Frondoza et al. 1996; Stading and Langer 1999). The cultivation of cell–polymer constructs in these systems has also been directly compared with a technically more complicated system. Constructs of bovine chondrocytes attached to a PGA matrix were created during a preculture period in a disc-like shape measuring 2×5 mm. They were then cultivated for a maximum of 8 weeks under various flow conditions in either static- or orbital-shacked petri dishes, in a static or stirred vessel system, or in the rotating vessel reactor as the third available system (Vunjak-Novakovic et al. 1996, 1999). In this technically more sophisticated vessel the constructs move freely within the culture medium during cultivation. The reactor is made of polycarbonate in a cylindrical form of 5.75 cm diameter and a volume of 110 ml. It rotates around its central axis at a speed of 15–30 rpm to keep the constructs floating in suspension. To prevent a leakage of culture medium the vessel is completely closed and is not equipped with any air ports. A gas mixture with 10% carbon dioxide is supplied by a hollow cylinder covered with a gas permeable silicone membrane instead, reaching along the axis in the center of the rotating cylinder with a diameter of 2 cm. The membrane itself does not rotate and causes the culture medium to form a well-defined laminar fluid flow and a field of shear forces in which the constructs are cultivated. The entire system can be operated in a temperature-controlled room, but has to be run in a repeated batch procedure (Freed et al. 1998; Obradovic et al. 1999; Vunjak-Novakovic et al. 1999). Chang et al. (2004) established an innovative double chamber stirring bioreactor to cultivate biphasic osteochondral grafts in a single apparatus. This device was built up of two tubular-shape glass chambers, each with a magnetic bar stirrer and sufficient ports for media exchange, gas ventilation, and sample aspiration. Each chamber was connected to the respective medium circulation system with chondrogenic or osteogenic media composition and contains its own magnetic stirring mechanism. The separation of both chambers is ensured by a silicon rubber septum with several holes which clamps onto the plastic tube coated biphasic composite scaffolds. These calcined bovine calcium-phosphate blocks with a diameter of 10 mm and a length of 20 mm were soaked in gelatin solution to form a chondral compartment approximately 3 mm in depth. Afterward, 10 million porcine chondrocytes were injected into this gelatin part, the composite was placed in the silicon septum and finally the cultivation period of up to 4 weeks was performed under constant magnetic stirring at 50 rpm and a weekly complete medium exchange. Besides systems which utilize a spinner flask or magnetic bar stirrer to induce shear stress on chondrocyte monolayer cultures, the principle and mechanism of a cone viscometer that can apply a wide range of shear forces without the presence of a force gradient was described by Bussolari et al. (1982). Herein, a small angled cone rotates with 100 rpm in maximum above a plate with 12 fitted cell-seeded glass coverslips with a diameter of 12 mm. In this first experimental set-up two subconfluent densities of 25.000 and 50.000 endothelial cells per cm^2 were subjected to 5 Dynes per cm^2 shear for 7 days. Smith and others performed several experiments in similar cone viscometer devices on high-density monolayer cultures of human and bovine chondrocytes for periods of 24, 48, and 72 h with fluid-induced shear of 1.6 Pa or 16 dynes per cm^2 (Mohtai et al. 1996; Smith et al. 1995, 2000b).

Systems applying perfusion

In perfusion systems it is essential to fix the cultivated cells and prevent them from invading into the culture medium flow. This can be achieved by embedding the cells in a polymer matrix or encapsulating them. Both strategies have been pursued in a perfusion system, where cells are embedded in a polymer matrix with an additional surrounding agarose capsule. Several of these constructs are cultivated in a cylindrical shaped glass reactor filled with culture medium. The culture medium is perfused unidirectionally from a medium reservoir throughout the cylindrical reactor with a flow rate of 0.016 ml min^{-1}. The reactor itself is operated in a standard cell culture incubator. Sterile filters in the medium reservoir enable a gas exchange with the surrounding atmosphere (Sittinger et al. 1994). Experiments with copolymer fleeces of vicryl and polydioxanon matrices soaked with poly-L-lysin or

collagen II have been performed. In these matrices human chondrocytes were embedded and cultivated 2 weeks under perfusion. The time span was extended to 70 days while using a biphasic system of a copolymer of PGA and PLA "Ethicon" fixed on calcium carbonate materials (Bujia et al. 1995; Kreklau et al. 1999; Sittinger et al. 1994). Another system has been created very similar to the bioreactor introduced above. The basic difference is the existence of a closed circuit for the culture medium. The inside of a cylindrical column made of glass, 1 cm wide and 10 cm long, is the real culture volume of the system. The column is filled with several cell/polymer constructs measuring 7 × 15 mm each without any further encapsulation. The culture medium is perfused from a reservoir throughout the column and the entire system with a flow rate of 330 ml per minute. Constructs of bovine chondrocytes in collagen sponges have been characterized in their response to perfusion over a culture period of 15 days with the help of this system (Mizuno et al. 2001). Pazzano and co-workers described a system and the biochemical effects of up to 4 weeks direct constant fluid flow perfusion stimulation on bovine chondrocytes seeded in PLLA/PGA scaffolds. Within a closed loop a peristaltic pump controls axial fluid velocity of 1 μm s^{-1} through ten of the cell seeded disks, which were situated in a clamping plate within the bioreactor vessel (Pazzano et al. 2000). A closed medium circuit for perfusion has been created in another bioreactor system as well. The system contains five small cultivation chambers with a volume of 1.5 ml each, made of polycarbonate. All five chambers are aligned in parallel and are perfused by culture medium from a reservoir with a flow rate of 50 ml min^{-1} driven by a single peristaltic pump. In each chamber cell/polymer constructs measuring 2 × 10 mm are cultivated under steady perfusion. This system has been used for the cultivation of rabbit chondrocytes on PGA scaffolds to form a cartilage substitute during a culture period of 28 days (Dunkelman et al. 1995). The same system configuration was utilized in 2002 by Davisson and associates to examine the effects of 7 days continuous perfusion, with two fluid velocities of 11 and 170 μl per minute, through PGA scaffolds with ovine chondrocytes (Davisson et al. 2002b). A more sophisticated perfusion bioreactor system with automated cell seeding of 3D scaffolds has been developed by Wendt and associates (Wendt et al. 2003). The device contains two glass columns, each of them with a polysulfone–teflon chamber with a height of 4 mm and a diameter of 8 mm at the bottom containing the constructs. These bottom bases are connected to each other by a glass U-tube. The optical sensors at the top level of each glass column detect the cell suspension and switch the vacuum, change the pump direction, i.e. the fluid flow from the affecting column to the opposite column which leads to an oscillating bi-directional perfusion flow through the cell-seeded scaffolds. This bioreactor design enables at first the seeding of porous ceramics, foams, or meshes with chondrocytes or bone marrow stromal cells followed by direct perfusion in a single-unit ensuring sterility and easier processing.

Systems applying tension

A reactor system has been developed in which the generation of small hydrostatic pressure creates mainly tensile forces. In this very sophisticated bioreactor both types of loading are closely related to each other because of the special construction principle. Chondrocytes can be grown as a monolayer and covered by culture medium within a petri dish. The petri dish is placed in a pressure chamber where a gas phase acts on both sides of the dish. The gas phase below the dish is visibly smaller and connected to the gas phase above only by small holes. Two valves control the inlet and outlet ports of the otherwise sealed chamber and create precise pressure protocols with nitrogen fed into the system. When pressure is applied from the outside, the pressure above the dish increases faster and is actually slightly higher for the first few moments. This short but uneven pressure distribution is due to a relative inaccessibility of the space below the dish where it takes longer to reach the same pressure. Depending on whether the bottom of the petri dish is made of a flexible material or not, the petri dish will bend and cause an accompanied stretching of the dish bottom or not. A strain gauge attached to the bottom of a flexible plastic dish or an inflexible glass dish quantifies these tensile forces. As a result of the stretching the cells that are attached to the dish bottom will be stretched as well. This system has been used to investigate the effects of tensile forces on the activity of mechanically sensitive ion channels. For that purpose pressure of 0.016 MPa and a frequency of 0.33 Hz has been applied over 20 min (Millward-Sadler et al. 2000; Wright et al. 1996, 1997).

In another system only very stable multilayer cultures can be subjected to tensile forces by stretching the multilayer itself. The system consists of a tissue culture incubator modified with a central rotating axle in it. The chondrocytes are precultured to the form of a multilayer. They are then placed ino a plastic culture flask and have to be fixed on one side with a stationary clamp and a movable clamp on the other side. The rotating axle is connected to the movable clamps and

enables the system to generate displacements of different amplitudes or speeds. With this system a multilayer culture of chicken chondrocytes has been subjected to 5.5% strain and frequencies of 0.2 Hz for 24 h to determine whether mechanical tension modifies the ECM (De Witt et al. 1984).

A system for a direct stretching of cells is also commercially available. Cells can be seeded into a 96-well plate coated with type I collagen and precultured there. The plates which can either have a flexible or inflexible bottom can be subjected to various tension protocols by an appropriate actuator. An advantage of this system is that it is highly automated and enables the investigation of tensile forces on a high number of samples in parallel at various tension protocols. The system has been used to stretch adherent cultures of bovine chondrocytes repeatedly for 3 s every 6 s or min with a maximum elongation of 17 or 5% elongation, respectively (Fukuda et al. 1997).

Systems applying hydrostatic pressure

In comparison to the compression systems, those that utilize hydrostatic pressure show mostly a much easier construction. In one such system the sample is kept in culture medium within a normal plastic test tube. The gas phase above the culture medium in the test tube is connected directly with another pressure chamber. In this second chamber two electromagnetic valves control the in- and outflow of a gas mixture and are therefore able to generate various pressure protocols. In this system the gas phase transmits pressure on the culture medium and the sample within. In some experiments intermittent hydrostatic pressure of up to 0.013 MPa was applied to chicken cartilage explants and cell suspensions of chicken chondrocytes to study its effects on the chondrocytes biosynthetic activities. The major disadvantage of this easy reactor system is the fragile components that only resist low pressure (van Kampen et al. 1985; Veldhuijzen et al. 1987). Other systems put the culture medium directly under pressure without the transmitter function of a gas phase above. The major part of one such reactor is a 0.5 l vessel made of steel connected to a hand-driven water pump. The pump is capable of creating intermittent pressure protocols with a maximum of 50 MPa. The inside of the vessel is filled completely with water and a syringe, sealed at the tip. The volume inside the syringe is the real culture volume containing the sample and culture medium. If the water pump compresses the water inside the steel vessel, the plunger of the syringe moves and transmits the hydrostatic pressure to the culture volume, while keeping the culture volume isolated. Advantageous in using steel vessels of this kind is the possibility to apply very high loads far higher than the loads occurring in vivo. In this system bovine cartilage explants were exposed to intermittent pressure of 5–50 MPa lasting 20 s or 5 min. The pressure increased with a speed of 5 Mpa s^{-1} and decreased with 5 Mpa s^{-1} (Hall et al. 1991). Two technically more advanced systems allow the sample pressurization within petri dishes. Common to both systems are the petri dishes filled with sample and culture medium, sealed with a gas—and fluid-impermeable but flexible membrane—and placed inside a steel pressure chamber. The pressure chamber with several sealed petri dishes in it can be filled with prewarmed water or equilibrated in a water bath as a whole. A hydraulic pump presses oil into a hydraulic cylinder either underneath or outside the chamber and creates the hydrostatic pressure. The pressure arising inside the chamber is transmitted by the flexible membrane to the culture medium and sample inside the petri dishes. Computer-controlled valves in the hydraulic system enable the precise application of various pressure protocols. Actually bovine explants and chondrocyte cultures have been precultured elsewhere before they were finally exposed for 1.5–20 h in the pressure chamber with protocols of 5 MPa and frequencies of 0.0034–1 Hz in one system (Lammi et al. 1994; Parkkinen et al. 1993). The second system exposed a high-density monolayer culture of adult bovine chondrocytes grown on poly-L-lysin pretreated plastic plates to intermittent sinusoidal pressure of 10 MPa at 1 Hz for periods of up to 24 h (Smith et al. 1996, 2000a) or 4 days (Ikenoue et al. 2003). The advantage of both systems lies once again in the ability to apply very high pressure, combined here with a very precise control unit. A disadvantage of this system like all other hydrostatic pressure systems introduced here is the fact that the culture medium cannot be changed during loading and the lack of a controllable gas supply. Besides these hydrostatic pressure devices for the stimulation of cartilage explants and chondrocyte monolayer cultures a couple of similar bioreactor designs have been built for the application of intermittent pressurization of 3D scaffolds. For example, Domm stimulated bovine articular chondrocytes embedded in alginate capsules for 3 weeks under partial pressure after limiting oxygen supply (Domm et al. 2000). Angele studied the effect of 1 Hz cyclic hydrostatic pressure on aggregates made of 200.000 human bone marrow-derived mesenchymal progenitor cells in a computer-controlled servopneumatic material testing system for up to one week with 4 h of stimulation each day (Angele et al. 2003); an adapted semi-sterile computer-controlled loading apparatus for the stimulation of mesenchymal progenitor cells seeded in

hyaluronan-gelatine composites, 4 mm thickness and 5 mm in diameter, has been established for mid-term pressurization of 7 days by Nerlich and Schumann (Nerlich et al. 2004).

Hydrostatic pressure systems with fluid exchange

There are two hybrid systems that enable the combination of high hydrostatic pressure together with perfusion for long-term experiments on 3D cell seeded scaffolds. A modified apparatus of Mizuno extends the advantages of the above-described direct perfusion system (Mizuno et al. 2001) by the application of static and 0.015 Hz cyclic hydrostatic fluid pressure of 2.8 MPa which enables long-term studies for up to 15 days on bovine articular chondrocytes in collagen sponges. Twenty-four of these sponges were perfused and a borosilicate class column was stimulated which is connected in a closed loop to the medium reservoir via a debubbler, to a single-piston cylinder pump, a pulse damper with pressure gauge and to a back pressure regulator connected to the control device (Mizuno et al. 2002). The culture chamber of another more sophisticated bioreactor system of Carver and coworkers is a steel vessel with a 10 ml volume, in which several cell/polymer constructs are arranged one over the other in culture medium. A peristaltic pump outside the steel vessel can perfuse all constructs completely and successively. They are fed with culture medium at a perfusion flow rate of 3 ml/min. The vessel's culture chamber can be separated completely from the perfusion system by air-driven valves. The second part of the system is an air-driven cylinder with a piston capable of applying a maximum hydrostatic pressure of 13.5 Pa on the culture volume. The bioreactor system either separates the culture chamber from perfusion and applies hydrostatic pressure or the culture chamber is perfused and supplied with fresh nutrients at normal pressure. This sophisticated bioreactor was used for long-term cultivations of equine chondrocytes in a PGA matrix for as long as 5 weeks. Various semi-continuous protocols generated conditions similar to the in vivo situation and were used to create tissue-engineered cartilage (Carver and Heath 1999a, b, c).

Direct compression

Systems for static compression of cartilage explants

A simple unconfined loading chamber with deadweight loads and a permeable filter as a force transmitter has been used by Burton-Wurster et al. (1993) to determine the effects of single load cycles with 0.025–1.2 MPa for 18–24 h of static unconfined compression on six canine full-thickness disks. The second experimental series tested the effect of a gradual intermittent compressive load with five cycles and 4 h on/20 h off-cycles. Another similar uniaxial unconfined compression device for the short-term mechanical loading of six bovine full-depth cylindrical cartilage explants with 5 mm diameter and 1–2 mm thickness was established by Valhmu (Valhmu and Raia 2002; Valhmu et al. 1998). This apparatus has been used to study the time-dependent kinetics of compression-induced signals and cartilage specific gene modulation after compressive stress of up to 500 kPa within the first 24 h of physiological strain application. Therefore, the explants were transferred to 60 mm diameter dishes containing 10 ml of media, followed by an equilibration period of 15 min between two porous sintered glass filters by a tare load of approximately 25 kPa. The static vertical load is subjected to the explants by adding free weights to the loading piston, which is guided by a linear bearing and connected to the corpus of the device. A similar, yet computer-based "load and displacement controlled device, LODEC" was described by the same laboratory in 1994 (Guilak et al. 1994) to determine the effects of 24 h of static unconfined compressive stress in seven levels between 0.001 and 1.0 MPa to bovine full-thickness articular cartilage specimens, 5 mm in diameter and 1–1.5 mm height. This closed-loop loading device controls a motorized micrometer with a displacement resolution of 1 μm and contains a high-accuracy in-line load cell with a force resolution of 0.01 N which enables the compression and mechanical testing of explants. Later on, Guilak et al. (Guilak 1995; Guilak et al. 1995) published in 1995 another device to determine the changes in the shape and volume of chondrocytes from canine osteochondral explants during the application of 15% compressive tissue strain. The set-up combines a confocal laser scanning microscope, a custom-designed viewing dish, two stainless-steel loading platens which clamp the hemicylindrical specimens, wherein one of them is driven by a high-resolution digital micrometer.

Systems for dynamic compression of cartilage explants

Cyclical uniaxial compressive loads of a greater magnitude may be generated with a somewhat more complex culture system. The first apparatus of Steinmeyer and associates, a polyethylene-lined titanium vessel contains a specimen holder for articular cartilage explants up to 10 mm in diameter and can hold 7 ml culture medium (Steinmeyer et al. 1993). There is no provision for medium exchange, but gas exchange is enabled by the inclusion of ports associated with the air space in

the chamber to which sterile filters are attached. Nevertheless, this system is limited to relatively short-term batch culture operation. The loading system is enabled by a spindle which passes through the vessel lid. Inside the vessel, the spindle is attached to a porous load plate. This assembly may be driven by a piston to generate loads with varying waveforms. Sinusoidal loads with a maximum pressure of 10 MPa are possible with frequencies of up to 10 Hz. In 1997 Steinmeyer (Steinmeyer 1997) described an improved articular cartilage explant loading system with enhanced biomechanical capabilities and computer-based controlling of relevant parameters, such as load waveform, force, frequency, and displacement ensuring metabolic activity and viability at the same time. In addition to static and intermittent loading—which the system also permits—cyclic sinusoidal loads over a frequency range of 0.001 to 5 Hz were studied in combination with a maximum pressure of 0.1 to 5 MPa during 10 days of culture. This advanced system has been used to study the influence of various compression profiles on the biosynthetic activities of steer cartilage explants (Sauerland et al. 2003; Steinmeyer and Knue 1997; Steinmeyer et al. 1999). A more robust version of this system incorporates a clamping ring to keep three canine cartilage explants in place and a load cell to measure actual pressure exerted on the sample. As a result of the increased robustness both larger maximal pressure and higher frequencies can be achieved. Peak pressure of up to 50 MPa, pressure "gradients" of 100 MPa s^{-1} and load cycles with hold and repeat steps in the range of 0.5–5 s can be produced. It can therefore be used to study the effects of abrupt heavy loading (Farquhar et al. 1996).

System for static compression of tissue-engineered constructs

Simple constant uniaxial compressive stress may be applied to cartilage cultures in 24 well co-culture plates by placing the constructs on the lower culture surface, fitting the inserts and then the lid above and placing a known mass on the lid. The insert transmits the compressive load to the construct. Constructs of murine embryonic mesenchymal cells embedded in a collagen gel have been cultivated under a static compression of 0.002 MPa for a period of 10 days in this system (Takahashi et al. 1998).

Systems for dynamic compression of tissue-engineered constructs

An alternative approach to expose cartilage cultures to compressive stresses involves a dual-plate polysulfone culture device, which enables 12 constructs to be cultured in a single culture space. Twelve perpendicular struts are located in each plate such that each construct is clamped between two opposed struts when the device is assembled. The magnitude of the compressive load is a function of the distance between the clamped plates. This is regulated by the thickness of inter-plate PTFE gaskets. The constructs are submerged in medium. There is a gas space above the cultures and typical culture dish gas exchange is possible. Constant or intermittent pressure of up to 0.77 MPa can be achieved with this multisample, batch culture device (Sah et al. 1989). Modifying this system to enable cyclic compressive loading was a significant task. Actuator-driven compression rods with integral micrometers were added. These modifications enable samples to be sufficiently and precisely located in the device such that the extent of deformation resulting from the applied loads can be controlled at the sub-μm level. At the same time, the overall size of the device was increased to enable 24 samples to be cultivated in parallel. As a result, this has become the first system under review which cannot be placed within a standard cell culture incubator. Additional features were therefore necessary. A heat exchanger was added for medium circulation and temperature control and a compressor and associated components were added to enable delivery of an appropriate sterile gas mix to the communal gas head space in the device. In addition to the gain in size and load/deformation control, this system also enables a cyclic load to be imposed on top of a constant load. Until now cartilage explants from several species and varying scaffold materials with embedded chondrocytes have been cultivated and stimulated under an impressive range of load regimes and culture conditions. The biosynthetic response to the various load regimes has been reported in many articles (Buschmann et al. 1995; Davisson et al. 2002a; Lee et al. 2003; Sah et al. 1990, 1991). Additional similar systems exist in which standard commercially available 24 well plates may be used. In one, the base of a 24 well plate is located within a sterile polymethyl-methacrylate box, which is, in turn, located within a standard incubator. An external actuator transmits a compressive load to each of 24 load plates (11 mm diameter) on struts that are attached to a main loading plate within the box. Compressive load is applied according to the extent of deformation. This system has been used to cultivate bovine chondrocyte/agarose constructs over a period of 2 days. Cyclic sinusoidal compressive loads (0.3–3 Hz) superimposed upon a constant load were generated with a maximum deformation of 15% (Lee and Bader 1997). Further, stimulation studies of Chowdhury

et al. (2003) explore the effects of continuous or intermittent compression on bovine chondrocytes embedded in agarose using various duty cycles of dynamic compressive loading, over a short-term culture period. In another (commercially available) system, cyclic loads with up to 0.015 MPa peak pressure and a frequency of 5.0 Hz can be generated via a pneumatic piston. The pressure is transmitted by several metal pins to the samples cultured in microwell plates. The associate control system enables many regimes of compressive loading. The system has been used to determine the ATP production of porcine chondrocyte pellets following compression (Graff et al. 2000). Besides these two stimulation systems which are suitable for standard tissue culture well plates, further custom-designed bioreactors for the dynamic mechanical compression of chondrocyte scaffolds are similar in their construction and effect. The loading apparatus of Hunter for the stimulation of chondrocyte-seeded collagen or fibrin gels (Hunter et al. 2002, 2004), the equipment of Elder for the compressive loading of chick limb bud-cells embedded in agarose gels (Elder et al. 2001; Elder et al. 2000), the device of Huang for the examination of combinatory effects of loading and TGF-β1 on the chondrogenesis of rabbit MSCs in agarose matrix (Huang et al. 2004a), and not to mention the mechanical loading system for chondrocyte loaded agarose disks (Mauck et al. 2000, 2002) and bilayer composite constructs (Hung et al. 2004) are all uniaxial compression systems which fit into standard incubators. They also offer control of imposed displacement and load response of the specimens, and contain similar loading platens which enter standard petri dishes to maintain sterility.

Systems for dynamic compression of tissue-engineered constructs under fluid exchange

A "Tissue Culture Unit" for the compressive deformation of cell-seeded specimens with implemented medium stirring mechanism has been developed by Demarteau et al. (2003a). The compressive strain of this computer-controlled bioreactor is generated by a microstepper motor which is connected via a positioning table, to a stainless steel rod applying a load cell to the six plungers in the cover lid of the culture chamber. To ensure contact of the internal plungers with the top of each construct, the initial height was regulated individually by external micrometer screws. Each base chamber contains six peripheral culture wells, each centered below the six plungers. The fluid exchange in the device is given by a medium exchange port which is directly connected to a central well of 32 mm in the base chamber. The integration of a magnetic bar in this well guarantees stirring of the medium without contact and enables long-term studies of static and dynamic compression of tissue-engineered cartilage. Recently, this apparatus has been used to study the metabolic effects of 72 h dynamic compression on human articular chondrocytes embedded in PEGT/PBT foam of 1 mm thickness and a diameter of 8 mm (Demarteau et al. 2003b). The constructs were subjected to 5% offset compression, then superimposed for 2 h to 5% sinusoidal compression at 0.1 Hz and 18 kPa stress followed by 10 h without deformation. Increased nutrient supply and waste transport around the specimens were ensured by the special magnetic bar stirrer mechanism during the 3 days of stimulation.

Systems for dynamic shear deformation of tissue-engineered constructs

There are two combinatory systems that enable the application of intermittent cyclic shearing forces and axial deformations. Frank et al. (2000) have developed a versatile shear and compression bioreactor to study the biosynthetic response of cartilage explants based on the compression system of Sah et al. (1989). This modified version of the static loading bioreactor with the above-described compression actor modules and non-rotating culture chamber lid contains a microstepper motor driven rotary position table which is connected to the polysulphone base of the chamber. The minimum theoretical rotational resolution of 0.0005° is measured by linear variable differential transformers. This set-up has been used in shear deformation studies on bovine cartilage disks from the femoropatellar groove with 3 mm in diameter and ~1 mm thickness to study the biosynthetic effects of 3% dynamic shear strain amplitude at frequencies between 0.01 and 1.0 Hz, partially under insulin-like growth factor I supplementation (Jin et al. 2001, 2003). Later on, this dual-axis bioreactor was used in dynamic compressive loading experiments without applying the sinusoidal rotation stress on 12 mm diameter chondrocyte-seeded peptide constructs and agarose hydrogels with strain amplitude of 2.5% superimposed on 5% static offset strain at a frequency of 1.0 Hz in a long-term study of Kisiday (Kisiday et al. 2004). Another biaxial tissue loading device by Waldman and associates (Waldman et al. 2004) is based on a modified, commercially available, dual-axis—compression and translational shear—Mach-1™ (Biosyntech, Laval, Quebec, Canada) mechanical tester. The sinusoidal compressive mechanical loading was conducted at 1 Hz for various durations and amplitudes between 5 and 20% displacement

in long-term, 8 weeks experiments. The force transmission from the load cell to the chondrocyte-seeded porous calcium polyphosphate scaffolds in a standard 24 well tissue culture well plate has been achieved by a custom designed culture plate top with porous titanium alloy loading platens. The translational shear actuator of the Mach-1™ device enables comparative studies on the biomechanical effects of intermittent shear—and/or compression stimulated tissues (Waldman et al. 2003a, b).

Systems for indirect dynamic compression of tissue-engineered constructs

A remarkable drawback of bioreactors which allow compressive stimulation is that the pressure mediators, which are driven pneumatically or by servo-motors, are mostly pistons, tappets, and other similar devices which are inserted into the bioreactor area where a preferably autologous cartilage transplant is situated, and afterward these put a defined pressure on the cell construct. Due to the necessary induction of the pressure applicator into the sterile cultivation system, the construction of closed pressure applicator bioreactors is very difficult, so that these systems are highly complex. Therefore, the use of these—potentially non-sterile—devices is only possible in basic research, because the use of these apparatus and methods in the medical field contradicts the present guidelines of GMP. Recently, only two indirect compression systems have been well described which try to overcome this engineering problem by the use of a magnetically driven pressure actuator. A simple, custom-made computer-based bioreactor device with magnetic pressure transmitters was established by Schumann (Schumann 2004). The apparatus allows a simultaneous compression of twelve cell-seeded specimens of Hyaff®-gelatine-composite-scaffolds with a diameter of 5 mm and height of 4 mm in commercially available 15 ml centrifuge tubes. Each tube also contains 4 ml media and a Teflon-coated permanent magnet bar with a dead weight of 16 g or 8 kPa, which leads to a uniaxial deformation of this scaffold of 40%. The cyclic compression with a frequency 0.33 Hz was applied 4 h every day for 1 week. A simple set-up of external electromagnets below each cultivation tube was controlled by a custom-made software program which ensured the magnetic repulsion of the dead weight from the cell-seeded constructs according to the respective stimulation frequency. The disadvantage of this device and other pressure simulation bioreactors is that the cell constructs cannot be directly perfused with culture medium during pressure force and the effect of multiple cell stimulation cannot be examined. Furthermore, the missing nutrient supply contradicts with the optimum metabolic exchange and the maximum synthesis of, e.g., extra-cellular matrix materials in cartilage cells. Another, more sophisticated computer-controlled bioreactor system with feedback control has been developed by "IMBIO-TOR"—Intelligent Mini Bioreactor—Consortium in the 5th EC Framework Programme (Shelton et al. 2004). The apparatus was designed for the production of three-dimensional, vital, and mechanically resistant matrix-coupled cell constructs, in which a dynamic mechanical stimulation and aseptic cultivation according to the GMP-regulations is possible. The main feature of the developed device is the location of the transplant in a sealed bioreactor that can be provided with multiple in vivo like stimuli. One of these stimuli is the direct perfusion of the spatial cell construct with a conditioned culture medium which on the one hand leads to the production of organo-typical shear forces and on the other hand admits an increased metabolic exchange. Typically, chondrocyte-seeded agarose constructs with 3 mm thickness and 5 mm in radius were selected as scaffold material for long-term studies of 15% compressive load. In the optimized design such a construct is mounted centrally on a small stage and forms the boundary between two compartments in a cylindrical polycarbonate bioreactor. The lower chamber with a radius of 3.5 mm serves as a distributor for incoming medium and supplements with flow rates of approximately 160 µl per min. The upper chamber with a radius of 7.5 mm fulfils a nutritional role during static culture periods and contains the outflow ports and the magnetic loading mechanism. A non-contact loading mechanism was essential for the closed operation of the device. The group developed a construct loading technology based on magnetic repulsion. A dynamic moving Teflon-coated NdFeB permanent magnet located within the sterilizable device is controlled by the movement of a second external magnet and generates vertical forces of up to 50 Newton's and a frequency of 4 Hz in maximum. This arrangement enables precise construct deformation without compromising the sterility of the grafts in the test system. A peripheral high-resolution strain gauge was used to verify the uniaxial force resulting from the acceleration of the magnet. The position of the loading plate in the mini bioreactor was detected by an inductive, analog displacement sensor. The reactor was incorporated in a closed tubing circuit with appropriate sensors for pH, pO_2, pCO_2, glucose, and lactate, miniaturized pumps and valves enabled automated direct perfusion, medium supplementation and replenishment and on-line sensing during loading while maintaining a sterile culture system (Schulz and Bader 2006).

From chondrocyte loaded laboratory devices to stem cell based bed-side manufacture?

Over the past few years some research groups have tried, at least in cooperation with biomedical companies, to develop bioreactors for the automatic production of human MACT transplants. The aim of the current efforts is directed toward translating corresponding laboratory prototypes into production systems to culture (and stimulate) autologous cell-based matrix-coupled articular cartilage which should be available as a tissue-engineered product for therapeutic use in OA diseases (Martin et al. 2004). The application and further development of corresponding research systems in bedsides apparatus should also enable the advantages of a complete monitoring of the culture conditions (e.g., temperature, ph, partial gas pressures) while guaranteeing an increased transport of nutrients and metabolites to and from the regenerated tissue by an (in)direct flow, transferred by shear stress (see "Systems applying shear forces") or by perfusion (see "Systems applying perfusion"). In particular, the additional application of a physiological stimulus such as hydrostatic pressure (see "Systems applying hydrostatic pressure") or even direct compression (see "Direct compression") within this production system should improve the quality of the transplant enormously. These advantages in production with in vivo adaptive physiological forces lead to a better differentiation of the chondrocytes resulting in an increased formation, composition, and arrangement of the matrix in cartilage regeneration. In addition, deformation in more fibrous cartilage tissue with inferior mechanical properties can be avoided as a result. Despite many biotechnological advantages in terms of an automated, autonomous, standardized manufacturing process in a closed environment there are currently no (large-scale) production facilities for 3D cartilage grafts in clinical practice. The problems in the envisaged translation of such systems involve on the one hand meeting current legal requirements and rules for manufacturing a patient specific medication by a TE company according to GMP which is ensured through a quality control/quality assurance program (QC/QA), similar to the validation process described by Mayhew and associates (Mayhew et al. 1998) for ACI in the company Genzyme Tissue Repair (Cambridge, USA). In addition to drug safety, a clinical production system must on the other hand combine an increased quality of tissue of the 3D transplants in a reproducible process with increased economic efficiency, compared to the standard procedure based on 2D-monolayer culture (ACT) or the matrix based technique (MACT) (Wu et al. 1999). There are currently two clinical concepts—with traditional expansion culture and without mechanical cell stimulation—for the GMP-adherent production of autologous transplants for OA diseases as described by industrial developers.

The closed bioreactor system propagated by Millenium Biologix (Zurich-Schlieren, Switzerland) called ACTES™ (Autologous Clinical Tissue Engineering System) is intended for a bed-site production of one and biphasic, osteochondral transplants for use mainly in the clinic. This automated apparatus should enable the insertion of a disposable biopsy chamber with the cartilage tissue biopsy. After the system has isolated the chondrocytes, these are then expanded in a further culture chamber inside the apparatus and then finally transported to a production chamber. Alongside a pure autologous chondrocyte cell transplant (ACT) one or more so-called Cartigrafts™ can especially be cultivated within this production vessel. These are cell-based biphasic, osteochondral transplants whereby the expanded cartilage cells are attached to one or more osteoconductive, synthetic bone scaffolds (Skelite™) and are then available for surgical operation for the OATS or for the mosaicplasty in the knee joint. An enormous technological advantage of this closed culture concept with its fully automatic, clinically internal implantation production is in the complete removal of the (up until now essential) costly GMP-manufacturing facility which will mean another step toward reducing costs for tissue-engineered transplants.

A further production concept for autologous patient-specific cell products from MSCs is described by Aastrom Biosciences, Inc. (Ann Arbor, USA, http://www.aastrom.com), with their AastromReplicell™ System. In this cell manufacturing system, a "tissue repair cell" production exists using a "single pass perfusion" in accordance with GMP compliance. Starting with bone marrow aspirates from patients' hip, progenitor and bone marrow-derived adult stem cell populations (bmMSCs) are expanded in this fully monitored system within 12 days which then provide therapeutical potential for multiple clinical applications. The key advantage for the use of MSCs as therapeutics for the repair of damaged or diseased tissues like vascular, fat, bone, and cartilage is based upon their enormous differentiation potential at the former (e.g., osteoarthritic) defect side. On the other hand, the main disadvantage of this system is the lack of complex colony development of the corresponding cell populations on 3D scaffolds in vitro and therefore no pre-differentiation, whether induced by growth factors such as medium supplements or by a physical stimulation.

During the past few years, MSCs from bone marrow have been shown as a high potential source of cells for cell-based therapeutic strategies (Baksh et al. 2004; Barry 2003a; Pittenger et al. 1999). The application of bmMSCs in cartilage and bone tissue engineering became a major area in regenerative medicine (Barry 2003b; Gao and Caplan 2003; Johnstone et al. 1998; Song et al. 2004). BmMSCs offer the opportunity to overcome the donor-site morbidity of primary chondrocytes and have demonstrated their chondrogenic differentiation potential when cultivated in various 3D scaffolds (Caterson et al. 2001; Lee et al. 2004; Li et al. 2005; Mauck et al. 2006) under the addition of a member of the transforming growth factor superfamily (Majumdar et al. 2001; Worster et al. 2001). Moreover, groups with well-accepted bioreactor systems for the stimulation of 3D cell loaded scaffolds have currently carried out extensive studies on the beneficial influence of dynamic mechanical loading on the chondrogenesis of 3D bmMSC loaded scaffolds (Angele et al. 2004; Huang et al. 2004b). However, the influence of mechanical strains on MSCs or progenitor cells during cell condensation and chondrogenesis is not well understood. Mechanical stress sensors were described, including mechano-sensitive ion channels, integrins coupled to cytoskeleton or stretch-activated enzymes, and several cell response pathways were detected (Hamill and Martinac 2001). Based on these results, models were proposed how the mechanical stress applied to the cell surface is transformed into chemical signals inside the cell (Ingber 2003a, b).

The research community is still in the development process for the regeneration of MSC-based grafts for the clinical treatment of OA diseases. These endeavors try to combine the use of MSCs in clinically approved scaffolding materials which should be cultivated, mechanically, stimulated and fully monitored under well-defined parameters in the above-mentioned closed GMP-compliance bioreactor systems.

A current promising R&D project from Germany aims for the "Multiparametric Monitoring and Steering of Mesenchymal Stem Cell derived Cartilage Formation in 3D Production Systems" (MS CartPro, http://www.MS-CartPro.de). The main objective of this multidimensional approach is the completion of the aseptic IMBIOTOR bioreactor system with its non-contact dynamical loading mechanism (Systems for indirect dynamic compression of tissue-engineered constructs) by the implementation of novel technologies for quality control and quality assurance. This GMP-capable device for cartilage production will be equipped with a non-invasive online monitoring system allowing for steered cultivation of MSC populations along protocols derived from a predictive bioinformatical model. For that purpose the project utilizes competences in proteo-toponomics, biophysics, genomics, lipidomics, and bioinformatics. The implementation of the non-invasive stem cell sorting technology by the microfluidic optical stretcher (MOS, US Patent 6,067,859) allows the gentle, marker-free analysis and sorting of promising stem cell (sub)populations while preserving their integrity. This system can measure under aseptic conditions the elasticity of a large number of patient specific MSCs from a biopsy in a short time by optically induced surface forces (Lincoln et al. 2004). The elasticity of the cytoskeleton is a physical property of cells that has been identified as a sensitive inherent marker for the state of differentiation of the cells (Guck et al. 2005). Another challenge is to monitor growth and differentiation processes of the MSC population in the closed production system online and non-invasive. This could be achieved for the synthesis of ECM components during chondrocytic differentiation by applying biophysical methods such as a high-resolution NMR method (HR-MAS NMR), matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS), combinatory phase sensitive scanning acoustic microscopy (PSAM), and confocal laser scanning microscope (CLSM). ^{13}C MAS NMR spectroscopy has a very high potential as a tool for non-invasive characterization of—bioartificial—tissue (Huster et al. 2004; Schulz et al. 2006). Several studies have demonstrated the detection of all ECM compounds in native cartilage and cell-based repair tissue by NMR spectroscopy. Depending on the NMR methodology, mobile GAGs and rigid collagen or hyaluronan structures can be studied individually on the same sample (Huster et al. 2002, 2005, Naji et al. 2000; Schiller et al. 2004a). In addition, NMR spectroscopy has been successfully applied to study the molecular dynamics of the macromolecular components of cartilage as a basis for the understanding of its viscoelastic properties (Zernia and Huster 2006). Moreover, MALDI-TOF MS can be applied to characterize cells or cultivation supernatants on the molecular level. It offers unmatched mass resolution and very high sensitivity (Schiller et al. 2001). Thus, it is well suited as a control method for MSC differentiation. Besides profiling the presence of selected marker proteins, lipid analysis, lipidomics, this technique provides further important information on changes of the cellular membrane (Schiller et al. 2004b). These changes are expected to correlate with the differentiation status of MSC as well (Bieberich 2004). During MSC cultivation and differentitation phases in the bioreactor system high resolution ultra-sound analysis will be applied to investigate

the morphology and subcellular elasticity of MSCs. A variety of schemes has been developed to monitor living cells by ultrasound in such a way that besides high lateral resolution (1 μm at 1.2 GHz) and height detection (nm-resolution) by 3D phase contrast imaging quantitative information on the mechanical properties can be derived (Ngwa et al. 2004). This includes the determination of the velocity of sound for longitudinal and transversal polarized waves from which the Poisson ratio and—with the aid of the density—also Young's modulus and the shear modulus can be derived. Furthermore, phase and amplitude mapping of single cells also enables by comparison to a model calculation the determination of the absorption, a viscoelastic property, and the determination of the density with high resolution (Wagner et al. 1999). Changes of these properties during MSC differentiation will be detected online, providing information about the actual cell states and enabling a donor specific steering of the process. A novel developed combined PSAM and CLSM with spectral fluorescence contrast is used to identify and characterize (as exemplified by: Laforsch et al. 2004) objects according to their optical properties and monitor the elastic properties on a single cell basis. Furthermore, a novel sensor microscopic imaging system based on ultrasonic tomography and holography in the frequency range from 200 to 600 MHz enables the determination of elastic properties in the volume with cellular resolution (Grill et al. 1999). The sucessful development of this multidisciplinary approach could improve the understanding of MSC self-organization by providing a comprehensive and quantitative characterization of the tissue stem cell system from gene via protein and cellular to the tissue level. On the one hand it will develop methods of non-invasive sorting, monitoring, and characterization of MSC during differentiation and lineage commitment and a first predictive bioinformatical model capable of generating protocols of optimized MSC culture. On the other hand, all these aspects will contribute to the development of a bioreactor system for clinical application which allows for a donor specific production of cartilage grafts or other cell-based therapies (e.g., biphasic implants for OATS) for the treatment of OA diseases under GMP conformity. This manufacturing bioreactor with the implemented mechanical stimulation and monitoring sensor system will be an expansion and differentiation product oriented instrument that includes a cell culture unit as a low cost consumable device. This will permit the on-site production in a fully closed sterile environment. The concept includes the de-centralized installation at a hospital site as a self-controlled system to provide low cost implants for patients in need according to the recommendations of the Joined Advisory Board of the German Societies for Traumatology and Orthopaedic Surgery (Behrens et al. 2004).

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