Susanne Grässel · Attila Aszódi *Editors*

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Volume 3: Repair Strategies and Regeneration

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Preface

The closing, third volume of the Cartilage book series is dedicated to provide an overview about the current procedures of articular cartilage repair. The text is designed to be of use to multiple medical and basic science disciplines as orthopedics, rheumatology, and trauma surgery and all basic and clinical investigators working in the field of cartilage regeneration. This volume covers various cartilage repair strategies including cell-based, biomolecule-modulated, gene transfer, and tissue engineering approaches.

Chapter 1 provides an overview of the current status and future perspectives of cartilage repair strategies. The authors, after giving a short introduction to the clinical relevance and pathology of cartilage injuries, summarize the principles of currently practiced cartilage repair modalities including palliative approaches, strategies based on bone marrow stimulation, whole-tissue transplantation, and tissue engineering strategy. Recent advances of stem cell-based therapies and biomaterial scaffold designs in cartilage repair strategies are also presented. The chapter opens a discussion of the remaining scientific and clinical challenges in cartilage repair, specifically highlighting the need of enhancement of tissue integration, maintenance of cell phenotype, prevention of OA progress, and simplification of surgical and rehabilitation procedures.

The next four chapters give details for cell-based cartilage regeneration strategies. Chapter 2 focuses on adult *mesenchymal stem cells* (MSCs) and their growth factor-modulated surface marker expression. The authors clarify the characteristics and the embryonic origin of cartilage progenitor cells and summarize the contribution of MSCs from different origins to cartilage repair. Finally, a few examples of promoting articular cartilage phenotype by growth factor administration, in relation to the modulation of surface marker expression, are given. Chapter 3 introduces the *chondrogenic progenitor cell* (CPC), a specific cell type bearing stem cell characteristics such as migratory activity, clonogenicity, and multi-potency. These cells, which are present in osteoarthritic cartilage tissue and involved in regeneration processes, provide a promising alternative approach for cartilage repair. Various factors modulating the chondrogenic potential of CPCs including transcription factors, cytokines, growth factors, extracellular matrix molecules, and calcium homeostasis are presented. Chapter 4 demonstrates the attractiveness of *induced pluripotent stem* (iPS) cells for cartilage regeneration, which originates from their immense expandability and their intrinsic ability to give rise to stable hyaline cartilage. The

application of iPS cells can overcome most problems of classical cell-based regeneration strategies such as the extremely limited supply of human articular chondrocytes and the restricted differentiation capacity of mesenchymal stem cells from bone marrow or adipose tissue. Beyond being a potential alternative cell source for articular chondrocyte implantation, iPS cells are particularly promising for in vitro modeling of genetic diseases and for drug testing. Reprogramming patient-specific cells with a genetic predisposition and engineering disease-specific genetic variations into healthy control iPS cells promise to recapitulate "diseases in a dish" more realistically than immortalized human cell lines and will be an invaluable complementation for animal models. Whether iPS cells will satisfy these tremendous expectations will depend on our ability to upscale iPS cell culture, to derive sufficient amounts of relevant cell types like chondrocytes from iPS cells with acceptable efforts, and to find clinically safe reprogramming techniques for iPS cell-based therapies. Chapter 5 provides a short overview about current procedures for cell-based treatment strategies like bone marrow stimulation techniques, osteochondral transplantation, and *autologous chondrocyte transplantation*. Requirements and outcome parameters for a successful treatment and future directions in cartilage regeneration are discussed. Finally treatment recommendations according to cartilage defect size and depth are given.

The following two chapters deal with the role of biologic agents for the regenerative process of cartilage injury. Chapter 6 describes the *growth factors* with the most promising in vitro and in vivo data in cartilage repair, namely, bone morphogenetic protein-7, transforming growth factor-β, fibroblast growth factor-18, connective tissue growth factor, insulin-like growth factor-1, and recent advancements with autologous solutions of growth factors, such as platelet-rich plasma. Each section provides a background on mechanism of action, summarizes pivotal basic science research, and describes the results of clinical application in animal and human models of chondral disease. In chapter 7, *platelet-rich plasma* (PRP), an autologous blood-derived concentrate rich in growth factors, is introduced. Currently PRP is the most exploited biological approach for conservative management (simple intra-articular injections) and as an augmentation during surgical procedures. It has been applied both to treat osteoarthritis and chondral/osteochondral lesions in different joints, with the primary aim of providing symptomatic relief and functional recovery, and to induce a positive modulation of the entire articular microenvironment. The authors summarize the clinical evidence available on the role of PRP to treat cartilage pathology, focusing in particular on the data coming from randomized controlled trials.

Subchondral sclerosis is one of the hallmark findings of osteoarthritis (OA) and has long been discussed as one of its causes. Chapter 7 focuses on the changes in the *subchondral bone*, which often precede cartilage destruction in the development of the disease. Integration of the so far published data including in vitro, in vivo, and mathematical work suggests a critical role for this tissue in nutrition and oxygen supply to the articular cartilage, which may become even more critical in energy-demanding processes of healing and regeneration. Indeed, the success of current predictive diagnostics like specialized MRI techniques and scintigraphy as well as

successful regenerative clinical therapies like microfracturing, AMIC, or NAMIC can be better explained if the subchondral bone is taken into account as a supply route for the cartilage. Consequently the subchondral bone has to be included into the diagnostic and therapeutic concepts aiming to regenerate lost or damaged cartilage for advanced diagnosis and treatment of OA.

The next two chapters provide the concepts of *gene therapy* and *tissue engineering* for cartilage repair. Gene therapy protocols are well suited to deliver genes coding for therapeutic factors over time in a spatially defined manner within sites of cartilage injury resulting from acute trauma or during osteoarthritis. The focus of Chapter 9 is to examine the benefits of gene therapy to improve cartilage repair in such lesions, based on promising experimental and clinical evidence in relevant models in vivo using growth, transcription, and signaling factors capable of stimulating the chondrogenic and chondro-reparative processes locally. A continuous, combined effort between scientists and orthopedic surgeons may allow to bring gene therapy from encouraging data at the bench to a successful, safe translation in the broadly affected human population. Chapter 10 summarizes several promising options to engineer articular cartilage-like constructs, ranging from applying biological factors to mechanical, magnetic, or even electrical stimuli. The paradigm of cartilage tissue engineering classically comprises three pillars: cells, scaffolds, and signals. As cell sources for cartilage repair are addressed by other chapters in this volume, the author focuses on the two remaining pillars. First, due to their importance for the subsequent tissue engineering path, scaffold-free and scaffold-based applications are distinguished. Although most classical techniques in the field are scaffold-based, relatively more attention is now paid to emerging scaffold-free methods as articular cartilage repair constructs. Only proper tissue organization will permit long-term functional durability, and mimicking tissue growth without artificial support structures holds a lot of potential. While the extracellular matrix is an integral aspect of the tissue properties, it also impedes the integration of the repair construct into the surrounding host tissue. Several approaches to tackle this dilemma are depicted. The importance to develop bioreactors is also emphasized as they are inevitable for the reproducible application of sophisticated mechanobiological stimulation regimes. In this context, the contribution of selected growth factors is described. At the end of the chapter, the importance of integrating multiple of these parameters into multimodal concepts for achieving phenotypic stability of the engineered cartilage-like constructs is addressed.

Finally, Chapter 11 focuses on different *animal models* which play an important role to test novel experimental strategies and reconstructive surgical treatments of focal articular cartilage defects. Such animal models need to reflect the different appearances and etiologies of cartilage defects, e.g., caused by trauma or osteoarthritis. Depth of articular cartilage defects plays an important role. Full-thickness chondral defects do not extend into the subchondral bone, while osteochondral defects penetrate the cement line and extend to the subchondral bone, thereby changing its structural integrity. Mice, rats, rabbits, goat, sheep, minipigs, and horses are representing good models, bridging the gap between in vitro studies and clinical experiments in human. Each of them has benefits and limitations. Evaluation

of cartilage repair may be performed using a large variety of methods, among which nondestructive evaluations and histological scoring, the latter being considered as the gold standard. As the available reconstructive surgical approaches for articular cartilage repair become increasingly complex, precise animal models to test and to translate new surgical techniques into appropriate clinical treatments are required.

Bringing together international experts from diverse fields of musculoskeletal research was a demanding task requiring patience and persistence. For that we are very grateful to the authors of this volume who managed to complete their chapters on time and who dedicated their spare time to writing their reviews.

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Overview: State of the Art and Future Prospectives for Cartilage Repair

1

Yangzi Jiang, Hang Lin, and Rocky S. Tuan

Abstract

Articular cartilage degeneration, for example, resulting from joint injury and trauma, has remained a major clinical challenge as cartilage does not have self-healing capability, and osteoarthritis (OA) often ensues. OA affects over 15% of the population, including 65% of those above 65 years of age, and is a major cause of physical disabilities. There is thus a need to develop treatment strategies that can effectively target prevention and/or blockage of early stage disease progress, rather than prosthetic replacement of the joint at the end stage. This chapter provides an overview of the state of the art and future prospectives of cartilage repair strategies. The clinical relevance and tissue pathology of cartilage injury are first introduced, covering the structure and function of cartilage tissue and evaluation and clinical management of cartilage injuries. Next, the principles and strategies of currently practiced cartilage repair are summarized, including palliative approaches (e.g., arthroscopic debridement/lavage), intrinsic repair (e.g., bone marrow stimulation technique—abrasion, drilling, and microfracture), whole tissue transplantation (e.g., osteochondral graft transplantation), and tissue engineering strategy (e.g., autologous chondrocyte implantation/transplantation). An overview of recent advances in cartilage repair strategies is presented, particularly the progress made in stem cell-based therapies and biomaterial scaffold designs. The chapter concludes with a discussion of the remaining scientific and clinical challenges in cartilage repair, specifically highlighting the need of enhancement of tissue integration, maintenance of cell phenotype, prevention of OA progress, and simplification of surgical and rehabilitation procedures.

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1.1 Introduction

1.1.1 Clinical Relevance and Needs of Cartilage Repair

Cartilage is the load-bearing surface of the synovial joint, or diarthrosis, which is generally considered a tissue with simple structure, as it is avascular and hypocellular and consists primarily of extracellular matrix (ECM) and a small amount of chondrocytes. The principal function of cartilage is to provide weight-bearing and mechanical support. However, cartilage has limited self-repair ability. Injury in cartilage often represents the initiation of joint degeneration and eventually leads to degenerative joint diseases, such as osteoarthritis (OA). OA is the most common cause of loss of mobility in elderly adults, and it has profound social, physical, psychological, and economical consequences (Callaghan 2003). Taking the United States as an example, OA affects 27 million adult Americans, including 65% of those above 65 years of age (Goldring 2006; Lethbridge-Cejku et al. 2003), and directly contributes to 9–10% of the disabilities (Felson 2004). There is no cure for OA, and osteoarthritic patients suffer from chronic pain and limited joint movement, distress and depression, and lost productivity. With the increasing aging demographics, OA is thus recognized as a significant global burden with clearly unmet clinical needs (World Health Organization 2002).

OA of the knee and hip joints are major causes of mobility impairment. The risk factors of OA include both intrinsic or progressive systemic factors, as well as factors that affect joint local mechanical environments (Fig. 1.1). Gender, family history, developmental joint growth, and shape abnormality are considered the intrinsic systemic factors, while progressive systemic factors, such as aging, hormonal status, nutrition, and lifestyle, vary among individuals. These systemic risk factors are related to the susceptibility to OA, but insults to the joint local mechanical environment, caused by overload (e.g., as a result of obesity), repetitive joint loading (e.g., in elite athletes), injury and trauma (e.g., from accident), or instability (e.g., resulting from joint surgeries), directly harm articular cartilage.

Current clinical OA management is mainly concerned with symptom reduction, e.g., pain, swelling, and stiffness, with oral nonsteroidal anti-inflammatory drugs (NSAIDs) being the most commonly used pharmacological treatment at mid-stage of the disease, and arthroplasty, an irreversible procedure, as the final solution to maintain joint function (Fig. 1.2). Consequently, it is highly desirable to develop treatment strategies that target the prevention and/or blockage of the progress of diseases in the early stage, rather than replacement of the joint at the end stage. Therefore, treatments that aim to repair cartilage defects have been under active investigation and are expected to provide more treatment choices to OA patients.

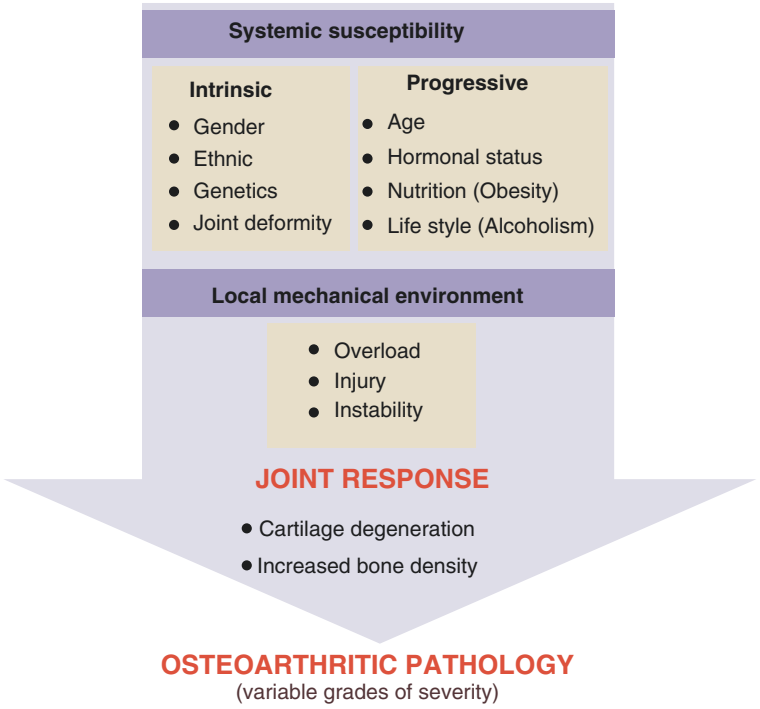

Fig. 1.1 Risk factors of osteoarthritis (OA) and the relationship between cartilage injury and OA

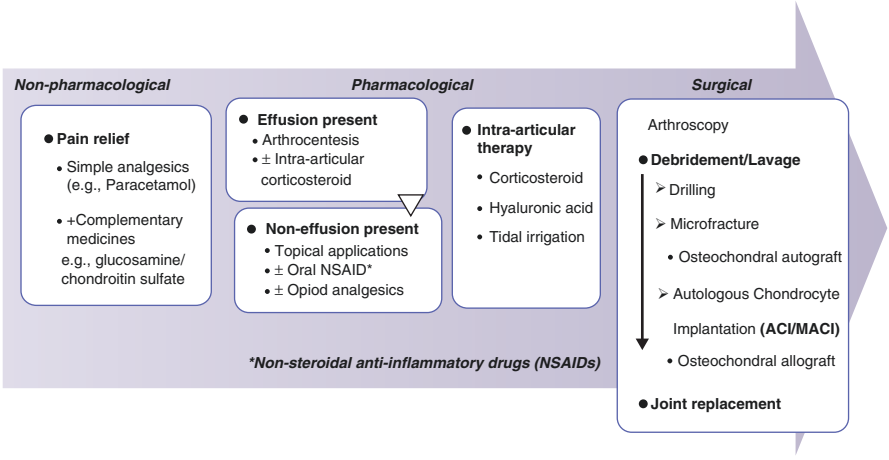

Fig. 1.2 Current OA clinical management and cartilage repair interventions. *ACI* autologous chondrocyte implantation, *MACI* matrix-associated autologous chondrocyte implantation

1.2 Cartilage Structure, Function, and Injuries

1.2.1 Cartilage Structure

Articular cartilage is the matrix-rich tissue that covers the surface of joints. With a thickness of 2–8 mm, cartilage is maintained by chondrocytes, which make up less than 10% of the tissue volume, whereas over 90% of the tissue is constituted by a unique cartilaginous extracellular matrix (ECM). In the articular cartilage, chondrocytes are distributed into four zones: (1) superficial zone, ~10–20% of the thickness, consisting of a thin layer of small, flattened chondrocytes arranged parallel to the surface; (2) middle or transitional zone, ~ 40–60% of the thickness, with chondrocytes that are spherical and separated; (3) deep or radial zone, ~30% of the thickness, consisting of large chondrocytes that form columns perpendicular to the surface; and (4) calcified zone, in which chondrocytes are hypertrophic and the matrix is calcified, representing a transition to subchondral bone (Fig. 1.3).

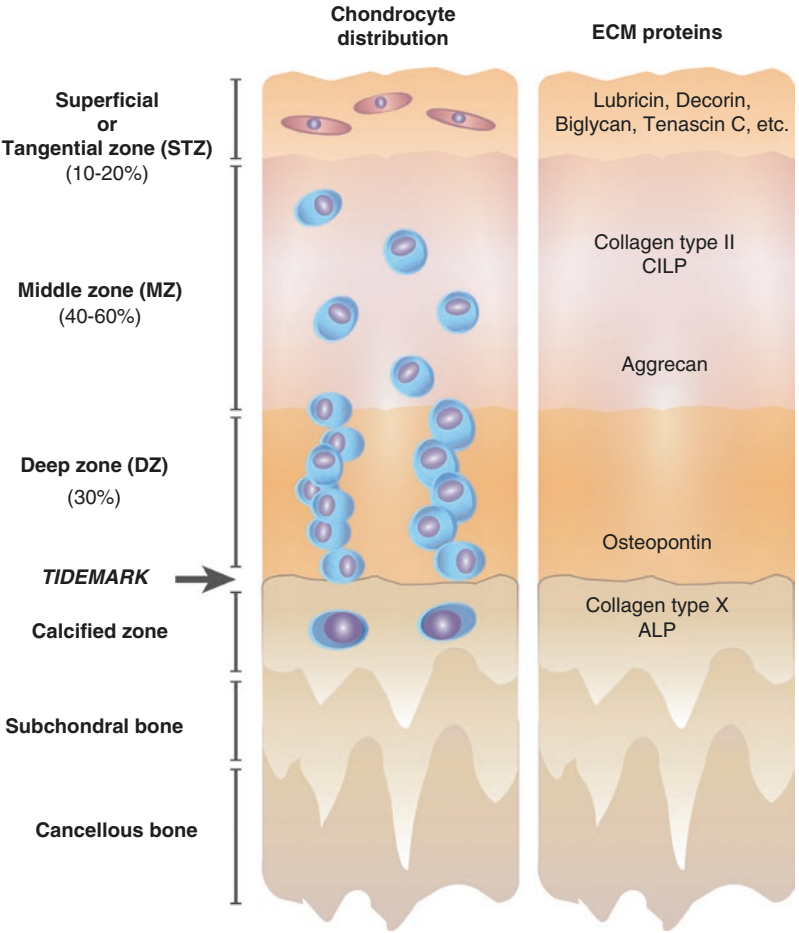

Fig. 1.3 Schematic diagram for longitudinal section of articular cartilage. *Left*, cell distribution; *right*, ECM component and distribution. *CILP* cartilage intermediated layer protein, *ALP* alkaline phosphatase

The major components of the ECM are collagen and proteoglycans (PGs). Collagen forms cross-banded fibrils, fibrils form meshes, and PGs are trapped in the mesh to hold water, responsible for the compressive strength of the cartilage ECM. In addition to collagen and PGs, other minor ECM components are localized to specific zones of the articular cartilage: (1) in the superficial zone, PGs that reduce friction are found, e.g., lubricin, which protects chondrocytes and cartilage surfaces, as well as inhibits synovial cell overgrowth (Rhee et al. 2005; Waller et al. 2013); decorin, biglycan, and tenascin C are also found in the superficial zone; (2) ECM proteins that are associated within the middle zone consist of collagen type II (Col2), aggrecan (ACAN), and cartilage intermediated layer protein (CILP); (3) in the deep zone, a zonal-specific protein, osteopontin, has been identified; and (4) in the calcified zone, collagen type X (Col10) and alkaline phosphatase (ALP) are found associated with chondrocyte hypertrophy and in the calcified matrix environment (Fig. 1.3) (Grogan et al. 2013).

The ECM within the articular cartilage may also be designated into three regions based on proximity to the chondrocyte—the pericellular, territorial, and interterritorial regions (Heinegard and Saxne 2011). Matrix content and the diameter of collagen fibrils differ in these regions. Collagen type VI (Col6) is deposited specifically in the chondrocyte pericellular region (Zhang 2015), whereas large collagen fibrils and the majority of PGs reside in the territorial and interterritorial regions. In terms of orientation, collagen fibrils are oriented mostly parallel to the surface in the superficial zone, obliquely in the middle zone, and perpendicular to the joint surface in the deep zone, which is suited to load transmission (Johnson and Pedowitz 2007).

Cartilage is classified into hyaline cartilage, fibrocartilage, and elastic cartilage, each with a unique ECM composition. Hyaline cartilage, which contains high amounts of Col2 and PGs, is formed during embryonic skeletogenesis, as an integral part of the endochondral skeleton in the form of the growth plate and articular cartilage, as well as in other tissues, such as respiratory tract cartilage, temporomandibular disc, etc. Fibrocartilage contains collagen type I (Col1), Col2, and PGs and is found largely in the intervertebral disc, pubic symphysis, and meniscus. Elastic cartilage contains elastic fibers (elastin), collagens, and PGs (Williams 2007) and is found mostly in the outer ear, Eustachian tube, and epiglottis. In this chapter, emphasis will be on articular cartilage which, with its poor self-healing ability, is a tissue of significant clinical relevance.

1.2.2 Cartilage Injury

Injury of cartilage may happen in different joints, usually initiated by direct microtrauma or trauma, or concomitant with other injuries to joint tissues, such as meniscus or anterior crucial ligament, leading to joint instability and axial malalignments. The location and severity of injury and the size of the defect are crucial for the selection of potential treatments. For example, defects in a relatively non-load-bearing area may not need immediate treatment, while treatments of concomitant injuries should co-address the damages to the meniscus, subchondral bone, and ligament/tendon and correct the axial malalignments to achieve optimal repair cartilage.

Cartilage defects can be detected by a number of methods. For example, the integrity of cartilage and subchondral bone can be observed by noninvasive magnetic resonance imaging (MRI) (Williams 2007), as well as via arthroscopic inspection. The severity of cartilage defects has been codified by the International Cartilage Repair Society (ICRS), which has provided a cartilage injury evaluation package to inform clinical decision-making (Mats Brittberg et al. 2000). In addition to the location and the size of the defect (Fig. 1.4), five levels of articular cartilage defects are defined: (*Level 1*) normal and intact articular cartilage superficial lesions; (*Level 2*) softening of cartilage surface and/or superficial fissures and cracks of cartilage surface; (*Level 3*) abnormal defects occupying <50% of cartilage depth; (*Level 4*) severely abnormal defects occupying >50% of cartilage depth, down to calcified zone, or down to the subchondral bone, or with blisters; and (*Level 5*) severely abnormal defects penetrating the subchondral lamella or subchondral bone (Fig. 1.4).

1.2.3 Clinical Outcomes of Treatments

Clinical outcomes of treatment of cartilage degenerative conditions are influenced by multiple factors, such as the general health status of the patient, the severity of the lesion, the specific treatment procedure, as well as the rehabilitation regimen. Some of the commonly used evaluations of joint health include physical examination, monitoring activities of daily living, kinematic assessment of joint functions, medical imaging such as magnetic resonance imaging (MRI) and computed tomography (CT), arthroscopic examinations, and tissue biopsy. Information collected from these procedures is used to form the basis of evaluation, such as described in Table 1.1.

1.3 Current Cartilage Repair Strategies

Current clinically available cartilage repair treatments may be classified into five different levels, and the decision-making is generally based on the severity of the injury, lesion size, patient health status, history, and preference and whether the purpose is to address the outcomes of a previous surgical intervention.

1.3.1 Palliative Approach: Arthroscopic Debridement/Lavage

The goal of the palliative approach is removal of the mechanically offensive tissue sources and the dead, damaged, and inflammation-affected tissues by debridement and lavage, to achieve pain relief. This symptomatic therapy can be effective for several months and occasionally a few years; however, it does not result in filling of cartilage lesions.

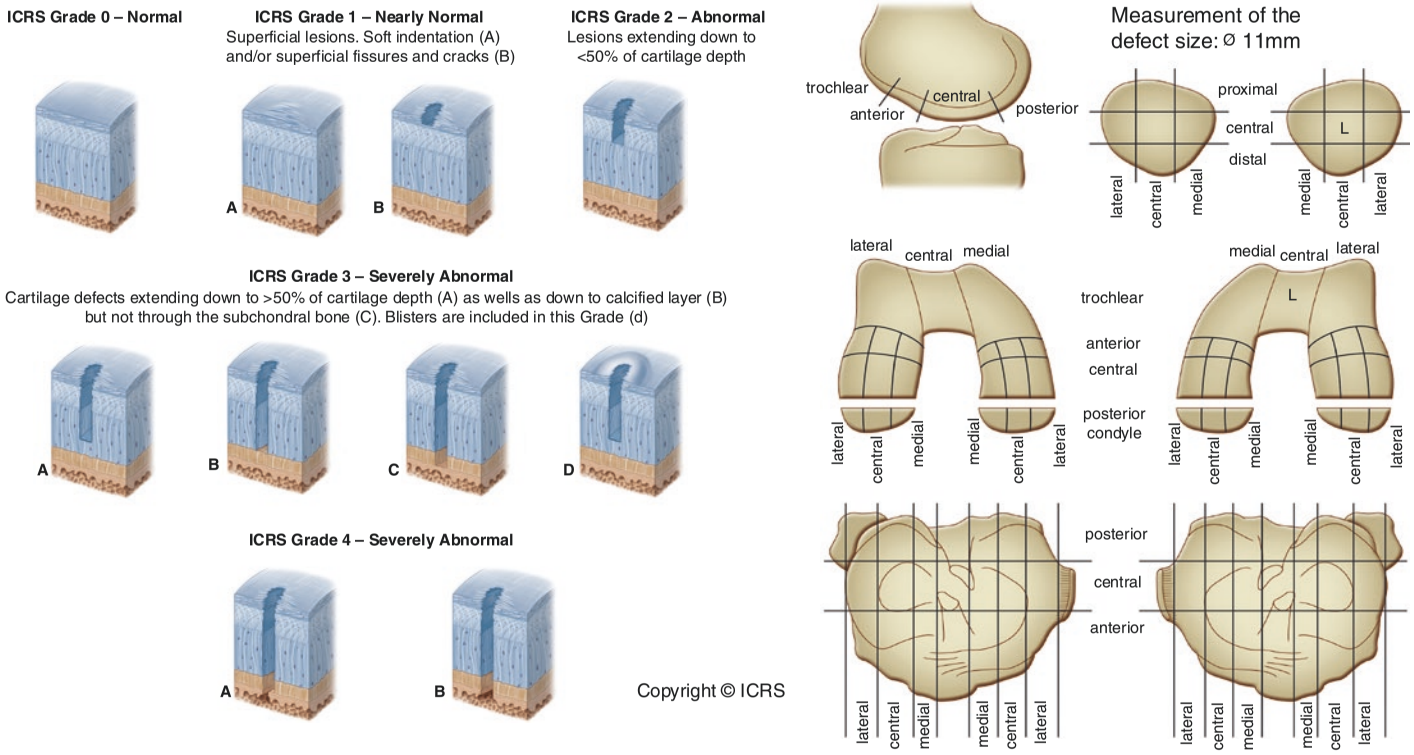

Fig. 1.4 Illustration of ICRS grades of cartilage defects and locations (Copyright @ ICRS; used by permission)

Table 1.1 Summary of current treatment strategies for cartilage injuries

Tissue repair/regeneration[a]	1. Palliative	2. Bone marrow[a] stimulation techniques		3. Whole tissue transplantation		4. Tissue engineering[a]
Interventions	Debridement/lavage	Drilling	Microfracture	Osteochondral autograft	Osteochondral allograft	Autologous chondrocyte implantation (ACI/MACI)
Demand	Low	High → Low		High	High	High
Lesion size (cm^2)	0–4	(0.5–2)–(4–6)		1–5	3–10[+]	2–12[+]
Known durability	<2 years	2–6 years		5–20 years	2–10 years	>20 years
Filling tissue	–	Fibrocartilage		Hyaline cartilage	Hyaline cartilage	Hyaline-like
Filling scale	–	Partial		Near total	Near total	Near total
Limitation	Temporary effect, no repair	Incomplete defect filling, injury of subchondral bone plate, low quality of repair tissue		Limited availability of healthy tissue; secondary injury	Limited availability; immune response; potential necrosis	Limited cell number; two-stage procedure

[a]Involving endogenous tissue repair/regeneration; [+]refers to other sizes beyond as indicated (Williams 2007; Chahal et al. 2013)

1.3.2 Intrinsic Repair: Bone Marrow Stimulation Technique—Abrasion, Drilling, and Microfracture

Cartilage defect has been shown to exhibit a healing response, particularly when associated with bone marrow or other neighboring tissues. The initiation of such a healing process is dependent on the depth of the lesion site and whether the defect is in communication with the bone marrow. When the lesion of the articular cartilage is located only in the superficial zone to middle/deep zone, termed as partial- and full-thickness cartilage defects, the defects generally do not exhibit any intrinsic healing. On the other hand, osteochondral defects, which violate the tidemark and penetrate into the subchondral bone and thus connect the defect to the bone marrow cavity, exhibit limited but definitive reparative capacity, most likely because the tissue defect permits bone marrow and marrow stromal cells to infiltrate into the lesion site to form a stem cell-rich fibrin clot to stimulate intrinsic repair (Jiang et al. 2010, 2011). In clinical practice, this intrinsic reparative property is exploited in the microfracture technique by drilling into the subchondral bone, to treat small size cartilage defect (usually 0.5–2 cm^2) (Mithoefer et al. 2005; Williams 2007). This approach, referred to as marrow stimulation, provides partial fill to the defects with fibrocartilage, and the known durability is 2–6 years. The marrow stimulation technique is thus safe and readily applicable in clinical practice and cost-effective as a first-line treatment for grade 3 or 4 lesions (Pestka et al. 2012; Knutsen et al. 2007). However, it is not applicable for larger defects with higher mobility demands, such as in young athletes, and the quality of the repaired tissue is unpredictable and variable, thus affecting the effectiveness of the second-line treatments, such as autologous chondrocyte implantation (see below), after the first microfracture treatment has failed (Pestka et al. 2012) (for details, see Chap. 5).

1.3.3 Whole Tissue Transplantation: Osteochondral Graft Transplantation

For those full-thickness defects larger than 2 cm^2 or associated with osteochondritis dissecans, transplantation of healthy cartilage and subchondral bone is one of the options. The result has been proven useful in clinical practice, and the source of the transplant tissue may be from the same person (autologous) or from other donors (allogeneic). This whole tissue transplantation approach is the biologic reconstructive method to salvage the failed first-line less invasive treatments.

In autologous osteochondral transplantation, or mosaicplasty, one or more osteochondral cylinders are collected from less weight-bearing areas of the joint surface or, less frequently, from the contralateral knee joint and then inserted singly or as multiple plugs into the defect area. This method thus creates a mosaic hyaline cartilage surface and may be considered as first-line treatment for patients of high mobility demand and with smaller lesions, but is generally limited by the availability of structurally and functionally adequate donor graft tissues (for details, see Chap. 5).

In practice, both fresh and cryopreserved frozen allografts are used. Fresh allografts provide higher cell viability, but host-graft immune response and possible disease transmission are possible concerns, whereas frozen allografts have lower viable cell yield. In comparison, as there is no donor site morbidity, allograft-based cartilage resurfacing technique has the longest history with good long-term results (Williams 2007; Sherman et al. 2014). However, the availability of allograft specimens is also limited, and the cost is significantly higher than autografts.

1.3.4 Tissue-Engineered Cartilage

To develop alternative methods of tissue transplantation, tissue engineering/regenerative medicine approaches have been explored and developed rapidly since the 1970s. The aim of tissue engineering is to replace diseased or lost tissue/organ with an engineered tissue, derived from cells, biomaterial scaffolds, and bioactive factors, for the purpose of restoring or establishing normal function (Mason and Dunnill 2008). The three key elements of tissue engineering are tissue-forming cells, structural scaffolds, and signaling molecules, the combination and application of which result in a functional tissue construct to promote tissue healing and regeneration (Kuo et al. 2006).

1.3.4.1 Autologous Chondrocyte Implantation/Transplantation (ACI/ ACT)

The concept of using autologous periosteal grafting to treat cartilage defects was first proposed for the repair of full-thickness defect in a rabbit model (O'Driscoll et al. 1986). Cultured autologous chondrocytes were later used in addition to the periosteum (Grande et al. 1987, 1989). In the early 1990s, ACI was applied for the first time in clinical practice to treat full-thickness chondral defects in the knee joint by the Peterson group (Brittberg et al. 1994), and a subsequent 10–20-year follow-up showed that 74% in 224 patients reported their status being as good or better compared to pre-ACI years (Peterson et al. 2010). At present, the autologous chondrocyte-based therapies are by far one of the most successful examples in the clinical application of the cell-based tissue engineering principle to achieve tissue regeneration.

The ACI technique is more complicated than microfracture or osteochondral transplantation, as it requires two separate surgeries and an intermediate step of in vitro cell culture. In brief, in the first surgery, a very small amount (~250 mg) of healthy cartilage is collected under arthroscopy from a presumably non-load-bearing area of the articular joint surface, and chondrocytes are isolated and culture expanded in the laboratory for ~3–6 weeks. The culture-expanded cells are then sent to the operating room and introduced into the cartilage defect area and sealed with a sutured autologous periosteal flap. With ACI, hyaline-like cartilage tissue is usually observed in the defects within a year, and this procedure can be used for the repair of larger defects ($\geq$10 cm^2). The overall therapeutic efficacy is 70–90% in 0–5 years, as evidenced by relief of symptoms and improvement of joint function

(Jiang et al. 2011). The therapeutic effects have been reported to last longer than the marrow stimulation technique (Zaslav et al. 2009) and also can be used for osteochondritis dissecans (OCD) knee lesions (Cole et al. 2012) (for details, see Chap. 5).

With advances in biomaterial development, the ACI technique has also evolved accordingly. Biomaterials have been used instead of autologous periosteal flap covers, thus eliminating the extra open injury sites of tissue harvesting and shortening the operation time. The biomaterials used are usually with high biocompatibility (e.g., membranes of collagen types I and III, and the cells are seeded on the biomaterials and delivered as a single piece of tissue-engineered construct. This technique is now referred to as matrix-associated autologous chondrocyte implantation (MACI) or tissue-engineered cartilage (TEC) transplantation (Jiang et al. 2011).

1.3.5 Total Joint Replacement

When the injury and disease are judged to have reached end stage, the final solution is to replace the damaged joint with a prosthesis that is often constructed from metal (e.g., cobalt-chromium or titanium-based alloys), plastic (e.g., ultrahigh molecular weight polyethylene), and/or ceramics. Total joint arthroplasty (TJA), generally considered as one of the most successful medical procedures, has a long clinical history, and the prostheses employed, e.g., for the knee and hip, are designed to replicate the movement of the normal joint. The most common complications to these otherwise highly effective but irreversible procedures include septic or aseptic periprosthetic osteolysis, the latter most likely caused by the accumulation of wear debris from the prosthesis, thus limiting the functional life expectancy of the prosthesis to less than 10–15 years (Tuan et al. 2008). For younger TJA patients (<40–50 years of age), a revision procedure is generally expected, thus presenting additional surgical challenges.

In Table 1.1, a summary of the current treatment strategies for cartilage injuries and concerns for decision-making in cartilage repair procedures is presented.

1.4 New Strategies Under Investigation

Although the outcomes of ACI/MACI have been mostly favorable, there are also significant limitations (Jiang et al. 2011), including the low number of harvested cells and potential leakage and uneven distribution of cells in the defect (Kim et al. 2010). ACI/MACI is also a long and complicated procedure with multiple steps, and the repair tissue is only hyaline-like but not completely hyaline cartilage, as collagen type I, a hallmark of fibrocartilage, can be found in the repair tissue. In addition, many patients with chronic OA may be unable to get the ACI treatment because of the lack of healthy donor tissue. To overcome these limitations, contemporary research efforts have actively focused on the optimization of cell sources,

identification and application of growth factors, and development of scaffolds with improved biomimetic properties. The following is an overview of the progress in these endeavors.

1.4.1 Cell-Based Therapies

1.4.1.1 Stem Cells

Stem cells present the most promising candidate cell type for cartilage tissue repair because of their self-renewal ability and chondrogenic potential—the ability to form cartilage. Stem cells in mammals may be divided into two broad types: embryonic stem cells (ESCs) and adult stem cells. ESCs are pluripotent stem cells derived experimentally by extraction from the inner cell mass of a blastocyst, while adult stem/progenitor cells are found in different adult organs and tissues. Pluripotency refers to the potential of a stem cell to differentiate into cells of all three germ layers—endoderm, mesoderm, and ectoderm. In addition to ESCs, induced pluripotent stem cells (iPS cells or iPSCs) have recently been generated from adult somatic cells by transcription factor gene-based reprogramming and exhibit similar pluripotency. In comparison, adult stem cells are multipotent, i.e., they can be induced to differentiate into the cell types that are closely related in developmental origin (e.g., from the same embryonic germ layer). Finally, progenitor cells are developmentally committed cells, with the ability to differentiate into only one or few cell types, referred to as exhibiting uni- or oligopotency (for details, see Chaps. 2 and 4).

Stem Cell Types Relevant to Cartilage Repair

Embryonic Stem Cells (ESCs) and Induced Pluripotent Stem Cells (iPSCs) Because of their highly uncommitted state, the pluripotent ESCs and iPSCs present more challenges in terms of being induced to differentiate into cartilage cells, compared to multipotent stem cells, such as adult stem cells. Generally, an additional step, for example, first turning pluripotent stem cells into a mesodermal lineage, is required (Diederichs and Tuan 2014), whereas mesoderm-like multipotent stem cells, such as bone marrow-derived mesenchymal stem cells, may be directly induced to undergo chondrogenic differentiation. Both ESCs and iPSCs have been shown to have chondrogenic capability in vitro (Yamashita et al. 2010; Hiramatsu et al. 2011) and in animals (Wakitani et al. 2004a; Toh et al. 2010; Dattena et al. 2009; Yamashita et al. 2013). However, some unsolved problems remain, e.g., differentiation efficacy, because not all of the cells contribute to hyaline cartilage formation (Dattena et al. 2009), and only selected clones/cell lines have been reported with cartilage matrix-forming ability (Yamashita et al. 2013). Another issue of concern is safety, in view of potential tumor formation and immunological rejection of allogeneic cells when allogeneic ESCs or iPSCs are introduced in vivo. More standardization and safer methods are clearly needed to bring these pluripotent stem cells into clinical use (for details, see Chap. 4).

Mesenchymal Stem Cells (MSCs) MSCs are currently the most promising therapeutic cells for cartilage repair, in view of their more defined and ready chondrogenic potential compared to ESCs/iPSCs and their increasingly apparent beneficial trophic bioactivities, such as anti-inflammatory activity (Tuan et al. 2003). MSCs can be isolated from bone marrow, adipose tissue, muscle, bone, synovium, and other adult tissues. Among cells derived from diverse tissue sources, autologous bone marrow-derived MSCs (BMSCs) hold the best potential of application because of their ready availability and high chondrogenic efficiency. BMSCs produce higher amount of cartilage matrix and promote higher cartilage recovery than MSCs isolated from periosteum, synovium, adipose tissue, and muscle (Li et al. 2011b). Synovium-derived MSCs (Vinardell et al. 2012), adipose MSCs, and muscle MSCs (Li et al. 2011b) exhibited chondrogenesis potential but also fibrous tissue formation. Clinical application of BMSCs for cartilage repair was reported by several groups (Teo et al. 2013; Kasemkijwattana et al. 2011; Wakitani et al. 2004b), and comparable results using BMSCs and ACI were reported at 2 years post-surgery (Nejadnik et al. 2010). Longer follow-up is needed to verify the level of efficacy.

Adipose-derived stem cells (ADSCs) are another promising, candidate MSC type, especially in terms of their availability. Adipose tissues contain over 100-fold of mononuclear, stem-like cells per volume than bone marrow aspirates (Pendleton et al. 2013). However, their chondrogenic potential appears to be lower compared to BMSCs (Hildner et al. 2011) and requires different chondroinductive factors, e.g., BMP6 (Estes et al. 2006; Diekman et al. 2010). Improving the cartilage-forming ability of these cells is thus required and is one of the challenges for their application for articular cartilage repair (for details, see Chap. 2).

MSCs isolated from other tissues surrounding the joint, such as synovium and periosteum, are also being investigated for articular cartilage repair (Koga et al. 2008). A summary of the in vitro and animal research models used for stem cell-based cartilage repair studies is presented in Table 1.2.

Tissue-Specific Stem Cells In many adult tissues (e.g., muscle), endogenous stem/progenitor cells are the cells that function to maintain tissue homeostasis, an inherent activity of tissue repair mechanism. Articular cartilage has traditionally been considered a physiologically self-renewing tissue. Interestingly, cartilage stem/progenitor cells (CSPCs) have recently been found within human articular cartilage (Williams et al. 2010; Quintin et al. 2010; Fickert et al. 2004; Alsalameh et al. 2004; Jiang and Tuan 2015). These cells are being characterized as stem/progenitor cells in vitro by virtue of their self-renewal ability, multipotent differentiation capability, and expression of stem cell markers. However, there is currently no single marker that could define CSPCs in vivo, and their derivation, fate, and lineage information are still largely unknown.

Chondrocytes in long-term culture are known to lose their cartilage matrix-forming ability, a process known as dedifferentiation, a process that limits ACI application; however, the discovery of the phenotypic plasticity of human articular chondrocytes (Tallheden et al. 2003) suggested a possible origin of tissue-specific stem/progenitor

Table 1.2 Cartilage and joint health evaluations

Usage	Evaluation	Characteristics	Tools	Reference
Regular	Short Form (SF)-36	Patient-reported survey, 36 items	–	Towbin et al. (1989)
OA	Kellgren and Lawrence Grading system (K&L)	OA pathology, indicated by radiographic changes (e.g., joint space narrowing, osteophytes, and bony sclerosis)	X-ray	Kellgren and Lawrence (1957)
	Criteria of American College of Rheumatology (ACR)	Includes laboratory tests such as synovial fluid observation, white blood cell count, and erythrocyte sedimentation rate	Lab tests	Moskowitz (2007)
	The Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC)	Survey assesses pain, stiffness, and physical function in patients with hip and/or knee OA	–	Kornfeld and Kornfeld (1985)
Joint function	Tegner Lysholm Knee Scoring Scale (Lysholm)	Knee function	–	Tegner and Lysholm (1985)
	International Knee Documentation Committee (IKDC) Score	Patient-reported score	–	Akagi et al. (2002)
	Activities of Daily Living (ADL) Score	Daily self-care activity and life quality	–	Irrgang et al. (1998)
	Modified Cincinnati Score (Cincinnati)	Function, pain, symptoms, (function in daily living, function in sport and recreation, and knee-related quality of life)	–	Noyes et al. (1983)
	Knee Injury and Osteoarthritis Outcome Score (KOOS)			Roos et al. (1998)
Cartilage defects	International Cartilage Repair Society (ICRS) Cartilage Injury Evaluation Package	Includes doctor score and patient score. Minimally invasive surgical procedure by arthroscopy, locating defects and intra-articular cartilage surface position and repair at the same time	Arthroscopy, histology, etc.	Gibson et al. (1998)
	Magnetic Resonance Observation of Cartilage Repair Tissue (MOCART) System	Noninvasive location of defects in cartilage and subchondral bone	Magnetic resonance imaging (MRI)	Marlovits et al. (2004)

cells in adult cartilage. Recently, a subgroup of stem/progenitor cells is found in chondrocytes isolated from adult articular cartilage, termed chondrocyte-derived progenitor cells (CDPCs), identified by their demonstrated dynamic change of phenotype from that of mature chondrocytes toward that of MSCs (Jiang et al. 2016) (for details, see Chap. 3). Upon low density and low glucose in vitro expansion, CDPC number was enriched in a shorter period of time, and these cells showed enhanced chondrogenic capability compared to human BMSCs. The CDPCs were tested in a MACI procedure in place of chondrocytes for the repair of large-sized cartilage defects (6–13 cm^2) in young patients, and 1-year follow-up showed pain reduction, joint function improvement, and hyaline cartilage filling (Jiang et al. 2016). The existence of CDPCs in articular cartilage suggests a novel approach to cartilage repair and provides a new view of the phenomenon of chondrocyte dedifferentiation that illustrates the importance of extrinsic factors that regulate cell fate in cartilage.

Signaling Factors: Stem Cell Differentiation and Cell Phenotype Maintenance

Knowledge gained from studies of embryonic development has revealed that skeletogenic differentiation of mesenchymal cells in vivo is an intricate process regulated by a variety of signals that act coordinately and sequentially at different stages of development to guide the formation of the hyaline cartilage and to maintain its differentiated state and function of the chondrocytes. Specifically, signaling factors, such as members of the transforming growth factor β (TGFβ) superfamily, are required. During embryonic development, chondrogenesis in the primordial limb bud begins with mesenchymal cell recruitment, proliferation, and condensation. Several growth factors and signaling molecules act in concert in the initiation of cell condensations, including TGFβ family members, such as the bone morphogenetic proteins (BMPs), fibroblast growth factor (FGF), and Wnt signaling. Specific extracellular matrix molecules are also involved in defining and enabling the transition from chondroprogenitor to chondrocytes; for example, fibronectin, hyaluronan, and collagen type I are involved in modulating cell-cell interaction during mesenchymal condensation, mediated via N-cadherin (Goldring et al. 2006; Shum et al. 2003; Wezeman 1998; DeLise et al. 2000; Gehris et al. 1996; Oberlender and Tuan 1994a, b) by regulating a key chondrogenesis-specific transcription factor, Sox9 (Akiyama and Lefebvre 2011).

As presented earlier, compared to the multipotent MSCs, ESCs/iPSCs are pluripotent cells; thus, deriving cartilage tissue from ESCs/iPSCs is more complicated than from MSCs, because they need to undergo additional commitment and differentiation steps. In general, differentiating ESC/iPSCs into cartilage involves a two-step differentiation strategy, combining culture conditioning and growth factor induction. ESCs/iPSCs are first differentiated into a multipotent state, followed by differentiation along the chondrocytic lineage (Hwang et al. 2008). This differentiation scheme is guided by the knowledge on embryonic mesodermal commitment, with the second step derived from known pathway of MSC chondrogenic differentiation. A number of growth factors have been shown to regulate MSC proliferation and chondrogenic differentiation, including members of the TGFβ superfamily, insulin-like growth factors (IGFs), FGFs, and platelet-derived growth factors

(PDGFs) (Heng et al. 2004; Bobick et al. 2009; Danisovic et al. 2012; Freyria and Mallein-Gerin 2012), and are being applied to promote chondrogenic differentiation of MSC-like cells derived from ESCs and iPSCs.

Among these growth factors, TGFβs and BMPs are the most important inducers of chondrogenic differentiation. In particular, BMP-2 and BMP-7 (also known as OP-1) have been approved by the FDA for clinical application (Alaoui-Ismaili and Falb 2009). Interestingly, MSCs from different tissue origins exhibit different growth factor requirements for chondrogenesis. For example, TGFβ1 and TGFβ3 are used to promote chondrogenesis of bone marrow-derived MSCs (Barry et al. 2001; Byers et al. 2008; Huang et al. 2009), whereas BMP6 is required for efficient stimulation of chondrogenesis with TGFβs for adipose-derived MSCs (Estes et al. 2006; Hennig et al. 2007), and BMP4, in combination with TGFβs, enhances the cartilage matrix-forming ability of bone marrow or cartilage-derived cells (Jiang et al. 2010, 2016). Different growth factor receptor repertoires likely contribute to the different biological characteristics of the MSCs derived from different adult tissue sources (for details, see Chap. 6).

1.4.1.2 Allogeneic Chondrocytes

Similar to allogeneic osteochondral transplantation, the use of allogenic chondrocytes is of potential for cartilage repair, in particular if they may be derived from ESCs and iPSCs. Previous studies have shown that pure allogeneic chondrocyte/tissue implantation evoked a systemic immunological reaction followed by rejection by the host (Moskalewski et al. 2002; Revell and Athanasiou 2009). A potential solution is the use of biomaterials as a vehicle for allogeneic chondrocyte delivery for cartilage repair, which may blunt the immune reaction (Fragonas et al. 2000). A clinical trial of 21 patients reported that patients implanted with allogenic chondrocytes cultured in alginate beads and delivered into osteochondral lesions showed apparent functional improvement, with a 19.5% failure rate at mean follow-up of 6.1 years, but without any signs of clinical deterioration or adverse reactions to the alginate beads/allogenic chondrocyte implantation (Almqvist et al. 2009; Dhollander et al. 2012). Such an approach may present practical possibilities for the introduction of different types of allogeneic cells for the repair of articular cartilage defects.

1.4.2 Biomaterial Scaffolds for Cartilage Repair

1.4.2.1 Current Biomaterials and 3D Scaffolds

In tissue engineering/regenerative medicine, biomaterial scaffolds are employed to supply mechanical support and cell growth niche, as well as deliver cells/signaling factors into the tissue defect sites to enhance repair. The scaffold design must meet the combined demands of material biocompatibility, mechanical property, capability of integration with target tissue, and maintenance of the bioactivities of implants (i.e., cell and growth factors). Because articular cartilage is bathed in synovial fluid and there is constant joint movement, cells for cartilage repair or regeneration must be delivered to the tissue site encased in a three-dimensional (3D) scaffold with adequate mechanical support. Table 1.3 presents a summary of the materials that

Table 1.3 Research models used in cell-based cartilage repair studies

	Models	Cell types	Reference
Animal models	Nude mice	iPSCs or iPSC-derived cells	Hiramatsu et al. (2011)
	Rat	Adipose-derived stem cells	Xie et al. (2012)
		ESCs or ESC-derived cells	Wakitani et al. (2004a) and Toh et al. (2010)
		Muscle-derived stem cells	Mifune et al. (2013) and Matsumoto et al. (2009)
		Bone marrow-derived stem cells	Park et al. (2006) and Yoshimura et al. (2007)
		iPSCs or iPSC-derived cells	Jiang et al. (2012)
	Rabbit	Bone marrow-derived stem cells	Koga et al. (2008), Murphy et al. (2003), Kayakabe et al. (2006), Guo et al. (2010), Li et al. (2011a), Yan and Yu (2007), Im et al. (2001), and Park et al. (2009)
		Adipose-derived stem cells	Fernandez et al. (2012), Koga et al. (2008), and Li et al. (2011a)
		Synovium-derived stem cells	Koga et al. (2008), Lee et al. (2013), Lee et al. (2012), Suzuki et al. (2012), and Pei et al. (2009)
		Periosteum-derived stem cells	Li et al. (2011a)
		Muscle-derived stem cells	Li et al. (2011a) and Koga et al. (2008)
		Umbilical cord-derived stem cells	Yan and Yu (2007)
	Goat	Bone marrow-derived stem cells	Sharma et al. (2007)
	Sheep	ESCs or ESC-derived cells	Dattena et al. (2009)
	Mini pig	Bone marrow-derived stem cells	Zhou et al. (2006)
		Synovium-derived cells	Pei et al. (2013)
Clinical studies	Clinical study/ case report	Autologous chondrocytes	Brittberg et al. (1994) and Peterson et al. (2010)
		Cartilage-derived progenitor cells	Jiang et al. (2016)
		Bone marrow-derived stromal/stem cells	Wakitani et al. (2002), Kuroda et al. (2007), Wakitani et al. (2004b), and Wakitani et al. (2007)
Non-animal models	Engineered tissue	Chondrocytes, bone marrow-derived stromal/stem cells	Rackwitz et al. (2014)
	3D cultures	Adult and embryonic-derived cells	Cheng et al. (2013), Tuli et al. (2004), Jiang et al. (2016), and Toh et al. (2010)

have been used for cartilage tissue engineering. Additional information is provided in a systematic review on scaffold-based repair for cartilage healing by Filardo et al. (2013a) and in a review on product development and clinical trial by Ahmed and Hincke (2010) (for further details, see Chap. 10).

1.4.2.2 Major Concerns in Scaffold Design

Biocompatibility Biocompatibility, referring to the property of a scaffold not harming cells or tissues or evoking severe immune reaction, is the fundamental requirement for the application of biomaterial scaffold application. A unique characteristic of articular cartilage is its avascularity, rendering it less immune reactive than most other tissues (Revell and Athanasiou 2009). The majority of biomaterials currently used in cartilage repair studies and applications are biocompatible, although undesirable reactions are sometimes observed depending on the type of polymer, cross-linking methods, and the nature of the by-products.

Mechanical Property The compressive modulus of native human articular cartilage ranges from 4.3 to 11.6 MPa (Shepherd and Seedhom 1999). Ideally, the grafted scaffolds should possess similar mechanical strength in order to withstand compressive loads immediately after surgery. As shown in Table 1.4, synthetic polymers generally have higher compressive modulus than native biomaterials. However, cell-binding ligands are absent in synthetic polymers; in addition, higher mechanical strength often is a consequence of a structurally dense composition, which is likely to limit nutrient penetration, cell growth, and neocartilage formation. Therefore, hybrid scaffolds that combine the properties and structures of both synthetic and native materials must be considered in the design and development of biomaterial scaffold for future cartilage repair applications (for details, see Chap. 10).

In Situ Fabrication One approach to improve the efficacy of cartilage repair is the formation of cartilage in situ, instead of implantation of engineered cartilage constructs produced from cells and scaffold cultured ex vivo. To allow in situ delivery of cells and growth factors with minimal surgical invasion, the use of injectable scaffold such as cross-linkable hydrogels represents another approach. Currently, there are several strategies to initiate and control the in situ cross-linking process:

1. Photo cross-linking. The basic principle of this method is to first derivatize vinyl groups onto the polymer and then use free radicals, produced from photoinitiator by light exposure (most often using UV light), to polymerize the monomers. Due to the potential cytotoxic effects of UV exposure on cells, visible light-activated cross-linking has recently received attention (Lin et al. 2013; Fairbanks et al. 2009).
2. Thermal cross-linking. This process involves the incorporation of thermosensitive polymers, such as poly(N-isopropylacrylamide-co-acrylic acid). The precursor solution is liquid at ambient temperature and undergoes gelation at around 37 °C (Klouda and Mikos 2008).

Table 1.4 Materials and products that have been used for cartilage repair

	Material	Monomer form or precursor	Sources	Compression modulus	Cell-binding ligands	Biodegradable	Clinical trial	Product name
Synthesized	Polylactic acid (PLA)/ polyglycolic acid (PGA)	Lactic acid/glycolic acid		20 kPa at 68% PLA in PGA mesh (Moran et al. 2003)	–	Yes	Yes	BioSeed-C (PLA, PGA copolymer)
	Poly(ethylene glycol)	Ethylene glycol		320 kPa at 100% (Riley et al. 2001)	–	–	Yes	ChonDux (with chondroitin sulfate)
	Poly(vinyl alcohol)	Vinyl alcohol		250 kPa at 20% (Holloway et al. 2010)	–	–	Yes	SaluCartilage
	Self-assembling peptide	RADA (Kopesky et al. 2010), RGDS (Miller et al. 2010), KLDL (Kopesky et al. 2011)		<2 kPa (Kisiday et al. 2002)	Yes	Yes	–	
	Agarose	Galactose and 3,6-anhydrogalactose	Marine algae	5–10 kPa at 2% (Huang et al. 2009)	–	–	Yes	Cartipatch (mixture of agarose and alginate)
Natural derived	Alginate	β-D-mannuronic acid and R-L-guluronic acid	Marine algae	20–30 kPa at 2% (Wong et al. 2001)	–	–	Yes	Cartipatch (mixture of agarose and alginate)
	Chitosan	D-glucosamine, *N*-acetylglucosamine	Mushroom, arthropod exoskeletons	20 kPa at 2% (Subramanian and Lin 2005)	–	Yes	Yes	BST-CarGel
	Collagen types I/III	Collagen 1α1, 1α2; collagen 3α1	Tendon or skin from human or animals	7 kPa at 0.7% (Li et al. 2012)	Yes	Yes	Yes	Chondro-Gide (with collagen III), CaReS, PureCol/VitroCol/ Nutragen

(continued)

Table 1.4 (continued)

	Material	Monomer form or precursor	Sources	Compression modulus	Cell-binding ligands	Biodegradable	Clinical trial	Product name
	Collagen type II	Collagen 2α1	Cartilage from human or animals	0.88 kPa at 1% (Pfeiffer et al. 2008)	Yes	Yes	–	
	Fibrin	Fibrinogen	Blood from human or animals	0.029 kPa	Yes	Yes	Yes	Tissucol
	Hyaluronan	D-Glucuronic acid, D-*N*-acetylglucosamine	Cartilage from human or animals	8–30 kPa (Bian et al. 2012; Toh et al. 2012)	Yes	Yes	Yes	Hyalograft C, HYAFF11
	Silk fibroin	Fibroin	Silkworms	13–34 kPa at 8% (Yan et al. 2012)	Yes	Yes	–	
	Decellularized matrix	Native ECM from cartilage	Cartilage from human or animals (Schwarz et al. 2012)	1.92 MPa (Schwarz et al. 2012)	Yes	Yes	–	
	Cell sheet	ECM secreted by cells	Stem cells (Ando et al. 2008) or chondrocyte (Mitani et al. 2009)	<5 KPa (Ando et al. 2008; Mitani et al. 2009)	Yes	Yes	–	

3. Enzymatic cross-linking. Peroxidases, such as horseradish peroxidase, catalyze oxidative polymerization of phenol groups in the monomers in the presence of H_2O_2 (Zavada et al. 2016).
4. pH-sensitive cross-linking.
5. Ionic interaction- and hydrophobic interaction-initiated cross-linking.
6. Chemical cross-linking. In general, scaffolds capable of in situ fabrication are preferred in terms of a better fit for the tissue defects prefabricated scaffolds.

1.5 Unsolved Problems and Challenges in Cartilage Repair

1.5.1 Tissue Integration

Integration of implant tissue is critical for cartilage repair, particularly in view of the presence of mechanical loading which will otherwise dislodge the implanted construct. Current methods to promote tissue integration include: (1) application of biocompatible glue such as fibrin (Bekkers et al. 2010), (2) suturing (Bryant et al. 2007), (3) pin or transosseous fixation (Zelle et al. 2007), (4) regenerative interaction, and (5) chemical cross-linking between the biomaterial scaffold and host tissue (Sharma et al. 2013). New strategies should encourage both inherent cell-based repair and external implants, for instance, the application of chemokines such as stromal cell-derived factor (SDF-1) to guide migration of the implanted cells to host tissue or recruit cells from host tissue (Zhang et al. 2013) and stimulating production of cartilage ECM to seal the gap between graft and tissue.

1.5.2 Maintenance of Cell Phenotype

A major current challenge is the long-term maintenance of the hyaline chondrocyte phenotype of the transplanted cells in vivo, particularly the differentiated stem cells. For MSC populations that have undergone chondrogenic differentiation, fibrous tissue formation or a hypertrophic phenotype is frequently seen. Chondrocyte dedifferentiation is generally thought to be the reason for fibrocartilage formation, which affects the quality and mechanical property of the repair tissue, while hypertrophy represents unregulated "maturation" of the chondrocytes along the endochondral ossification pathway as seen in long bone development. The host environment also greatly influences the phenotypes of the transplanted cells and the repaired tissue. Thus, host matrix, growth factor composition, as well as mechanobiological signals all play important roles in phenotype maintenance and production of the new cartilage ECM.

Three-Dimensional Environment A three-dimensional (3D) production of the environment is believed to be critical for the maintenance of the chondrocyte phenotype. Thus, experimentally, 3D cultures such as pellets (Anderer and

Libera 2002) or alginate hydrogel encapsulated cultures (Henrionnet et al. 2012) are commonly used. The current trend of stem cell-based strategies is to deliver chondrogenic inductive factors together with cell seeding or to deliver the chondrogenic inductive growth factors to the local stem cells, in order to guide proper tissue repair. These factors can be directly injected or embedded in the scaffolds; however, due to the short half-lives of bioactive factors (mostly proteins), and their rapid diffusion or clearance from the host environment, high doses and/or repeated administrations are often required. New methods of encapsulation of biomolecules are being developed to achieve spatiotemporally controlled, sustained release of biofactors to recapitulate the cell-cell paracrine signaling responsible for promoting and directing tissue repair (Cleaver and Melton 2003; Werner and Grose 2003). For example, micro- and nanoparticle-based control-release systems can provide protection against drug degradation/inactivation and achieve a localized, sustained release (Tayalia and Mooney 2009; Alvarez et al. 2015).

Bioreactor Technology Advances in bioreactor technology provide another approach to assess the proper microenvironment conditions, including oxygen concentration, medium composition, and mechanical stimulation (Mabvuure et al. 2012), that are required to enhance tissue neogenesis. In particular, mechanical stimulation at an appropriate range and mode could enhance cartilage matrix deposition, e.g., dynamic cyclic loading results in the increase of ECM synthesis in engineered cartilage (Spiller et al. 2011; Saini and Wick 2003) (for details, see Chap. 10).

Gene-Based Delivery of Growth Factors Another developing approach is to achieve growth factor delivery using plasmid or viral gene vectors incorporated in cells or biomaterials. The genetically modified cells could maintain production of the signaling factors transiently or permanently after transplantation (Koria 2012). For example, a cell-mediated gene therapy system for the repair of knee arthritis, in which allogenic chondrocytes express TGF-β1, is in Phase II clinical trial (TissueGene) (Ha et al. 2012). However, in general, the safety of viral vector usage and controlled gene expression requires further investigation (for details, see Chap. 9).

1.5.3 Simplifying Surgical and Rehabilitation Procedures

ACI and MACI both involve two-stage surgeries with inherent complexity. A single-stage point-of-care strategy is thus preferred and ideally involves a single application of growth factors, biomaterial scaffolds, and cells ready to be used at the same time of harvest (Stanish et al. 2013). Such a technique would require "smart" scaffolds that can provide sufficient mechanical support to allow immediate mechanical loading after surgery, in order to create an environment that enhances posttransplantation tissue repair. Growth factors also need to be incorporated efficiently and at

optimized levels. For cells, higher yield harvesting, efficacious sourcing, and optimal chondrogenic capacity are needed.

1.5.4 Prevention of OA Progress

Beside the local repair event in the cartilage defect, the ultimate goal of cartilage repair is to reestablish joint health and prevent OA progress. For this purpose, it is envisioned that an appropriate anti-inflammatory therapy is needed either concurrent with or subsequent to the regenerative procedure, as well as proper mechanical conditions and tissue environment.

1.5.4.1 Establishing Correct Joint Mechanical Environment

Overloading or an abnormal mechanical environment could be harmful to a healthy joint, not to mention the injured tissue. It is noteworthy that ACI/MACI cannot improve joints that have undergone previous or combined meniscectomies or ACL surgeries (Filardo et al. 2013b), and a high body mass index (BMI) had a deleterious effect on the clinical outcome of ACI/MACI (Jaiswal et al. 2012). A stable and healthy mechanical environment is thus the first consideration before joint resurfacing. For example, varus deformities of 5° and more were considered for high tibial osteotomy in patients with cartilage defects (Bode et al. 2013). Another example is that a meniscus transplantation is needed in combination with ACI/MACI in meniscus-deficient knees (Bhosale et al. 2007).

1.5.4.2 Rebuilding Subchondral Bone

Cartilage defects are often accompanied by subchondral bone lesions, such as in osteochondritis dissecans (OCD). Subchondral bone pathology is involved in the progression of OA both biochemically and mechanically (Cox et al. 2011; Pan et al. 2009). In large osteochondral defects, the lack of subchondral bone self-repair can result in the absence of surface cartilage repair (Madry et al. 2010). Subchondral bone remodeling is a primary cofactor to consider during cartilage repair in joint function restoration; for example, some MACI procedures for OCD are performed after bone grafting (Ochs et al. 2011; Vijayan et al. 2012; Filardo et al. 2012). The implantation of biomaterial scaffolds to facilitate subchondral bone regeneration was also found to have an overall beneficial effect on osteochondral regeneration (Jiang et al. 2013).

1.5.4.3 Anti-Inflammatory Actions

The host tissue microenvironment is critical in regulating the biological activities of the transplanted cells. Thus, an inflammatory host environment could compromise cartilage ECM production by the transplanted cells during repair due to the presence and action of pro-inflammatory factors. For example, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are well-known OA mediators, and pro-inflammatory factors are elaborated in the OA joint that inhibit cartilage ECM generation and promote chondrocyte apoptosis (Tetlow et al. 2001). The application of

neutralizing antibodies and inhibitors of pro-inflammatory factors for OA is under active investigation, such as intra-articular injection of TNF-α neutralizing antibody (Urech et al. 2010) or interleukin-1 receptor antagonist (IL-1Ra) (Whitmire et al. 2012). It is believed that these agents can act by altering and neutralizing the pro-inflammatory environment to promote the reparative activities of the transplanted cells/tissue. Interestingly, BMSCs or ADSCs have been clearly shown to exhibit immunoregulatory characteristics (Tuan et al. 2003; Bartholomew et al. 2002; Aggarwal and Pittenger 2005; Bailey et al. 2010), rendering them as promising anti-inflammatory cell candidates for both OA and tissue repair. However, the exact approaches to balance their differentiation ability and immunoregulatory activities and how long the effect will last still require further investigation.

Conclusion

The significant disease burden of OA, which affects up to 15% of the populace, underscores the need for novel and effective therapies. The emerging discipline of tissue engineering and regenerative medicine has expanded the therapeutic vista by offering the potential of "bio-resurfacing" that may restore both structure and function to the diseased joint. To realize the promise of this exciting approach, challenges related to cells (e.g., sourcing, expansion, differentiation, and phenotype stability), scaffold (e.g., biocompatibility, mechanical properties), and chondro-supportive biofactors (e.g., composition, delivery), as well as long-term safety, must be overcome by developing effective and efficacious technologies, which depends critically on active interaction and collaboration among scientists, engineers, and clinicians.

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MSC Populations for Cartilage Regeneration

2

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Abstract

Adult mesenchymal stem cells (MSCs) have an excellent capacity to repair tissues since they can proliferate and differentiate to form various tissues, cartilage included. Moreover, MSCs are potentially accessible in high quantities with low donor site morbidity and reasonable cartilage-forming capacity. In 1998, Johnstone et al. (Exp Cell Res 238(1):265–272) were the first that proposed an effective protocol to chondrogenically differentiate MSCs by using transforming growth factor-β (TGF-β), now used by many groups in the world and since then hardly changed. However, MSCs are a heterogeneous population, and the amount

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and type of cartilage formed are strongly influenced by intra- and inter-donor variation. In this chapter, we mainly focused on surface markers and their modulation by growth factors. We aim to first clarify the characteristics and the embryonic origin of cartilage progenitor cells (chondroprogenitor), then to summarize the characteristics and the contribution to cartilage repair by MSCs from different origins both in vivo and in vitro, and finally, to show a few examples of promoting articular cartilage phenotype by growth factor administration, in relation to the modulation of surface marker expression. With the exception of the next section focused on embryology, our interest was posed specifically on MSCs from human origin.

2.1 Embryonic Origin of Chondroprogenitor Cells (CPCs)

Adult joints contain progenitor cells (Im 2016). Some insight into the origin, fate, and function of such progenitor cells, including those associated with articular cartilage, has been gained through multiple studies utilizing various conditional reporter mice. Whether these adult progenitor cells are derived from interzone cells, the compact layer of mesenchymal cells from which synovial joints and their specialized tissues arise (Holder 1977), remains to be clarified.

Interzone cells emerge at sites previously occupied by chondrocytes (Craig et al. 1987; Nalin et al. 1995). This led to the hypothesis that interzone cells represent dedifferentiated chondrocytes. Sox9 and Dcx lineage-tracking studies using mouse embryos, however, demonstrate that while articular and growth plate chondrocytes arise from a common population of mesenchymal progenitors, articular chondrocytes arise from distinct populations of these mesenchymal progenitor cells (Soeda et al. 2010; Zhang et al. 2011). This hypothesis is supported by the observed migration of joint progenitor cells into the prospective joint site from flanking regions (Hyde et al. 2008; Pacifici et al. 2006; Koyama et al. 2007; Li et al. 2013).

Indian hedgehog null ($Ihh^{-/-}$) mouse embryos lack joints in their skeletal elements, which remain completely cartilaginous (Koyama et al. 2007). In $Ihh^{-/-}$;Gdf5 mouse embryos, in which Gdf5 is selectively expressed by interzone cells, Gdf5-expressing cells were observed at respective joint sites, but not within the future joint site itself; instead they flanked and surrounded the uninterrupted joint site (Koyama et al. 2007; Storm and Kingsley 1996). The absence of a joint in $Ihh^{-/-}$ mouse embryos may arise from the inability of the Gdf5-expressing cells to migrate into the prospective joint area.

Recruitment and immigration of surrounding cells into the interzone are also supported by studies involving the TGF-β type II receptor that is essential for joint formation, especially in the hands/feet (Spagnoli et al. 2007; Li et al. 2013). At E13.5, Tgfbr2-positive cells are present in the dorsal and ventral regions of the joint but completely absent from the central region of the interzone. By E16.5 and postnatally, positive cells are present in various joint tissues, although not in articular cartilage. Tgfbr2-deficient mouse embryos display fusion of digit joints, a

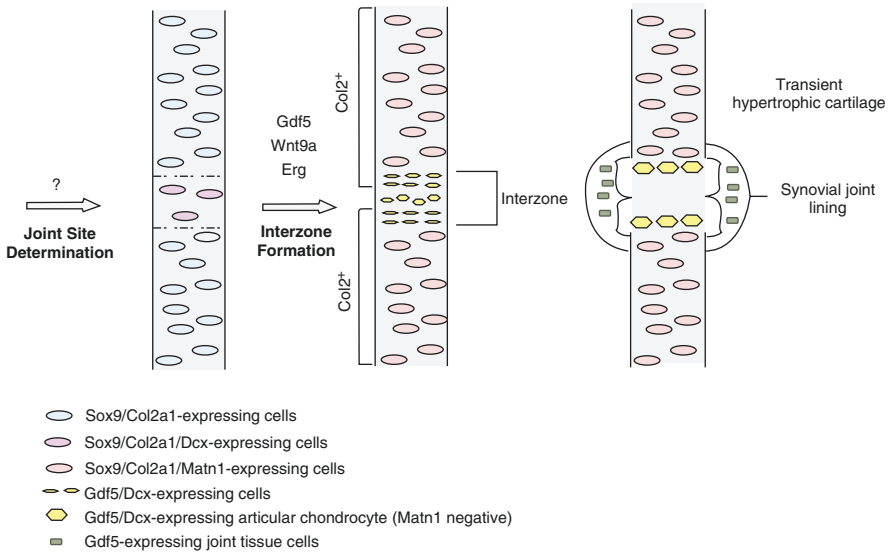

Fig. 2.1 Model of joint development. Unknown upstream mediators determine the location of the future joint site along *Sox9/Col2a1/Dcx*-expressing cells within the uninterrupted cartilaginous template. Soon after, the interzone mesenchymal population is defined and morphologically observable among *Sox9/Col2a1/Matn1*-expressing cells. *Gdf5/Dcx*-expressing cells differentiate to form the articular chondrocytes of the synovial joint. Remaining interzone cells are involved in forming other joint tissues and structures (Adapted from Decker et al. 2014)

phenotype which can be rescued by blocking the MCP-5 receptor CCR2, further supporting the need for low expression of Mcp5 for interzone and joint formation (Longobardi et al. 2012). Moreover, Tgfbr2-expressing cells present in the adult joint are observed, through BrdU-labeling experiments, to constitute slow-cycling stem/progenitor cells (Li et al. 2013).

Thus, along the initial uninterrupted Sox9/Col2/Dcx-expressing cartilaginous anlagen, the future joint site develops, the site itself identified by as yet unknown upstream morphogenetic and determination mechanisms. Soon after, the interzone mesenchymal population is specified through Gdf5 expression with concomitant cell recruitment and maintenance of Dcx expression. Dorsal and ventral flanking cells activate Tgfbr2 expression and articular chondrocytes and then arise from Gdf5/Dcx-expressing cells with a Sox9/Col2a1+/Matn1 history (Fig. 2.1).

2.2 In Vivo Presence of MSCs in Adult Joints

Endogenous stem or progenitor cells contribute to maintenance of healthy tissues by acting as reservoirs of repair cells or as immunomodulatory sentinels to reduce inflammation. In a joint environment, these cell populations exist in tissues adjacent to articular cartilage, including the articular cartilage itself, bone marrow, synovium,

synovial fluid, and infrapatellar fat pad (Im 2016). A great deal has been learnt in recent years about the isolation and characterization of endogenous MSCs from these tissues. The characteristics of endogenous stem or progenitor cell populations from these tissues are discussed below.

2.2.1 Characteristics of MSCs In Vivo

2.2.1.1 Articular Cartilage-Derived Chondroprogenitor cells (CPCs)

Chondrocytes are the most abundant cells within articular cartilage. A small population of these chondrocytes constitutes the chondroprogenitors (Dowthwaite et al. 2004; Khan et al. 2009). CD166 is the most broadly used biomarker for the identification and localization of these progenitor cells in human articular cartilage with high chondrogenic potential (Pretzel et al. 2011; Swart 2002). One study investigated the zonal distribution of $CD166^{+}$ CPCs in articular cartilage, where it was verified that these cells were almost exclusively located in the superficial and middle zones (Pretzel et al. 2011). Notably, 99% of the mesenchymal progenitor cells in that study co-expressed CD166 with another surface marker: the TGF-β co-receptor CD105. Indeed, the superficial zone seems to be of central importance for the growth of articular cartilage in young animals (Hunziker et al. 2007) and presumably relies on a progenitor cell population located in this zone (Hayes et al. 2001). Apart from CD166, also expression of Notch-1 (Dowthwaite et al. 2004) or triple positivity for CD44/CD151/CD49c (Grogan et al. 2007) or CD9/CD90/CD166 (Fickert et al. 2004) has been shown to isolate chondroprogenitors from articular cartilage.

2.2.1.2 Bone Marrow-Derived MSCs (BMSCs)

Bone marrow (BM)-derived MSCs are most extensively studied and best characterized. Several markers were identified that are suitable to isolate MSCs directly from fresh bone marrow samples including antibodies specific for a variety of cell surface molecules, CD49a, CD63, CD73 (SH3/SH4), CD90, CD105 (SH2), CD106, CD140b, CD146, CD200, CD271, CD349, TNAP, Hsp90, GD2, TM4SF1, and NG2 as well as orphan antigens defined by antibodies STRO-1 and 3G5 (Lv et al. 2014; Harichandan et al. 2013; Harichandan and Buhring 2011). In other approaches, BMSCs were enriched using negative selection, by depletion of hematopoietic cells, employing markers such as CD14, CD34, CD45, and/or CD235 (glycophorin A) and other lineage-negative markers (Harichandan and Buhring 2011).

CD271 is the most widely used marker for the characterization and purification of primary human bone marrow MSCs (Alvarez-Viejo et al. 2015). MSCs reside in $CD271^{bright}$ cells, while $CD271^{dim}$ cells comprise $CD45^{+}$ hematopoietic cells (Buhring et al. 2007). More selective markers for MSC isolation than CD271 are SUSD2 (W5C5) and CD140b, as they are expressed on $CD271^{bright}$, but not $CD271^{dim}$ cells (Sivasubramaniyan et al. 2012, 2013). Another well-known marker for BMSCs is Stro-1. But it is unsuitable as a sole marker to separate MSCs from its harboring tissue, as greater than 95% of Stro-1^{+} cells in the human BM are glycophorin A expressing nucleated erythroid cells (Simmons and Torok-Storb 1991). Delorme

et al. (2008) reported CD73, CD130, CD146, CD200, and integrin a5/β5 as markers to enrich colony-forming unit fibroblasts (CFU-Fs) from bone marrow-derived mononuclear cells (MNCs), while other known MSC markers CD49b, CD90, and CD105 showed less enrichment. The neural ganglioside GD2 was introduced by Martinez et al. (2007) as a stand-alone marker to isolate BMSCs. However, in another study, GD2 was found to be expressed only in cultured MSCs and not in primary MSCs (Sivasubramaniyan et al. 2013).

Several groups have reported that BMSCs are heterogeneous with respect to their growth and differentiation potential (Lv et al. 2014). However, little information exists about markers that discriminate between anatomically and functionally distinct MSC subsets. A few studies have defined CD146 and SSEA-3 as markers of perivascular BMSCs while CD56 and CD166 as markers of endosteal BMSCs (Sivasubramaniyan et al. 2012; Tormin et al. 2011). In addition, $CD56^+$ BMSCs give rise to osteoblasts and chondrocytes but not to adipocytes (Battula et al. 2009), while CD56-BMSCs give rise to osteoblasts and adipocytes but poorly to chondrocytes. This suggests that apart from the distinct surface antigen expression profile and differentiation potential, bone-lining and perivascular MSCs may also have distinct, yet to be identified, functional characteristics/properties.

2.2.1.3 Synovium-Derived MSCs (SM-MSCs)

In 2001, De Bari et al. introduced synovium as a source of MSCs that possess chondrogenic potential (De Bari et al. 2001). Recent work has shown subpopulations of MSCs to express different surface markers, such as CD105, CD166, CD90, CD9, and CD271 (Chang et al. 2013; Ogata et al. 2015; Van Landuyt et al. 2010). Chang et al. (2013) reported that CD105- and CD166-enriched cells derived from human synovium may be valuable sources for cartilage regeneration due to their enhanced chondrogenic potential. The most widely used BMSC marker CD271 is shown to be present in a specific subpopulation of inflamed synovium but not at all or barely present in expanded cell populations from the same patients (Van Landuyt et al. 2010; Ogata et al. 2015). In another study, Van Landuyt et al. (2010) reported the detection of CD34 expression on a subpopulation of $CD271^+$ MSCs, but the chondrogenic differentiation ability of this population is not known. So far, CD90 is the most commonly used marker for isolation of SM-MSCs. It has been used in combination with CD9/CD166 or CD271 to isolate a subpopulation of highly chondrogenic SM-MSCs (Ogata et al. 2015; Fickert et al. 2003). But these studies did not characterize the chondrogenic ability of all the MSC subpopulations from synovium, thus questioning the reliability of CD90 or CD271 in isolating highly chondrogenic SM-MSCs.

2.2.1.4 Synovial Fluid-Derived MSCs (SF-MSCs)

SF-MSCs form a pool of highly clonogenic cells with chondrogenic potential and are present both in healthy and OA joints (de Sousa et al. 2014; Jones et al. 2008). The exact origin of SF-MSCs is unclear. Morito et al. showed that they originate neither from BM nor from circulating MSCs but probably from the synovium or cartilage (Morito et al. 2008; Lee et al. 2012). The phenotype of freshly isolated human SF-MSCs is not yet known.

2.2.1.5 Infrapatellar Fat Pad-Derived MSCs (IF-MSCs)

Subcutaneous human adipose tissue is an interesting source of multipotent progenitors (Zuk et al. 2002). In recent days, also human infrapatellar fat pad is newly gathering attention as a source of MSCs (Khan et al. 2012). Jurgens et al. (2009) characterized stem cells in the freshly isolated stromal fraction based on the presence of the early marker CD34 and the absence of the endothelial marker CD31. These $CD34^{+}CD31^{-}$ cells were characterized further and shown to be additionally positive for the stem cell-associated markers CD29, CD54, CD90, CD105, and CD166. Others have also characterized the phenotype of IF-MSCs but only looked into culture-passaged cells. Thereby, certain markers such as CD34 were found to be negative, whereas in fact they are present on the surface of freshly isolated cells and lost upon culturing (Wickham et al. 2003). In another study, a small subpopulation of human IF-MSCs has been shown to express the pericyte marker 3G5, and anatomically these cells are localized to the perivascular region (Khan et al. 2008). In a recent study, CD44 was used to isolate IF-MSCs, and these freshly isolated IF-MSCs have been shown to have enhanced chondrogenic potential in vivo when seeded on a cartilage-extracellular matrix-derived scaffold (Almeida et al. 2015). These findings indicate that infrapatellar fat pad may be a promising cell source for endogenous MSCs.

2.2.2 Contribution to Repair by MSCs In Vivo

Until now, there is no clear blueprint of the host cell sources that participate in in situ cartilage regeneration. But more new knowledge is being gained in this field starting from the work of Hunziker and Rosenberg (1996) where for the first time a combination of fibrin, cells, and growth factors was used to repair a cartilage defect. For instance, Lee et al. (2010) showed that the articular surface of the synovial joint can be regenerated with TGF-β3 infused in a bioscaffold composite of poly-ε-caprolactone and hydroxyapatite. Though the source of endogenous stem cells bringing about the repair is not known, they speculate that the endogenous cells are derived from stem or progenitor cells of synovium, bone marrow, infrapatellar fat pad, and perhaps vasculature.

First approaches to heal cartilage by in situ regeneration date back as early as 1959 (Muller and Kohn 1999). The Pridie technique was directed at recruitment of BMSCs to cartilage defects by drilling small holes into the subchondral bone that underlies regions of damaged cartilage. It was refined later on, by reducing the size of the perforations, and was called microfracture technique which is now a frequently performed and well-studied procedure (Richter 2009; Steadman et al. 2001). While microfracture is restricted to lesions of 1–4 cm^2 size, autologous matrix scaffolds that induce chondrogenesis were developed and used to treat larger defects in combination with microfracture (Kramer et al. 2006) (for details see Chap. 5). Development of scaffolds with different materials and biophysical properties containing bioactive factors aiming to attract endogenous MSCs, especially BMSCs, is a growing field of research. In a study, Koelling et al. (2009) found some

evidence that chondrogenic cells migrate from the bone marrow to the cartilage defect through breaks in the tidemark. Hence, for full-thickness defects, the contribution of BMSCs is most likely, but for partial thickness defects, where direct access of BMSCs to the joint cavity is restricted, MSCs from synovium (Hunziker and Rosenberg 1996) and joint fat pads could be involved.

Cells derived from human synovium were shown to have the highest chondrogenic potential among the various mesenchymal tissue-derived cells, indicating a possible source for cartilage repair (Sakaguchi et al. 2005). Nishimura et al. (1999) showed that explants of synovium embedded in agarose undergo chondrogenesis when cultured in the presence of TGF-β1. The data indicate a possible synovial origin for the chondrocytic cells. Furthermore, these data are consistent with the clinical findings of synovial chondrogenesis leading to synovial chondromatosis (Nishimura et al. 1999). It was recently demonstrated by Kurth et al. (2011) that there is an in vivo presence of slow-cycling SMSCs in synovium of murine knee joints. Through iododeoxyuridine labeling and surface staining, these cells were shown to increase in number after cartilage injury, showing their activation in response to cartilage damage. Hence, homing of stem cells from synovium to sites of cartilage injury is an appealing concept that warrants further investigation.

Cartilage-derived progenitors have been observed in human, equine, and bovine articular cartilage (Jiang and Tuan 2015) and have been identified as slow-cycling cells by pulse-chase experiments in a murine model (Candela et al. 2014). In a recent study, Tong et al. (2015) confirmed the existence of CPCs in situ for the first time in murine model, both in normal and OA articular cartilage, and showed that these CPCs were activated from resting state in OA. Regarding the ability of articular cartilage to self-repair, they speculate that this ability probably depends on the presence of CPCs, as the number of transient-proliferating CPCs is synchronous with the OA progression in the early stage.

In the infrapatellar fat pad, MSCs are localized in the perivascular region, and hence, they can infiltrate into the synovial fluid through the synovium and participate in cartilage repair. The direct involvement of IF-MSCs in cartilage repair in situ is yet to be explored.

MSCs are present in the SF from knee joints of healthy individuals as well as from individuals with OA, RA, ligament injury, and meniscal injury (Jones et al. 2004, 2008; Matsukura et al. 2014; Morito et al. 2008). Diseased or injured joint SF has greater MSC numbers than healthy joint SF (Jones et al. 2004; Matsukura et al. 2014; Morito et al. 2008), with total number positively correlating with the period of injury (Morito et al. 2008) or severity of OA (Jones et al. 2004; Lee et al. 2012; Sekiya et al. 2012). These SF-MSCs are possibly derived from dislodged synovial fragments. This is particularly noteworthy since synovial stem cells have been proposed to contribute to spontaneous cartilage repair (Hunziker and Rosenberg 1996; Kurth et al. 2011), and synovial fluid could therefore be viewed as a possible conduit for their passage (Jones et al. 2008). Taken together, these findings provide a platform to explore the role of SF-MSCs in superficial cartilage homeostasis and repair.

2.3 MSCs In Vitro

It has been proposed that the best markers for identification of progenitor cells may be different between freshly isolated and culture-expanded cells. The first definitive markers of MSCs in vitro were proposed in the pioneering study of Pittenger et al. (1999) and included CD105 (SH2) and CD73 (SH3). These two markers alongside CD90 remain the primary molecules used to identify MSCs by the International Society for Cellular Therapy (ISCT) which also advices that MSCs should be negative for the expression of CD11b or CD14, CD19 or CD79a, CD34, CD45, and HLA-DR (Dominici et al. 2006). This is primarily to allow the exclusion of hematopoietic cells which may contaminate MSC cultures.

Cultured MSCs, however, are uniformly and strongly positive/negative for many markers regardless of their passage or time in culture. That many markers are expressed at similar levels in early- and late-passage MSCs indicates their value may be limited to basic MSC characterization only. The following is a concise review of in vitro markers of MSCs from different tissue sources (Table 2.1) and their correlation with chondrogenic potential.

2.3.1 Articular Cartilage-Derived MSCs (AC-MSCs)

Barbero et al. (2003) were the first to describe the existence of a subpopulation of cells within the dedifferentiated adult human articular chondrocyte population that, at clonal level, possess properties of MSCs, including tri-lineage differentiation potential. Hoechst 33342 dye uptake (Grogan et al. 2009) and adhesion to fibronectin (Williams et al. 2010) are utilized in an attempt to isolate this population of cells from the chondrocyte population. Equally, these cells can be isolated by their ability to migrate out of explants in vitro, although only from damaged cartilage (Koelling et al. 2009), in which they are present in higher numbers (Alsalameh et al. 2004).

AC-MSCs migrate from diseased cartilage in response to nerve growth factor (NGF) (Jiang et al. 2015) raising the possibility that these NGF-responsive cells are native $CD271^{+}$ cells, as CD271 expression is not expressed during in vitro expansion (Jiang et al. 2015; Koelling et al. 2009). In contrast, almost all clonal cells obtained by adhesion to fibronectin (Williams et al. 2010) and that have high chondrogenic capacity express CD49e (Grogan et al. 2007). Clonal cells with high chondrogenic capacity also express CD49c, CD49f, CD166, CD44, CD90, and CD151 higher than clonal cells with low chondrogenic capacity (Grogan et al. 2007); singly sorted $CD49c^{Bright}$ and $CD44^{Bright}$ cells have greater chondrogenic capacity than unsorted chondrocyte populations (Grogan et al. 2007).

Notch-1, Stro-1, and CD106 are highly expressed on clonal cells compared to articular chondrocytes (Ustunel et al. 2008; Williams et al. 2010); however, they are too widely expressed on mature chondrocytes in vivo to be useful (Grogan et al. 2009). Similarly, despite a CD105/CD166-enriched adherent population having chondrogenic capacity comparable to BMSCs, these markers are also expressed in vivo (Alsalameh et al. 2004). The combination of CD9/CD90/CD166 to obtain cells with chondrogenic differentiation capacity after culture may prove useful; however, further investigation is warranted (Fickert et al. 2004).

Table 2.1 Summary of the markers expressed by human MSCs in vitro

Marker	Percentage of positive cells and reference
B2microglobulin	BM: +++ [7]
BMPR1A	SF: – [16]
CXCR4	SF: – [16] FP: – [1]
D7-FIB	BM: +++ [7–8] SM: ++ [12] SF: ++ [16] FP: +++ [2]
FLK-1	BM: – [15] ± [9] SM: ± [9] SF: – [16]
Glycophorin A	BM: – [7] SM: – [11]
HLA-I	BM: +++ [7]
HLA-II	BM: – [7]
HLA-DR	BM: – [4] SM: – [11] SF: – [4] FP: ± [3–4]
KDR	SM: – [15] FP: – [1]
STRO-1	AC: ± [19] BM: – to + [15], ± to +++ [9] SM: – [11], – to + [15], ± to +++ [9] SF: + to ++ [16]
TGFβR11	BM:– [8] FP: – [2]
CD3	BM: – [7]
CD9	BM: +++ [7] FP: + [3]
CD10	BM: – to + [15], ± to +++ [9] SM: – [15], +/1 to ++ [9], ± to + [11], + to ++ [15] SF: – to + [16] FP: [3]
CD11a	SM: – [11] FP: – [3]
CD11b	BM: – [7] FP: – [3]
CD11c	FP: – [3]
CD13	BM: ± to + [7], +++ [4,8] SM: + to +++ [11], ++ to +++ [12] SF: +++ [4] FP: +++ [2,5]
CD14	BM: – [7] SM: – [11] FP: – [3]

(continued)

Table 2.1 (continued)

Marker	Percentage of positive cells and reference
CD15	BM: − [7]
CD16	BM: − [7]
CD18	BM: − [7] FP: − [3]
CD19	BM: − [7]
CD28	BM: − [7]
CD29	BM: +++ [7] SM: ++ to +++ [15] FP: ++ [3,5]
CD31	BM: −[7,10], − to ± [4,9] SM: − [11], − to ± [9] SF: − [4,16] FP: − [3,4], ± [5]
CD34	BM: − [4, 6–10], ± [9] SM: − [13], − to ± [9], − to + [11] SF: − [16,18], − to ± [4], + [17] FP: − [2], ± [1,3,4,6], + [5]
CD36	BM: − [4,7] SF: − [4] FP: ± [4]
CD38	BM: − [7]
CD44	BM: +++ [6,7,8,9,10] SM: ++ [10,14], ++ to +++ [11,15], +++ [9, 13] SF: ++ to +++ [16,18], +++ [4, 17] FP: +++ [2,3,4,5,6]
CD45	BM: − [6,7,8,9,10] SM: − [10,11,13], − to + [12], ± [9] SF: − [16,18] FP: − [1,2,3,5]. ± [6]
CD49a	BM: ++ to +++ [7] SM: − to ++ [15], ± to + [11]
CD49b	BM: ± to + [7]
CD49c	BM: ++ to +++ [7], +++ [4] SF: ++ to +++ [4] FP: +++ [4]
CD49d	BM: + to ++ [4] SM: − [11] SF: ++ [4] FP: +++ [4]
CD49e	BM: ++ to +++ [7] FP: ++ [3]
CD49f	BM: − [7], + [4] SF: + [4] FP: + [4]
CD50	BM: − [7] FP: ± [3]

Table 2.1 (continued)

Marker	Percentage of positive cells and reference
CD51	BM: +++ [7]
CD54	BM: ± to ++ [7], ± to +++ [9], + [4,10] SM: ± to +++ [9], + [15] SF: + [4], ++ [16] FP: ± [3], + to ++ [4]
CD55	BM: +++ [7] SM: − [11]
CD56	BM: − [7], ± [6] SM: − [11] FP: −[6], ± [3]
CD58	BM: ++ to +++ [7]
CD59	BM: +++ [4,7] SF: +++ [4] FP: +++ [3,4]
CD61	BM: − [7]
CD62	SM: − [11]
CD62e	BM: − [7] FP: + [3]
CD62L	BM: − [7]
CD62P	BM: − [7]
CD68	SM: − [11]
CD71	BM: − [7], + to ++ [4] SF: ± [4] FP: [4]
CD73	BM: +++ [4,8] SM: + to ++ [11,13], ++ to +++ [12,14,15] SF: ++ to +++ [18], +++ [4] FP +++ [1,2,4]
CD90	AC: +++ [19] BM: +++ [4,6,7,9,10] SM: ++ [10,13,14,15], +++ [9] SF: + to ++ [16], ++ [18] FP: +++ [1,4,5,6]
CD95	BM: − to ± [7] SF: +++ [4,17,18]
CD102	BM: − [7]
CD104	BM: − [7]
CD105	AC: ++ [19] BM: ++ to +++ [7,10], +++ [4,6,8,9] SM: + to +++ [13], ++ [12], ++ to +++ [10,14,15], +++ [9,11] SF: − to + [18], + to ++ [16], +++ [4] FP: ++ [5], +++ [1,2,3,4,6]
CD106	BM: − to + [15], ± to +++ [9], ++ [4], ++ to +++ [7] SM: − [11], − to + [10, 12,15], ± to +++ [9], + [13] SF: − [16], − to ± [4] FP: ± [4], + [2,3,5]

(continued)

Table 2.1 (continued)

Marker	Percentage of positive cells and reference
CD117	BM: − [7,8,10], − to ± [4,9] SM: − [10,11], − to ± [9] SF: − [4,16] FP: − to ± [4]
CD133	BM: − [7,8] SM: − [11] FP: − [1,2]
CD133/1	BM: − [4] SF: − [4] FP: − [4]
CD140b	BM: +++ [4] SF: +++ [4] FP: +++ [4]
CD146	BM: ++ [8] SM: − to + [12] FP: + [2]
CD147	BM: ++ to +++ [15] SM: ++ to +++ [15], +++ [9] SF: ++ [16]
CD151	BM: +++ [4,8] SM: ++ to +++ [14] SF: +++ [4] FP: ++ [5], +++ [2,4]
CD166	AC: ++ [19] BM: ± to + [7], ± to +++ [9], ++ [15], +++ [4,8] SM: ± [11], ± to +++ [9], + to ++ [10,13], ++ to +++ [12,15], ++ [14] SF: − to + [16], +++ [4] FP: ++ [3,5], +++ [2,4]
CD271	BM: − [8], − to ± [4,7,9,10], + [15] SM: − to ± [9], − to + [10,12], + [13] SF: − [4,16] FP: − to ± [4], + [2]

Percentage of positive cells: (+++) = 80–100%, (++) = 40–80%, (+) = 15–40%, (±) = 5–15%, (−) = 0–5%. When the authors reported different percentage of expression following the MSC expansion in vitro, a range of expression is indicated in the table. Due to the large number of literature available, for some of the listed markers only a representative selection of the literature is reported. References (see the reference paragraph for more details): [1] Lopez-Ruiz E. et al., *Osteoarthritis and Cartilage* (2013), [2] Jones E.A. et al., *Arthritis Rheum* (2004) and *Arthritis Rheum* (2008), [3] Wickham M.Q. et al., *Clin Orthop Relat Res* (2003), [4] Alegre-Aguaron E. et al., *Cells Tissues Organs* (2012), [5] Lopa S. et al., *Eur Cell Mater* (2014), [6] Ding D.C. et al., *Cell Transplant* (2015), [7] de la Fuente R. et al., *Exp Cell Res* (2004), [8] English A. et al., *Rheumatology* (2007), [9] Shirasawa S. et al., *J Cell Biochem* (2006), [10] Sakaguchi Y. et al., *Arthritis Rheum* (2005), [11] Jo C.H. et al., *Cytotherapy* (2007), [12] Karystinou A. et al., *Rheumatology* (2009), [13] Arufe M.C. et al., *J Cell Biochem* (2010), [14] Jones E. et al., *Ann Rheum Dis* (2010), [15] Han H.S. et al., *J Orthop Res* (2014), [16] Morito T. et al., *Rheumatology* (2008), [17] Lee D.H. et al., *Osteoarthritis and Cartilage* (2012), [18] Matsukura Y. et al., *Clin Orthop Relat Res* (2014), [19] Ozbey O. et al., *Acta Histochem* (2014).
AC articular cartilage-derived MSCs, *BM* bone marrow-derived MSCs, *SM* synovium membrane-derived MSCs, *SF* synovial fluid-derived MSCs, *FP* infrapatellar fat pad-derived MSCs

2.3.2 Bone Marrow-Derived MSCs (BMSCs)

The studies of Johnstone and Pittenger were the first to demonstrate and characterize individual clonal BMSCs and their chondrogenic or multi-lineage potential (Johnstone et al. 1998; Pittenger et al. 1999), establishing that not all clonal cells are capable of chondrogenesis. Colony cells were uniformly positive for CD105 (SH2), CD73 (SH3), CD29, CD44, CD71, CD90, CD106, CD120a, and CD124 and negative for CD14, CD34, and CD45, similar to their parent culture. From the beginning, therefore, these surface markers appeared not suitable for exclusive identification of BMSCs with high chondrogenic potential. Indeed, many of the surface markers characterized for BMSCs are acquired during culture and remain consistently expressed (Alegre-Aguaron et al. 2012). This is in contrast to the chondrogenic potential of these cells, which decreases with culture (Banfi et al. 2000; Bonab et al. 2006). Furthermore, CD105-sorted populations have been shown to possess similar chondrogenic potential and are comparable to the total, unsorted populations (Majumdar et al. 2000; Cleary et al. 2016).

Conversely, other markers, such as CD106, have been identified which appear more sensitive to culture conditions, including passage number, with declining expression in later passages (Fukiage et al. 2008). Moreover, CD106 may be a marker of MSC differentiation potential as it is strongly downregulated after chondrogenic differentiation (Gronthos et al. 2003). CD271 expression, present on a small percentage of freshly isolated BMSCs, also decreases with culture. However, this decrease occurs almost immediately during the first passage (Jones et al. 2006; Quirici et al. 2002; Mifune et al. 2013). Both $CD271^+$ and $CD271^-$ BMSCs, though, are capable of healing chondral defects in vivo, although $CD271^-$ repair ECM is less rich in proteoglycans (Hermida-Gomez et al. 2011; Mifune et al. 2013). Similarly, $CD146^+$ MSCs derived from bone marrow have higher CFU-F ability and proteoglycan content when compared to the total or $CD146^-$ population (Hagmann et al. 2014; Sacchetti et al. 2007; Kaltz et al. 2010). These observations require further investigation, however, as no difference in CFU-F potential and differentiation potential has been reported (Espagnolle et al. 2014).

2.3.3 Synovium-Derived MSCs (SM-MSCs)

Although similar to MSCs derived from other sources, synovium-derived MSCs often seem to possess superior chondrogenic potential and higher CFU-F numbers (Jo et al. 2007; Sakaguchi et al. 2005; Shirasawa et al. 2006). In vitro, chondrogenically differentiated synovium-derived MSCs acquire the expression of markers associated with the stable cartilage phenotype of articular cartilage-derived cells. This phenotype, however, is only transient, and chondrogenically differentiated SM-MSCs fail to form stable cartilage in vivo (De Bari et al. 2004).

Surface marker expression is characteristic of MSCs and is relatively stable during culture, with the exception of the first passage; CD14, CD34, CD45, and HLA-DR disappear, CD105 and CD166 appear, and CD10, CD13, CD44, CD49a,

and CD73 increase. Thereafter, only mild fluctuations in expression occur (Jo et al. 2007; Nagase et al. 2008). A $CD9^+/CD90^+/CD166^+$ population of synovium-derived cells, the total frequency of which increases with culture, represents an osteochondral progenitor population. There is, however, no difference between this and the unsorted population (Fickert et al. 2003). The $CD34^-/CD44^+/CD90^+$ subpopulation too possesses multipotent differentiation potential (Lee et al. 2012).

CD44 and CD90 expression alone correlates positively with chondrogenic potential (Jo et al. 2007; Jones et al. 2010), with CD90 expression correlating with pellet weight (Jo et al. 2007). CD90 is also expressed on >80% of $CD271^+$ MSCs from synovium (20–30% of the total population) which, along with $CD73^+$ cells, have better chondrogenic potential (Arufe et al. 2010; Harvanova et al. 2011). Indeed, $CD73^+$ cells also displayed the best osteogenic phenotype indicating that this surface protein is likely a marker of osteochondral cells. $CD105^+$ subpopulations also show chondrogenic potential, with expression also on >80% of $CD271^+$ cells (Arufe et al. 2009, 2010). A population of $CD271^+/CD90^+$ in combination with CD73, CD44, or CD105, therefore, may prove the most chondrogenic. Moreover, $CD14^+$ cells dampen the chondrogenic capacity of SM-MSCs. Depletion of this subpopulation is, therefore, likely to improve the chondrogenic potential of the starting population (Han et al. 2014).

2.3.4 Synovial Fluid-Derived MSCs (SF-MSCs)

Several studies have demonstrated no difference in chondrogenic potential for MSCs derived from SF, BM, and SM (Kurose et al. 2010; Lee et al. 2012). Indeed, SF-MSCs are assumed to be derived from either BM or SM, with current evidence weighing in the favor of an SM origin (Jones et al. 2004; Matsukura et al. 2014; Morito et al. 2008; Sekiya et al. 2012). However, others indicate that differences in chondrogenic potential may exist between SF-derived MSCs and MSCs derived from other sources, including having weaker chondrogenic potential than BM- or IF-derived MSCs (Alegre-Aguaron et al. 2012).

Akin to MSCs derived from other tissue sources, a proportion of cultured SF-MSCs can be $CD271^+$ (Jones et al. 2004), although at levels lower than MSCs from BM (Alegre-Aguaron et al. 2012; Jones et al. 2004). Similarly, cultured SF-MSCs express different levels of CD117, CD106, CD71, CD54, CD49c, HLA-DR, CD34, CD166, and CD133/1 to BMSCs (Alegre-Aguaron et al. 2012); SF-MSCs increase their expression of CD34 and CD49d, while decreasing their expression of CD71, CD106, and CD271 (Alegre-Aguaron et al. 2012).

While no difference is observed in the ability of $CD90^+$ and $CD90^-$ SF-MSCs to undergo osteogenesis or adipogenesis, greater chondrogenic potential exists in the $CD90^+$ fraction. This potential, however, is not superior to that of the total, unsorted population (Krawetz et al. 2012). $CD105^+$ SF-MSCs are also capable of tri-lineage differentiation (Harvanova et al. 2011), but further studies are required to understand whether this fraction is superior to the total or negative fraction.

2.3.5 Infrapatellar Fat Pad-Derived MSCs (IF-MSCs)

The presence of MSC-like cells within a cellular population derived from the infrapatellar fat pad (IPFP) was first described by Wickham et al. (2003). Such cells can be isolated in higher quantities from the IPFP than from other tissue sources (Khan et al. 2007) and are phenotypically comparable to MSCs, including their differentiation and CFU-F ability (English et al. 2007). IF-MSCs, however, have been described to maintain their chondrogenic differentiation capacity longer in culture than MSCs derived from other tissues (English et al. 2007). Moreover, several studies have identified higher chondrogenic capacity in this MSC source (Ding et al. 2015; English et al. 2007; Khan et al. 2008; Lopa et al. 2014). IF-MSCs seem to lack age-related declines in proliferative and differentiation potential (Khan et al. 2008), and similar to other MSC populations, when expanded in the presence of fibroblast growth factor-2 (FGF2), proliferation and chondrogenic potential is enhanced (Khan et al. 2008).

Cell surface marker expression of these cells indicates a profile similar, although not identical, to other MSC populations; IF-MSCs express relatively higher levels of CD34, CD45 (Ding et al. 2015), CD271 (English et al. 2007), and CD106 (Mochizuki et al. 2006; Lopa et al. 2014), with lower expression of CD146 (English et al. 2007) and CD10 (Mochizuki et al. 2006). The maintenance of CD271 expression on a subpopulation of cultured IF-MSCs raises the possibility that in vivo, MSCs present in the infrapatellar fat pad also express CD271 (English et al. 2007). This observation remains unclear, however, as CD271, CD45, and CD34 expression on cultured IF-MSCs is often described as negative (Khan et al. 2007, 2012; English et al. 2007). 3G5 is also observed on a subpopulation of these cultured IF-MSCs (Khan et al. 2007, 2008, 2012) showing that pericytes are present in the infrapatellar fat pad.

2.4 Manipulation of Joint Stem Cell Phenotype In Vitro by Growth Factors

Growth factors are often used as media supplement for MSC expansion. The first growth factor tested on human MSCs was FGF2 in 1997 (Martin et al. 1997), and few years later, its role in enhancing chondrogenic capacity was established (Mastrogiacomo et al. 2001; Tsutsumi et al. 2001). Although the capacity of FGF2 to improve proliferation and chondrogenic capacity of MSCs is evident and acknowledged by the scientific community, very little is known about the role of FGF2 and other growth factors in influencing surface marker expression.

Addition of FGF2 decreases the expression of CD146 and alkaline phosphatase (ALP) on BMSCs, and the effect seems reversible after removal of the growth factor, possibly indicating a direct effect of FGF2 on CD146 expression. The effect of FGF2 on CD146 and ALP expression was observed on both the whole population of BMSCs (Gharibi and Hughes 2012; Hagmann et al. 2013b) and on the CD146+ subpopulation (Sacchetti et al. 2007). The group of Paolo Bianco further studied the

effect of FGF2 and other growth factors on preselected CD146+ BMSCs, observing that FGF2 and platelet-derived growth factor-BB (PDGF-BB) also reduce the expression of CD105 and CD49a, and TGF-β enhances CD63 and α-SMA expression (Sacchetti et al. 2007). Epidermal growth factor (EGF) slightly increases ALP expression, and WNT3a treatment did not significantly influence cell surface marker expression (Gharibi and Hughes 2012). Surprisingly, none of those studies related the effect of growth factors on surface marker expression with the chondrogenic capacity of the cells. This was evaluated by Hagmann et al. (2013a) in a study comparing the use of different expansion media on BMSCs. However, the media used were supplemented with several different factors, and therefore, it is not possible to conclude on the effect of a single growth factor on surface marker expression or chondrogenesis. Only more recently, we observed that the expansion of BMSCs in the presence of WNT3a influenced the chondrogenic capacity of the cells and also their surface marker expression (Narcisi et al. 2015). However, the only direct effect observed was an enhanced number of CD271+ cells found in the cells treated with WNT3a. All the other markers tested were either unchanged by the treatment (CD73 and CD146) or maintained longer during the expansion in vitro (CD90, CD105, CD166), but they were not regulated directly. The ability of the BMSCs treated with WNT3a to enhance or retain the expression of certain surface markers in culture was linked by the authors to the capacity of the WNT3a-stimulated BMSCs to maintain, over multiple passages in vitro, a robust chondrogenic capacity.

Considering the other cell sources, to our knowledge only one article underlined the effect of growth factors on surface marker expression, demonstrating that FGF2 does not alter the expression of CD13, CD29, CD44, CD90, CD105, 3G5, STRO-1, CD34, and CD56 in IF-MSCs (Khan et al. 2008). However, again, no correlation with the chondrogenic capacity was reported.

Conclusion

In this chapter we provided an overview of the relation between surface marker expression and chondrogenic capacity of human adult MSCs from different sources. Since a large number of studies are available, in particular for BMSCs, we had to make a selection of the literature. However, two clear messages should be evident: (1) surface marker expression of cells in vivo strongly depends on tissue and cell localization, and (2) in vitro surface marker expression of expanded MSCs with high chondrogenic capacity is different between MSCs isolated from different sources. Several attempts to isolate, characterize, and purify populations of MSCs either directly from the tissue or after expansion in culture have not led to a clear description of MSCs with enhanced chondrogenic capacity. Moreover, especially for the culture-expanded MSCs, the use of different isolation techniques or expansion media could strongly influence the outcome. It is also interesting to note that MSCs derived from cartilage (CPCs), infrapatellar fat pad (IF-MSCs), and synovium (SD-MSCs) are still poorly characterized compared to BMSCs, and, moreover, only a very limited number of studies have directly compared the chondrogenic capacity of selected populations from different sources. Despite the use of surface markers for cell selection, the resulting

populations are still heterogeneous, which might explain the lack of success in finding the most suitable marker to select the best chondrogenic population of cells directly from the native tissue or after expansion in vitro. Therefore, researchers are continuously looking for new surface markers or new combinations of markers. However, we are convinced that additional efforts in exploring the use of alternative systems to purify or select chondrogenic cells with methods not necessarily based on surface marker expression might be required.

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Chondrogenic Progenitor Cells and Cartilage Repair

3

Diana Oellerich and Nicolai Miosge

Abstract

Osteoarthritis and rheumatoid arthritis are the two most common diseases of joints causing cartilage and bone destruction leading to the loss of joint function. Causal therapies are still challenging, and to date various cell biological methods applying chondrocytes or mesenchymal stem cells only generate fibrocartilaginous repair tissue with less good mechano-biological properties.

In osteoarthritis and rheumatoid arthritis, a specific cell type with stem cell characteristics including migratory activity, clonogenicity, and multipotency could be characterized, which we named "chondrogenic progenitor cells." These cells, involved in regeneration processes, are largely unsuccessful mainly because they produce collagen type I. Manipulation of these progenitor cells, which are already present in diseased cartilage tissue, could be a promising approach for cartilage repair. Several chondrogenic pathways and interacting partners have been already identified. The transcription factors Runx2 and Sox9 play an important role by influencing the collagen II production. Interleukins and TGF-β might also play an important role in the regulation of Runx2. Furthermore, it could be shown that various other factors like mechanical stimulation or components of the pericellular matrix prompt chondrogenic progenitor cells to trigger chondrogenesis. The differentiation potential of chondrogenic progenitor cells seems to be affected by calcium homeostasis, including calcium regulatory mechanisms. However, several challenges remain regarding the elucidation of the regulatory pathways that determine chondrogenic progenitor cells to become more chondrogenic.

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3.1 Osteoarthritis and Rheumatoid Arthritis

Osteoarthritis (Fig. 3.1) is a whole-organ disease, also affecting the synovium, subchondral bone, and meniscus (Goldring and Otero 2011; Englund et al. 2012; Goldring and Goldring 2010). As a degenerative joint disease, it shows a progressive loss of the articular cartilage (Loeser et al. 2012). The synovium is responsible for producing inflammation mediators, which can also be found in osteoarthritis (Bougault et al. 2012). Modified cell-matrix interactions result in destroyed tissue integrity and primarily affect the hyaline cartilage. However, this also applies to other tissues, especially the subchondral bone. An eburnation of the subchondral bone can arise (Goldring and Otero 2011; Goldring and Goldring 2010). An abnormal remodeling of the subchondral bone often leads to a thicker, but mechanically less stable, tissue (Lories and Luyten 2011). Osteoarthritis often originates from

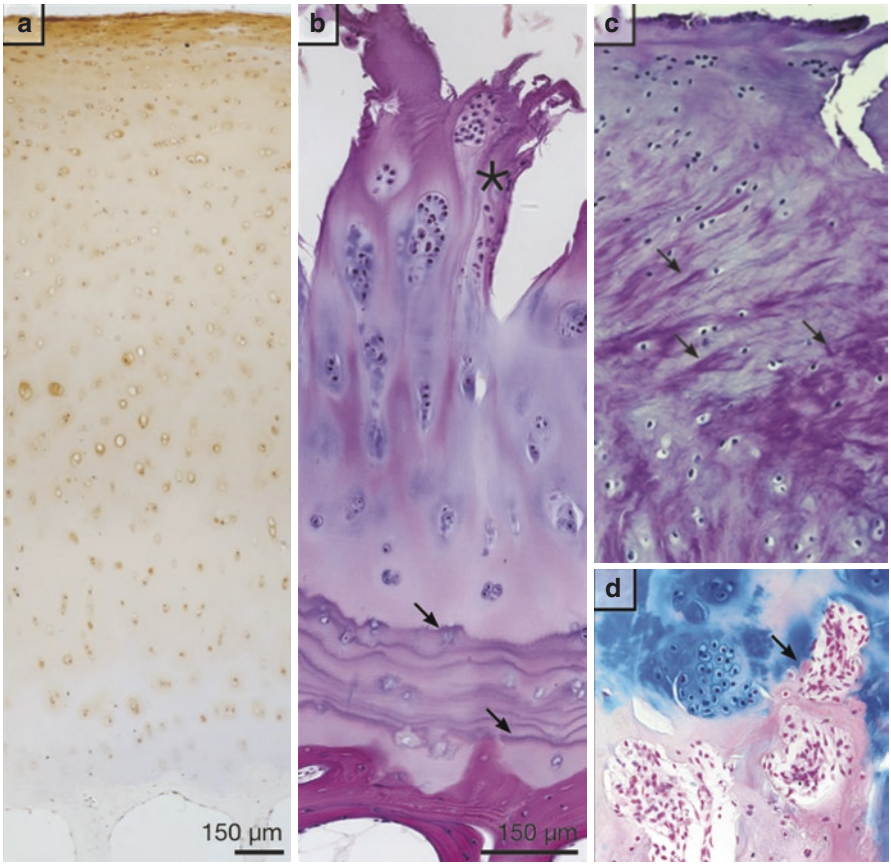

Fig. 3.1 (**a**) Immunohistochemistry for biglycan in healthy human articular cartilage of the knee joint. (**b**) Histology of late-stage OA, please note the numerous tidemark duplications and the deep surface fissures. (**c**) Fibrocartilaginous repair tissue, where the chondrogenic progenitor cells are found. (**d**) Breaks in the tidemark with mesenchymal tissue entering the cartilage tissue

meniscal lesions (Englund et al. 2012). Even when meniscal substitutes, such as allografts or bioengineered substitutes, are used, there is no protective effect against the development of osteoarthritis (Hommen et al. 2007). No markers have been discovered to diagnose early stages of osteoarthritis. At present, no therapy to treat the causes of osteoarthritis is available that could promise a complete cure (Lohmander and Roos 2007; Lohmander et al. 2014; Musumeci et al. 2014). In the late stages of refractory osteoarthritis, knee replacement is the gold standard of treatment (Johnson and Hunter 2014).

Various cellular mechanisms cause rheumatoid arthritis and result in cartilage and bone destruction. Inflammation plays an important role in the disease process. The inflamed synovium produces a tumor-like pannus tissue, which destroys the cartilage tissue. Proinflammatory cytokines, such as IL-1β, IL-6, TNF-α, and matrix metalloproteinases, are produced by fibroblast-like synoviocytes and synovial macrophages (Karouzakis et al. 2006). The RANKL-dependent induction of osteoclasts is primarily responsible for bone destruction (Kim et al. 2014). In the synovium in rheumatoid arthritis, CD4+ T cells accumulate (Franz et al. 1998; Blaschke et al. 2003; Toh and Miossec 2007).

3.2 Regeneration Attempts in Cartilage Tissue

Chondrocytes are the only cell source found in healthy articular cartilage (Muir 1995; Kock et al. 2012). These produce collagen type II, which together with aggrecan is mainly responsible for the high mechanical resilience of articular cartilage tissue. Cartilage has a very low potential for intrinsic self-repair and regeneration in its mature tissue, because chondrocytes are believed to have no capacity for migration, proliferation, and repair (Tew et al. 2001; Johnstone et al. 2013; Redman et al. 2005).

Most surgical therapies aim to stimulate cells from the bone marrow by microfracture, abrasion arthroplasty, and Pridie drilling (Buckwalter and Mankin 1998; Hochberg et al. 2012; Lohmander and Roos 2007; Minas 1999; Steadman et al. 2002; Steinwachs et al. 2008; Muller and Kohn 1999). However, these treatments attempt to support fibrocartilaginous repair tissue and cannot induce hyaline cartilage (Ronn et al. 2011; Becher et al. 2010). Other treatment options such as osteochondral autologous transplantation or autologous chondrocyte implantation also try to recover the articular surfaces (Muller et al. 2010; Vasiliadis et al. 2010).

3.2.1 Mesenchymal Stem Cells and Osteoarthritis

Tissue regeneration should focus on generating a repair tissue, which exerts the same mechano-biological properties and assimilates with the native tissue (Redman et al. 2005). Currently, one focus uses stem cell-based therapies to induce regeneration. The use of mesenchymal stem cells targets restores hyaline articular cartilage. Mesenchymal stem cells can be found in differentiated tissues and fulfil

various tasks in the adult human body. They are characterized by their multi-lineage potential, which was demonstrated first by Pittenger et al. (1999), who isolated mesenchymal stem cells from bone marrow. Currently, mesenchymal stem cells can be obtained from diverse adult tissues (Kuhn and Tuan 2010). They have the capability to differentiate into various mesenchymal phenotypes including muscle, ligament, tendon, adipose, stroma, bone, and cartilage (Cai et al. 2004; Caplan 2007). Furthermore, they possess immunomodulating properties, anti-inflammation effects, and self-renewal capacities (Bonfield et al. 2010; Chamberlain et al. 2007; Chen and Tuan 2008). Stem cells are located in a yet-to-be-defined niche and remain quiescent except for rare cell divisions (Fuchs et al. 2004).

One of the first attempts to achieve cartilage repair in osteoarthritis was performed by Wakitani et al. in 1994. Large, full-thickness defects of the articular cartilage in the knees of rabbits were repaired by osteochondral progenitor cells (Wakitani et al. 1994). Later, a clinical trial was designed to investigate the influence of mesenchymal stem cells on damaged human cartilage. Human autologous culture expanded bone marrow mesenchymal cells were transplanted into the osteoarthritic knee joints of patients. A cartilage-like tissue formed after 42 weeks. However, symptoms were not significantly improved (Wakitani et al. 2002).

Further essential findings concerning stem cell therapy of osteoarthritis were obtained by Murphy et al. (2003). They explored the role of implanted adult mesenchymal stem cells in tissue repair and regeneration in an injured joint in a goat model. Regeneration of the meniscal tissue was stimulated, and its progressive destruction was prevented. However, none of the stem cells were located in the diseased cartilage tissue.

Another study investigated the benefit of mesenchymal stem cell treatment in patients suffering from moderate to late-stage osteoarthritis. No complete regeneration of cartilage and no efficient long-term success were obtained (Davatchi et al. 2011; Cucchiarini et al. 2014).

3.2.2 Mesenchymal Stem Cells and Rheumatoid Arthritis

Rheumatoid arthritis involves chronic inflammation of the synovium, which induces cartilage and bone erosion (De Bari 2015). Mesenchymal stem cells can also be found in the synovium (De Bari et al. 2001). In rheumatoid arthritis, the immune modulatory properties of mesenchymal stem cells seem to be very important (El-Jawhari et al. 2014). The effects of mesenchymal stem cells seem to be repressed by the inflammatory milieu. Rheumatoid arthritis patients show a lower prevalence of mesenchymal stem cells than is seen in osteoarthritis patients (Jones et al. 2004). Furthermore, there is a negative correlation between the chondrogenic and clonogenic capacities of synovial mesenchymal stem cells and the magnitude of synovitis in rheumatoid arthritis (Jones et al. 2010). In addition, there is an interdependency between infiltrating inflammatory/immune cells and resident fibroblast-like synoviocytes. A proliferation of fibroblast-like synoviocytes, the major pathogenic component in rheumatoid arthritis, is observed in rheumatoid arthritis (Li and

Makarov 2006). These cells support the development of harmful pannus, which results in damaged articular cartilage and bone (Naylor et al. 2013). A recent study demonstrated that interactions between fibroblast-like synoviocytes and mesenchymal stem cells are possible. The placental growth factor, which occurs at higher levels in joints suffering from rheumatoid arthritis, could attract bone marrow mesenchymal stem cells to the synovium, where interactions with the resident fibroblast-like synoviocytes may lead to angiogenesis and chronic synovitis by further enhancing the secretion of placental growth factor (Park et al. 2014). However, the relationship between fibroblast-like synoviocytes and mesenchymal stem cells remains unclear. It might be possible that they represent different functional stages of the same lineage or that they represent the same cell type with functional specialization and diversification according to their positional information and environmental cues (De Bari et al. 2001).

Recent clinical studies have explored the benefit of mesenchymal stem cell treatment in rheumatoid arthritis patients. The intravenous injection of umbilical cord mesenchymal stem cells in addition to disease-modifying antirheumatic drugs induced a significant clinical improvement in patients suffering from active rheumatoid arthritis and in whom conventional treatment was ineffective (Wang et al. 2013). However, larger multicenter clinical studies are needed to provide safe treatment recommendations.

3.3 Chondrogenic Progenitor Cells

Restoring fully functional hyaline cartilage has not been achieved to date via chondrocytes or mesenchymal stem cells. Various experiments and methods have only generated fibrocartilaginous repair tissue instead of stable hyaline cartilage (Cucchiarini et al. 2014). Fibrocartilaginous repair tissue shows morphologically distinct cell types (Kouri et al. 1996). In the late stages of osteoarthritis, single chondrocytes and cells, which are organized in aggregates, are found. Furthermore, comprised chondrocytes undergoing a degenerative process exist in all zones of the cartilage. The most common cells are *elongated secretory type 2 cells*, which display a secretory phenotype (Kouri et al. 1996). These cells have been named *fibroblast-like chondrocytes* (Tesche and Miosge 2005). Koelling et al. (2009) were able to demonstrate that these cells show typical stem cell characteristics, including clonogenicity, multipotency, and migratory activity, and named them *chondrogenic progenitor cells*. They are also referred to as *osteochondroprogenitor cells* (Khan et al. 2009). In osteoarthritis and rheumatoid arthritis, these chondrogenic progenitor cells (Table 3.1) are involved in regeneration efforts that are largely unsuccessful in diseased cartilage tissue (Schminke and Miosge 2014).

Recently the presence of migratory progenitor cells in diseased tissues has been explored because they may play an important role in tissue regeneration and could be a promising target for cell-based therapy. Migratory progenitor cells show stem cell characteristics and possess great chondrogenic potential. The migratory potential is an important feature. The mechanism of cell migration can be found in numerous

Table 3.1 Mesenchymal stem cells and chondrogenic progenitor cells under investigation for cartilage repair (modified from Muhammad et al. 2013)

Cell types	Mesenchymal stem cells	Chondrogenic progenitor cells
Origin	Adult tissue	Osteoarthritic cartilage
Self-renewal	Slightly limited self-renewal	Limited self-renewal
Differentiation potential/ preclinical aspects	Multipotent; mainly differentiation into the cell types of the mesodermal lineage	Multipotent; already determined to the osteochondrogenic lineage
Stem cell marker positivity (CD, Stro-1)	Stro-1, CD13, CD29, CD44, CD49a, **CD73**, **CD90**, **CD105**, CD114, CD166 (while no expression of CD14, CD19, CD34, and CD45)	Stro-1, CD13, CD29, CD44, **CD73**, **CD90** (while no expression of CD18, CD31, CD34, CD117, CD271)
Immunity/preclinical aspects	Less immunogenic; difficult to maintain undifferentiated in cell culture	Unknown; easy to isolate and differentiate into chondrocytes

biological processes (Theveneau and Mayor 2013). Migration is a relevant characteristic of epithelial cells, i.e., during wound healing of the skin (Blanpain and Fuchs 2014). Migration is also indispensable to mesenchymal stem cells. Diverse repair processes in one's lifetime are dependent on the migration of these cells (Sohni and Verfaillie 2013). Hematopoiesis and bone regeneration require cell migration (Sahin and Buitenhuis 2012; Pignolo and Kassem 2011). Additionally, progenitor cells, which are involved in basic biological processes of the stem cell niche, migrate (Augello et al. 2010). Progenitor cell populations that were generated from patient tissue from late stages of osteoarthritis possessed great migratory potential, at least in vitro and ex vivo (Schminke and Miosge 2014; Muhammad et al. 2013).

Chondrogenic progenitor cells show stem cell marker positivity for Stro-1 and CD29 and also for CD13, CD44, CD73, and CD90. They are negative for CD31, CD34, CD117, and CD271. Cells isolated from the superficial zone of healthy cartilage tissue in vivo also show stem cell marker positivity and could be related to chondrogenic progenitor cells. So far, this observation has not been confirmed (Dowthwaite et al. 2004). Chondrogenic progenitor cells in the late stages of osteoarthritis exhibit multi-differentiation potential to become adipocytes, cells of the osteoblastic lineage and chondrocytes. They can be cloned and expanded for up to 60 population doublings. Chondrogenic progenitor cells can exist as cells of the chondrogenic lineage if they are simply placed in 3D alginate culture (Koelling and Miosge 2009).

3.3.1 Chondrogenic Progenitor Cells from Osteoarthritic Patients (CPCs)

Chondrogenic progenitor cells are a subpopulation of cells that are localized in the repair tissue of advanced stages of osteoarthritis (Koelling et al. 2009). The

compositions of collagens change in the late stages of osteoarthritis. Collagen types I and III are found in the fibrocartilaginous cartilage (Sandell and Aigner 2001; Poole 1999). Furthermore, a reduction of collagen type II is observed by quantitative immunohistochemistry (Miosge et al. 1998). In contrast to the microarray experiments, mRNAs of cartilage-specific collagens are upregulated, and increased anabolism is observed (Aigner et al. 2006). The change of the matrix composition may have a distinct influence on the deficient functioning of the repair tissue. Further investigations showed an increased expression level of proteoglycans such as biglycan, decorin, and perlecan (Tesche and Miosge 2005; Bock et al. 2001). This might reflect compensation for the loss of matrix molecules and the stabilization of the extracellular matrix (Tesche and Miosge 2005).

The transcription factors *runt-related transcription factor 2* (Runx2) and *sex-determining region Y-box 9* (Sox9) play an important role in the regulation mechanisms of chondrogenic progenitor cells (Koelling et al. 2009; Koelling and Miosge 2010). Sox9 is involved in the development of chondrocytes, operates the synthesis of cartilage-specific matrix components, and inhibits the beginning of chondral ossification (de Crombrugghe et al. 2001). Runx9 coordinates the development of osteoblasts and is essential for bone formation (Stein et al. 2004).

An ex vivo experiment using siRNA in three-dimensional culture downregulation of the osteogenic transcription factor Runx2 resulted in a simultaneous upregulation of the chondrogenic transcription factor Sox9. Hence, COL2A1 mRNA was detected (Koelling and Miosge 2010; Koelling et al. 2009; Muhammad et al. 2014). A Sox9 knockdown results in reduced aggrecan and Runx2 expression (unpublished observation). Mass spectrometry analysis was used to identify proteins, which are involved in the signal transduction and transcription of Sox9 and Runx2. Overexpression of DDX5, HSPA8, RAB5C, and YWHAE resulted in enhanced gene expression of Sox9. HSPA8 also enhanced the gene expression of Runx2, which was downregulated by YWHAE, and the chondrogenic potential of the chondrogenic progenitor cells was increased. A knockdown of LEMD2 and TMPO leads to an upregulation of Sox9. Further indicators of increased chondrogenic potential were the enhanced expression of the extracellular component ACAN and the decreased expression of COL1A1.

The pericellular matrix with laminins and nidogen-2 may also play an important role in the regulation mechanisms of chondrogenic progenitor cells. It has been shown that chondrogenic progenitor cells produce high levels of laminin-α1, laminin-α5, and nidogen-2 in their pericellular matrix. Laminin-α1 regulates collagen expression by enhancing collagen type II and decreasing collagen type I expression. Nidogen-2 upregulates Sox9 expression. A knockdown of nidogen-2 results in reduced Sox9 expression and enhanced Runx2 expression. Laminins and nidogen-2 guide chondrogenic progenitor cells toward chondrogenesis (Schminke et al. 2016a). This fact highlights the importance of the extracellular matrix components on chondrogenic progenitor cells and in stem cell biology (Fuchs et al. 2004; Fuchs 2008). The population of chondrogenic progenitor cells is not present in healthy cartilage.

Mechanical stimulation has an influence on chondrocytes. The primary cilium, a mechanosensor, also seems to be involved in mechano-transduction in chondrocytes.

The mechanical load enhances ciliogenesis in the growth plate. The expression and localization of key members of the Ihh-PTHrP loop is altered, resulting in decreased proliferation and a switch from proliferation to differentiation. Abnormal chondrocyte morphology and organization is also observed (Rais et al. 2015; Muhammad et al. 2012).

Furthermore, calcium signaling is important for chondrogenesis. A recent study suggested that calcium homeostasis, including calcium regulatory mechanisms, has an influence on the differentiation potential of chondrogenic progenitor cells. An autocrine/paracrine purinergic mechanism plays an important role in driving calcium oscillations in these cells (Matta et al. 2015). Furthermore, the external influence of the sympathetic nervous system on chondrogenic progenitor cell-dependent chondrogenesis is important. A norepinephrine-dependent inhibition of chondrogenesis and acceleration of hypertrophic differentiation was recently discovered (Jenei-Lanzl et al. 2014).

3.3.2 Chondrogenic Progenitor Cells from Rheumatoid Arthritis Patients

Chondrogenic progenitor cells can also be isolated from diseased cartilage tissue of patients who suffer from rheumatoid arthritis. Interleukins, which are commonly found in the inflamed rheumatoid tissue of affected joints, exert a negative influence on these cells. This results in a less chondrogenic phenotype. High levels of matrix metalloproteinases and proinflammatory cytokines, such as tumor necrosis factor α (TNF-α), which are influenced by IL-17, are produced by these chondrogenic progenitor cells. IL-17 A/F leads to the upregulation of Runx2 protein and enhanced IL-6 protein and MMP3 mRNA levels. Blocking antibodies against IL-17 improved the repair potential of the progenitor cells. When chondrogenic progenitor cells are treated with the antihuman IL-17 antibody secukinumab or the anti-TNF-α-antibody adalimumab, a reduction of the proinflammatory IL-6 protein levels and a positive influence on the secretion of anti-inflammatory IL-10 protein are observed. Runx2 protein is also reduced by the same antibodies, which promote chondrogenesis. The chondrogenic capacity of the chondrogenic progenitor cells can be improved again by anti-inflammatory agents. Again, progenitor cells are distinguished by their high migration potential. They are able to repopulate diseased cartilage tissue ex vivo. Inflammatory mediators have a remarkable influence on these progenitor cells and their ability to migrate (Schminke et al. 2016b).

3.3.3 Meniscus Progenitor Cells (MPCs)

In the inner, avascular part of diseased human menisci, meniscus progenitor cells can be found. They are normally distinguished by the production of collagen type I, and they display a fibrocartilaginous nature and high migration potential (Muhammad et al. 2014). During the investigation of diseased human menisci from

patients who were suffering from advanced stages of osteoarthritis, strongly affected menisci exhibit a downregulation of TGF-β and Smad2, resulting in the upregulation of Runx2. These facts support the assumption that meniscus progenitor cells also underlie a fine-tuned interaction between Runx2 and Sox9. Chondrogenic differentiation can be initiated by a knockdown of Runx2, which enhances p-Smad2. On the other hand, BMP2 stimulation of meniscus progenitor cells results in lower Smad2 levels and supports a change of the cells toward the osteogenic lineage (Muhammad et al. 2014). Additionally, a study in mice ascertained that TGF-β signaling directs knee morphogenesis and is important for meniscus development (Pazin et al. 2012).

Conclusion

Regenerative therapies aim to substitute diseased tissue with native-like functional tissue. Until now, nearly all attempts have only managed to achieve the generation of a fibrocartilaginous repair tissue instead of fully functional, collagen type II-enriched hyaline cartilage. To date, treatment approaches using mesenchymal stem cells have not obtained satisfying long-term results. A new strategy is not to transplant stem cells into diseased cartilage tissue but to manipulate resident cells with stem cell characteristics, which are already present in situ and are active in their physiological response to the cell biological stimuli of the diseased tissue (Muhammad et al. 2013). Chondrogenic progenitor cells could be a promising target for cartilage repair (Fig. 3.2).

Published successes provide insight into the chondrogenic pathways and interacting partners of chondrogenic progenitor cells. Two master regulators, Runx2

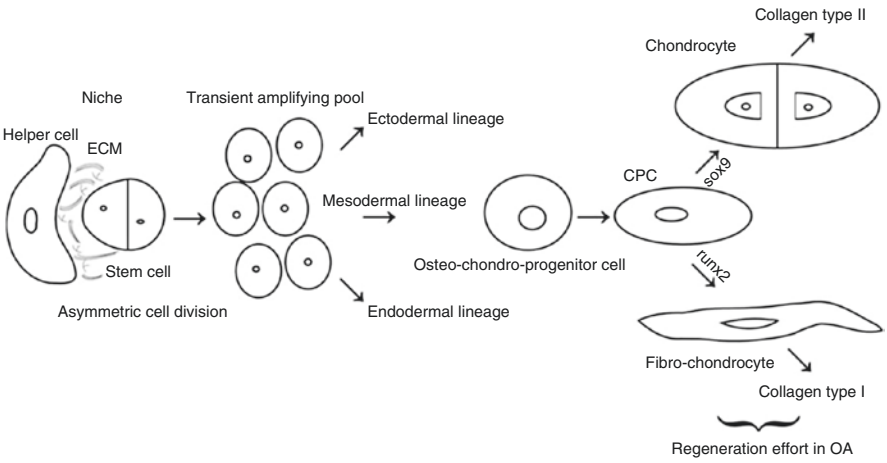

Fig. 3.2 The concept of chondrogenic progenitor cells in situ. Cells derived from the stem cell in its niche are already pre-determined as osteochondroprogenitor cells. In OA cartilage, under the control of runx2, they become collagen type I producing cells. If these cells would be targeted in situ to switch to collagen type II production by enhancing Sox9 expression, they would help to regenerate a more hyaline-like cartilage tissu

and Sox9, have already been identified. A knockdown of Runx2 leads to the upregulation of Sox9, aggrecan, and collagen type II (Koelling et al. 2009). The pericellular matrix with laminins and nidogen-2 is also involved in regulation mechanisms. Laminin-α1 and nidogen-2 guide chondrogenic progenitor cells toward chondrogenesis (Schminke et al. 2016a). Furthermore, mechanical stimulation has an influence on the chondrogenic differentiation potential of chondrogenic progenitor cells. An important role is played here by the primary cilium, which is necessary for mechano-transduction in chondrocytes (Rais et al. 2015; Muhammad et al. 2012). Calcium homeostasis may have autocrine/paracrine purinergic mechanisms that affect the calcium oscillations in these cells (Matta et al. 2015). In addition, the sympathetic nervous system was induced to have a norepinephrine-dependent inhibition of chondrogenesis (Jenei-Lanzl et al. 2014). Interleukins, especially IL-17, influence chondrogenic progenitor cells through the upregulation of Runx2 and drive them toward a less chondrogenic phenotype. Repair potential can be improved again by blocking antibodies against IL-17 in rheumatoid arthritis (Schminke et al. 2016b). The downregulation of TGF-β is also associated with an upregulation of Runx2 in meniscus progenitor cells (Muhammad et al. 2014).

Against the background outlined here, further investigations should focus on manipulating chondrogenic progenitor cells in situ with the help of small modifying molecules to improve their chondrogenic potential. It remains to be demonstrated whether manipulated chondrogenic progenitor cells produce an extracellular matrix, which provides repair tissue with better mechanical stress resistance than fibrocartilaginous tissue. Moreover, there is still a lack of knowledge about the behavior of manipulated chondrogenic progenitor cells in vivo. It has to be shown that they survive and maintain their favorable characteristics in the hostile microenvironment of the diseased organ (Koelling and Miosge 2009). In addition, the influence of age, gender, and body weight should not be disregarded (Gharibi et al. 2014; Murphy et al. 2002). Overall, further knowledge and understanding of the mechanisms that contribute to the regulation of stemness, multipotency, and differentiation have to be accomplished to allow a “restitutio ad integrum” in diseased cartilage tissue.

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Induced Pluripotent Stem Cells and Cartilage Regeneration

4

Solvig Diederichs and Wiltrud Richter

Abstract

Induced pluripotency has attracted enormous scientific and public attention and has quickly entered all fields of research not only those that had so far lacked an easily accessible cell source for cell therapies and in vitro models. The attractiveness of induced pluripotent stem (iPS) cells for cartilage regeneration roots from their immense expandability and their intrinsic ability to give rise to any adult tissue including stable hyaline cartilage. Thus, iPS cells offer to overcome the extremely limited supply of human articular chondrocytes and the restricted differentiation capacity of mesenchymal stem cells from bone marrow or adipose tissue that differentiate along the endochondral pathway and form mineralized bone upon ectopic implantation. Beyond being a potential alternative cell source for articular chondrocyte implantation, iPS cells are particularly promising for in vitro modeling of genetic diseases and for drug testing. Reprogramming patient-specific cells with a genetic predisposition and engineering disease-specific genetic variations into healthy control iPS cells promises to recapitulate "diseases in a dish" more realistically than immortalized human cell lines and will be an invaluable complementation for animal models. Whether iPS cells will satisfy these tremendous expectations will depend on our ability to upscale iPS cell culture, to derive sufficient amounts of relevant cell types like chondrocytes from iPS cells with acceptable efforts, and to find clinically safe reprogramming techniques for iPS cell-based therapies.

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4.1 Introduction

The era of induced pluripotent stem cells (iPS cells) started with the breakthrough report by Takahashi and Yamanaka who reprogrammed somatic cells back to pluripotent stem cells by retrovirally overexpressing a defined set of transcription factors (Takahashi and Yamanaka 2006). The search for such factors present in the egg cytoplasm had been initiated by John Gurdon who managed to obtain normally developed *Xenopus* frogs by transferring somatic intestinal cell nuclei into an egg, thus proving that some components within the egg cytoplasm were capable to reprogram a somatic nucleus to pluripotency. Yamanaka's cumbersome exclusion experiments surprisingly uncovered that only four factors—Oct3/4, Sox2, Klf4, and c-Myc—were sufficient to initiate transcription of the complete pluripotency network within somatic cells, to erase the epigenetic marks accrued during differentiation and to, thus, reprogram committed cells back to pluripotency. For proving that cellular differentiation is not an irreversible process and that mature cells can be reprogrammed to become pluripotent, John Gurdon and Shinya Yamanaka were awarded the 2012 Nobel Prize for Physiology or Medicine. Extensive research is currently going on in the new field of induced pluripotency addressing various aspects of iPS technology, such as cell sourcing, reprogramming efficiency, and the development of sophisticated reprogramming strategies. Furthermore, elucidation of the pluripotency transcription network that enables stem cells to infinitely proliferate and maintain the undifferentiated status is ongoing.

The simplicity and reproducibility of cellular reprogramming by ectopic expression of few defined transcription factors has stimulated iPS cell research to spread beyond basic stem cell science into applicational research. Soon, iPS cells became a universal tool holding prospect for cell therapy, for diagnosis and drug development, and even for the rescue of endangered species (Fig. 4.1). Thus, iPS cells also entered the field of regenerative medicine where iPS cells represent the only practical cell source, e.g., in neurobiology or cardiology. Cartilage regeneration may also benefit from iPS cells since they promise to overcome limitations of the current cell sources which are articular chondrocytes and mesenchymal stromal cells. Chondrocyte availability suffers from the invasiveness of tissue harvest requiring creation of a new defect in a tissue with low intrinsic regeneration capacity. Thus, only small amounts of cartilage tissue can be harvested for cell isolation, and chondrocytes need to be expanded in vitro in order to provide sufficient cell quantities. Upon in vitro culture, however, chondrocytes dedifferentiate and lose their capacity to form cartilage, thus severely limiting the amount of chondrocytes available for cell therapy and research. The high proliferative capacity of stem cells offers to overcome the limited availability of primary chondrocytes.

Multipotent mesenchymal stromal cells (MSCs) that can be isolated from bone marrow (Bianco and Gehron 2000; Caplan 1991), adipose tissue (Zuk et al. 2001), or umbilical cord blood (Erices et al. 2000) are currently the best described stem cell source for cartilage regeneration and closest to clinical translation. The harvest procedures for bone marrow and adipose tissue are less invasive than an articular cartilage biopsy, and MSCs can be expanded for some passages before they lose their

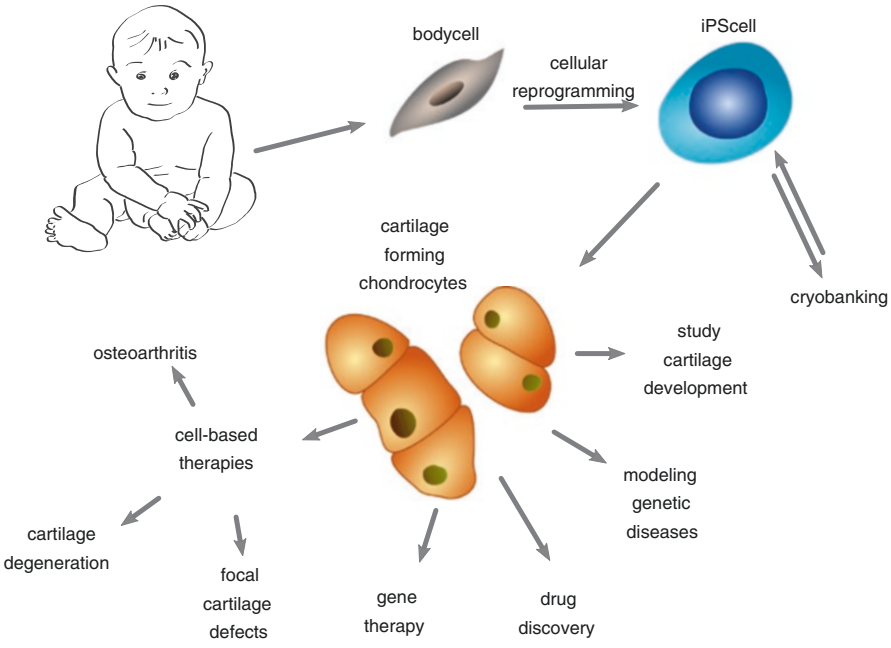

Fig. 4.1 Promise of iPS cells for cartilage regeneration. Body cells like fibroblasts, lymphocytes, or cord blood cells can be reprogrammed into iPS cells that can be cryopreserved. Upon need, iPS cells can be revitalized and differentiated into chondrocytes for cell-based therapies. Importantly, iPS cells are particularly promising for basic research to elucidate cartilage development and pathomechanisms of genetic cartilage diseases in order to develop novel treatment strategies

chondrogenic capacity (Bruder et al. 1997; Narcisi et al. 2015). Although bone marrow-derived MSCs are considered to contain skeletal stem cells (Bianco 2014) which are the developmental progenitors of articular chondrocytes, the cartilage they form in vitro is not hyaline but comprises also fibrous components (collagen type I), becomes hypertrophic, and expresses markers of calcifying cartilage (Dickhut et al. 2010; Pelttari et al. 2006). Upon ectopic implantation, such cartilage becomes mineralized and forms endochondral bone (Pelttari et al. 2006; Janicki et al. 2010). Thus, MSCs seem to be primed for endochondral development and incapable to form stable hyaline cartilage in vitro. The intrinsic capability of iPS cells to form any adult tissue including hyaline articular cartilage and their unlimited proliferative capacity thus make iPS cells a highly attractive cell source that could potentially provide access to ample amounts of in vitro-generated cartilage. Furthermore, iPS cells offer the new possibility of in vitro disease modeling, for which both primary chondrocytes and MSCs are inapplicable, because upon genetic manipulation and subcloning they easily lose their capacity to form cartilage.

Thus, iPS cells are attractive for cartilage regeneration because their abundant availability may solve the limited supply of articular chondrocytes, their intrinsic capacity to form stable hyaline cartilage may overcome the endochondral priming

of MSCs, and their stable clonal expansion capacity makes in vitro models accessible for elucidating pathomechanisms and for drug development and high-throughput screenings. In the following sections, the status and recent major achievements to utilize iPS cells for cell-based therapies, to derive therapeutically relevant cells from iPS cells, and to establish iPS cell-based in vitro disease models will be presented, and future perspectives will be discussed.

4.2 Making iPS Cells Available for Cell-Based Therapies

Optimal cell sourcing is a central question for all cell-based therapies. In line with the current trend of personalized medicine, autologous strategies are preferred over allogeneic application. But even if virtually any cell is reprogrammable, it practicality requires a cell source that is easily accessible by a noninvasive procedure. Skin fibroblasts that were originally used by Yamanaka have probably been reprogrammed most frequently, but a skin biopsy can cause scar formation and is afflicted with the risk of infection. Thus, skin fibroblasts may not necessarily be the best tissue source for reprogramming. Peripheral blood on the other hand is more easily accessible, and blood withdrawal is less invasive than a skin biopsy and very well accepted by most patients. T lymphocytes have been reprogrammed with good yields (Okita et al. 2013; Seki et al. 2011), and the rearrangements of the T-cell receptor DNA which are specific for each individual T lymphocyte make the clonality of the resulting iPS cells easy to monitor under GMP culture conditions. Thus, in those scenarios where personalized iPS cell-based therapies are supposed to be generated, peripheral blood holds prospect to become a popular and easily accessible source.

However, all cells accrue genetic mutations during their lifetime, and these mutations persist upon reprogramming (Gore et al. 2011) and may increase the risk of tumorigenicity. Thus, embryonic cells would be preferable over adult cells making cord blood cells highly attractive for reprogramming because of their developmental youth and low genetic modification rate (Giorgetti et al. 2009; Haase et al. 2009; Takenaka et al. 2010). Of course, autologous cord blood is currently not available for every patient, but cryopreservation of cord blood has become increasingly popular, and cord blood banks are growing in many countries. In the future, iPS cells could be routinely generated and cryopreserved from cord blood to provide a cell pool for biologic treatment strategies in any organ system including cartilage. Banking iPS cells instead of cord blood will also facilitate a more prompt availability. Indeed, generating a validated and well-characterized iPS cell line is a time-consuming process that can take months and should, thus, preferentially be already accomplished when a diagnosis calls for cell-based therapy. Of course, the generation of therapeutically active cells or cartilage tissue from iPS cells to treat injured or degraded cartilage will also take a few weeks, but this appears an absolutely acceptable period to wait for implantation of a cartilage regenerate.

Generating iPS cell banks to cover the complete population, however, might seem enormously challenging, and thus, despite a general preference for autologous

strategies, feasibility and practicality aspects support allogeneic approaches. Especially for regenerating cartilage, which is avascular and alymphatic and has been suggested to be immune privileged to some degree (Elves and Zervas 1974; Hunt et al. 2014; Levy et al. 2013), allogeneic treatment might be well tolerated. Japanese stem cell researchers are already establishing an iPS cell bank containing HLA-homozygous donor cells under the assumption that matching at least the three major HLA markers will result in less immune rejection (Takahashi and Yamanaka 2013; Taylor et al. 2012; Turner et al. 2013; Yamanaka 2012). Of course, matching major HLA types such as HLA-A, -B, and -DR might not completely rule out an immune reaction when other HLA loci or minor histocompatibility antigens mismatch (Tsumaki et al. 2015; Turner et al. 2013). Future studies will reveal whether the potential immune privileges of cartilage may allow imprecise donor matching, thus increasing the availability of suitable donor cells and overcoming potential graft shortage. Of course, allogeneic iPS cell banks would ideally only contain cells from healthy donors. This criterion may prove challenging for cord blood cells, since donors might seem healthy at birth but can still develop degenerative or other diseases with age. Our continuously advancing ability to assess the cellular genetic and epigenetic profile together with our progressing insight into disease-causing cell transformations will help setting up sophisticated quality controls to ensure graft safety.

In summary, their accessibility and genetic integrity make cord blood cells an ideal source for generating iPS cells. When an autologous approach is preferred but autologous cord blood is not available, peripheral blood seems the most obvious alternative choice for conversion into personalized iPS cells. To accelerate the availability of cell-based therapies upon need, iPS cell banks are highly promising. Practicality and feasibility have encouraged allogeneic instead of autologous approaches. An allogeneic iPS cell bank is currently being established in Japan and is meant to largely cover the population with 100 HLA-homozygous cell lines. Thus, having allogeneic iPS cells available for cartilage regeneration seems only a small step away. In the future, iPS cells could be generated and banked routinely and could then upon need provide an autologous cell pool for biologic treatment strategies in any organ system including cartilage that might be injured or diseased throughout life.

4.3 Therapeutic Cells from iPS Cells

The euphoria of having a universal cell source available intuitively suggests utilizing these cells therapeutically for cell and tissue transplantation strategies. Their unlimited proliferative capacity is one main quality defining iPS cells. However, the disadvantage of this highly praised property is that upon implantation, iPS cells could potentially proliferate without inhibition and form tumors. Indeed, the formation of teratomas—benign tumors containing tissues originating from all three germ lines—is the most stringent quality test for newly reprogrammed human iPS cell lines to verify their pluripotency. Thus, iPS cells cannot be implanted directly but

must be differentiated into offspring with a restricted proliferative capacity, and contamination of these progeny with pluripotent stem cells must be excluded.

The most obvious strategy to utilize iPS cells for cell implantation therapy in cartilage is to differentiate them into cartilage-forming chondrocytes. It took surprisingly long until the first study reported human iPS cell chondrogenesis (Medvedev et al. 2011) 4 years after human iPS cells were generated for the first time (Park et al. 2008; Takahashi et al. 2007; Yu et al. 2007). In this study by Medvedev et al., iPS cells were initially differentiated as embryoid bodies—small cell aggregates in which tissues from all three germ lines are formed spontaneously like in an embryo but far less coordinated (Medvedev et al. 2011). Cells dissociated from these embryoid bodies were subsequently subjected to chondrogenic stimuli in 3D culture where they were supposed to self-aggregate and deposit cartilaginous matrix. Hallmarks of chondrogenesis were indeed detected, but the amount of proteoglycans and collagen type II remained disappointingly low. Several alternative methods to induce in vitro chondrogenesis of human iPS cells have been reported since then. Most of them omitted embryoid body culture arguing that this method is uncontrollable and overly laborious. Co-culture with chondrocytes was a plausible attempt based on the assumption that direct cell-cell contact and/or the soluble factors produced by chondrocytes might be able to instruct iPS cells to differentiate efficiently into the chondrogenic lineage (Qu et al. 2013; Wei et al. 2012). However, the cartilage formation efficiency of such chondrogenic iPS cell offspring still remained low. Moreover, the instructive influence of chondrocytes remained debatable, because the switch of culture conditions even without the influence of chondrocytes is known to induce iPS cell differentiation and the studies did not include a chondrocyte-free control group. Compared to the strong induction of spontaneous iPS cell differentiation by discontinuing high-dose bFGF treatment and increasing medium exchange intervals (Diederichs and Tuan 2014; Frobel et al. 2014; Guzzo et al. 2013; Lian et al. 2010), the actual effect of co-culture with chondrocytes appears minor.

In vitro recapitulation of embryonic cartilage development is currently considered to be the most promising and successful method for iPS cell differentiation (Umeda et al. 2012; Wu et al. 2013; Yamashita et al. 2015). This strategy requires sequential induction of multiple developmental phases including a mesendodermal and a mesodermal stage before induction into the chondrogenic lineage. Two recent publications suggest that such an approach may be capable to induce formation of stable hyaline cartilage from human iPS cells with a quality that might well match that of primary chondrocytes (Umeda et al. 2012; Yamashita et al. 2015). Wnt agonists and activin A are typically used to enhance formation of a mesodermal subpopulation, but the optimal growth factor cocktail has yet to be found. Thus, both studies relied on an enrichment strategy to exclude mis-differentiated cells that would otherwise impede efficient chondrogenesis. The Nakayama group enriched cells expressing platelet-derived growth factor receptor-α (PDGFRα) but not vascular endothelial growth factor receptor-2 (FLK1 or KDR) (Umeda et al. 2012). These surface receptors had been suggested in previous studies with mouse embryonic stem cells to define the mesodermal lineage (Tanaka et al. 2009). The Tsumaki

group chose an approach that did not rely on an uncertain correlation between surface markers and chondrogenic capacity. Instead, they argued that embryonic cartilage formation is initiated by mesenchymal condensation and consequently isolated such condensations early after chondrogenic induction of mesodermally pre-differentiated human iPS cells (Yamashita et al. 2015). The chondrogenically induced human iPS cells deposited ample cartilaginous matrix rich in collagen type II and proteoglycans in both studies. Fibrous collagen type I and hypertrophic collagen type X that usually both accompany collagen type II deposition during chondrogenesis of human MSCs seemed to remain largely absent. In vivo, the Tsumaki group reported that their human iPS cell-derived cartilage nodules remained stable for up to 12 months at ectopic sites in immune-deficient mice. Moreover, neocartilage survived and integrated into focal cartilage defects in both immune-deficient rats and in immunosuppressed mini-pigs without any signs of tumor formation.

Thus, first highly promising reports suggest that hyaline cartilage can be generated from human iPS cells via a mesodermal pre-differentiation followed by chondrogenic induction. Qualitatively, the iPS cell-derived chondrocytes seemed to match the chondrogenic capacity of primary chondrocytes and to outperform MSCs that can only be induced to form fibrous and hypertrophic cartilage in vitro. However, the work has now to be repeated by other groups with independent iPS cells from different sources. Moreover, optimal mesodermal stimuli have yet to be found in order to obtain homogenous differentiation, and enrichment strategies which make the protocols impracticable for routine high-throughput application have to be avoided.

Instead of completing chondrogenesis and generating cartilage-producing chondrocytes, the differentiation process of iPS cells could also be arrested at a specific (meta-)stable intermediate that comprises the capacity to mature after implantation and give rise to therapeutically active cells under orthotopic instruction. IPS cell-derived mesenchymal progenitor cells (iMPCs) could potentially represent such a therapeutically interesting intermediate. iMPCs are immature cells in a transitional state of development (between stem cells and terminally differentiated cells), and their restricted capacity to self-renew and differentiate into only musculoskeletal tissues should reduce tumorigenicity. Thus, iMPCs may be highly attractive with regard to safety aspects and therapeutic applicability. Various methods have been reported to generate iMPCs including embryoid body outgrowth culture (Diederichs and Tuan 2014; Frobel et al. 2014; Koyama et al. 2013; Li et al. 2010), spontaneous differentiation of iPS cells (Diederichs and Tuan 2014; Frobel et al. 2014; Guzzo et al. 2013; Hynes et al. 2014; Kang et al. 2015; Lian et al. 2010), and co-culture with MSCs (Diederichs and Tuan 2014) (Fig. 4.2). iMPCs resemble MSCs by expressing a similar surface marker profile ($CD44^+$, $CD73^+$, $CD90^+$, $CD105^+$, $CD34^-$, $CD45^-$) and a general capacity for in vitro differentiation into the three mesenchymal lineages (cartilage, bone, fat; Fig. 4.3). Thus, iMPCs and equivalent derivatives from embryonic stem cells have frequently been termed “MSCs” (Chen et al. 2010; Hynes et al. 2014; Kang et al. 2015; Kimbrel et al. 2014). Direct comparison with MSCs from the same donor, however, showed that despite evident similarities, iMPCs are not completely

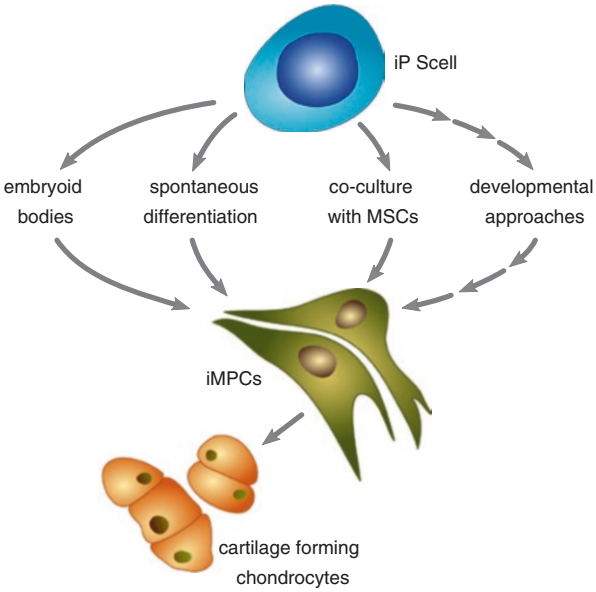

Fig. 4.2 Strategies to generate iMPCs from iPS cells

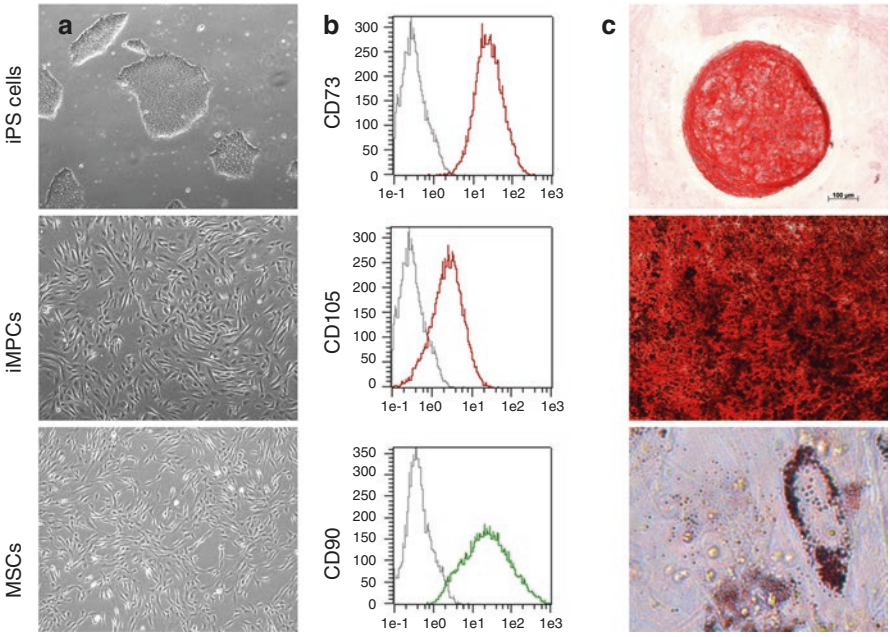

Fig. 4.3 iMPCs are MSC-like cells. (**a**) Morphology of iPS cell colonies, iMPCs and MSCs, as indicated. (**b**) iMPCs express the surface markers CD73, CD105, and CD90 that are typical for MSCs. (**c**) iMPCs are capable to differentiate in vitro into the three mesenchymal lineages and deposit collagen type II after chondrogenic stimulation (*top*), mineralize their matrix under osteogenic stimulation (Alizarin red staining, *middle*), and form intracellular lipid vesicles upon adipogenic stimulation (Oil Red O staining, *bottom*)

equivalent to MSCs (Diederichs and Tuan 2014; Frobel et al. 2014). The most striking differences were their DNA methylation profile, which was still more closely related to iPS cells than to MSCs (Frobel et al. 2014), and their inferior inducibility to differentiate into the three mesenchymal lineages (Diederichs and Tuan 2014; Frobel et al. 2014). Stimuli that easily induced MSC differentiation were less efficient for iMPCs (Diederichs and Tuan 2014), and this was also true for chondrogenesis which was less robust with iMPCs than with MSCs when induced with the same stimuli. Although additional treatment with BMPs can enhance iMPC chondrogenesis, deposition of cartilaginous matrix was still unsatisfactory (Guzzo et al. 2013; Saitta et al. 2014), and the ideal chondrogenic stimuli for iMPCs remain to be discovered. Whether the orthotopic articular environment in vivo might be able to more efficiently instruct iMPCs to differentiate into chondrocytes and form stable hyaline cartilage has yet to be investigated. In addition to finding adequate chondrogenic stimuli, iMPC generation strategies could also be adapted to yield more potent iMPC populations that can be more easily induced to differentiate. This overlaps with the developmental strategies mentioned above where growth factor cocktails are investigated with the aim to enhance formation of mesodermal/mesenchymal subpopulations without yielding an ideal combination yet.

In the recent years, cell implantation research has faced a paradigm shift with regard to the supposed activity and function of implanted cells. The original idea that implanted cells actively generate new tissue and their differentiation into tissue-forming cells is the major requirement for stem cells has been challenged by observations of beneficial effects of cell therapies even without long-term cell engraftment (Boukhechba et al. 2011; Giannoni et al. 2010; Niemietz et al. 2014; Seebach et al. 2014; Zimmermann et al. 2011). The paracrine activity of implanted cells secreting cytokines including growth and differentiation factors as well as immunomodulatory substances becomes increasingly recognized (Caplan and Dennis 2006; Liang et al. 2014; Ranganath et al. 2012). Thus, the major contribution of transplanted cells might be to control the inflammatory response, to attract endogenous repair cells to the defect site, and to support the intrinsic regenerative capacity. This general reformation of cell therapy will also significantly influence iPS cell research, and, consequently, the paracrine activity of iPS cell derivatives is currently being investigated (Lian et al. 2010). The presently most interesting questions are whether iPS cell-derived chondrocytes or iMPCs stably engraft into a cartilage defect, whether their paracrine activity might outweigh their capacity to form cartilage, and which iPS cell descendent is most potent to regenerate cartilage.

In summary, highly promising results have been achieved for differentiating iPS cells into chondrocytes that form stable hyaline cartilage of high quality without undesired fibrous or hypertrophic features. The next step will be to simplify the current differentiation strategies and to improve their efficiency in order to yield therapeutically active cells with feasible efforts and costs. The key will thereby be to either find factors stringent enough to force the complete iPS cell population into homogeneous differentiation or else to develop culture conditions

that automatically enrich for specific subpopulations—for example, by size exclusion of cell condensates—without manual intervention or sorting.

Instead of completely differentiating iPS cells into chondrocytes, mesenchymal intermediates like iMPCs may be simpler to generate in shorter time. Whether iMPCs may become an attractive therapeutic cell source will depend on our ability to find stimuli that reproducibly induce iMPCs with a robust chondrogenic activity and/or a high paracrine activity. In comparison to bone marrow-derived MSCs, that seem to be primed for endochondral development, iPS cells and their offspring should overcome the risk of ectopic bone formation in the joint. Moreover, given the biologic variability of MSC activity, which is strongly dependent on donor age (Dexheimer et al. 2011; Majors et al. 1997; Muschler et al. 2001), general health status (Murphy et al. 2002), and harvest procedure (Muschler et al. 2001), iPS cells may in the future comprise a more robust and less vulnerable cell source. Thus, iPS cells are attractive for cell-based cartilage regeneration and promise to overcome limited supply and activity of currently available cell sources.

4.4 Disease in a Dish: In Vitro iPS Cell Models

Generating in vitro disease models is often considered to be the most promising future iPS cell application. For elucidating pathomechanisms and for drug development, stable cell lines can be utilized for a multitude of experiments and screens. The cumbersome process of iPS cell generation that might be uneconomic for treating just one specific patient can here become profitable. Animal models have enormously contributed to elucidate pathomechanisms and will certainly remain indispensable for preclinical drug testing in the near future. In vitro models are, however, highly valuable complements because they allow screens of large substance libraries. Moreover, they represent the only possibility for preclinical tests and investigations in the human system which can considerably deviate from the regulatory mechanisms observed in animal models that often recapitulate human diseases incompletely. Because of the differences in size and movement between mice and human, the cartilage composition deviates. Mouse cartilage is necessarily much thinner (0.03 mm on the femur) than human cartilage (2.2 mm on the femur), and chondrocytes represent 15–40% of the mouse femoral cartilage volume but only 2% in humans (Aigner et al. 2010). Thus, human chondrocytes produce far more cartilaginous matrix than mouse chondrocytes. Like for cell-based therapies, primary tissue-specific cells like chondrocytes would be ideal for disease models but are not available in sufficient amounts. The obtainable number and the expandability of disease-specific chondrocytes that could potentially be isolated from specific patients are severely limited and allow only very few experiments. MSCs, that are available in larger quantities and can be expanded to some degree without losing quality, cannot be differentiated into stable hyaline cartilage in vitro. Immortalized cell lines are available in ample

quantities allowing large screens, but their phenotype and behavior are often abnormal, thus limiting the transferability of observations from immortalized cell lines to equivalent tissue cells.

Human iPS cells offer the attractive possibility to derive disease-specific cell lines with unlimited expandability. They can thus substantially contribute to the investigation of pathomechanisms and to the development of novel drugs not only for common diseases but also for rare disorders that have been hard to investigate. This includes of course osteoarthritis, the most frequent degenerative joint disease, but also the numerous hereditary osteochondral dysplasias which result from genetic disorders causing defective cartilage and bone differentiation, formation, and growth (Ikegawa 2006). Malformations can be manifest already at birth as in achondroplasia and Kniest dysplasia that cause dwarfism, as well as in the lethal forms of achondrogenesis, thanatophoric dysplasia, and campomelic dysplasia. Other dysplasias like multiple epiphyseal dysplasia cause early onset of osteoarthritis or cause anarchical cartilage and fibrous tissue-like enchondromatosis or multiple osteochondromatosis where multiple cartilage cysts (enchondroma) or bone tumors (exostoses) are formed. For many dysplasias, the disease-causing mutations are already known (Ikegawa 2006). The exact mechanisms, on the other hand, how, for example, defective collagens cause the multiple symptoms of collagenopathies, or how a gain of function mutation of an FGF receptor can result in dwarfism or lethal respiratory insufficiency, and how such conditions can be treated, are currently largely unknown. IPS technology now offers the possibility to generate and study cell lines carrying the disease-causing mutations. In addition to reprogramming patient-specific cells, novel gene editing methods allow introducing genetic defects into well-characterized iPS cell lines. This will considerably increase the comparability between independent studies and the quality of the results. Since iPS cells are not committed to a certain developmental lineage, the effects of a certain disease or drug can be studied in various cells or tissues with an identical genotype that are derived from the same iPS cell clone.

The main challenge when attempting to establish an iPS cell-based in vitro model is to establish a phenotype that correctly recapitulates the disease in vitro. This is one major limitation for studies attempting to establish an iPS cell-based model for osteoarthritis which is not necessarily caused by a genetic defect but rather by multiple concurrent factors including age, mechanical overload, or trauma and genetic predisposition. Thus, even iPS cells reprogrammed from osteoarthritic chondrocytes have so far not been shown to recapitulate any relevant disease mechanism in the dish (Kim et al. 2011; Lee et al. 2014; Wei et al. 2012). Obviously, genetic diseases where the causative mutation is known like skeletal dysplasias seem far better suitable for iPS cell models. However, even with known genetic mutations, obtaining a phenotype can prove challenging, because the genetic mutation alone might not be sufficient to induce a functional defect under basal culture conditions without additional stressors (Kim et al. 2013). Diseases that manifest after childhood or at an advanced age might be hard to recapitulate in short-term culture. To imitate aging, cellular stressors could be applied or trophic factors

withdrawn. Such strategies may help establishing an iPS cell model even for osteoarthritis in the near future.

With respect to diseases caused mainly by genetic defects, first very promising results have been reported. In a remarkable study, the Tsumaki group has reprogrammed fibroblasts from patients with thanatophoric dysplasia and achondroplasia caused by FGFR3 gain of function mutations (Yamashita et al. 2014). During in vitro differentiation, the disease-specific iPS cells were less able to form cartilaginous tissue, exhibited decreased proliferation, and increased apoptosis. The phenotype was rescued by knocking down the causative FGFR3 activity, thus proving that the FGFR3 mutation in these cells caused the phenotype. Once they had verified that the diseased iPS cells recapitulated the main abnormalities typical for patients and models of FGFR3-related diseases, the authors went on to screen for substances capable to rescue the diseased phenotype and found statins to improve cell survival and increase proliferation and cartilage formation. In a chondrodysplasia mouse model, statins were subsequently found to rescue reduced bone growth. Statins are clinically approved to reduce cholesterol levels but have so far not been considered for the treatment of chondrodysplasia. Thus, this iPS cell model has enabled the discovery of a new treatment strategy, has given first insights into the mechanisms of statin effects on chondrodysplasia cells, and has a high potential for further mechanistic studies of FGFR3-related chondrodysplasia.

Another interesting study focused on neonatal-onset multisystem inflammatory disease (NOMID) which is an inherited auto-inflammatory disease caused by NLRP3 mutations (Yokoyama et al. 2015). Utilizing their NOMID iPS cells as an in vitro disease model, the authors assessed the question why the epiphyseal overgrowth in NOMID is resistant to anti-IL-1β therapy while systemic inflammation can effectively be controlled with anti-IL-1β treatment. The data suggested that in chondrocytes with disease-causing mutations in NLRP3, the chondrogenic master transcription factor SOX9 was abnormally strongly expressed, induced by the cAMP/PKA/CREB signaling pathway, and this may cause overproduction of extracellular matrix independently of the NLRP3 inflammasome. This study excellently demonstrated the strength of iPS model to elucidate mechanisms and to thus promote the development of new therapies. Another very interesting aspect of this study was the derivation of isogenic healthy control cells enabled by the genetic mosaicism of the disease. In such cases like NOMID, the primary patient cell population comprises both wild-type and mutated cells, and, thus, iPS cell clones can be derived that differ only in the altered chromosome or mutated gene and the integration sites of the introduced pluripotency factors. Indeed, isogenic cells seem the ideal controls, given the increasingly realized fact that every person carries disease-relevant single-nucleotide polymorphisms (Abecasis et al. 2010) meaning that essentially no iPS cell line could be defined as "healthy." Such isogenic controls can also easily be obtained when the disease-causing mutation is introduced into a well-described control iPS cell line or when the mutation of a patient-derived cell line is corrected via gene editing techniques.

A third insightful study established an iPS cell model for type II collagenopathy (Okada et al. 2015), which comprises a number of diseases caused by

heterozygous mutations in the collagen type II gene. While previous investigations had been limited by difficulties to obtain live chondrocytes from patients, the authors generated iPS cells from dermal fibroblasts. In addition, they also directly induced the chondrocyte phenotype in diseased fibroblasts by ectopically expressing two pluripotency factors KLF4 and C-MYC in combination with the chondrogenic master transcription factor SOX9. This technique of direct conversion of fibroblasts into chondrocytes is another potential cell source for cartilage regeneration. Bypassing the stage of pluripotency, direct conversion certainly promises to be a quicker way of generating chondrocytes from an unrelated cell source than reprogramming and subsequent differentiation into chondrocytes. However, only at the iPS cell stage, cells are indefinitely expandable, and thus limited cell numbers become an issue yet again. The authors used direct lineage conversion into so-called iChon cells complementary to the iPS technology. They showed that both diseased iChon cells and chondrocytes differentiated from diseased iPS cells recapitulated the expected pathological features. Collagen type II secretion was severely limited and accompanied by increased expression of stress markers of the endoplasmic reticulum as well as increased apoptosis. Cartilage in teratomas generated from diseased iPS cells in vivo resembled the pathological cartilage tissue in patients with type II collagenopathy. Intracellular retention of collagen type II was accompanied by an apparently decreased density of the extracellular matrix. In general, the amount of cartilage in disease-specific teratomas was decreased compared to teratomas obtained from control iPS cells. Beyond successful recapitulation of the disease phenotype, the data gave new insights into the pathomechanisms of type II collagenopathy and suggested that cellular stress and apoptosis are the central issues of this disorder rather than chondrogenic maturation which seemed to be less affected. Moreover, chemically increasing protein folding seemed to reduce cellular stress and apoptosis, while challenging the cells by forcing chondrogenic differentiation and collagen type II synthesis further increased cell stress and apoptosis.

This study demonstrated that the iPS cell technology can be successfully complemented by direct lineage conversion that can quickly provide a certain amount of cells for a number of assays. Generating iPS cells and subsequent differentiation into the cells of interest can take several months, but the infinite expansion capacity of iPS cells enables high-throughput drug screening. Moreover, disease-specific teratomas even allow in vivo studies in human tissues to test specific drugs and novel therapies.

In summary, in vitro iPS cell models are particularly valuable for cartilage diseases like skeletal dysplasias that are directly caused by genetic variations. Pathomechanisms that are not correctly recapitulated in animal models can be investigated in vitro, and high-throughput screenings can be performed to find new therapeutic substances. Novel gene editing methods enable generating adequate controls, and first studies have already yielded promising results by finding additional effective therapeutics (Yamashita et al. 2014) or by enlightening the mechanism behind a phenotype resistant to current therapy (Okada et al. 2015; Yokoyama et al. 2015).

4.5 Challenges and Gaps of Knowledge

The new field of iPS research has evolved tremendously in the past years. The possibilities of a universal cell source with infinite expandability that can be generated by simply overexpressing a defined set of transcription factors currently seem tremendous. Certainly, to harness the full potential of iPS cells, we need a more detailed understanding of the highly artificial and massively invasive cellular reprogramming process. Stringent quality controls and elucidation of the pluripotency network will help minimizing iPS cell variability that can arise from diverse origins. These include the variable presence of preexisting mutations in the originating cells (Gore et al. 2011), incomplete reprogramming including retained epigenetic memory and aberrant reprogramming of DNA methylation (Lister et al. 2011), newly arising mutations and changes of gene copy number during reprogramming and culture (Gore et al. 2011; Laurent et al. 2011), as well as aberrations in imprinting (Trounson et al. 2012). Of course, such variants can considerably affect the capacity of iPS cells to differentiate into cells desired for cell-based therapy or disease models. Our continuously advancing ability to assess whole-genome DNA methylation, genome-wide expression, and the complete cellular metabolome will help setting up sophisticated quality controls and thus to standardize cellular reprogramming (Muller et al. 2011).

With regard to clinical safety, a plethora of new techniques has been developed to overcome the potential risks of iPS cells. To avoid retroviral transgene delivery that results in random integrations into the genome, thus risking insertional mutagenesis and proto-oncogene activation, non-integrating vectors can be used. Adenoviral vectors (Stadtfeld et al. 2008), nonviral and episomal vectors (Yu et al. 2009), expression plasmids (Okita et al. 2008), and piggyBac transposition (Woltjen et al. 2009) have already been successfully applied for cellular reprogramming. Also, the Cre-recombinase system (Soldner et al. 2009) can be used to excise the transgenes after iPS cell generation when the endogenous pluripotency network is activated. Small molecule-based chemical reprogramming (Hou et al. 2013; Shu et al. 2013), protein-based strategies (Zhou et al. 2009), and introduction of microRNA clusters (Anokye-Danso et al. 2011; Lin et al. 2008) promise to eliminate many drawbacks by completely avoiding genetic manipulation and the ectopic expression of oncogenes.

Significant progress has also been made with regard to the inherent risk of tumorigenicity which requires all iPS cells to be removed from cell-based therapeutics before implantation. Sophisticated purification strategies using lineage-specific reporters (Tabar and Studer 2014) enable identification and enrichment of specific cells, i.e., cells committed to a certain lineage or residual pluripotent cells (Tang et al. 2011). Also, so-called suicide genes have been proposed for eliminating all transplanted cells in case of any adverse effects (Di Stasi et al. 2011).

Furthermore, xeno-free and GMP-compliant culture conditions have been developed in order to generate clinical-grade iPS cells (Nakagawa et al. 2014). Suspension culture and large-scale culture conditions are being tested for iPS cells and their offspring in order to overcome the current elaborate culture in small colonies and to provide large cell quantities (Haraguchi et al. 2013; McLaren et al. 2013; Olmer et al. 2012).

In summary, the major current challenges are the considerable variability of independently derived iPS cells and the generation of sufficient amounts of relevant iPS cell progeny with acceptable efforts. For clinical application, safety issues are of major concern, and insertional mutagenesis, potential tumorigenesis, and ectopic tissue growth need to be controlled. Sophisticated technologies and continuously increasing insights into stem cell biology have already brought us close to overcoming these hurdles in the near future.

4.6 Summary

IPS cells are on the verge of becoming a valuable tool for cartilage regeneration. First, iPS cell-based in vitro models of genetic cartilage diseases have resulted in novel insights into pathomechanisms induced, for example, by a constitutively active FGF receptor or misfolded collagen. A new treatment strategy for FGFR-related skeletal dysplasia with statins has been proposed and can now be tested. The promising results of these first cartilage-related iPS cell-based disease models will certainly spur the generation of models for other cartilage diseases including dysplasias caused by mutations in other collagens (types IX, XI), SOX9, filamin B, COMP, matrilin, PTHR1, or the sulfate transporter SLC26A2. High-throughput screens that had so far relied on immortalized cell lines with atypical phenotypes can now be performed with human disease-specific cells. Also in vivo tests with human tissues formed in iPS cell-derived teratomas are possible. Thus, the true strength of iPS cells for cartilage regeneration currently is to serve as the most realistic human in vitro model for disease mechanisms as well as for normal human development. Thus, iPS cell research will increase our understanding of the processes and regulatory mechanisms during chondrogenic differentiation, cartilage formation, and tissue homeostasis and will teach us to selectively manipulate these processes.

In addition, iPS cells are also highly attractive for cell-based therapies and offer to overcome the invasive tissue harvest procedure and limited supply for autologous chondrocyte implantation. Whether mesenchymal intermediates like iMPCs may replace iPS cell-derived chondrocytes as the most attractive therapeutic cells will depend on our ability to improve iMPC generation strategies to give rise to homogeneous, highly active cells. Stringent standardization of reprogramming and iMPC derivation could be envisioned to yield “super MSCs” with high regenerative capacity that is not afflicted with endochondral priming and donor variability. Of course, the regulatory hurdles for novel cell therapies are high and have so far prevented the translation of MSC-based therapies into clinical application. In contrast to MSCs, however, iPS cells are the only potential cell source for life-threatening diseases like cardiac insufficiency, kidney failure, Parkinson’s disease, or stroke damage, and such fatal maladies might spur the approval for clinical application. Our recent advances to generate clinical-grade iPS cells are very promising, and the establishment of iPS cell banks as well as first in man clinical phase I trials for macular degeneration indicates that iPS cell therapy might one day become reality.

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Cell-Based Cartilage Regeneration

5

Johannes Zellner and Peter Angele

Abstract

Since decades various regenerative cell-based treatment options have been developed for cartilage repair. With the introduction of the autologous chondrocyte transplantation, also large-sized chondral defects can be successfully addressed. This chapter gives a short overview about current procedures for cell-based treatment strategies like bone marrow stimulation techniques, osteochondral transplantation, and chondrocyte transplantation. Requirements and outcome parameters for a successful treatment and future directions in cartilage regeneration are discussed. Finally treatment recommendations according to cartilage defect size and depth are given.

5.1 Introduction

Articular cartilage injuries are common. They can result from acute traumatic injuries, posttraumatic or early degenerative changes, osteochondritis dissecans, or avascular necrosis. Numerous reports analyzing high numbers of arthroscopies show cartilage lesions in up to 60% of the patients (Widuchowski et al. 2007). The incidence of chondral injuries indicates the high impact on the society, as it is

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generally agreed that the persistence of cartilage defects is a risk factor for joint dysfunction, which finally may lead to severe osteoarthritis.

Cartilage lesions can remain symptomless over a long period of time. This can cause a delayed diagnosis and late treatment of cartilage injuries, which may have negative consequences for joint recovery. Clearly, this emphasizes the importance of an adequate regenerative treatment of cartilage lesions at the earliest time point in order to prevent onset and development of osteoarthritis (Zellner et al. 2015).

Since decades regenerative treatment options for small- and middle-sized cartilage lesions were developed like, e.g., Pridie drilling or microfracture. With the introduction of the "autologous chondrocyte transplantation (ACT)" technique by Brittberg et al. in 1994, also large-sized cartilage defects can be successfully addressed via a regenerative approach (Brittberg et al. 1994).

Recently many randomized clinical trials investigated the efficiency and the quality of different cartilage repair procedures. Most of them enrolled young and active patients with "ideal" chondral defects that were focal and isolated with clearly defined borders (Lattermann and Luckett 2011). However, in reality, most patients that present with clinically symptomatic chondral lesions do not fulfill these criteria. Consecutively a detailed analysis and assessment of the cartilage defect and all underlying pathologies should be performed. Specific comorbidities have to be taken into account prior to performing regenerative cartilage repair as they may require additional concomitant or staged surgical procedures.

All cell-based cartilage repair strategies like bone marrow stimulating techniques or autologous chondrocyte transplantation require a correction of the comorbidities like malalignment, meniscal deficiency, instability, or pathologies of the subchondral bone. Only if the comorbidities are addressed sufficiently, the chance for appropriate cartilage regeneration is achievable.

Axis deviations can cause overload of an affected joint compartment. For cartilage treatment, malalignment needs be corrected to restore normal load distribution that allows the repair tissue to adjust to physiological loads. Corrected patellofemoral and tibiofemoral alignment improves clinical outcome when realignment operations are performed concurrently with the cartilage repair or as a staged procedure (Behery et al. 2014).

Another factor contributing to successful cartilage regeneration is the meniscal status. As the menisci are critical for shock absorption and load distribution in the knee joint, meniscal deficiency also affects cartilage regeneration (Makris et al. 2011). Meniscal lesions should be treated adequately in combination with cartilage regeneration. Because of the direct correlation between the lost amount of meniscus tissue and the increase of load on the surrounding cartilage, as much meniscus substance as possible should be restored (McDermott and Amis 2006) which can be achieved by suturing or limited partial resection. In case of previous subtotal meniscectomy, also meniscal supplementation or allograft transplantation should be discussed in order to restore a normal joint physiology for cartilage regeneration.

It has been clearly shown that joint instability contributes to a significant increase in cartilage lesions. In the long term, ACL insufficiency is a negative predictor for development of knee osteoarthritis. Therefore the correction of ligamentous

instability by ligament reconstruction is a mandatory requirement for regenerative cartilage treatment.

Besides the alignment, meniscal integrity, and knee stability, the status of the subchondral bone is crucial for a successful cartilage repair. It has been shown that the adjacent bone quality affects the regeneration of the cartilage defect (Gomoll et al. 2010). So the state of the subchondral bone needs also to be taken into account for planning a regenerative cartilage repair procedure.

In conclusion, analysis, evaluation, and correction of all comorbidities and underlying pathologies are mandatory requirements for a good clinical outcome after cell-based cartilage regeneration procedures.

5.2 Regenerative Treatment Options for Cartilage Repair

5.2.1 Bone Marrow Stimulation Techniques

Due to their simplicity and low costs, bone marrow stimulation techniques are the most commonly used procedures among regenerative options for cartilage treatment worldwide. Developed by Steadman in the 1980s as an enhancement of tissue response techniques like drilling (Pridie) and abrasion arthroplasty (Johnson), this widely used procedure is generally regarded as safe and effective. Cartilage repair with the microfracture technique involves several systematic steps, including debridement to a stable cartilage margin creating a stable defect containment, careful removal of the calcified cartilage layer with special curettes or shavers, and homogeneous microfracture penetrations within the cartilage defect with specific awls perpendicular to the subchondral bone plate. This procedure results in a complete defect filling by a well-anchored mesenchymal clot (Mithoefer et al. 2009b) (Fig. 5.1).

The aim is to recruit bone marrow cells via creating a communication between cartilage lesions and subchondral bone to get access to potential cartilage precursor cells. Stem cells migrate from the marrow cavity to the fibrin clot of the defect and promote the formation of a fibrocartilaginous tissue (Marcacci et al. 2013). Arthroscopically performed microfracturing is a cost-saving procedure with a low complication rate and mainly successful for small defects. However the development of tissue hypertrophy or formation of soft scar tissue that lacks the mechanical characteristics of hyaline cartilage are disadvantages of this specific treatment (Fortier et al. 2012).

According to the recent literature, good indications for treatment of chondral lesions with microfracture are:

- Small defect size (<3 cm^2)
- Full-thickness, traumatic cartilage defects (Outerbridge grades III and IV) with full containment
- Intact articulating joint surface
- Patient age between 18 and 50 years

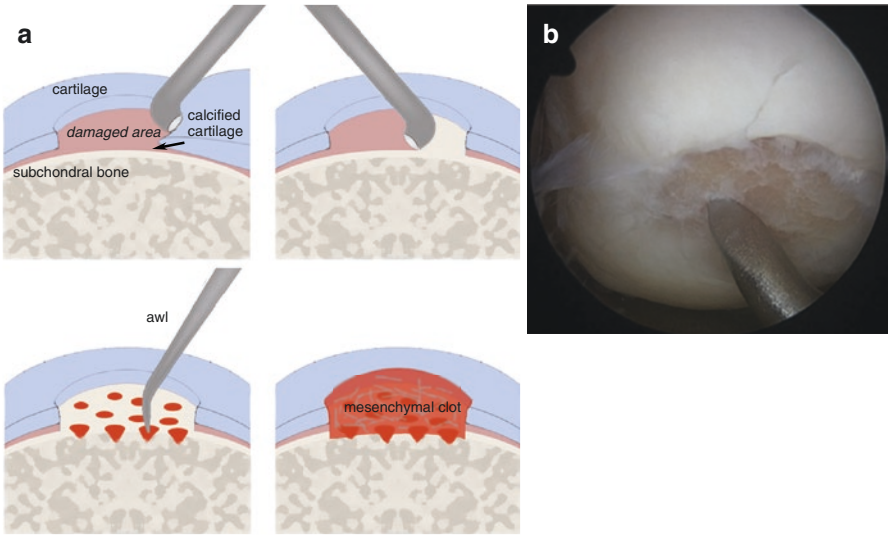

Fig 5.1 (**a**) Schematic drawing of cartilage repair using the microfracture technique. After debridement in order to create stable defect edges (*upper left*), the calcified cartilage layer is removed (*upper right*), and microfracture penetrations are placed in the defect with a distance of 3–4 mm (*lower left*), resulting in defect filling with a mesenchymal clot (*lower right*) (adopted from Mithoefer et al. 2009b). (**b**) Arthroscopic view of a microfracture procedure for treatment of a full-size cartilage defect at the lateral femoral condyle after preparation of stable defect edges (containment)

- No or corrected concomitant morbidity (axis deviation, instability, meniscus injuries, subchondral bone pathology)
- Full range of motion of the affected joint

An important factor for a successful outcome after microfracture treatment seems to be the postoperative rehabilitation protocol. Partial weight bearing with no more than 20 kg and the use of CPM is recommended for 6 weeks without limitation of the range of motion (Steadman et al. 2001).

In a systematic review, Mithoefer et al. (2009b) observed that microfracturing provides effective functional improvement for at least 2 years. In smaller defects, microfracture shows promising results concerning mobility, reduction of pain, and return to sport (Kon et al. 2011). Positive prognostic factors for a successful cartilage treatment with microfracture are size smaller than 3 cm^2, BMI less than 30 kg/m^2, femoral defects, age younger than 40, interval of pain less than 1 year, and no previous knee surgery. However, recent reports show that over time the results of microfracture are getting worse especially in active patients and larger chondral defects 5 years after the procedure. Additionally, the effects of microfracture are patient age related, meaning that older patients do not seem to profit at the same extent from this specific treatment as compared to young patients (Kreuz et al. 2006; Mithoefer et al. 2009a). The repair tissue response is often unpredictable; fibrous, soft, spongiform tissue combined with central degeneration is frequently found, and

patients may have to adjust their activity level to that of their knee function (Nehrer et al. 1999). Another reason for the deterioration of the clinical outcome after microfracture over time might be the development of subchondral sclerosis and cysts or the formation of intralesional osteophytes. Consecutively a complication rate of up to 50% after microfracturing is described in literature after 5 years. Recent results published in the literature recommend using these procedures only for the treatment of acute and small lesions and not in large cartilage defects anymore.

Further developments in the field of bone marrow stimulating techniques try to overcome the shortcomings of the procedures. In combination with the microfracture technique, coverage of the prepared and treated chondral defect site by a biomaterial is becoming more and more popular. This enhanced procedure was first described by Gille et al. (2010). The so-called autologous matrix-induced chondrogenesis (AMIC) reveals promising results in terms of functional outcome. In a prospective study, Gille et al. investigated 27 patients up to 62 months with a mean defect size of 4.2 cm^2. 87% of the patients showed an increase in functional outcome scores like ICRS, Tegner, Cincinnati et al. compared to the preoperative status. In another study, the same authors detected a significant decrease of pain in the VAS after 1 and 2 years postoperatively (Gille et al. 2013). Kusano et al. (2012) also detected significant improvements in functional scores and pain reduction after 29 months, but MRI findings showed generally incomplete or inhomogeneous tissue filling. Comparing AMIC with the original microfracturing technique, Anders et al. (2013) found no significant differences in the IKDC or Cincinnati score at 1- or 2-year follow-ups. A recent study has shown an improvement in repair tissue quality by enhancing microfracture with a chitosan-based biomaterial (BST-CarGel; Piramal, Laval, Quebec, Canada) (Stanish et al. 2013). Mixed with autologous blood, it stabilizes the clot and enhances marrow-derived repair in the microfractured cartilage lesion. Using this technique, Stanish et al. observed an equivalent clinical benefit compared to microfracturing alone, but a greater defect filling and superior repair tissue quality in MRI evaluation. Further studies and long-term results will show whether enhanced microfracture techniques are really capable to overcome the shortcomings of the original procedure regarding the development of intralesional osteophytes or formation of subchondral cysts. However, there is doubt whether these modifications make microfracture-based techniques more appropriate for treatment of large-sized chondral defects.

Recently the technique of microfracturing has been modified to a microdrilling method. The idea of drilling holes through the damaged cartilage area into the subchondral bone marrow space to stimulate repair tissue was first described by Pridie. Thermal necrosis was a potential disadvantage that could affect the outcome. The improved modern microdrilling version with arthroscopically applicable narrow-caliber drills up to 4 mm in depth is more reproducible and less traumatic. In an animal model, Chen et al. compared this “micro-Pridie” drilling method histologically with standard microfracturing. While microfracture caused compacted bone formation around the created holes that sealed them off from viable bone marrow, drilling cleanly removed the bone from the holes and provided access channels to marrow stroma. Heat necrosis was not seen in the drilling group (Chen et al. 2009).

Furthermore Eldracher et al. demonstrated improved osteochondral repair by application of smaller drill holes that reflect the physiological trabecular distance in a translational sheep model. They conclude to use small-diameter bone-cutting devices for subchondral drilling (Eldracher et al. 2014). However, no prospective clinical trial has shown significant improvement of the microdrilling method over the original microfracture technique yet.

5.2.2 Autologous Osteochondral Transplantation

Focal (osteo-)chondral defects may also be addressed with osteochondral autograft transplantation (OAT). It is the only method to transfer native hyaline articular cartilage into the defect area. Harvesting and subsequent implantation of autologous osteochondral plugs is performed in a one-step procedure. The plugs are frequently taken via a small incision from a non-weight-bearing area such as the medial or lateral margin of the trochlea or the intercondylar notch. This procedure guarantees a tissue transfer of viable osteochondral units that aims to integrate via bone-to-bone healing, since the mature cartilage tissue has limited healing potential and rarely fully heals and integrates with surrounding cartilage. The fast bone-to-bone integration allows a rehab program with a rapid increase in weight bearing. In the early 1990s, Hangody conceived and perfected the mosaicplasty technique, which uses multiple small-diameter osteochondral plugs that can be implanted also through an arthroscopic approach, and good results have been reported at long-term follow-up, particularly for defects up to 4 cm^2 (Hangody et al. 2010). Especially deep focal chondral defects which affect the subchondral plate or small cartilage lesions with pathologies of the subchondral bone like cysts may be responsive to a treatment with OAT. In controlled randomized prospective studies, Gudas et al. (2012) showed significantly better clinical results after 12, 24, and 36 months comparing OAT versus microfracture. Compared to other regenerative treatment options, OAT requires the shortest postoperative time of partial weight bearing during the rehabilitation period. Consecutively, time to return to sport is diminished. However, with increasing defect size, complication rate rises due to integration problems and donor site morbidity. Therefore, treatment of chondral defects larger than 3–4 cm^2 with OAT is no longer recommended in literature.

5.2.3 Autologous Chondrocyte Transplantation

The treatment of choice for large full-thickness articular cartilage defects is the matrix-guided autologous chondrocyte transplantation (MACT).

Brittberg et al. (1994) first introduced the technique of the autologous chondrocyte transplantation (ACT) in 1994. Particularly for treatment of cartilage defects larger than 3 cm^2, the ACT method revealed superior long-term success (Bentley et al. 2012). The conventional technique is accompanied with periosteum harvest and fixation over the cartilage defects via large skin incisions. Autologous

chondrocytes were injected underneath the periosteal flap. Hypertrophy of the periosteum with high rate of revision arthroscopies and the risk of transplant failure of up to 20% were major drawbacks of the conventional autologous chondrocyte transplantation technique.

MACT was developed to address these problems. In a first arthroscopy, small osteochondral plugs are taken from the non-weight-bearing cartilage adjacent to the lateral femoral notch. Then the chondrocytes are isolated, cultured, and seeded on biodegradable scaffolds. Approximately 3 weeks after the first arthroscopy, the cell-seeded scaffolds are implanted into cartilage defects. Therefore, the lesion is prepared by removal of the calcified cartilage layer and creation of containment with stable rims of the defect. The cell-matrix construct is then fixed in the defect with sutures or biodegradable devices like plugs or anchors (Fig. 5.2).

Another technique uses self-adhering chondrospheres to fill the defect. These further developments of the ACT technique enable to minimize the incision and to perform the procedure in a "mini-open" way or arthroscopically. Consecutively the rehabilitation time was reduced and the complication rate diminished.

With the new MACT technique, also some other disadvantages of the ACT were eliminated (Harris et al. 2011). The rate of hypertrophy of the transplant that may be caused by the biologically active periosteal flap was reduced by the matrix-guided technique.

An advantage of the second and third generation of the autologous chondrocyte transplantation is the scaffold-based technique that also simplified the surgical procedure. The biomaterial represents a temporary 3D structure of biodegradable

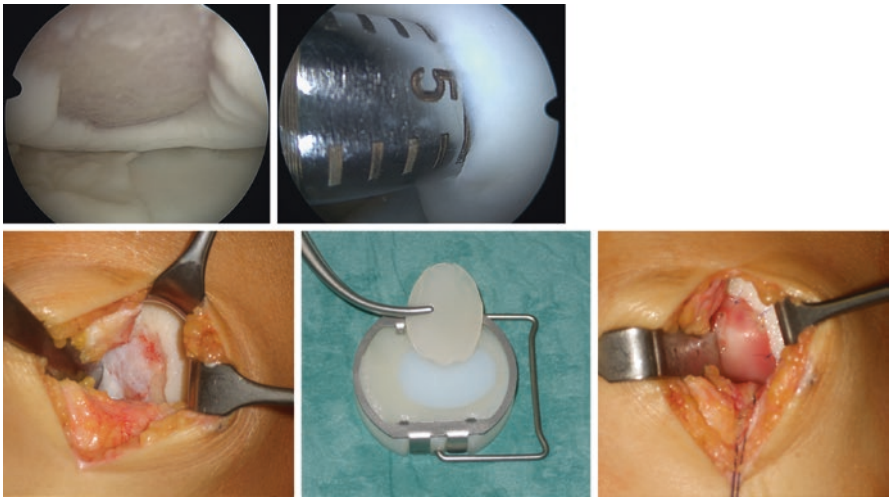

Fig. 5.2 *Upper row*: arthroscopic evaluation of a full-size cartilage defect at the lateral femoral condyle and harvesting of osteochondral plugs from the medial edge of the lateral notch border for further culture of autologous chondrocytes. *Lower row*: 3 weeks after cell harvest, the defect at the lateral femoral condyle is prepared via mini-arthrotomy. After cutting the autologous chondrocyte transplant to the correct size, the cell-seeded implant is placed into the defect and fixed with sutures

polymers, which favors the growth of the specific cartilaginous cell type. An ideal scaffold should mimic the biological and structural properties of native cartilage in order to enable cell infiltration, attachment, proliferation, and differentiation. The matrix should be biocompatible and biodegradable in order to support initial tissue formation and then to be gradually replaced by the regenerated tissue. The different three-dimensional biomaterials support the redifferentiation process, cell protection in the initial phase, and a homogenous cell distribution in the defect.

Compared to other reconstructive therapy options for cartilage defects like microfracturing, MACT restores the cartilage defect up to date with the best quality of the regenerated tissue (Vavken and Samartzis 2010).

Especially for full-thickness cartilage defects larger than 4 cm^2, MACT is the recommended therapy in literature (Niemeyer et al. 2016). Other cartilage therapy procedures failed to improve the clinical outcome of cartilage defects of that size.

In a controlled randomized prospective study for large-sized chondral defects (4–10 cm^2), the outcome after MACT was significantly better after 2 years compared to microfracture (Basad et al. 2010). Similar long-term results were seen for active patients comparing MACT with microfracture. In another randomized prospective study, Crawford et al. saw significantly more therapy responder in the MACT group compared to the microfracture group after 6, 12, or 24 months. These results correlated to the clinical and functional outcome of the patients in the KOOS and IKDC scores (Crawford et al. 2012).

The reason for the superior results after MACT compared to microfracture might be the better defect filling, the histological results, and the lack of osteophytes in the defect site or the regenerated tissue, which can be predominantly detected 4 or 5 years after microfracture (Zellner et al. 2015).

However, if microfracture fails as primary procedure for treatment of a chondral defect, the risk of treatment failure after the secondary performed MACT increases significantly. For that reason some authors do not recommend microfracture as a first-line treatment especially for larger defects. On the other hand, there are reports in literature, which reveal good results of MACT even as a second-line therapy procedure. Additionally the age-related effects of a cartilage therapy seem to be less significant with MACT in comparison to microfracture.

In a controlled randomized prospective study, Bentley et al. (2012) showed significantly better outcome results after ACI compared to OAT. The best clinical results of MACT were observed for traumatic chondral lesions and for osteochondrosis dissecans. On the other hand, degenerative cartilage defects and chronic lesions are still difficult to treat, especially when patients with a long history of pain show a significantly worse outcome after MACT (Angele et al. 2015).

In a published study, Vanlauwe et al. compared ACI with microfracture and showed a significant improvement of patients' outcome after MACT when the symptoms of the cartilage lesion did not last more than 3 years. On the other hand, in patients with clinical symptoms more than 3 years, ACI failed to improve the functional outcome significantly compared to microfracture (Vanlauwe et al. 2011).

The earlier a biological cartilage repair is performed, the better are the clinical results. Consecutively primary cartilage defects should be treated as soon as possible to improve the long-term outcome.

Another problem for biological cartilage repair is the localization of the defect. Results of all treatment options behind the patella are worse than in other parts of the knee joint (Niemeyer et al. 2011). Also in the original description of the ACI technique, Brittberg et al. observed significant more treatment failures for defects in the patellofemoral compartment. Probably the special biomechanical situation in the retropatellar area is the reason for the higher rate of cartilage treatment failure. As this is not a problem of a specific cartilage repair procedure, the necessity arises to address all pathologies for a successful cartilage treatment behind the patella. Comparable to malalignment of the leg axis, knee instability, or meniscal tears in the femorotibial compartment, all pathologies like maltracking of the patella or dysplasia in the retropatellar area should be corrected.

As mentioned above the status of the subchondral bone is crucial for successful cartilage regeneration. For deep osteochondral defects like in osteochondritis dissecans, the MACT can be combined with bone augmentation like cancellous bone grafting or autologous bone transplantation, e.g., from the iliac crest. After reconstruction of the osseous part, the defect is covered by MACT.

Macroscopic and histological findings play an important role after MACT. For the evaluation of the quality of the regenerated tissue, not only histological findings but also the amount of defect filling, the surface quality, and the integration into the surrounding native cartilage are important (Nehrer et al. 1999).

It has been shown that complete defect filling with differentiated tissue correlates with good clinical results. On the other hand, incomplete defect filling with undifferentiated scar tissue reveals unsatisfying scoring results with ongoing pain and worse joint function of the patients (Henderson et al. 2007). This effect can be particularly seen in larger chondral defects. In a pilot study, we reported that the transplant quality is adequate at the time of surgery of MACT. We retrospectively reviewed 125 patients with large localized cartilage defects (mean defect size 5 cm^2) of the knee who were treated with MACT. Portions of the cell-matrix constructs that were not implanted in the cartilage defects were further cultured and tested for their potential to form articular cartilage. In vitro assessment of the cell-matrix implants showed chondrogenic differentiation with positive staining for glycosaminoglycans and collagen II in all cultures. Enzyme-linked immunosorbent assay confirmed an increase of collagen II production. Clinically, we observed an improvement in median IKDC score from 41 to 67 points at the last follow-up indicating that cartilage extracellular matrix deposition shows adequate implant quality for MACT at the time of implantation and justifies the use for treatment of large cartilage defects (Zellner et al. 2013).

Besides regulatory restrictions and high costs, a disadvantage of today's autologous chondrocyte transplantation is the necessity of two steps for the surgical procedure. After cell harvest a certain time of cultivation and expansion of the chondrocytes is mandatory prior to the application. Consecutively, the patient needs two operations plus the phenotype and quality of the transplanted chondrocytes

might be affected. Future directions are aiming for the development of one-step procedures.

Appropriate cell types might help to affect the complexity of ACI and simplify surgical procedures. Alternative cell sources are allogenic cells or mesenchymal stem cells. Allogenic chondrocytes can help to reduce donor site morbidity. In combination with a biocompatible and chondroinductive matrix, allogenic chondrocytes harvested from neonatal donors or from donor's knee joints within 24 h of death may be used in a single-stage procedure (Farr et al. 2014). Preliminary results demonstrated a safe and effective treatment for cartilage defects with a mean lesion size of 2.7 cm^2. Clinical outcomes showed significant improvement over baseline and favorable histological repair tissue 2 years postoperatively. Dhollander et al. reported of midterm results after implantation of alginate beads containing human mature allogenic chondrocytes in cartilage lesions of the knee. Twenty-one patients were followed for an average period of 6.3 years, and a significant improvement in WOMAC and VAS scores was observed. However, four failures occurred and MRI evaluation with the MOCART score only revealed moderate values (Dhollander et al. 2012).

Autologous adult mesenchymal stem cells (MSCs) are a potential cell source for a single-step cell-based treatment of large cartilage defects. MSCs have a better proliferation rate than chondrocytes and a high potential for differentiation into several lineages including chondrogenesis. Autologous MSCs can derive from many sources. Particularly, bone marrow-derived MSCs (BMSCs) combine many advantages as they are easy to isolate and to store. Extensive preclinical and clinical work has shown that BMSCs can differentiate into cartilage among other tissues. Other potential sources for MSCs are adipose tissue, muscle, synovium, periosteum, and umbilical cord. Nejadnik et al. (2010) analyzed the clinical outcome of patients treated with autologous BMSCs compared to patients treated with first-generation ACI for large cartilage defects in the knee. After 2 years a similar functional outcome regarding IKDC, Lysholm, or Tegner scores was found. The authors concluded that using BMSCs for articular cartilage repair is as effective as chondrocytes. In addition, it required one less knee surgery, reduced costs, and minimized donor site morbidity. However, in some countries, regulatory burdens might be a problem for implementing the use of autologous mesenchymal stem cells into daily clinical practice.

5.2.4 Current Treatment Recommendations for Chondral Injuries

In their current review, Niemeyer et al. (2016) provide a concise overview on important scientific background issues and the results of clinical studies discussing advantages and disadvantages of ACI and other cartilage treatment options. They describe the biology and function of healthy articular cartilage, the present state of knowledge concerning potential consequences of primary cartilage lesions, and the suitable indication for ACI. Based on current evidence, an indication for ACI is given

for symptomatic cartilage defects starting from defect sizes of more than 3–4 cm^2 in the case of young and active sports patients at 2.5 cm^2. Smaller lesions are supposed to be treated by bone marrow stimulating techniques like microfracturing. However, the status of the subchondral bone will influence the decision-making process for cartilage therapy. Smaller defects with pathologies of the whole osteochondral unit are best treated with OAT. For large and deep osteochondral lesions, a combination of MACT and bone augmentation techniques is the favorable treatment option.

Conclusions

With increasing knowledge cell-based cartilage regeneration becomes a more and more routinely used technique with well-predictable outcome and results. As research activities are increasing in the field of regenerative joint therapy, recent developments help to overcome remaining limitations step by step. Simplification of regulatory burdens is needed to transfer rising knowledge and developments into daily clinical practice.

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Growth Factors in Cartilage Repair

6

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Abstract

The articular cartilage does not heal completely after injury, predisposing patients to accelerated progression of degenerative joint disease. While surgical intervention can address chondral defects and yield positive functional outcomes, substantial research has gone into the use of growth factors to augment cartilage repair and preclude or postpone the need for operative management. This chapter describes the growth factors with the most promising in vitro and in vivo data in cartilage repair, namely, bone morphogenetic protein-7, transforming growth factor-β, fibroblast growth factor-18, connective tissue growth factor, insulin-like growth factor-1, and recent advancements with autologous solutions of growth factors, such as platelet-rich plasma. Each section provides a background on mechanism of action, summarizes pivotal basic science research, and describes the results of clinical application in animal and human models of chondral disease.

6.1 Introduction

The optimization of cartilage repair both in the setting of acute, post-traumatic chondral injury and in halting the progression of chronic degenerative disease remains a challenge to clinicians and researchers alike. Cartilage tissue is

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avascular, aneural, and alymphatic and receives all nutrients from the synovium via diffusion. The articular cartilage therefore does not heal completely after traumatic injury. Instead, spontaneous repair of cartilage produces tissue that fails to integrate with surrounding native cartilage and is inferior in both structure and function. The result is a nonuniform articular joint lining with greater susceptibility to inflammation, further injury, and progression of osteoarthritis (OA). Epidemiological consequences are profound, as knee OA affects between 19–28% of Americans over age 45 (Felson et al. 1987; Jordan et al. 2007). Of this number, 13–18% of patients had an identifiable acute trauma to the joint (Kern 1988), and data shows that post-traumatic arthritis can develop within 10 years of the initial insult (Roos et al. 1995). While arthroplasty addresses end-stage disease, there remains a window for intervention with biologic agents to prevent progression of OA after initial chondral injury. Ideally, such intervention must recreate a cartilage that is thin and compression resistant, has a low coefficient of friction, distributes load, and lasts for many years. To date, the use of recombinant growth factors has yielded appreciable cartilage repair both in vitro and in vivo and holds significant clinical promise for patients suffering from chondral injury.

Growth factors are circulating, biologically active polypeptides that stimulate and promote chondrocyte growth and differentiation (Goldring et al. 2006). They drive chondrocyte synthesis of extracellular matrix components, such as proteoglycans, aggrecan, and type II collagen. Aside from anabolic effects, many growth factors also have anti-catabolic effects on cartilage tissue by decreasing expression of local cytokines such as interleukin-1 (IL-1) and matrix metalloproteinases (MMPs) (Fortier et al. 2011; Pascual-Garrido and Chubinskaya 2015; Chubinskaya et al. 2011). Growth factors do not function in a vacuum, but rather have complex signaling interactions with other mediators. As described by Giannoudis, an accurate model for cartilage repair also takes into account chondrocyte interactions with the synovial environment and host bone, all of which are affected by mechanical forces and circulating cytokines (Fig. 6.1) (Giannoudis et al. 2008). For example, the presence of inflammatory cytokines (IL-1) and oxidative stress has recently been shown to decrease chondrocyte responsiveness to growth factor augmentation (Elshaier et al. 2009; Loeser et al. 2014). Thus, maximizing the chondroprotective capacity of growth factors necessitates understanding not only the expression of growth factors in healthy and in diseased cartilage but also their complex interactions with the surrounding synovial environment. The goal of this chapter is to familiarize the reader with growth factors showing the greatest clinical promise in cartilage repair and to provide an update on evidence supporting their use in chondral disease.

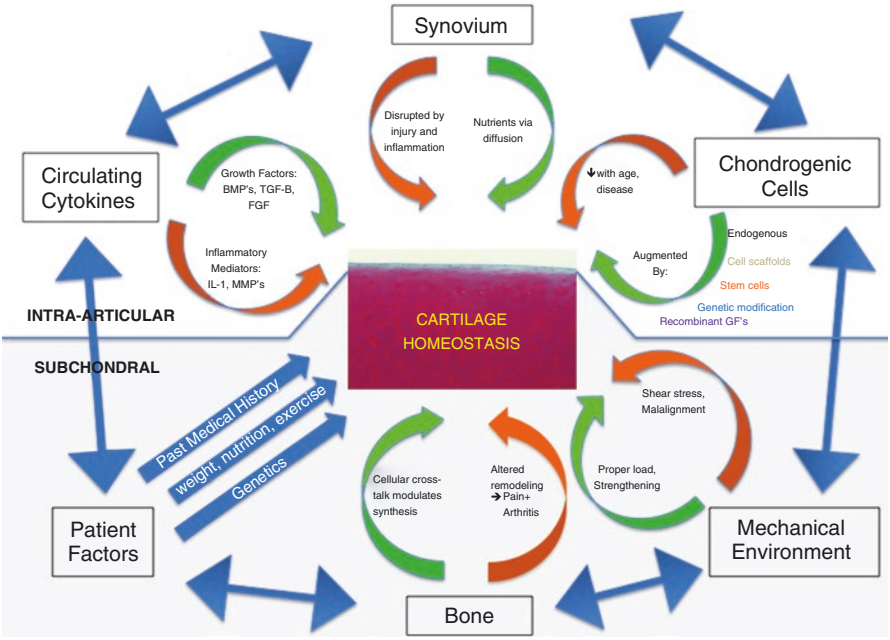


Fig. 6.1 Evaluation and treatment of articular cartilage defects have shifted toward treating the entire joint as an organ. In order to maintain integrity over time and to heal after minor damage, articular relies on interactions with several different local and systemic factors

6.2 Bone Morphogenetic Proteins

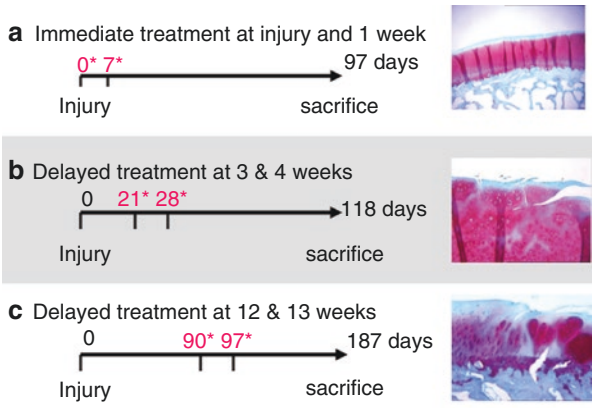
Of all growth factors, bone morphogenetic proteins (BMPs) have the most robust evidence from both in vitro and in vivo studies supporting their use for cartilage regeneration and repair. BMPs are members of the transforming growth factor-β (TGF-β) superfamily that have wide-ranging biological activities, including the regulation of cellular proliferation, apoptosis, differentiation and migration, embryonic development, and the maintenance of tissue homeostasis during adult life (Goumans and Mummery 2000; Massague and Chen 2000; Itoh et al. 2000). In chondrocytes, BMPs stimulate cartilage synthesis and decrease activity of catabolic cytokines, such as IL-1, IL-6, IL-8, MMP-1, and MMP-13 (Badlani et al. 2009; Elshaier et al. 2009; Chubinskaya et al. 2007b, 2008; Im et al. 2003). The ability of BMPs to induce an anabolic response in cartilage in vitro has been documented using different BMPs in multiple species, including human, bovine, rat, rabbit, and mouse, and a variety of culture conditions.

Though the BMP family has several growth factors, most data to date explores the use of BMP-7, also known as osteogenic protein 1 (OP-1). In vitro augmentation of both human and animal chondrocytes with BMP-7 has yielded increased production of cartilage-specific extracellular proteins, such as collagens type II and VI, aggrecan, decorin, fibronectin, and hyaluronan [HA] via upregulation of enzymes such as hyaluronan synthase (Nishida et al. 2000b; Chubinskaya et al. 2007a). When applied to other cell types in the knee, BMP-7 has been shown to increase ECM synthesis in synovial and bone marrow-derived MSCs, both alone and in combination with TGF-β (Miyamoto et al. 2007; Shen et al. 2010). This profound anabolic response stems from BMP-7 regulatory properties as a modulator of other growth factors, such as insulin-like growth factor-1 and fibroblast growth factor, as well as their receptors, kinases involved in signaling, inhibitory binding proteins, and downstream transcription factors (Chubinskaya et al. 2011). Furthermore, BMP has been shown to restore tissue responsiveness to IGF-1 (Chubinskaya et al. 2007a). BMP-7 also downregulates catabolic mediators (IL-1, IL-6, IL-8, IL-11, and tumor necrosis factor [TNF]-α) and inhibits both baseline and cytokine-induced expression of MMP-1 and MMP-13 (Im et al. 2003). Lastly, it modulates expression of receptors for certain matrix components, such as CD44 (Nishida et al. 2000b), and the synthesis of chondrocyte cytoskeleton proteins, such as talin, paxillin, and focal adhesion kinase (Vinall et al. 2002), bolstering the cartilage scaffold and strengthening newly formed tissue. While several growth factors have shown decreased efficacy with aged or diseased chondrocytes, BMP-7 induces an anabolic response across a variety of age groups and different stages of OA (Chubinskaya et al. 2007b). Despite its anabolic capacity, BMP-7 has not been shown to induce chondrocyte hypertrophy or other changes in chondrocytic phenotype, nor have BMP-7-treated animal knees displayed any histological evidence of uncontrolled fibroblast proliferation or radiographically detectable osteophyte formation (Fortier et al. 2011).

Animal studies have provided substantial evidence for the use of BMP-7 in vivo. A study of New Zealand white rabbits with femoral condyle defects showed extensive regeneration of both subchondral bone and a hyaline-like cartilage layer when treated with BMP-7 compared to fibrocartilage tissue fill-in defects left empty or treated with collagen only (Grgic et al. 1997). In a sheep study, continuous presence of BMP-7 led to markedly improved gross and microscopic cartilage healing of focal condylar and trochlear defects, suggesting the growth factor attracted mesenchymal-like cells originating from the synovium into the defect area (Jelic et al. 2001). In addition to applications of recombinant BMPs, rabbit studies evaluating the local application of a BMP gene to periosteal-derived allogenic mesenchymal stem cells via a retroviral vector showed complete or near-complete bone and cartilage regeneration of osteochondral defects at 8 and 12 weeks (Mason et al. 2000). In a rabbit model of ACL tears, BMP-7 injections promoted significantly improved tissue healing and prevented progression of OA compared to placebo injection (Hayashi et al. 2008). BMP-7 has also been tested in conjunction with

existing cartilage restoration surgery, improving gross and histological outcomes with mosaicplasty (osteochondral autograft) (Shimmin et al. 2003) and with microfracture (Kuo et al. 2006). Though BMP-7 appears effective in several different treatment models, the timing of BMP-7 application after joint injury affects its efficacy. In a model of post-traumatic osteochondral defects, Hurtig and colleagues showed increased cartilage healing and chondroprotective effects of BMP-7 augmentation both immediately and at 3 weeks after injury, but this effect was diminished at 12 weeks after injury (Fig. 6.2) (Hurtig et al. 2009). This finding suggests that a window of opportunity exists shortly after cartilage injury where growth factor intervention used as a disease-modifying agent may prevent progression of post-traumatic OA. At later time points post-injury, the natural history of disease may become more challenging to overcome with growth factor therapy alone.

Human trials of intra-articular BMP-7 injection have been underway over the past half-decade. In 2010, Hunter and colleagues published results of a phase I randomized controlled trial of various dosing regiments of BMP-7 injections in patients over 40 years old with symptomatic knee OA (Hunter et al. 2010). Despite higher rates of injection at the site of pain, there were no differences in toxicity or adverse events between BMP-7 and placebo. Furthermore, patients receiving BMP-7 injections at midrange dose reported a symptomatic improvement and anti-pain effects, though this was not the primary objective of the study. Unfortunately, a phase II clinical OA study did not appear to be successful; however, the reasons for such outcome have yet to be determined.



Adapted form *Hurtig et al, 2009, JOR*

Fig. 6.2 Timing of the effect of BMP-7 after impact injury in sheep. Growth factor application either immediately (**a**) or within 1 month (**b**) of traumatic chondral injury yields excellent cartilage repair on safranin O histology. Delayed growth factor application after 4 months (**c**) yields suboptimal cartilage repair, suggesting that there is a window for growth factor augmentation of cartilage repair shortly after chondral injury

6.3 Transforming Growth Factor-β

TGF-β is a cytokine secreted by many cell types; it plays crucial roles in cell proliferation, differentiation, development, apoptosis, tissue homeostasis, and the immune system. Signaling occurs primarily through the SMAD pathway, which involves a heterotetrameric complex that acts as a transcription factor via phosphorylation cascades. Among its three common isoforms, TGF-β1 has been mostly studied in chondrogenesis. TGF-β1 has been shown to stimulate chondrocyte synthetic activity and to decrease the catabolic activity of interleukin (IL)-1 and tumor necrosis factor (TNF)-α (Lotz et al. 1995; Lires-Dean et al. 2008). TGF-β1 helps to maintain chondrocyte characteristics during in vitro culture by promoting cell proliferation and extracellular matrix (ECM) protein synthesis and through inhibition of MMPs to protect normal morphology (Blaney Davidson et al. 2007). Furthermore, TGF-β1 stimulates chondrogenesis of synovial lining cells and of bone marrow-derived mesenchymal stem cells (Fan et al. 2010; Kurth et al. 2007). Studies have demonstrated significant enhancement of cartilage repair with TGF-β1 application in scaffolds applied to defects (Abe et al. 2003; Diao et al. 2009) and in human mesenchymal stem cells transfected with TGF-β1 genes via an adenovirus (Qi et al. 2013; Lee et al. 2001).

As with BMP-7, cartilage regeneration efforts utilizing TGF-β have moved forward into human trials over the past few years. Springing from research by Noh and colleagues on the ability of genetically engineered chondrocytes virally transduced with TGF-β1 (GEC-TGF-β1) to repair articular cartilage defects in animals, TissueGene-C has emerged as a leading growth factor in human trials for knee OA (Noh et al. 2010). The safety and biologic activity of injectable GEC-TGF-β1 were evaluated in a 2012 phase I trial by Ha and colleagues in patients with advanced knee OA (Ha et al. 2012). There were no severe adverse effects related to the GEC-TGF-β1 treatment reported, and the most common adverse effect was effusion. Ten of twelve patients showed improvements in clinical scores at 6 months and improvements in range of motion and pain up to 1 year. Since then, two phase II trials have investigated the efficacy and outcomes of intra-articular injectable GEC-TGF-β1 for knee OA. Cherian and colleagues recently conducted a prospective, multicenter, double-blinded, placebo-controlled, randomized study of GEC-TGF-β1 in the knees of patients with grade 3 OA and showed improved responses on the International Knee Documentation Committee (IKDC) score and Visual Analogue Scale (VAS) at 1-year follow-up and decreased need for analgesics (Cherian et al. 2015). Similarly, Lee and colleagues recently published results of another phase II trial of GEC-TGF-β1 injections into the knees of patients with OA yielding improved IKDC and VAS pain scores at 6 months follow-up (Lee et al. 2015). Given such positive results this year, GEC-TGF-β1 appears to hold significant promise as an injection therapy for moderate knee OA. Further, longer follow-up and phase III trials are needed to better define appropriate indications and dosing regimens for patients with chondral disease.

6.4 Fibroblast Growth Factors

The fibroblast growth factor (FGF) family plays important roles in human embryonic development, cell growth, morphogenesis, tissue repair, tumor growth, and invasion. FGFs are heparin-binding proteins and interact with heparan sulfate proteoglycans on the cell surface for signal transduction (Friedl et al. 1997). In total, 22 members of the FGF family have been identified in humans. The FGF receptor family has four members: FGFR1, FGFR2, FGFR3, and FGFR4. Human articular chondrocytes express all four receptors but contain significantly higher concentrations of FGFR1 and FGFR3 compared with FGFR2 and FGFR4 (Yan et al. 2011). FGFs are important regulators of cartilage development and homeostasis (Ellman et al. 2008). Three members of FGF family, FGF-2, FGF-8, and FGF-18, have been investigated for their role in cartilage homeostasis.

The role of FGF-2 in the production of the ECM in cartilage is controversial. FGF-2 was initially suggested to stimulate a robust cartilage repair response (Henson et al. 2005). However, strong mitogenic effects of FGF-2 lead to clustering of chondrocytes and uneven production of ECM due to low levels of collagen type II (Ellman et al. 2008). Furthermore, FGF-2 has been shown to suppress aggrecan and type II collagen and to promote the expression of aggrecanase and TNF-α receptors (Ellman et al. 2008; Im et al. 2008). FGF-2 also inhibits the stimulatory effect of bone morphogenetic protein-7 (BMP-7) and insulin-like growth factor-1 (IGF-1) on chondrocyte proteoglycan synthesis (Loeser et al. 2003, 2005). Ultimately, these findings have precluded FGF-2 from further consideration as a biological treatment in OA (Ellman et al. 2013).

While FGF-2 is mostly known for its pro-catabolic responses in cartilage, FGF-18 has repeatedly been shown to exert strong anabolic effects in chondrocytes and chondroprogenitor cells, leading to enhanced chondrogenic cell differentiation and type II collagen production (Ellsworth et al. 2002; Moore et al. 2005; Bradley et al. 2015). FGF-18 signaling through FGFR3 promotes chondrocyte proliferation at early embryonic stages. When development is complete, signaling through the same receptor works to suppress chondrocyte proliferation and prevent hypertrophic differentiation (Liu et al. 2002, 2007). The ability of FGF-18 to promote chondrocyte proliferation and the production of type II collagen and proteoglycan has been shown in vitro using adenovirus-mediated transfer of FGF-18 into the pinnae of nude mice (Ellsworth et al. 2002). Similarly, Barr et al. demonstrated an anabolic in vitro effect of recombinant human FGF-18 (rhFGF-18) on damaged cartilage, as rhFGF-18 increased aggrecan synthesis and reduced collagen breakdown in response to damage (Barr et al. 2014). In vivo studies began in rats, with Moore and colleagues investigating the utility of FGF-18 injections in a rat meniscal tear model of OA (Moore et al. 2005). Intra-articular injection of FGF-18 provided substantial cartilage production and reduced cartilage degeneration in OA. In an ovine model of chondral defects, augmentation of microfracture surgery with intra-articular injection of rhFGF-18 improved quality and quantity of repair tissue in chondral defects (Power et al. 2014).

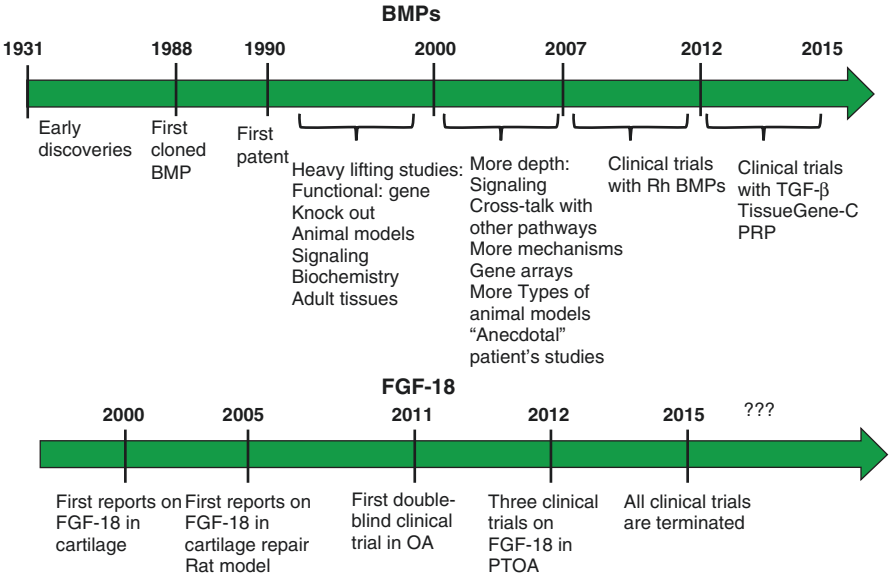


Fig. 6.3 A chronological timeline of advances in research of two growth factors with high potential in cartilage repair

Over the last decade, FGF-18 injection trials have commenced in humans (Fig. 6.3). Two phase II trials investigating the use of FGF-18 in patients with focal chondral defects were closed due to low enrollment. However, a randomized, double-blind, placebo-controlled, proof-of-concept trial recently investigated the efficacy and safety of recombinant human FGF-18 (sprifermin) in the treatment of symptomatic knee OA (Lohmander et al. 2014). There were no reports of local or systemic safety problems associated with any dose of sprifermin, and systemic levels of sprifermin were below detectable levels. Sprifermin was associated with statistically significant, dose-dependent reductions in loss of both total and lateral femorotibial cartilage thickness and volume on quantitative MRI, as well as reductions in radiographic joint space narrowing in the lateral femorotibial compartment. There was no significant relationship between treatment group and reduction in central medial femorotibial compartment cartilage thickness as measured by quantitative MRI. However, while all groups had improved WOMAC pain scores, patients receiving the 100 μg dose of sprifermin had significantly less improvement at 12 months compared with those receiving placebo. Despite this, positive imaging results and lack of toxicity in this phase I study suggest that FGF-18 may be a promising therapy for OA.

6.5 Connective Tissue Growth Factor

Connective tissue growth factor (CTGF, also known as CCN2) is an extracellular protein of the CCN family. Together, this group comprises extracellular matrix-associated heparin-binding proteins that play an important role in the regulation of

cellular proliferation, migration, adhesion, survival, differentiation, and synthesis of extracellular matrix proteins. Among these members, CTGF has been studied widely in chondrogenesis. CTGF plays a crucial role in the development of skeletal tissues, namely, by driving condensation of mesenchymal cells and regulating chondrocyte proliferation and differentiation (Nishida et al. 2000a; Song et al. 2007). CTGF promotes proliferation and cartilage matrix formation in the growth plate of the cartilage (Nakanishi et al. 2000) but has not been shown to cause hypertrophy or calcification in articular cartilage chondrocytes (Nishida et al. 2002).

In vivo studies exploring the use of CTGF in animal models of OA have emerged over the past decade. Such studies have further illustrated its role in promoting chondrocyte proliferation and differentiation during chondrogenesis, as CTGF knockout mice exhibited an expanded growth plate hypertrophic zone and impaired endochondral ossification (Ivkovic et al. 2003; Kawaki et al. 2008). Conversely, Tomita and colleagues demonstrated that overexpression of CTGF accelerated the process of endochondral ossification by promoting the proliferation and differentiation of growth plate chondrocytes (Tomita et al. 2013). Furthermore, cartilage-specific overexpression of CTGF in transgenic mouse studies slowed progression of age-related osteoarthritic changes in articular cartilage (Itoh et al. 2013). When applied to a full-thickness cartilage defect model, Nishida and colleagues showed that local administration of recombinant CTGF with gelatin hydrogel stimulated cartilage repair in a rat model (Nishida et al. 2004). Similarly, bone marrow mesenchymal stem cells transfected with CTGF recently provided hyaline-like cartilage regeneration similar to normal cartilage in a rabbit model of focal articular cartilage defects (Zhu et al. 2014). Such studies suggest a critical role for CTGF in both the protection and regeneration of articular cartilage. Though further testing is needed to clarify the safety and efficacy of this growth factor in cartilage disease, CTGF remains a promising therapeutic target for cartilage regeneration.

6.6 Insulin-Like Growth Factor-1

Insulin-like growth factor-1 (IGF-1) is a growth factor that is essential in embryonic tissue development and for growth and maintenance of mass throughout all stages of human life. Similar to BMP-7, IGF-1 has been shown to have robust anabolic and anti-catabolic effects. IGF-1 is required to maintain integrity of healthy articular cartilage, as rats with IGF-1 deficiency develop greater articular osteoarthritic cartilage degeneration in the knee than controls (Ekenstedt et al. 2006). The role of IGF-1 in healing of cartilage defects has best been demonstrated in a horse model, both by supplementing chondrocytes with IGF-1 (Fortier et al. 2002) and by genetically modifying chondrocytes with an IGF-1-encoded adenovirus (Goodrich et al. 2007). Unlike BMP-7, however, human chondrocytes have demonstrated a decreased response to IGF-1 with increasing age (Loeser et al. 2000, 2002) and with advanced OA (Schalkwijk et al. 1989). IGF-1 also appears to be more affective as a driver of synthetic function and less effective at preventing catabolism in advanced disease (Morales 2008). As such, there have not been any trials to date in humans with IGF-1 for the treatment of OA or focal chondral defects.

6.7 Platelet-Rich Plasma

While most studies to date have focused on individual growth factors, the use of combined autologous solutions of multiple growth factors, namely, in the form of platelet-rich plasma (PRP), has gained enormous popularity in the past decade. PRP is defined as plasma with a minimum twofold increase in platelet concentration above baseline levels or greater than 1.1×10^6 platelets/μL (Miller et al. 2007). Its use stems from the well-defined role of platelets in wound healing, initially through clot formation with fibrin to close wounds and ending with the release of factors involved in angiogenesis, inflammation, and the immune response (Nurden et al. 2008). Densely packed α-granules in platelets release a multitude of growth factors, including FGF, TGF-β, platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF), that modulate inflammation, attract local stem cells and fibroblasts, and trigger a regulated proliferative healing response (Smyth et al. 2009). In vitro studies of PRP on cartilage have yielded extremely positive results, as stem cells buffered in PRP had increased proliferation and chondrogenic differentiation (Mishra et al. 2009). Akeda and colleagues showed that porcine chondrocytes cultured in PRP displayed increased DNA content and proteoglycan and collagen type II synthesis (Akeda et al. 2006). Importantly, PRP did not affect the types of proteoglycans or collagen produced, suggesting that chondrocytes remain phenotypically stable when enriched with PRP. When compared to hyaluronic acid in co-culture with osteoarthritic chondrocytes, PRP-enriched samples resulted in a significant reduction in MMP expression, increased hyaluronan (HA) synthase expression by synoviocytes, and increased aggrecan production (Sundman et al. 2014). Aside from aiding in tissue healing, this data suggests that PRP may play a chondroprotective role as well (for details see Chap. 7).

With such overwhelmingly positive in vitro results, animal and human studies utilizing PRP have progressed rapidly. In rabbits, PRP-treated osteochondral defects showed greater cartilage regeneration and production of ECM than placebo (Sun et al. 2010). Early human knee injection trials with PRP showed improved pain and patient-reported outcome scores when compared with HA (Kon et al. 2010). In 2012, Sanchez and colleagues conducted a randomized, controlled trial comparing PRP to HA and achieved the same results at 6 months post-injection (Sanchez et al. 2012). In longer follow-up studies (12 months), symptomatic improvements in patients with knee OA have been also documented (Gobbi et al. 2012). Multiple randomized controlled trials have since shown benefits of PRP compared to other available therapies (Cerza et al. 2012). Furthermore, PRP has not been limited to the knee, as injections were shown to be effective in osteochondral lesions of the talus compared to HA (Mei-Dan et al. 2012). Unfortunately, latest studies have yielded mixed results, as a randomized controlled trial by Filardo and colleagues found no difference in outcomes between HA and PRP (Filardo et al. 2015). This finding is in agreement with an earlier study by the same group showing no superiority of PRP over HA (Filardo et al. 2012). However, a recent systematic review of PRP injections in the knee found that intra-articular injections are overall a viable therapy for patients with mild OA (Campbell et al. 2015). This group found that PRP injections carry a slightly higher risk of local adverse reactions after multiple injections and

work best in patients with early degenerative disease. Ultimately, more trials are needed to determine the proper patient cohort, dosing strategy, and injection frequency of PRP.

Conclusion

In summary, tremendous progress has been made in harnessing the potential of growth factors for cartilage repair, both in regard to halting deterioration following post-traumatic focal defects and in preserving cartilage from long-term degenerative changes. Of the individual growth factors, BMP-7, TGF-β, and FGF-18 appear to have the most short-term promise for future clinical studies. However, it has become increasingly clear that complete cartilage repair will not stem from the addition of a single growth factor, but rather a combination of growth factors (Fortier et al. 2011). This has been shown by the fact that BMP-7 produces better cartilage repair when applied in combination with TGF-β or IGF-1 than it does on its own (Loeser et al. 2003; Chubinskaya et al. 2007a) and by in vitro evidence that IGF-1, TGF-β, and FGF-2 regulate each other's gene expression and subsequent protein production (Shi 2009). Thus, it comes as no surprise that combinations of growth factors, such as those in platelet-rich plasma, may yield better and more sustainable clinical outcomes in patients with focal cartilage defects or OA, especially at early stages of disease.

Several unanswered questions remain in regard to the use of growth factors for cartilage repair. Aside from which growth factors produce optimal regeneration, it is unclear whether clinicians and researchers should strive to boost endogenous growth factor production or augment with recombinant, exogenous growth factors. Future short-term goals include obtaining a better understanding of the pathophysiology of cartilage degeneration so that growth factor therapy can be tailored to various stages of the healing process. Optimal doses and formulations must be determined in order to maximize clinical response and minimize side effects. Growth factors must be studied further in hostile, inflammatory environments to better understand their efficacy in disease states. This will likely underscore a difference in potential therapy for post-traumatic chondral defects versus therapy for chronic degenerative joint disease. Relationships between industry and academia must be fostered transparently, as previous clinical trials have come to a halt after suboptimal results. Ultimately, the solution will likely involve partnerships with regulatory agencies to move new technologies forward efficiently. Future clinical trials must be conducted with carefully selected patient cohorts. Lastly, costs of therapies will have to be reduced so that these may become financially feasible options for patients.

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The Role of Platelet-Rich Plasma in Cartilage Repair

7

Elizaveta Kon, Giuseppe Filardo, Berardo Di Matteo, and Maurilio Marcacci

Abstract

The use of biological agents to positively modulate articular homeostasis and provide a stimulus to tissue healing is a growing field in orthopedics. Among the available strategies, platelet-rich plasma (PRP), an autologous blood-derived concentrate rich in growth factors, is currently the most exploited biological approach for conservative management (simple intra-articular injections) and as an augmentation during surgical procedures. It has been applied both to treat osteoarthritis and chondral/osteochondral lesions in different joints, with the primary aim of providing symptomatic relief and functional recovery, and also to induce a positive modulation of the entire articular microenvironment. In this chapter, we analyze the clinical evidence available on the role of PRP to treat cartilage pathology, focusing in particular on the data coming from randomized controlled trials.

PRP is a novel technology, and, based on the available evidence, there is an overall support on the safety of this biological approach but still no conclusive

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evidence about its real efficacy in the management of cartilage pathology, especially when applied during surgical procedures. Looking at conservative application, PRP proved to be better than placebo but not clearly superior to more traditional approaches such as viscosupplementation.

The improvement in this field will pass through the understanding of the interaction dynamics between PRP and the etiopathogenetic/regenerative cartilage processes, as well as the identification of the best PRP features and application modalities, together with the identification of the patient and lesion type that may benefit from this treatment.

7.1 Platelet-Rich Plasma: Why Using It in Cartilage Pathology?

A healthy joint requires a well-controlled balance between molecular signals regulating anabolic and catabolic activities. This balance is determined both at the level of single cells and also at the whole tissue architecture, consisting of complex interactions among all the different articular tissues such as the cartilage, bone, synovium, ligaments, tendons, and menisci (Lories 2008). Several factors may come into play to impair the maintenance of joint homeostasis, and this process leads to the development of chondral or osteochondral lesions and, in case of chronicity, to the onset of osteoarthritis (OA) (Heijink et al. 2012; Hunter and Felson 2006), which is a degenerative disorder of the entire joint characterized by loss of articular cartilage, subchondral bone remodeling, osteophyte formation, synovial hyperplasia, changes in synovial fluid, fibrosis of joint capsule, and changes in menisci and ligaments (Lotz and Carames 2011).

Currently, several treatment options have been proposed to mitigate the symptoms related to cartilage lesions and/or OA and to improve joint function including changes in lifestyle, regular exercises, specific rehabilitation protocols, dietary supplements, "conventional" pharmacologic agents (such as NSAIDs), and also minimally invasive treatments consisting in intra-articular injections, the most common being corticosteroids and hyaluronic acid (Reginster et al. 2015; Cutolo et al. 2015; Jacobs et al. 2014). However, none of these treatments is fully effective, and the outcome is never clearly predictable: furthermore, the majority of these conservative options are not able to act on the inner mechanisms responsible of the tissue damage, and, therefore, they have minimal or no role in modifying the course of the disease. In recent years, research has focused on the possibility of modulating overall joint tissue homeostasis through the application of biological agents which could promote healing minimizing the risks and adverse events related to the use of traditional "on-the-shelf" products (Filardo et al. 2016; Kon et al. 2012).

In this scenario, the most exploited strategy is platelet-rich plasma (PRP), an autologous blood derivative containing a higher platelet concentration than whole blood. Platelets have been documented to act as a reservoir of many

autologous growth factors (GFs), which serve as the rational for their concentration and the production of PRP: a safe and easily available method to have bioactive molecules delivered into the lesion site, with the aim of enhancing the healing process and driving it toward a better quality of tissue repair with improved biomechanical properties. More in detail, activation of platelets contained in PRP induces the release of several GFs that are stored in a-granules, such as platelet-derived growth factor (PDGF), transforming growth factor (TGF)-ß, insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), and many other bioactive and anti-inflammatory molecules as well (Boswell et al. 2012; Xie et al. 2014) (Fig. 7.1). These agents, released at the site of injury in high concentrations, may promote tissue healing by stimulating cell proliferation, chemotaxis, cell differentiation, and angiogenesis and also by modulating inflammatory and catabolic molecules involved in the cartilage degenerative processes (Braun et al. 2014).

PRP can be easily prepared from venous blood even in an outpatient setting, and it can be delivered via a simple intra-articular injection or during surgical procedure as an augmentation. This appealing treatment option has encountered a large success among clinicians and has been increasingly used in the last decade, with several industries introducing their "proprietary" kits for PRP production. Despite the widespread of this biological treatment in orthopedics, clinical results are sometimes contradictory with no clear treatment indications, due to low-level clinical studies and the lack of understanding of the mechanism of action of this blood derivative (Kon et al. 2013).

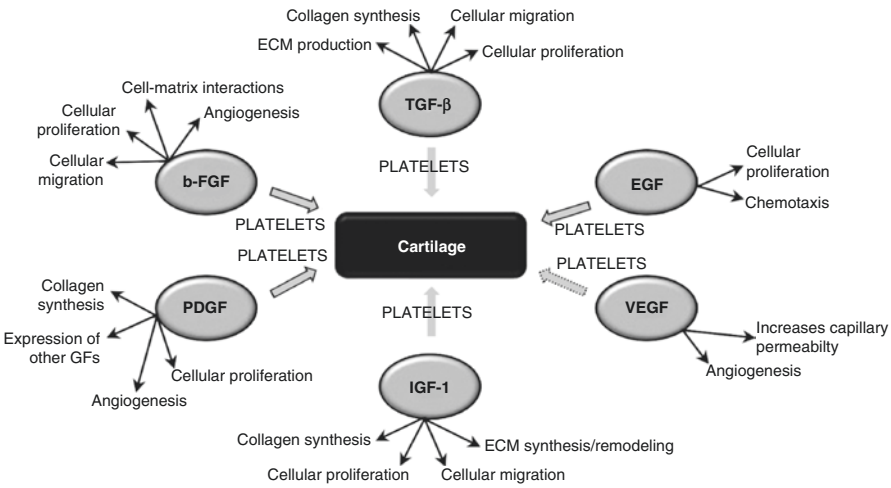

Fig. 7.1 Role and action of the most important platelet-derived growth factors in cartilage healing

7.2 Review of Current Clinical Evidence

7.2.1 Knee

7.2.1.1 Conservative Management

Among the many studies dealing with PRP injective intra-articular application, just a few randomized controlled trials (RCTs) have been published. Sanchez et al. (2012a) investigated the efficacy of single-spinning leukocyte-free PRP compared to HA in 153 patients evaluated up to 6 months of follow-up. The only aspect where a superiority of PRP was found was the percentage of responders (patients with at least 50% of pain reduction), which was significantly higher in the PRP group. Besides this finding, the study did not show that PRP in moderate/severe OA was more effective than HA. Similar considerations were made by Filardo et al. (2015), according to the results of their randomized double-blind trial including 192 patients evaluated up to 12 months and comparing PRP and HA: no statistical intergroup difference was reported at any follow-up evaluation. Conversely, Cerza et al. (2012) treated 120 patients by either autologous conditioned plasma (ACP, a low-concentrated PRP without leukocytes) or HA. Surprisingly, the ACP group showed a significantly better performance than HA in all groups of treatment, including patients affected by grade III knee OA. Furthermore, the clinical gap between treatments increased over time in favor of ACP. Finally, a recent randomized trial by Patel et al. (2013) was the first to test PRP versus saline. Seventy-eight patients affected by Kellgren grade I–III OA were included and treated bilaterally with one injection of PRP, two injections of PRP (3 weeks apart), or one injection of saline. Despite the low number of patients included, a significant difference was observed between PRP and saline solution in terms of clinical outcome. Interestingly, no difference was reported among patients who received one or two PRP injections. Clear superiority of PRP compared to saline was also reported in the RCT recently published by Smith (2016) on 30 patients followed up to 12 months: the biological approach outperformed viscosupplementation at any follow-up evaluation.

Only one RCT (Forogh et al. 2016) investigated the comparison between PRP and corticosteroid: 41 patients were treated by either a single injection of PRP or corticosteroids. Even in this case, both at 2 and 6 months' follow-up, PRP provided better results than corticosteroids.

7.2.1.2 Augmentation in Knee Surgery

Dhollander et al. (2011) treated five symptomatic osteochondral defects of the patella with microfracturing followed by the application of a collagen I/III scaffold membrane. PRP was administered beneath the membrane at the interface with the microfractured subchondral bone. The final follow-up was 24 months after the surgical procedure. Clinical results after 2 years were satisfactory both in terms of pain relief and functional improvement, and MRI evaluation showed good quality of the repair tissue. A further study by Siclari et al. (2012) showed the efficacy of a polyglycolic acid/hyaluronan scaffold immersed in PRP for treating full-thickness chondral defects of the knee: 52 patients were treated arthroscopically by perforations

and scaffold implantation. At 1-year follow-up, a significant clinical improvement was observed in all KOOS subcategories. Osteochondral lesions were treated by Buda et al. (2010), who used an innovative arthroscopic one-stage approach based on autologous bone marrow-derived mesenchymal stem cells (BMDCs), PRP, and, alternately, porcine collagen powder or hyaluronic acid (HA) membrane to create a scaffold: 20 patients were treated and followed up for 24 months with IKDC and KOOS scores combined with MRI analysis. Besides the significant improvement in clinical scores, interesting correlations were found: combined surgery slowed down recovery although, at final evaluation, similar results were obtained with respect to those of patients without combined procedures; hyperintense MRI signal of repair tissue was correlated with poorer clinical results. In general, these preliminary reports suggested good results, but the low scientific level of these papers and even more the concomitant application of different treatments hinder the possibility to understand the real role of PRP.

7.2.2 Ankle

7.2.2.1 Conservative Management

Conservative application was tested in a prospective study of Mei-Dan et al. (2012) who compared the efficacy of hyaluronic acid (HA) and PRP in 30 patients (15 per group) affected by talar osteochondral lesions not responsive to other previous conservative management. Patients were allocated to receive three-weekly intra-articular injections of HA (2 ml each) or PRP (2 ml each) and were evaluated up to 28 weeks of follow-up. Investigators used AHFS, AOFAS, and VAS for pain, stiffness, and function scores. Results were statistically significant, and PRP proved to be more effective in controlling pain and reestablishing function.

7.2.2.2 Augmentation in Ankle Surgery

Surgical application of PRP in talar osteochondral lesions has been exploited by Giannini et al. in two different studies (Giannini et al. 2009, 2010). They described the first clinical application of an innovative arthroscopic one-stage technique involving autologous mesenchymal stem cells (MSCs), PRP, and, alternately, porcine collagen powder or HA membrane. The procedure consisted of harvesting bone-marrow derived cells from the posterior iliac crest of the patients through a traditional marrow needle. A total of 60 ml bone marrow aspirate was collected and immediately put into a cell separator-concentrator to obtain a final 6 ml of concentrated MSCs. At this point, a collagen powder or the hyaluronic acid membrane could be used. In case of the former, 2 ml of MSCs concentrate were added to 1 g of collagen powder and 1 ml of platelet-rich fibrin gel (obtained the day before surgery). In case of HA membrane, it was cut to match the size of the talar osteochondral lesion and then covered with 2 ml of MSCs concentrate and 1 ml of platelet-rich fibrin gel. The total procedure was conducted via ankle arthroscopy, and, after the preparation of the lesion, the biological composite was located onto the defect through a cannula, using a probe to obtain the best possible fitting.

The first clinical trial (Giannini et al. 2009) involved 48 patients (average age = 28.5 years) affected by focal lesions (average size = 2.1 cm^2) evaluated at 6, 12, 18, and 24 months of follow-up through the AOFAS score. A significant increase of this parameter was already registered 6 months after the surgical procedure, and this result was later confirmed up to the final follow-up. The rate of return to high impact sports activity was satisfying, with more than 75% of the patients back at the 11 months follow-up. Investigators found a correlation between clinical outcome and lesion size, with lower results for defects >3 cm^2, and it was also shown that previous surgery negatively affected the outcome. On the other hand, no influence was observed for lesion depth, neither difference was seen according to the scaffold used (collagen powder or HA membrane). Five second-look arthroscopies were performed at 1-year follow-up: in two cases, biopsies were taken revealing, after histological and immunohistological analysis, the presence of new cartilage tissue with various degrees of tissue remodeling toward the hyaline type. The overall findings made possible to assert that this novel approach could stimulate tissue regeneration with interesting clinical efficacy, with results even comparable to the ones of autologous chondrocyte implantation (ACI) but without the double surgical time and the inherent stress for the patient. To this particular aspect, the same authors dedicated a further study (Giannini et al. 2010) focusing on the comparison of the MSCs + PRP + scaffold technique with open and arthroscopic ACI. Eighty-one patients were included in this analysis, 10 of which treated with open ACI, 46 with arthroscopic ACI, and 25 with bone marrow-derived mesenchymal cells (BMDCs) "one-step" technique. Clinical results were compared up to 3 years of follow-up. AOFAS was the instrument adopted for clinical evaluation together with radiographical analysis. The clinical improvement in each subgroup was significant, and no intergroup difference was observed, thus confirming the possibility of matching the effectiveness of chondrocyte transplantation through a single-step procedure. X-rays showed no sign of osteoarthritis progression; MRI revealed a good rate of defect filling and integration of the newly regenerated tissue within the surrounding tissue. Another aspect worth of consideration is the economical issue: in fact, authors pointed out that their one-step regenerative technique costs less than half of the traditional arthroscopic ACI.

7.2.3 Hip

7.2.3.1 Conservative Management

Few studies have been published on this particular topic. The first one, authored by Battaglia et al. (2011), reported the results of PRP ultrasound-guided injective treatment in 20 patients affected by hip OA (Kellgren-Lawrence Score from I to III): three intra-articular injections 2 weeks apart were performed with patients followed up for 1 year. The clinical outcome was positive, but a worsening occurred 3 months the final evaluation, thus confirming the time-dependent effect of PRP. Similar results were confirmed in a later randomized study by the same authors (Battaglia et al. 2013), where PRP was compared to HA. One hundred patients were divided

into two treatment groups, receiving three intra-articular injections: a comparable clinical benefit was documented in both groups, without significant difference, and the same trend of worsening results over time was registered. Sanchez et al. (2012b) treated 40 patients affected by OA with 3-weekly ultrasound-guided injections of PRP. Evaluation was carried out for 6 months using the WOMAC, Harris, and VAS scores for pain. Satisfactory results were reported with a significant reduction in pain level at the first evaluation after 6 weeks, which was confirmed at the final 6 months' follow-up. Functional recovery was encouraging as evaluated through a specific subscale of the WOMAC score. However, 11 out of 40 patients did not have any beneficial effect after injective treatment: in these cases, a metal resurfacing was required. Recently, a RCT was published by Dallari et al. (2016) who randomized 111 patients in three different treatment groups: one to receive 3-weekly HA injections, one to receive 3-weekly PRP injections, and the last to receive 3-weekly PRP+HA injections. They found that PRP-treated patients were the best responding group at 12 months evaluation in terms of functional recovery and pain control, without any significant contribution from the addition of HA to the platelet concentrate.

7.3 Discussion

PRP technology is facing a remarkable widespread in everydays' clinical practice, both as an augmentation during surgery and as a conservative approach for treating disparate musculoskeletal conditions (Vannini et al. 2015; Di Matteo et al. 2015). For what concerns the current understanding on the potential and feasibility of applying PRP in the management of cartilage pathology, looking at the surgical application, it is not possible to draw definite conclusions about the efficacy of this treatment. Based on the current evidence, it is very difficult to identify how much PRP might contribute to determine the clinical outcome with respect to the surgical treatment performed alone, and more comparative studies aimed at assessing the specific role of PRP are needed. Furthermore, in many cases, PRP is administered together with other biological augmentation methods, such as mesenchymal stem cells or bioengineered scaffolds, so it is even more difficult to determine the contribution of PRP as such. The studies currently available are just case series treating disparate conditions in biomechanically very different joints, so the overall evidence is very limited and should be considered with caution. Moreover, follow-up evaluation is mainly at short term, and further studies are needed to determine the persistence of the reported good clinical outcome. In the near future, PRP will be more and more widely used in cartilage regenerative techniques, but, despite being safe, according to the present evidence, there is still no recommendation for using PRP as an augmentation during surgical procedures (Perdisa et al. 2014).

Considering PRP for conservative management, many preliminary studies have already confirmed the safety of PRP and also suggested promising clinical outcome, but also a gradual worsening over time. Two studies comparing PRP and saline for the treatment of knee cartilage pathology have been published (Patel et al. 2013;

Smith 2016), and in both of them, it appeared clearly that the biological solution provided significantly better outcome. However, the real challenge comes when taking into account the randomized trials comparing PRP to viscosupplementation, which is currently the most adopted injective treatment worldwide. Actually it should be pointed out that each trial employed a different type of PRP. The study authored by Filardo et al. (2015), which had the longest follow-up evaluation of patients treated (1 year), reports no overall difference between PRP and HA in terms of clinical outcome and objective measurements at any follow-up evaluation. Therefore, in the case of more advanced signs of OA, PRP does not seem to be superior to viscosupplementation, a conclusion that was also reached in the study by Sanchez et al. (2012a), who were able to demonstrate just a better percentage of "responders" (patients with at least 50% reduction in pain) in PRP group compared to viscosupplementation. Surprisingly, Cerza et al. (2012) reported significantly better results for ACP even in grade III OA: worthy of consideration is the fact that they used different blood-derived products with evaluation limited up to 6 months of follow-up.

All the randomized trials deal with the application of PRP in patients affected by different stages of the disease, from chondropathy to severe OA. Therefore, no conclusions can be drawn about the possibility of applying this approach to a specific phase of cartilage degenerative pathology: subgroup analysis does not allow, in any of the trials published, sufficient statistical strength to provide a real clinical indication. What emerges can be considered just a "suggestion" to avoid the indiscriminate use of PRP, which seems to offer clinical benefits but cannot yet be considered a first-line treatment for knee cartilage pathology (Di Matteo et al. 2016). The situation is not clearer when looking at different joints, due both to the paucity of literature and the low scientific quality of studies published. Just one RCT has been published investigating the potential of PRP in treating osteochondral defects of the talar dome (Mei-Dan et al. 2012), but the small number of patients included and the follow-up topped at 7 weeks are major limitations. Hip OA has been the subject of four studies, two of them (Battaglia et al. 2013; Sanchez et al. 2012b) characterized by a low number of patients included (about 40); the most recent one included more than 100 patients (Dallari et al. 2016), divided into three treatment groups, to explore the efficacy of PRP against HA and also the potential of the combination of PRP+HA. The best performance, at 1-year evaluation, was achieved by the PRP group, and no contribution was due to the addition of HA to the blood derivative: in any case, this study is not sufficient to fully endorse the application of PRP in hip OA, also considering the controversial results reported by previous smaller trials. At present, the main problem related to PRP is the immense inter-product variability (Tschon et al. 2011). Cellularity is maybe the most debated aspect: leukocytes, monocytes, macrophages, and other cells are present in variable proportions according to the production method PRP was adopted from. In particular, the possible negative results of leukocytes have been investigated by in vitro studies that showed deleterious effects in terms of inflammation, cell migration, and matrix molecule formation/degradation (Assirelli et al. 2015; McCarrel et al. 2012). However, despite these findings, up

to now, no clinical trial has shown the superiority of leukocyte-poor PRP with respect to leukocyte-rich formulation, thus further underlining the complexity of the in vivo environment and the pathogenic mechanisms of cartilage disease that can hardly be mirrored during in vitro trials. Beyond cellularity, several other variables have to be considered, such as the preparation procedures, activation methods, storage modalities, application protocols, and many other aspects that might not be of secondary importance for determining PRP properties and clinical efficacy. The number of variables to take into account is so high that it is likely to hypothesize that certain PRP formulations may be not ideal for the treatment of cartilage pathology with respect to others, and therefore research should move toward in identifying the best PRP features that could promote cartilage healing and joint homeostasis. At the current state of art, we are far from reaching this goal, and clinicians should remember that using an autologous blood-derived product is not, in any case, a guarantee of safety and efficacy. The number of names and acronyms encountered searching for studies on this biological treatment approach, such as PRP, PRGF, ACP, PL, etc., clearly represents the complexity of this field and explains the difficulties in the literature analysis, study comparison, and understanding of some contradictory results. There have been many attempts in the past to classify PRP products (Dohan Ehrenfest et al. 2009; Mautner et al. 2015), but the biological nature of this autologous approach does not help standardization, and, at present, there is no useful classification to be adopted in clinical practice. Even identifying macro-categories (Dohan Ehrenfest et al. 2010) has more theoretical value than a practical utility: the range of products that can be obtained is tremendously wide, and so the potential clinical usage, that it is difficult to put order in this complex and rapidly evolving field. However, new insights into the importance of technical and biological variables are now emerging (Salamanna et al. 2015; Marmotti et al. 2015) and will probably help to optimize platelet concentrates for this clinical application in the future. Until a new generation of products specifically conceived for intra-articular use is developed, physicians should be aware of the current options and their potential and limitations.

Conclusion

PRP is a novel technology, and the literature on cartilage treatment can rely only on a few high-level trials: there is an overall support on the safety of this biological approach but inconclusive evidence about its real efficacy in the management of cartilage pathology, especially when compared to more traditional treatment options. The improvement in this field will pass through the understanding of the mechanism of interaction between PRP and the etiopathogenetic/regenerative cartilage processes, as well as the identification of the best PRP features and application modalities, together with the identification of the patient and lesion type that may benefit from this treatment. Until further high-level studies will confirm the indications, results, and limitations suggested by the available literature, PRP should be considered as a second-line treatment and performed within controlled studies, rather than applied indiscriminately in the clinical practice.

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Subchondral Bone in Articular Cartilage Regeneration

8

Arndt F. Schilling

Abstract

Subchondral sclerosis is one of the hallmark findings of osteoarthritis (OA) and has long been discussed as one of its causes. Indeed, the changes in the subchondral bone often precede cartilage destruction in the development of the disease. Integration of the so far published data including in vitro, in vivo, and mathematical work suggests a critical role for this tissue in nutrition and oxygen supply to the articular cartilage, which may become even more critical in energy demanding processes of healing and regeneration.

Indeed, the success of current predictive diagnostics like specialized MRI techniques and scintigraphy as well as successful regenerative clinical therapies like microfracturing, AMIC, or NAMIC can be better explained if the subchondral bone is taken into the account as supply route for the cartilage.

Consequently, subchondral bone has to be included into the diagnostic and therapeutic concepts aiming to regenerate lost or damaged cartilage for advanced diagnosis and treatment of OA.

8.1 The Multi-tissue Human Joint

The mammalian locomotion system has evolved for movement at very low energy costs (Pontzer and Kamilar 2009). In the human bipedal locomotion, relatively high forces have to be transmitted from one moving bone to the next. The hip joint is already loaded with three times bodyweight when walking (Bergmann et al. 2016), and much higher forces (>9× bodyweight) are seen in sports (McNitt-Gray et al.

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1993). A multilayered tissue structure that is constantly adapted to its current use has evolved to meet these demanding requirements. Far away from the joint, the diaphyses of the long bones resemble tubes with a strong cortical ring, which encases the embedded bone marrow, reflecting their primary loading in the direction of the long axis. Closer to the joints starting in the metaphyses, the major muscles insert, and the pattern of loading changes to multidirectional, which is reflected by a change in the bone structure to trabecular bone with less cortical casing. On top of the metaphysis resides the epiphysis. It develops from a secondary ossification center and consists of trabecular bone, blending into calcified cartilage with its cover consisting of hyaline cartilage (Fig. 8.1).

Between the calcified cartilage and the uncalcified cartilage is the so-called tidemark, a zone of distinct glycoprotein profile marking the zone of mineralization (Lyons et al. 2005). The combination of calcified cartilage and underlying trabecular bone is referred to as "subchondral bone" (Fig. 8.1). Interestingly, impulsive forces that act on the joint during movement are mainly attenuated in this bone and not in the cartilage, although the bone is roughly ten times stiffer (Radin and Paul 1970). The main function of articular cartilage is therefore reducing friction. The excellent tribological properties of hyaline cartilage make it possible to move the articulating bones relative to each other while transducing the high loads between them. This allows energy efficient movements with multiple degrees of freedom while preserving the cartilage (Chen et al. 2013).

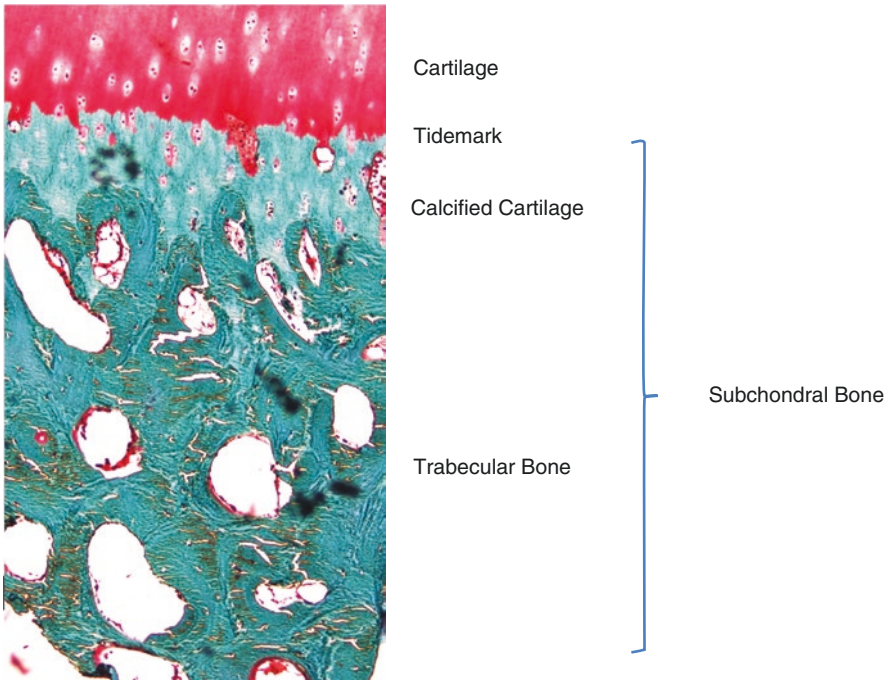

Fig. 8.1 Histological section of the osteochondral area

8.2 Cartilage Injury and Lack of Regeneration

Injury to or degradation of the articular cartilage consequently leads to breakdown of this sophisticated, concerted locomotion system. Loss of cartilage results in increased friction between the articulating bones which leads to inflammation and thereby loss of more cartilage completing the vicious circle (Glyn-Jones et al. 2015). The patients experience loss of mobility and pain. There is a general consensus that cartilage injuries usually do not heal. Surprisingly, it has been shown that chondrocytes have the ability to generate new cartilage tissue if the environment is permitting this (Hoenig et al. 2011, 2013). Furthermore, the development of osteophytes with full cartilage cover in joint disease shows a general ability of forming articular cartilage de novo (Gelse et al. 2003). So why is there usually no regeneration of cartilage defects in vivo? A possible road to the answer for this question may lie in the observation that tissue healing is associated with a high demand for energy that needs to be satisfied. Indeed, tissues with a rich blood supply (e.g., liver, oral cavity) heal well, while chronic wounds are usually found in patients with impaired blood supply to the wounded areas (peripheral vascular diseases, diabetics, smokers, burn patients) (Varricchi et al. 2015; Wu et al. 2007).

8.3 Nutrition of Cartilage: Synovial Nutrition (SyN) vs. Subchondral Nutrition (SuN)

Healthy articular cartilage does not contain blood vessels. It consists only of chondrocytes embedded in extracellular matrix produced by these chondrocytes (Hoenig et al. 2013). Consequently, nutrition and oxygen for the metabolism of the cells inside the cartilage must be maintained by diffusion, and the diffusing nutrients have to reach the cartilage somehow. Possible routes for this traffic of nutrients would be from the synovia, supplied by the vessels into the joint capsule (synovial nutrition: SyN), or through the subchondral bone supplied by the epiphyseal plexus (subchondral nutrition SuN) or both (Ingelmark and Saaf 1948) (Figs. 8.2a,b). The notion that cartilage may be supplied by the synovial fluid alone (SyN) is mainly based on experiments with young and adult rabbit joints (Hodge and McKibbin 1969). These experiments clearly show that in young rabbits, a route through the subchondral bone exists which is however completely closed in the adult animals. This lead the authors to the conclusion that nutrition of the cartilage in adult animals can only be mediated through the synovial fluid.

In humans however, there is intraosseous and extraosseous blood supply to the distal femoral condyles (Reddy and Frederick 1998). Holmdahl and Ingelmark described two types of channels connecting the medullary cavity through the subchondral bone with the cartilage: (1) “ampulla-like” with a diameter of ca. 40 μm and (2) “canal-like” or “dendritic” with a diameter of around 15 μm (Holmdahl and Ingelmark 1950). For human joints, it was demonstrated that a fluorescent dye instilled into the marrow space can be found in the cartilage after 16 h and completely penetrates the cartilage after 48 h (Greenwald and Haynes 1969). In a

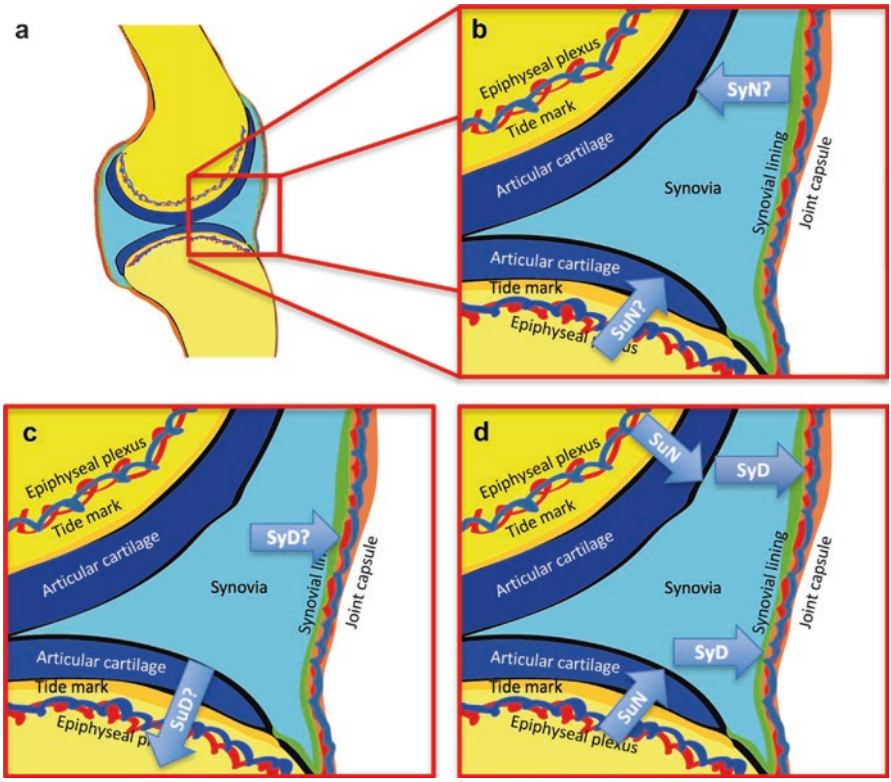

Fig. 8.2 (**a**) Schematic representation of possible routes of nutrition (**b**) and disposal (**c**) for the cartilage; *SyN* synovial nutrition, *SuN* subchondral nutrition, *SuD* subchondral disposal, *SyD* synovial disposal; (**d**) SuN-SyD Hypothesis

modeling study, Zhou et al. showed that a favorable oxygen concentration around 5% throughout the cartilage can only be maintained by diffusion, if the lower part of cartilage is supplied by the bone route (Zhou et al. 2004, 2008). Indeed, gadolinium (Gd(DTPA)2) penetrated into cartilage from the articular surface, when injected intra-articularly, and from both sides when injected intravenously. Furthermore, intravenous injection showed a shorter overall penetration time (Bashir et al. 1997), suggesting a more efficient supply through vessels of the subchondral bone (SuN). The vascularity of the subchondral bone was found to be age dependent and location dependent (Lane et al. 1977); more vessels are present in the more loaded areas of the articular surfaces, which suggests a better supply of the cells that experience a higher load assuming subchondral nutrition (SuN). The density of cells in the cartilage however increases with distance from the subchondral bone (Stockwell 1971). If the cells are viewed separately, this could be interpreted as an indication that the nutrition at the surface is higher and therefore there are more cells in this area. The cell density of a tissue is determined by relative ratios of cells and ECM assuming that either a high cell density means that there are many cells or that there is little ECM. However,

in taking the production of the extracellular matrix (ECM) by the cells into account, a different picture emerges: the expression of collagen II by chondrocytes rises with rising oxygen tension, nearly doubling from 0% oxygen to 20% oxygen (Grimshaw and Mason 2001). If we assume a SuN route, the oxygen tension would be high close to the subchondral bone; thus, the cells would produce more collagen II in this area and therefore stand further apart leading to lower cell density at the subchondral bone border. With rising distance from the subchondral bone, oxygen tension would drop, eventually leading to hypoxia and decrease of collagen II production, which would lead to downregulation of matrix synthesis and therefore indirectly increase the cell density at the joint surface. This is also in accordance with a higher collagen II content in smaller vertebral disks (Boubriak et al. 2013). Interestingly, while in different species the thickness of articular cartilage in the knee ranges over a factor of nearly 50× (0.05–2.3 mm), the number of cells over a square millimeter of subchondral bone is surprisingly constant (25,500 ± 8800 cells/mm^2), suggesting that a given surface area of subchondral bone only has the ability to supply a given amount of cells. If O_2 would be transported by the synovial fluid (SyN), there would be no obvious reason for such a relationship. Furthermore, more synovial fluid should increase the oxygen tension in the joint. Surprisingly, however, the amount of synovial fluid in the joint is inversely related with its oxygen tension (Richman et al. 1981). This observation of lower levels of oxygen in joints with more synovial fluid can hardly be explained with changes in influx. Increased influx of freely diffusing fluid from the circulation should bring in more oxygen, not less. There are two possible ways to explain these findings. First, patients with higher volumes of synovial fluid may have an even higher metabolism of the cells in the joint (e.g., due to inflammatory processes), with higher counts of leucocytes in the joint. There was however no correlation between white blood cell counts and oxygen consumption in these experiments (Richman et al. 1981). Second, a diminished disposal of synovial fluid may be responsible.

8.4 Waste Disposal in the Joint: Subchondral Disposal (SuD) vs. Synovial Disposal (SyD)

In this case, the increase in volume would be due to longer clearance time bringing the synovial fluid in contact with the oxygen consuming cells for a longer time thereby explaining why pO_2 is lower in patients with more fluid in the joint. As the cells in the joint have a metabolism (although a slow one), the waste products have to be disposed of. Indeed, the published data suggests a weak positive correlation between the amount of synovial fluid and CO_2 waste, supporting a role of waste disposal from the joint and synovial efflux (Richman et al. 1981). In healthy joints, there is efficient clearance of the synovial fluid with half-lives of intra-articularly injected drugs in the range of hours (Owen et al. 1994). This clearance is dependent on particle size and health status of the joint (Pradal et al. 2016). For clearance routes again, there are the same two possible options conceivable: through the subchondral bone (subchondral disposal (SuD)) or through the synovial capsule

(synovial disposal (SyD)) (Fig. 8.2c). Ingelmark and Saaf already showed in 1948 that micrometer-sized particles injected into the joint space can be found after several loading cycles of the joint in the synovial membrane but not in the cartilage (Ingelmark and Saaf 1948). However, when they injected the particles into the bone marrow, they could find them in different zones of the cartilage, dependent on the movement of the joint, suggesting a transport mechanism from the subchondral bone through the cartilage into the synovia. It is hardly conceivable how other waste products should diffuse against this flow. Further favoring a possible role in waste disposal, the human synovium has a relatively large surface area (277 cm^2 in the knee) with capillaries that form delicate anastomosing loops and a general histological appearance resembling rather mesenterial tissue than pleura (Davies 1946).

Taken together, while there is currently a common belief in a combined role for synovial nutrition and waste disposal through dialysis of serum in the joint capsule (SyN-SyD), the published data allow to hypothesize a SuN-SyD nutrition/disposal pattern: flow of fluid and nutrients in healthy joints from the epiphyseal plexus, through the cartilage, and waste disposal by resorption through the large surface of the mesentery-like synovial capsule (Fig. 8.2d).

8.5 Changes of Subchondral Bone in Age and Joint Disease

If we assume a SuN-SyD nutrition/disposal, pathological changes in the subchondral bone and/or synovial capsule should lead to distinct changes in the joints leading to the same outcome: degradation of the cartilage. Synovitis is only present in 50% of patients with early OA but in nearly all patients with late-stage OA (Haywood et al. 2003). So, in half of the patients, the disease starts either in the subchondral bone or directly in the cartilage. The resulting cartilage degradation and ensuing inflammation then probably lead to synovitis in the process. Although there are a number of pharmaceutical approaches to counteract inflammation in joint disease, so far this approach was only effective against pain, but did not reverse the disease (for review see Philp et al. 2016). A possible reason for this might be lack of stratification of these patients, as probably only 50% of patients with leading inflammation would benefit from the treatment and the combination with patients without this indication would presumably generate impaired statistical power leading to nonsignificant results.

Changes in subchondral bone as a cause for rather than an effect of cartilage degradation have been discussed at least since 1970 (Radin et al. 1972; Radin and Paul 1970). In healthy joints, the thickness of the calcified cartilage decreases with age from 200 μm at the age of 20 to 100 μm at the age of 90, while the number of tidemarks increases from one to three in the same time frame (Lane and Bullough 1980). A common finding of joint injury and OA is sometimes a painful "bone bruise" originating from a localized increase in water content of a trabecular region detectable in MRI (Fig. 8.3).

It is commonly explained as intratrabecular bleeding of micro-injuries of the subchondral bone. Considering a Sun-SyD pattern, this phenomenon could also be

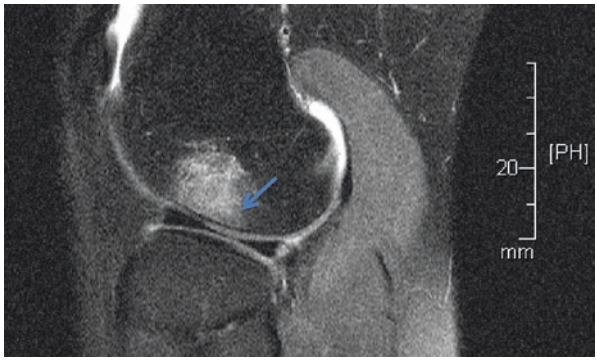

Fig. 8.3 Bone bruise

a posttraumatic congestion of the micro-channels that supply the SuN route, leading to buildup of fluid before the block. This would also explain the associated pain as induced by increased pressure in the affected bone. Indeed, the penetration of fluorescent dye from the marrow significantly reduces in a specimen with arthritic changes of the joint when compared to normal controls (Greenwald and Haynes 1969). Subchondral sclerosis, a thickening of the subchondral zone which is a hallmark feature of osteoarthritis, would have a similar effect. Indeed, there is an inverse correlation between OA and osteoporosis (Bergink et al. 2005). Microarray gene expression analysis of osteoarthritic bone suggests altered bone remodeling through WNT, TGF-beta, and BMP signaling (Hopwood et al. 2005, 2007) leading to increased bone formation. The bone tissue mineralization in the subchondral bone is decreased in OA, most probably reflecting this increased buildup of new bone (Cox et al. 2012). Furthermore, in osteoarthritic men, serum RANKL levels drop with age (Findlay et al. 2008) possibly leading to decreased bone resorption in the subchondral bone.

8.6 Regeneration of Cartilage and the Role of Subchondral Bone

If indeed the subchondral bone plays an important role in the pathophysiology of cartilage degeneration, it may also play a role in cartilage regeneration. Currently, different treatment options came up, some of which influence the bone and therefore presumably also subchondral bone.

8.6.1 Pharmacotherapy of Bone Turnover

Subchondral bone turnover is increased 20-fold in OA (Bailey et al. 2004) which can be visualized by MRI/99^{mTc}-DPD-SPECT/CT (Maas et al. 2015). The progression of joint space narrowing can be predicted by scintigraphy of the subchondral bone (Dieppe et al. 1993). Blocking of bone turnover by inhibition of osteoclasts

can prevent the progression of arthritic changes in rats (Hayami et al. 2004). There are a number of available drugs that can influence this pathway in clinical trials (Karsdal et al. 2008), but so far the results mainly point to a positive effect on osteoarthritic pain (Varenna et al. 2015; Davis et al. 2013). This may in part be due to the slow remodeling of the bone. Laslett et al. report a first positive trend of bisphosphonate treatment on joint space narrowing after 4 years of treatment (Laslett et al. 2014). So it may take time until positive effects of inhibition of remodeling become visible.

8.6.2 Changing the Biomechanics Leading to Unloading of the Subchondral Bone

Lifestyle Modification Weight loss in obese patients can improve the symptoms of osteoarthritis (Gudbergsen et al. 2012). It can be hypothesized that weight loss and the subsequent decrease of biomechanical forces in the joints may lead to changes in the subchondral bone. As weight loss in these patients is associated with a multitude of beneficial effects and there is no data on subchondral bone in these patients available, this is only speculation.

Surgery There are different surgical techniques for changing the biomechanics of the joint in OA. It has been shown that open-wedge osteotomy changes the subchondral bone structure (Ziegler et al. 2015). The change of the alignment of the joint through wedge osteotomy or reorientation of the acetabulum has been shown effective in the treatment of different forms of OA (Spahn et al. 2013; Hartig-Andreasen et al. 2012). Furthermore, open-wedge osteotomy (Kesemenli et al. 2013) has also been shown to resolve painful bone marrow edema, supporting the Sun-SyD hypothesis.

8.6.3 Reopening the SuN Pathway

The most commonly applied surgical procedure in early OA is microfracturing of the subchondral bone also called bone marrow stimulation technique (BST) (Min et al. 2013) (for further details, see also Chap. 5). It is sometimes combined with the use of biomaterials (AMIC, NAMIC) (Benthien and Behrens 2015; Anders et al. 2013). The conceptual framework underlying this procedure assumes that by opening the barrier to the bone marrow, this allows mesenchymal stem cells to repopulate the cartilage and repair the tissue (Min et al. 2013; Shapiro et al. 1993). While this procedure has very high success rate in a first-line treatment, it does not seem to perform as well in a salvage situation (Truong et al. 2014) although the amount of recruited stem cells stays the same. Differences in subchondral bone repair following first-line and salvage procedure may explain these clinical findings, if reopening of the SuN route is assumed as underlying mechanism. The scar tissue that is formed in the first-line procedure would need a different microfracture

technique than the original subchondral bone. This is similar to scar surgery, where the results of scar incision are worse than the results of scar excision (Davids et al. 2016).

There are at least two recent reports of cartilage regeneration after complete cartilage loss. First, using a combination of high tibial wedge osteotomy together with subchondral drilling and stem cell therapy, it was possible to regenerate cartilage from grade 4 bone-on-bone lesions (Saw et al. 2015). Second, even more promising, temporary surgical joint distraction alone leads to symptomatic and structural improvement in end-stage knee osteoarthritis (Wiegant et al. 2013) which was comparable to the results of open-wedge osteotomy (van der Woude et al. 2016). Both cases can be explained by structural optimization of the subchondral bone allowing SuD. However, this hypothesis will have to be further tested in the coming years.

Conclusion

Regeneration of lost cartilage is possible and is clinically performed worldwide. A prerequisite for eventual cartilage healing seems to be a favorable environment especially of the subchondral bone, but also of the synovial capsule. Many findings can be explained, if a physiologic flow of nutrients and oxygen from the subchondral bone through the cartilage into the synovial capsule is hypothesized. Closer inspection of the physiology and pathology of the delicate subchondral structures and their connection with the overlying cartilage may lead to better individualized treatments for patients with cartilage defects.

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Gene Transfer Strategies for Articular Cartilage Repair

9

Magali Cucchiarini

Abstract

Gene therapy protocols are well suited to deliver genes coding for therapeutic factors over time in a spatially defined manner within sites of cartilage injury resulting from acute trauma or during osteoarthritis. The focus of this chapter is to examine the benefits of gene therapy to improve cartilage repair in such lesions, based on promising experimental and clinical evidence in relevant models in vivo using growth, transcription, and signalling factors capable of stimulating the chondrogenic and chondro-reparative processes locally. A continuous, combined effort between scientists and orthopaedic surgeons may allow to bring gene therapy from encouraging data at the bench to a successful, safe translation in the broadly affected human population.

9.1 Introduction

Articular cartilage defects like after trauma or during osteoarthritis (OA) have a limited capacity for self-repair. The idea of applying gene transfer strategies to enhance cartilage repair by local application of therapeutic (growth, transcription, signalling) factors originates from the possibility to extend therapeutic transgene expression and subsequent effects over time compared with the injection of recombinant agents showing relatively short half-lives. Increasing, promising experimental data have shown the benefits of providing such therapeutic sequences using various gene transfer systems in relevant in vitro, in situ, and in vivo models of focal

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and OA cartilage lesions via direct (cell-free) and indirect (cell-associated) procedures, allowing to rejuvenate the affected cells and to improve the repair of the damaged tissues. The availability of clinically suited vectors and of workable strategies may allow in a close future to provide adapted, effective novel options to treat cartilage injuries in patients.

9.2 Articular Cartilage Repair

Adult hyaline cartilage, the tissue that affords a smooth gliding of articulating surfaces and protects the subchondral bone against mechanical stress in the joint, has a limited intrinsic ability to heal in the absence of vascularization that may provide chondro-regenerative cells such as progenitor cell populations in response to injury (Heijink et al. 2012; Pape et al. 2010). The articular cartilage is formed by chondrocytes embedded in a self-produced, complex extracellular matrix in different layers, with a calcified basal layer towards the underlying subchondral bone. The cartilage matrix contains proteoglycans bound to 70–80% water, collagen fibrils (mostly type II but also type VI, IX, XI, and XIV collagen), and other molecules such as the cartilage oligomeric matrix protein (COMP), link protein, decorin, fibromodulin, fibronectin, and tenascin. Cartilage lesions can be circumscribed (focal defects) or generalized (osteoarthritis, OA, a chronic, inflammatory/catabolic whole degenerative joint disorder with a gradual degradation of the cartilage, subchondral bone, synovium, ligaments, tendons, and muscles) (Madry et al. 2012; Heijink et al. 2012; Loeser et al. 2012).

The repair of cartilage is considered as the production of a tissue that shares structural similarities with the hyaline articular cartilage. Regeneration, however, is a complete reproduction of the hyaline cartilage. Natural repair of chondral defects occurs by migration of cells from the synovial membrane but leading to a tissue that does not integrate with the surrounding cartilage and to larger defects. Osteochondral defects are filled with a clot from the bone marrow containing chondrogenically and osteogenically competent bone marrow-derived mesenchymal stem cells (BM-MSCs), but again a fibrocartilaginous repair tissue is produced with early signs of OA. OA cartilage has also restricted repair capabilities leading to an irreversible degradation of the cartilage and a remodelling of the osteochondral unit.

Current interventions for chondral defects include marrow stimulation (subchondral drilling, microfracture, and abrasion arthroplasty), and the transplantation of autologous chondrocytes with or without supportive matrix (autologous chondrocyte implantation, i.e. ACI) and those for osteochondral defects is based on the implantation of uninjured osteochondral cylinders and of subchondral bone grafts combined with ACI (Madry et al. 2011a) (for details, see Chap. 5). OA treatments are either conservative (non-pharmacological and pharmacological options using non-steroidal anti-inflammatory drugs, opioid analgesics, or intraarticular corticosteroid or hyaluronic acid (HA) injections) or surgical like by osteotomy (Madry et al. 2011a).

As none of these procedures are capable of fully managing any of these lesions, novel, effective options are needed to improve cartilage repair, like those afforded by gene therapy that may need to take into account the differences between the nature, size, number, and location of the injury and the stage of the disease (Madry and Cucchiarini 2013, 2015; Frisch et al. 2015b; Cucchiarini et al. 2014; Madry et al. 2011b; Cucchiarini and Madry 2005).

9.3 Gene Therapy: Principles and Experimental Approaches for Cartilage Repair

Gene therapy aims at treating human disorders by applying gene transfer techniques in patients in vivo. Foreign genes may penetrate the target cell to reach the nucleus where they stay extrachromosomal (more or less stable episomal forms) or become integrated within the host genome in specific or unspecific locations (stable forms) as features inherent to the class and biology of the vector employed. Targeting by gene transfer usually affects a high number of cells that are sufficient for the production of the transgene product and for consequent therapeutic applications. The best characterized vectors available for gene therapy protocols mostly include nonviral constructs and approaches and viral vectors (Table 9.1) (Cucchiarini and Madry 2005; Madry and Cucchiarini 2013, 2015; Frisch et al. 2015b; Madry et al. 2011b).

Table 9.1 Common vectors available for gene therapy

Types		Advantages	Limitations	Integration
Nonviral vectors		• Not infectious • Not toxic • Easy to produce • Large capacity	• Low efficiency • Short-term expression	–
Viral vectors	Adenovirus	• High efficiency	• Replication competence • Toxic • Immunogenic • Short-term expression	–
	Retro-/*Lentivirus*	• High efficiency • Long-term expression	• Replication competence • Insertional mutagenesis	Yes
	HSV	• High efficiency • Large capacity	• Cytotoxic • Short-term expression	–
	rAAV	• High efficiency • Long-term expression • Low immunogenic	• Difficult to produce • Size limitation	Mostly episomal

Abbreviations: *HSV Herpes simplex virus*, *rAAV* recombinant adeno-associated virus

9.3.1 Gene Transfer Vectors

9.3.1.1 Nonviral Systems

Transfection is the transfer of foreign genes via nonviral vectors (cationic lipids and liposomes, polymers, polyamines, polyethylenimines, nanoparticles). These systems are large, easy to generate, and safe (lack of acquiring replication competence like for viral vectors, no immunogenicity), allowing for repeated administration. Yet, they display a relatively low efficacy compared with viral vectors. Also, as mostly episomal forms, they commonly promote short-term transgene expression. These vectors are thus rather employed in indirect gene transfer protocols by implantation of ex vivo modified cells in the recipient.

9.3.1.2 Viral Vectors

Transduction is the term characterizing viral-based gene transfer. Viral vectors employ their natural entry pathways in the cells. Adenoviral vectors have a high gene transfer efficacy, enabling direct approaches in vivo, but they are highly immunogenic and remain episomal, restricting transgene expression to only about 1–2 weeks. Retroviruses instead can integrate into the host genome, allowing for the maintenance of the transgene over time. Still, integration is associated with a risk of insertional mutagenesis and of tumour gene activation. In addition, these vectors transduce only dividing cells (progenitor cells but not differentiated chondrocytes and bone cells) at a restricted host range, making them more adapted for indirect, ex vivo approaches, further allowing to increasing their otherwise low gene transfer efficacy. Lentiviral vectors that derive from the *Human immunodeficiency virus* (HIV) instead can integrate in nondividing cells at higher efficiency, but concerns remain due to the potential for insertional mutagenesis and to the problem of introducing material carrying HIV sequences. Large *Herpes simplex virus* (HSV) vectors can target nondividing cells, but still induce cytotoxic responses and allow only for very transient transgene expression. Small recombinant adeno-associated viral (rAAV) vectors have various advantages as they are safe and low immunogenic in the complete absence of viral gene sequences. This is particularly important as cartilage lesions are not life-threatening problems. They can transduce both dividing and nondividing cells and are mostly kept as very stable episomes, allowing for long-term transgene expression.

9.4 Strategies to Improve Cartilage Repair

Target cells relevant for cartilage repair permissive to gene transfer include articular chondrocytes to repopulate the injured cartilage, bone cells (osteoblasts/osteocytes to reconstruct the subchondral bone), synoviocytes (as a source of therapeutic factors), other affected cells (meniscal fibrochondrocytes, tendon/ligament cells, muscle cells), and progenitor cells (BM-MSCs that may commit towards mesodermal cell lineages

as isolated/expanded suspensions or directly in marrow aspirates/concentrates) (Arai et al. 2000; Baragi et al. 1995; Cucchiarini et al. 2014; Doherty et al. 1998; Gouze et al. 2002; Hildebrand et al. 1999; Madry et al. 2004a; Madry and Trippel 2000; Mason et al. 2000; Nita et al. 1996; Orth et al. 2014; Rey-Rico et al. 2015a; Stender et al. 2007). These cells may be directly genetically modified within the lesions by direct gene transfer vector administration or first isolated and/or expanded for indirect gene transfer ex vivo prior to reimplantation. This may offer to employ other cell types such as MSCs from other tissues like the adipose tissue, synovium, periosteum, and perichondrium (Cucchiarini and Madry 2005; Madry and Cucchiarini 2013, 2015; Cucchiarini et al. 2014; Frisch et al. 2015b; Madry et al. 2011b). Various approaches have been developed experimentally to deliver therapeutic gene sequences in sites of articular cartilage injury (Fig. 9.1), among which administration of a therapeutic

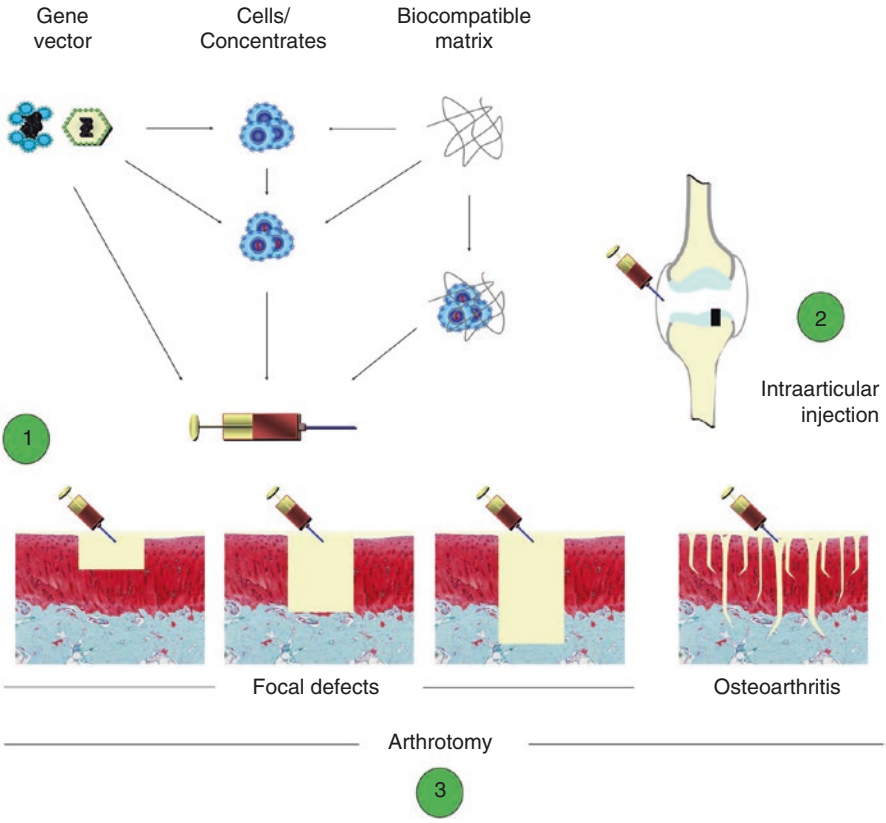

Fig. 9.1 Experimental approaches for the delivery of therapeutic gene sequences in sites of articular cartilage injury. (*1*) Preparation of a therapeutic composition with gene vectors, cells (suspension or as concentrates), and a biocompatible matrix. The composition may be provided (*2*) intraarticularly or (*3*) via arthrotomy in either focal defects or in OA cartilage

compound intraarticularly or via arthrotomy using either the gene vector itself or genetically modified cells as a suspension or using a biocompatible matrix (Cucchiarini and Madry 2005, 2014b; Johnstone et al. 2013; Madry and Cucchiarini 2013, 2015; Cucchiarini et al. 2014; Frisch et al. 2015b; Madry et al. 2011b).

9.4.1 Gene Therapy for Cartilage Repair: Evidence In Vitro

Pathways that might be targeted to improve cartilage repair by gene therapy include the activation of cell proliferation and survival, the stimulation of anabolic responses, and the prevention of inflammation and tissue degradation via single or combined approaches.

9.4.1.1 Activation of Cell Proliferation and Survival

These processes may be stimulated by gene transfer of mitogenic growth factors (insulin-like growth factor I (IGF-I), basic fibroblast growth factor (FGF-2), bone morphogenetic proteins (BMPs), transforming growth factor beta (TGF-β)) (Madry et al. 2001, 2004b; Cucchiarini et al. 2009, 2011; Weimer et al. 2012; Shi et al. 2013; Neumann et al. 2013; Venkatesan et al. 2013; Frisch et al. 2014a, b, 2015a), telomerase (hTERT) (Piera-Velazquez et al. 2002), inhibitors of apoptosis (bcl-2) (Surendran et al. 2006), or of the heat shock protein 70 (HSP70) (Grossin et al. 2006). Most remarkably, delivery and overexpression of the key IGF-I and TGF-β factors via potent rAAV-mediated gene transfer allowed for a stable reproduction of the cell proliferative indices in human OA articular cartilage explant cultures in situ, reaching levels and patterns typical of normal cartilage over an extended period of 90 days when the cells are embedded in their natural matrix (Weimer et al. 2012; Venkatesan et al. 2013).

9.4.1.2 Stimulation of Anabolic Responses

Production of matrix components may be enhanced by application of sequences for the matrix molecules themselves or of the enzymes that synthesize them (Venkatesan et al. 2004), of pro-anabolic growth and signalling factors (IGF-I, FGF-2, BMPs, TGF-β, parathyroid hormone-related peptide (PTHrP), Indian hedgehog (Ihh)) (Smith et al. 2000; Nixon et al. 2000; Shuler et al. 2000; Mi et al. 2000; Madry et al. 2001; Brower-Toland et al. 2001; Palmer et al. 2005; Cucchiarini et al. 2005; Ulrich-Vinther et al. 2005; Wang et al. 2011; Weimer et al. 2012; Steinert et al. 2012; Neumann et al. 2013; Venkatesan et al. 2013; Frisch et al. 2014a, b, 2015a), or of tissue-specific transcription factors (sex-determining region Y-type high mobility group box—SOX family, zinc-finger protein 145 (ZNF145)) (Li et al. 2004; Cucchiarini et al. 2007; Liu et al. 2011; Rey-Rico et al. 2015a; Shi et al. 2015).

Among these many factors, here again administration of IGF-I and TGF-β sequences via rAAV vectors may be best suited in light of reports showing close-to-normal, long-term reconstruction of the extracellular matrix in human OA articular cartilage for at least 90 days in situ (Weimer et al. 2012; Venkatesan et al. 2013), together with the use of the cartilage-specific SOX9 transcription factor that also exhibits highly effective chondro-regenerative activities that are essential for cartilage repair (Cucchiarini et al. 2007; Rey-Rico et al. 2015a).

9.4.1.3 Prevention of Inflammation and Tissue Degradation

Reduction of catabolic processes may be achieved with inhibitors of matrix-degrading enzymes (Kafienah et al. 2003) and of pro-inflammatory cytokines (interleukin-1 receptor antagonist—IL-1Ra) (Baragi et al. 1995; Roessler et al. 1995; Glass et al. 2014). Glass et al. recently provided interesting evidence that combining gene therapy with functional tissue engineering provided powerful tools to produce cartilage with immunomodulatory properties via scaffold-mediated IL-1Ra gene transfer in MSCs, permitting chondrogenesis upon pathologic activation by IL-1 (Glass et al. 2014). Prevention of osteophyte formation in OA may be also considered like by application of antagonists of the TGF-β/BMP pathway (latency-associated peptide—mLAP-1, Smads) (Scharstuhl et al. 2003) or of an IL-1Ra formulation (Fernandes et al. 1999).

9.4.1.4 Combined Approaches

Multifactorial approaches have been attempted by cotransfer of activators of proliferative/anabolic processes (IGF-I/FGF-2/BMPs/SOX) (Ikeda et al. 2004; Cucchiarini et al. 2009; Orth et al. 2011; Shi et al. 2012, 2013) or of activators of anabolic/proliferative pathways with inhibitors of catabolism (IGF-I/FGF-2/IL-1Ra) (Nixon et al. 2005; Haupt et al. 2005; Chen et al. 2010). Approaches that aim both at counteracting deleterious inflammation while restoring the altered metabolic balance might be desirable to address the spectrum of pathomechanisms triggered in sites of cartilage injury. Specifically, several groups showed that cells cotransduced with IGF-I/IL-1Ra via adenoviral vectors were more potent to reverse IL-1-mediated proteoglycan depletion in cartilage compared with single gene treatments (Nixon et al. 2005; Haupt et al. 2005; Chen et al. 2010).

9.4.2 Gene Therapy for Cartilage Repair: Evidence In Vivo

Both direct and indirect gene transfer strategies have been tested in animal models of cartilage injury to provide therapeutic genes for enhanced cartilage repair. Direct strategies are less invasive, but necessitate that the vectors are adapted to effectively reach the target cells within their dense matrix. In this case, the small rAAV vectors are probably the most adequate gene vehicles to achieve this goal. Indirect strategies might be more desirable to repopulate tissue lesions, having the additional advantages of introducing modified cells rather than free vector particles while permitting extensive control of the cells prior to reimplantation and allowing the use of biocompatible scaffolds.

9.4.2.1 Direct Gene Transfer Strategies

Such approaches have been tested to enhance cartilage repair in experimental models of focal defects (Cucchiarini et al. 2005, 2013; Morisset et al. 2007; Cucchiarini and Madry 2014a; Griffin et al. 2015) and of OA (Fernandes et al. 1999; Frisbie et al. 2002; Grossin et al. 2006; Chen et al. 2008, 2010; Hsieh et al. 2009, 2010; Shen et al. 2011; Oh et al. 2012; Santangelo et al. 2012; Zhang et al. 2015) using

sequences for growth factors (IGF-I, FGF-2, TGF-β) (Cucchiarini et al. 2005; Chen et al. 2010; Cucchiarini and Madry 2014a; Griffin et al. 2015; Zhang et al. 2015), IL-1Ra (Fernandes et al. 1999; Frisbie et al. 2002; Chen et al. 2010; Santangelo et al. 2012; Zhang et al. 2015), SOX9 (Cucchiarini et al. 2013), HSP70 (Grossin et al. 2006), silencers of NF-kappaBp65 (Chen et al. 2008), inhibitors of inflammatory pain processes (pro-opiomelanocortin, POMC) (Shen et al. 2011), antagonists of the canonical Wnt pathway (Dickkopf1, Dkk-1) (Oh et al. 2012), kallistatin or angiogenic inhibitors (thrombospondin-1, TSP-1) (Hsieh et al. 2009, 2010), or combined approaches (IGF-I/IL-1Ra, TGF-β/IL-1Ra) (Morisset et al. 2007; Zhang et al. 2015), allowing for improved cartilage repair in these different systems. Most significantly, treatment of experimental focal (osteochondral) defects by direct application of an rAAV vector carrying the potent SOX9 transcription factor improved the processes of cartilage repair in rabbit knee joints for a stable period of 16 weeks without detrimental effects (Cucchiarini et al. 2013). Regarding OA, therapeutic success has been reported by various groups based on the application of IL-1Ra-coding nonviral and adenoviral vectors both in OA guinea pigs, rabbits, and horses, promoting a net reduction in the disease severity together with improvements in cartilage preservation (Fernandes et al. 1999; Frisbie et al. 2002; Santangelo et al. 2012; Zhang et al. 2015).

9.4.2.2 Indirect Gene Transfer Strategies

Administration of genetically modified cells has been also reported to treat experimental models of focal defects (Mason et al. 2000; Lee et al. 2001; Hidaka et al. 2003; Georgi et al. 1992; Madry et al. 2005, 2013; Guo et al. 2006; Kaul et al. 2006; Kuroda et al. 2006; Goodrich et al. 2007; Evans et al. 2009; Vogt et al. 2009; Che et al. 2010; Ivkovic et al. 2010; Noh et al. 2010; Liu et al. 2011; Orth et al. 2011; Ortved et al. 2015; Sieker et al. 2015) and of OA (Bandara et al. 1993; Zhang et al. 2004; Matsumoto et al. 2009) using genetic modification of chondrocytes (Hidaka et al. 2003; Madry et al. 2005, 2013; Kaul et al. 2006; Goodrich et al. 2007; Che et al. 2010; Noh et al. 2010; Orth et al. 2011; Ortved et al. 2015), synoviocytes (Bandara et al. 1993; Zhang et al. 2004), various progenitor cells as suspensions or as marrow aspirates/concentrates (Mason et al. 2000; Katayama et al. 2004; Guo et al. 2006; Kuroda et al. 2006; Vogt et al. 2009; Matsumoto et al. 2009; Ivkovic et al. 2010; Liu et al. 2011; Sieker et al. 2015), and tissue grafts (Evans et al. 2009) to overexpress growth factors (IGF-I, FGF-2, BMPs, TGF-β) (Mason et al. 2000; Lee et al. 2001; Hidaka et al. 2003; Madry et al. 2005, 2013; Guo et al. 2006; Kaul et al. 2006; Kuroda et al. 2006; Goodrich et al. 2007; Evans et al. 2009; Vogt et al. 2009; Che et al. 2010; Ivkovic et al. 2010; Noh et al. 2010; Ortved et al. 2015), IL-1Ra (Bandara et al. 1993; Zhang et al. 2004), SOX9 (Liu et al. 2011), Ihh (Sieker et al. 2015), the cartilage-derived morphogenetic protein 1 (CDMP-1) (Katayama et al. 2004), IGF-I/FGF-2 (Orth et al. 2011), IL-1Ra/IL-10 (Zhang et al. 2004), or BMP-4/sFlt-1 (an antagonist of the vascular endothelial growth factor, VEGF) (Matsumoto et al. 2009), allowing for improved cartilage repair in these various models. Significant advances have been made in experimental cartilage repair when implanting IGF-I-expressing vectors in articular chondrocytes or BMP-2-modified

tissues (fat, muscle, fibrin clots) in focal defects in rabbits and horses using nonviral, adenoviral, retroviral, and rAAV vectors (Goodrich et al. 2007; Evans et al. 2009; Vogt et al. 2009; Madry et al. 2013; Ortved et al. 2015), leading to improved tissue healing. In OA models, mostly synovial cells overexpressing IL-1Ra via retroviral vectors have been provided to prevent cartilage breakdown in OA rabbits (Bandara et al. 1993; Zhang et al. 2004), while injection of muscle-derived MSCs producing BMP-4/sFlt1 in OA rats led to durable cartilage repair (Matsumoto et al. 2009).

9.5 Gene Therapy: Current Advances in Clinical Trials for Cartilage Repair—Perspectives

Compared with the large body of available experimental data, relatively few trials have been initiated to treat patients with focal cartilage lesions and OA, reflecting the difficulty to translate research ideas and visions into clinical and commercial reality (Evans et al. 2012, 2013; Cucchiarini et al. 2014; Bara et al. 2015). While no trials are ongoing for focal defects, phase I and II clinical trials have been recently published to treat OA patients by injecting retrovirally modified chondrocytes to produce TGF-β1 (Ha et al. 2012, 2015; Lee et al. 2015; Cherian et al. 2015), showing a trend towards efficacy with improvements in pain, function, and physical ability. Yet, in light of multiple reports showing adverse effects of TGF-β in experimental models in vivo (synovial inflammation and fibrosis, osteophyte formation) (van Beuningen et al. 1998; Bakker et al. 2001; Mi et al. 2003; Blaney Davidson et al. 2007; Remst et al. 2013), other approaches might be necessary to improve the safety of the outcomes and prevent such detrimental, undesirable effects in patients.

Remarkably, rAAV vectors have emerged as well-suited systems for clinical applications as these vectors are highly effective in direct, less invasive protocols compared with retroviral vectors and considered as safe delivery compounds, receiving market authorization by the European Union Medicines Agency's Committee for Medicinal Products for Human Use (CHMP) for the treatment of lipoprotein lipase (LPL) deficiency (Glybera®) (Yla-Herttuala 2012; Buning 2013). Interestingly, a phase I study by direct administration of an rAAV IL-1Ra construct has been initiated to treat OA patients (Evans et al. 2013). It remains to be seen whether such a trial will allow for successful outcomes as a major concern to the clinical use of this vector class is the significance of pre-existing and induced immune responses against the viral capsid proteins of AAV, with a clear impact on clinical efficacy and safety (Ferreira et al. 2014a, b). While an rAAV-based gene therapy might be provided in conditions of immunosuppression to improve efficacy (Ferreira et al. 2014b), the safety of the immunosuppressed patients remains a concern as OA is a nonlethal disease. A newly developed, highly potent approach to circumvent these issues is to provide rAAV using effective controlled release strategies by delivery of the vectors from adapted biomaterials as a means to mask the immunogenic capsid epitopes in the host without impairing the efficacy of gene transfer and target cell modification (Rey-Rico et al. 2015b, c; Rey-Rico and

Cucchiarini 2015; Diaz-Rodriguez et al. 2015). Tissue-engineering strategies may also prove beneficial to improve the repair of cartilage lesions and thus address the challenge of providing functional, adapted cartilage replacement therapies as largely tested in preclinical settings in relevant models (Johnstone et al. 2013; Cucchiarini and Madry 2014b; Madry and Cucchiarini 2014). Eventually, as complete regeneration of an original hyaline cartilage has not been reported thus far in preclinical models, further research is clearly needed to identify the most effective genes or combinations of genes for therapy, suggesting that a better understanding of the joint pathologies is essential by continuous efforts between orthopaedic surgeons, scientists, and regulatory organizations to advance the current approaches and trials in patients (Cucchiarini et al. 2014).

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Tissue Engineering Strategies for Cartilage Repair

10

Holger Jahr

Abstract

Cartilage repair addresses several facets of the diversity in our present approaches to regenerate articulating surfaces. The following chapter now summarizes several promising options to engineer articular cartilage-like constructs, ranging from applying biological factors and/or mechanical, magnetic, or even electrical stimuli. The paradigm of cartilage tissue engineering classically comprises three pillars: cells, scaffolds, and signals. As cell sources for cartilage repair are addressed by other chapters in this volume, the next pages will focus on the two remaining pillars: first, due to their importance for the subsequent tissue engineering path, scaffold-free and scaffold-based applications are distinguished. Although most classical techniques in the field are scaffold based, relative more attention is now paid to emerging scaffold-free methods as articular cartilage repair constructs. Only proper tissue organization will permit long-term functional durability, and mimicking tissue growth without artificial support structures holds a lot of potential. While the extracellular matrix is an integral aspect of the tissue properties, it also impedes the integration of the repair construct into the surrounding host tissue. Several approaches to tackle this dilemma are depicted. The importance to develop bioreactors is also emphasized as they are inevitable for the reproducible application of sophisticated mechanobiological stimulation regimes. In this context, the contribution of selected growth factors is described. Towards the end of the present chapter, the importance of integrating multiple of these parameters into multimodal concepts for achieving phenotypic stability of the engineered cartilage-like constructs is addressed.

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10.1 Introduction

Of the three types of cartilage (i.e. elastic, fibro-, and hyaline), this chapter will focus on hyaline articular cartilage due to its socioeconomic relevance and its importance in withstanding mechanical loads.

Chondrocytes reside in a complex, multiphasic extracellular matrix (ECM) described by three principal phases: a solid phase, a fluid phase, and an ion phase (Mow et al. 1999). Their interaction results in a complex mechano-electrochemical environment in which chondrocytes are exposed to multiple biophysical stimuli including mechanical forces (i.e. tension, compression, shear), fluid flow, hydrostatic and osmotic pressure gradients, and electrokinetic events (Brady et al. 2015b). The mechanism by which these cells convert mechanical signals into biochemical responses is called mechanotransduction (Ingber 2006), and it is well accepted that chondrocytes directly respond to mechanical forces (Wong and Carter 2003; Brady et al. 2015b).

Our current knowledge of these biophysical cues can be readily applied to engineer neocartilage. However, hyaline cartilage remains one of the most challenging tissues to replace, owing to its mechanical strength at articulating surfaces. Limited biomechanical properties and poor post-implantation integration with the host are major challenges in cartilage tissue engineering (TE). To this end, cartilage TE aims at delivering ex vivo generated constructs that are biologically mature and mechanically functional from day one to potentially decrease the recovery time for the patient.

In this chapter, general strategies for cartilage TE will be covered. While most commonly used adult stem cells for cartilage TE may be MSCs derived from bone marrow (BMSC), adipose (ADSC), and synovium (SDSC), appropriate cell sources for cartilage TE are dealt with in other chapters in this volume (details see Chaps. 2–4). While scaffold-based techniques are only briefly described, emphasis is put on recent scaffold-free approaches. The importance of tissue structure and bioactive compounds is briefly elaborated. Different currently reported bioreactor concepts to realize cartilage TE are discussed as is the role of mechanobiological aspects. Towards the end, other physicochemical factors are revealed as are issues with their integration and phenotypic stability upon implantation.

10.2 Medical Need for Articular Cartilage Tissue Engineering

Degeneration of articular hyaline cartilage may occur due to trauma and metabolic or mechanical deficits leading to osteoarthritis. Although current options for cartilage repair are reasonably effective to alleviate pain, they have their respective limitations (Huey et al. 2012). A major clinical need thus exists for cartilage repair and regeneration, but identifying optimal cell types and robust pretreatment conditions remains challenging. Currently, pre-culture under chondrogenic conditions seems necessary to maintain a long-term chondrocytic phenotype.

Thus, effects of growth factors (GFs) known to play key roles in cartilage development, either alone or in combination, have been investigated for maintenance of the chondrogenic phenotype and for promoting cartilage formation in vitro.

10.3 General Strategies for Cartilage Tissue Engineering

Besides a plethora of biological factors, mechanical, magnetic, and electrical stimuli have been used to promote proliferation, differentiation, and maturation of chondrocytes within established dose parameters or "biological windows". While cells are crucial for tissue regeneration as they are responsible for synthesizing the ECM molecules that reshape the tissue structure and confer mechanical properties, for deeper insights, the reader is referred to more specialized chapters in this volume.

Articular cartilage withstands compressive, tensile, and shear loads as part of its function, and it is also lubricious, resulting in a tissue with very low coefficient of friction. Cartilage's biochemical content, primarily water, collagen, and proteoglycans (PGs), and the specific organization of these molecules within the ECM allow for appropriate load distribution and transmission. One of the major design criteria in cartilage TE is therefore the creation of neocartilage with biomechanical properties that will withstand the demanding mechanical environment in vivo. Proper organization of the neocartilage is anticipated to allow for long-term functionality and durability.

10.4 Scaffolds for Cartilage Tissue Engineering

10.4.1 Scaffold-Based Applications

The choice of biomaterials and scaffold design is an important consideration for successful cartilage TE. Briefly, natural materials, like agarose, alginate, hyaluronic acid, fibrin, collagen derivatives (e.g. gelatine), or de-cellularized matrices, are being used (Raghunath et al. 2007). Though being generally attractive, disadvantages like inferior mechanical strength, antigenicity, and potential risk of disease transfer as well as rapid and variable host-specific degradation times have to be considered.

As the encapsulation forces of hydrogels, i.e. hydrophilic polymers with high water content and elastic nonadhesive properties, encourage cells to assume chondrocytic morphologies, they have been frequently applied in cartilage TE. However, their swelling from water uptake and inferior mechanical properties is a drawback.

With synthetic materials, on the other hand, scaffold properties are much easier to control and modify. The currently used product portfolio encompasses polyhydroxy acids (e.g. PLLA, PGA, PCL), poly(ethylene)glycol, or elastomeric polyurethanes (Raghunath et al. 2007; Camarero-Espinosa et al. 2016). Most, if not all, synthetic biopolymers lack bioactive molecules that promote cell attachment, proliferation, and differentiation. By combining materials, synthetic biopolymers can

potentially be tailored to provide the bulk properties, degradation profiles, and structures, while natural materials could provide bioactive molecules needed to activate desired signalling pathways. An exponentially increasing number of natural, synthetic, or composite polymeric scaffold materials with or without bioactive coatings or structural surface treatment exist. As these cannot be reviewed in the present chapter, the reader is referred to excellent overview articles (Darling 2013; Camarero-Espinosa et al. 2016).

10.4.2 Scaffold-Free Techniques

The traditional scaffold-based TE paradigm consists of cells, signals, and scaffolds (Fig. 10.1), but recent scaffold-free approaches consisting of just cells and signals can also be attractive. To make a clinically relevant scaffold-free tissue, TE

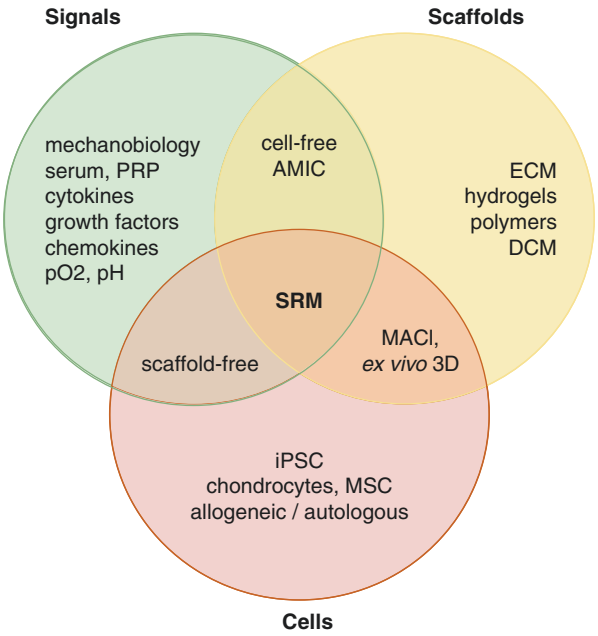

Fig. 10.1 Traditional cartilage TE paradigm. Cells, signals, and scaffolds are classical pillars of the three main cartilage regenerative strategies: (1) scaffold-based chemotaxis of host cells, (2) cell-seeded (biomimetic) scaffolds in combination with diverse signals, and (3) cell-free techniques. Recently, scaffold-free methods gained special attention. MSCs and chondrocytes (or combinations of both) appear to be best described cell sources. Neocartilage can be cultured ex vivo under different extracellular (i.e. biological, biophysical, mechanobiological) stimulation protocols for which bioreactor systems may be required. Modified after Makris et al. (2015). *AMIC* autologous matrix-induced chondrogenesis, *ECM* extracellular matrix, *DCM* de-cellularized ECM, *iPSC* induced pluripotent stem cells, *MACI* matrix-assisted autologous chondrocyte implantation, *MSC* mesenchymal stem cells, *pO2* partial oxygen pressure, *pH* potentia hydrogenii, *PRP* platelet-rich plasma, *SRM* synergistic regenerative medicine

considerations must include cell sourcing, stimulation of tissue-specific ECM production, and tissue organization. As scaffold-free approaches lack the exogenous material of scaffold-based methods, the resulting engineered cartilages (neocartilages) unfortunately commonly require large cell numbers. On the other hand, by mirroring cartilage development, only cell-secreted ECM contributes to neotissue properties. As cartilage is heterogeneous and anisotropic, achieving proper tissue morphology and organization is crucial for success (DuRaine et al. 2015).

This may be addressed by moulding or in vitro confining, assembling tissues as building blocks, and promoting their fusion to form higher-order structures. Applying mechanical stimulation to mature, the ECM may create functional anisotropy in these constructs (MacBarb et al. 2013). DuRaine and colleagues recently reviewed three promising scaffold-free TE principles: cell sheet engineering, aggregate engineering, and self-assembling process (DuRaine et al. 2015).

Cell sheets are cohesive monolayers and well known for their application as skin transplants from keratinocytes. This idea can be applied to chondrocytes, too, with subsequent draping, layering, or rolling to increase the application spectrum. While a phase III clinical trial using sheets of expanded juvenile allogeneic chondrocytes has been started (DuRaine et al. 2015), the technique has its limitations: chondrocytes are known to dedifferentiate during monolayer culture and progress towards a fibroblastic phenotype (Darling et al. 2009). In addition, harvesting and production of thicker, multicellular tissues may require substantial handling, too.

Aggregate constructs (also referred to as pellet cultures) commonly form by applying a rotational force to cells in suspension (Furukawa et al. 2003). This method is used to (re)differentiate cells or to form cartilaginous microtissue. The phenotype of chondrocyte aggregates is similar to that of native cartilage and thus thought to mirror cell aggregation and matrix production during cartilage development.

Recently, pellet culture of human MSCs in a demineralized bone matrix was used to produce engineered articular cartilage with a physiologically relevant compressive Young's modulus of about 800 kPa and an equilibrium friction coefficient of ~0.28 (Bhumiratana et al. 2014). Limitations of this technique are rather uncontrolled and nonhomogeneous shapes (Gigout et al. 2009) and potentially compromised core cell viability with subsequent loss of cell-type homogeneity. Also here, a phase III clinical trial was started in Europe, and 1-year follow-up results after treatment of full-thickness patella-femoral or femoral-condylar defects are promising (Fickert et al. 2012).

Self-assembling differs from aggregate formation in the way the tissue forms and the properties of the resulting ECM. This technique is applicable to different cell sources and the ECM composition, and its organization in the neocartilage appears similar to that of native cartilage (DuRaine et al. 2015). The adaptability and reproducibility of self-assembling processes make them highly promising to produce tissues with physiologically relevant properties. While successfully producing functional neocartilage, drawbacks are that (1) cells must be amenable to producing large amounts of ECM and (2) survive minimal cell-substrate interactions as the ECM accumulates during the initial phase of the self-assembling process. In

addition, self-assembling requires huge amounts of cells (Huey et al. 2012) which, in turn, currently requires monolayer expansion with subsequent redifferentiation. Also here, construct size of the engineered tissue is limited by diffusion (Leddy et al. 2004). However, self-assembling recapitulates developmental processes while allowing to control tissue geometry to create constructs with biologically reminiscent properties (Ofek et al. 2008).

10.5 Mimicking Tissue Structure and Properties

Articular cartilage withstands compressive, tensile, and shear loads and also has a very low coefficient of friction. Cartilage's biochemical content, primarily water, collagen, and PGs, and the organization of these molecules allow for appropriate load distribution and transmission. Therefore, one of the major design criteria in cartilage TE is the creation of neocartilage with biomechanical properties that will withstand the demanding mechanical environment in vivo.

Reported mechanical properties of natural cartilage include an aggregate modulus (0.1–2 MPa), hydraulic permeability (10^{-16} – 10^{15} m^4/Ns), compressive Young's modulus (0.24–0.85 MPa), Poisson's ratio (0.06–0.3), tensile equilibrium modulus (5–12 MPa), tensile Young's modulus (5–25 MPa), tensile strength (0.8–25 MPa), equilibrium shear modulus (0.05–0.4 MPa), complex shear modulus (0.2–2.5 MPa), and a shear loss angle (10°–15°) (Little et al. 2011). Its coefficient of friction ranges from 0.03 to 0.06 (DuRaine et al. 2009).

Therefore, proper tissue organization is believed to permit long-term functionality and durability of the neocartilage.

10.6 Bioactive Approaches

10.6.1 Growth Factors and Mechanical Stimuli

Improving the tensile properties of neocartilage has been one of the major challenges in cartilage TE (Makris et al. 2014b). Among the different GFs used in articular cartilage engineering, transforming growth factor (TGF)-β may be most prominently improving tensile properties, with 30 ng/ml continuously applied TGF-β1 increasing tensile properties by twofold as a result of increased collagen synthesis (Elder and Athanasiou 2009b; Mauck et al. 2003).

TGF-β1 is also able to enhance the stimulatory effect of dynamic, unconfined compression (i.e. 0–10% strain, 1 Hz) on chondrocytes seeded in agarose hydrogels by increasing collagen production by approximately twofold (Mauck et al. 2003). TGF-β1 or insulin-like growth factor (IGF)-1 resulted in a three- to fourfold increase in the aggregate modulus for both GFs, as well as in collagen and glycosaminoglycan (GAG) synthesis, respectively (Mauck et al. 2003). TGF-β3 release from poly(lactide-co-caprolactone) (PLCL) scaffolds increased the compressive properties of the engineered cartilage gradually from ~300 kPa at 4 weeks to ~400 kPa at

8 weeks and to ~550 kPa at 12 weeks (Kim et al. 2015). It is, however, noteworthy that, in general, changes in biomechanical properties do not scale linearly with changes in biochemical components. This suggests an important role for interactions between matrix components and matrix organization in determining biomechanical tissue properties. While, in cartilage TE, using anabolic GFs in combination with mechanical cues is still a promising approach to enhance the compressive properties of neocartilage, improvements in tensile properties due to these stimulation regimes are usually substantially lower than values derived from native cartilage (Elder and Athanasiou 2008).

As a result, biophysical agents have been investigated to further improve the tensile properties of neocartilage. As an example, chondroitinase ABC (c-ABC) cleaves chondroitin and dermatan sulphate and depletes GAGs (Natoli et al. 2009b; Asanbaeva et al. 2007), which upon recovery during subsequent in vitro culture restore the compressive mechanical properties (Natoli et al. 2009a, b). Counterintuitively, persistent effects of c-ABC treatment with enhanced collagen production and subsequently increased tensile properties were observed (Natoli et al. 2009a), and multiple c-ABC applications had an additive effect (Natoli et al. 2009a; O'Connell et al. 2014) of, most likely, biophysical nature (Responte et al. 2012). At this point, additional studies are required to elucidate the role of c-ABC in restoring tensile properties in engineered cartilage.

10.6.2 Gene Therapy

An increasing number of potentially therapeutic genes have emerged as tools in cartilage TE. These genes may not only be administered to cells as soluble factors but also through ex vivo or in vivo approaches using viral and non-viral vectors. The latter would allow for sophisticated and potentially better controllable, inducible longitudinal expression. Gene transfer may further occur through gene-activated matrices, thereby employing state-of-the-art scaffolds. Although gene therapy has converged with cartilage TE in recent years, genetic approaches bear certain risks which have to be well balanced against their potential benefits. For a recent comprehensive overview, please see elsewhere (Hu 2014).

10.7 Bioreactors

Most TE is performed ex vivo, while some promising in vivo bioreactor concepts have also been reported (Stevens et al. 2005), at least for the bone. In cartilage TE, bioreactors have been mainly used for three applications: (1) cultivation/proliferation of (suspended) cells prior to seeding into scaffolds, (2) maintaining vitality of cells upon seeding on/into porous scaffolds, and (3) achieving specific physical stimulation (Gelinsky et al. 2015; Martin et al. 2004; Hansmann et al. 2013). It is difficult to manually expand cell under good manufacturing practice (GMP) conditions, a prerequisite for clinical translation, and expansion bioreactors are

cost-efficient and safe alternatives (King and Miller 2007). Large-scale production of MSCs has been recently reviewed (Jung et al. 2012), with microcarrier seeding technologies reported by Chen et al. (2013). Physical stimulation in bioreactors can be "passive" or "active", where passive could be the stiffness of the matrix the cells adhere to (Rehfeldt et al. 2007) and active may refer to mechanical, electrical, or magnetic stimulation. MSC differentiation, for example, is strongly influenced by microenvironmental stiffness and mechanical cues (Wang and Chen 2013) alike. Anyhow, giving a concise overview of the available bioreactor options is difficult due to the rapidly developing field, and a good overview has recently been published (Jin et al. 2015). Bioreactor systems play an important role in TE, as they enable reproducible and controlled changes in specific environmental factors. They can provide technical means to perform controlled studies to understand specific biological, chemical, or physical effects. To date the goals and expectations of bioreactor development have been fulfilled only to some extent, as bioreactor design in TE is very complex and still at an early stage of development.

10.7.1 Mechanobiological Aspects

While several other mechanical tissue properties of cartilage are rather well described, studies also reporting tensile or shear properties are surprisingly hard to find.

Tensile strains, inherent in articular cartilage, are a major contributor to the mechanical functionality of this tissue. "Biaxial" tension, or stretch, has been applied through thin, cell-seeded membranes in either radial or circumferential direction (Fan and Waldman 2010). Here, not only the magnitude of stain but also its nature and the differentiation state of the chondrocytes do matter (Das et al. 2008b). Although being biologically relevant, several technical challenges restrict the use of Flexcell-like systems (Jansen et al. 2004, 2006; Garvin et al. 2003), and tension bioreactors will thus not be further discussed.

Shear is a naturally occurring physiological condition of mechanical stimulation in a functional joint. Three general categories of "high-shear" bioreactors (i.e. contact shear, fluid shear, and perfusion shear) have been investigated in cartilage TE. As yet, employing *contact shear* or sliding shear in cartilage TE has been reported in only a few instances, and the interested reader is therefore referred to the work by Wang and colleagues (Wang et al. 2013, 2014) or a recent excellent overview (Darling 2013). Although some reports for articular cartilage exist, *fluid shear* appears typically rather to be applied in vascular TE (Darling 2013), where much work has gone into optimizing cell seeding of scaffolds in, e.g. spinner flasks. Both positive and negative results are reported, depending on the level of shear applied. *Perfusion shear* can be simply applied by flowing media steadily through a chamber containing cells or cell-seeded scaffolds. Of note, direct perfusion bioreactors can be employed to align cells in the direction of flow (Pazzano et al. 2000), but even very low shear levels (0.092 Pa or 0.92 dyne/cm^2) can adversely affect cells (Goodwin et al. 1993) and chondrocytes appear to be particularly sensitive to turbulences (Darling 2013).

Cyclic compression and *hydrostatic pressure* are the most common mechanical stimulation techniques in cartilage TE (Gelinsky et al. 2015). Rather broad regimes ranging from 0.0001 to 3 Hz and compressive stresses ranging from 0.1 to 24 MPa and between 0.1 and 25% of strain have been described (Freyria et al. 2005).

Hydrostatic pressure (HP), similar to direct compression, is a stimulus that improves the compressive and biochemical properties of engineered cartilage (Elder and Athanasiou 2009a). Combined with TGF-β3 in a chondrogenesis model using MSCs, intermittent HP of 10 MPa at 1 Hz stimulated mRNA expression of SOX-9, collagen II, and aggrecan (Miyanishi et al. 2006). Combined with bone morphogenic protein (BMP)-2 and IGF-1, 10 MPa static HP in neocartilage, engineered from articular chondrocytes, increased aggregate and tensile modulus values by 17 and 30%, respectively, were reported (Elder and Athanasiou 2008). The same HP regimen also improved aggregate and tensile moduli when applied with TGF-β1. Since HP can be used before engineered cartilage develops robust mechanical characteristics, HP can be applied earlier than direct compression during TE of articular cartilage.

Microgravity High-shear perfusion can successfully stimulate cartilage matrix production, but the resulting tissue is often of a fibrous nature. In contrast, slower fluid flow seems to have a stimulatory effect on ECM synthesis while preserving the chondrocyte phenotype. Dynamic laminar flow in rotating (wall) bioreactors provides efficient oxygen supply and presents an attractive tool for TE studies (Darling 2013).The major difference between rotating bioreactors and past perfusion systems is the reduction in shear force. Stress exerted on a construct rotating at 19 rpm was calculated to approximately 0.15 Pa (Freed and Vunjak-Novakovic 1995), which is 300 times higher than that measured on microbeads but also significantly lower than that in fluid flow bioreactors (Darling 2013). The mass transfer enhancement by the rotation seems crucial to the success of this bioreactor type in cartilage TE (Marlovits et al. 2003a, b). Considering the scaffold-bioreactor compatibility, physical, mechanical, and material characteristics of the used scaffold material logically exclude certain types of bioreactors, while rotating bioreactors are generally saved to use for a wider range of applications. A potential limitation of this type of bioreactor is the random motion of the scaffolds in the vessel.

Mechanotransduction through the cytoskeleton may begin with integrin-mediated adhesions that transmit forces from the ECM to cytoskeletal filaments (Wang et al. 2009). Wang et al. postulated that cadherins may have a role in mechanotransduction due to their close interaction with both integrins and kinase receptors. Along this line, disturbances of microtubule organization further prevented the stimulatory effect of HP loading on PG synthesis (Jortikka et al. 2000). Specifically, microtubule depolymerization induced by nocodazole, an anti-polymerization agent, inhibited the usually observed increase in PG synthesis upon applying 5 MPa cyclic hydrostatic pressure at 0.5 Hz (Jortikka et al. 2000).

It should also be noted that even sophisticated bioreactor systems rarely apply only a single type of mechanical stimulus. From a biological point of view, this may more closely approximate the native environment as articular cartilage likely

experiences multiple types of loading at a time but also obstructs drawing hard conclusions about the primary stimulating force. While it is generally accepted that mechanotransduction pathways are involved in articular cartilage homeostasis, the exact role of mechanosensors in this process is yet to be determined. Current knowledge indicates the beginning of an exciting era where modulating these pathways would potentially result in significantly improved properties of engineered cartilage.

10.7.2 Physicochemical and Other Factors

While traditionally being studied rather in bone TE, electrical and magnetic stimulation protocols found their way into cartilage TE, too. Recent clinical benefits in relieving pain have been reviewed by Zeng and colleagues (Zeng et al. 2015). A hypothesis is that electromagnetic pulses may control inflammation and stimulate anabolic activities. Our knowledge of electromechanical signals in articular cartilage was recently excellently reviewed by Brady et al. (2015a, b), also describing the effect of biophysical, magnetic, and electrical stimulation regimes in cartilage TE.

pH Control of chondrocyte pH (pH(i)) determines ECM metabolism, and several transporters have been implicated in this process, which further revealed cartilage zone dependency in situ (Simpkin et al. 2007; Hall et al. 1996; Browning and Wilkins 2004). Next to increasing the concentrations of cations and increasing extracellular osmolarity, daily joint loading also reduces extracellular pH in cartilage (Wilkins et al. 2000), further pointing to an important regulatory function of pH in controlling cartilage homeostasis. Bioreactors are well suited to control several microenvironmental parameters simultaneously and were employed to show that even subtle fluctuations in extracellular pH (and oxygen tension) influence chondrocyte metabolism and marker expression on mRNA and protein level (Das et al. 2008a, 2010b).

Hypoxia In most tissues, "hypoxia" is a non-physiological stage and might inhibit robust matrix synthesis and cell proliferation. In contrast, low oxygen tension is a more physiological environment not only to BMSCs (Das et al. 2010a) but certainly also to adult cartilage-derived cells. In cartilage, chondrocytes appear adapted to this *physoxia* (i.e. physiologically low oxygen pressure). However, higher oxygen tensions, promoting chondrocyte proliferation, may be used to stimulate cellular expansion when seeding scaffolds (Schrobback et al. 2012). Once sufficient cells have populated the scaffold, chondrogenic differentiation can be promoted, or the chondrocytic phenotype be stabilized, by switching to lower oxygen tensions (Das et al. 2008a, 2010b; Heywood et al. 2010; Malda et al. 2004; Saini and Wick 2004). To this end, a high adaptability of specialized bioreactors in a standardized production process seems a promising strategy to engineer cartilage under reduced oxygen tension (Portner et al. 2009). In multiple studies, chondrocytic gene expressions of

collagen II and aggrecan were elevated in 5% oxygen compared to 20% oxygen, whereas expression of collagens I and X was suppressed (Das et al. 2010b; Schrobback et al. 2012) and TGF-β signalling was shown to participate in preserving the chondrocyte phenotype under hypoxia (Das et al. 2015). It should be noted that even for chondrocytes, excessively hypoxic environments may compromise glycosaminoglycan (GAG) synthesis (Murphy and Sambanis 2001). Next to a direct role of partial oxygen pressures in cartilage TE, manipulating nuclear factor (erythroid-derived 2)-like 2 activity and thus redox homeostasis may also hold promises for cartilage TE (Jahr 2015).

Osmolarity Tonicity is a measure of the effective osmotic pressure gradient, and chondrocytes are cells under such a constant pressure in situ (Urban 1994). Chronic exposure to anisotonic conditions through cyclic changes in extracellular osmolarity during daily joint loading subjects chondrocytes to swelling or shrinking, respectively. Intriguingly, Leijten and coworkers identified BMP and WNT signalling antagonists, like frizzled-related protein (*FRZB*), as natural inhibitors of hypertrophic differentiation (Leijten et al. 2013). In their study, effects of intermittent cyclical loading (0.5 MPa, 10 N) at 0.33 Hz and increasing medium osmolarity to 380 mOsm/kg had a very similar effect on FrzB mRNA abundances (Leijten et al. 2013). Sub-physiological osmolarities (i.e. plasma level osmolarities around 280 mOsm/kg) rather appear to encourage proliferation of chondrocytes while inhibiting ECM production (Xu et al. 2010). In contrast, hyper-osmolarity stabilizes SOX-9 mRNAs (Tew et al. 2009). This for chondrocytes rather *physiological* osmolarity is able to improve chondrocyte marker expression in human chondrocytes (van der Windt et al. 2010a). Under such conditions, pharmacological intervention, e.g. calcineurin inhibition, can selectively improve anabolic chondrocyte markers while suppressing catabolic ones in vitro (van der Windt et al. 2012). While osmolarity also seems to be an easy means to modulate in vitro chondrogenic differentiation capacity of progenitor cells (Caron et al. 2013b), calcineurin inhibitors may promote chondrogenic marker expression through stimulating the TGF-β pathway (van der Windt et al. 2010b). Of note, using an agonist of the transient receptor potential cation channel subfamily V member (TRPV)4 in combination with hyperosmotic stimulation synergistically improved compressive and tensile stiffness of engineered neocartilage (Lee et al. 2014). Recently, it was further shown that osmolarity directly beneficially influences chondrocyte repair after injury of human articular cartilage (Huang et al. 2015) by, among others, protecting against apoptosis (Amin et al. 2008). This may thus not only have implications for cartilage TE but for cartilage repair surgery, too (Eltawil et al. 2015). The emerging "channelome" of chondrocytes identified several candidates that potentially mediate osmotic stress response and volume changes in these cells (Barrett-Jolley et al. 2010) and paved the road for future improvements in this direction.

As the field of TE matures, more sophisticated bioreactors, enabling largely automated, non-supervised, and feedback-regulated parameter controls, will become available. Co-applying compressive and frictional forces, for instance, seems a promising future strategy (Stoddart et al. 2006; Shahin and Doran 2015).

Despite the progress in engineering biologically functional cartilage tissue in vitro with the aid of signalling molecules and bioreactors, challenges remain for its successful clinical translation. Issues often arise after the transplantation of engineered tissue in vivo, including phenotypic instability and poor integration.

10.8 Phenotypic Stability

The phenotypic instability of engineered cartilage is currently still a clinical problem. Undesired cell phenotypes potentially lead to the formation of tissues with deficient biological and biomechanical functions, often resulting from fibrous cartilage formation. In stem cell-based treatments, in vitro chondrogenesis often results in hypertrophic differentiation with increased type X collagen expression in vivo, which may cause mineralization of the engineered construct (Vinardell et al. 2012). To suppress hypertrophic differentiation, several molecules have been explored; in addition to parathyroid hormone (PTH) and parathyroid hormone-related peptide (PTHrP), BMP-7 may inhibit collagen X expression in human MSCs while further inducing chondrogenic differentiation (Caron et al. 2013a). As also osteoarthritic chondrocytes often express collagen X (von der Mark et al. 1992) and this phenotype may be induced by several cytokines, GFs, or ECM degradation products (Pitsillides and Beier 2011), cell-based TE constructs are at risk facing a similar fate upon implantation in an inflamed environment. Understanding corresponding signalling pathways will be pivotal towards overcoming the challenge of phenotypic instability of engineered cartilage.

10.9 Integration

Finally, failure of integration between engineered tissue and surrounding native cartilage continues to be a fundamental problem in the field. Cartilage lesions can also extend into the subchondral bone, making cartilage to cartilage, cartilage to bone, and bone to bone integration processes relevant for the clinical translation of cartilage implants. Several factors can influence the ability of the repair tissue to integrate with the native cartilage, such as cell death at the wound edge, the phenotype of the cells in the implanted tissue, and donor age (Khan et al. 2008). In addition, the degree of maturation of engineered constructs affects integration (Obradovic et al. 2001). Traditionally, cartilage TE aimed at stimulating the production of collagens and GAGs at levels similar to the native tissue. Ironically, the ECM can prevent adhesion and diffusion of cells and matrix proteins (Hunziker and Kapfinger 1998; Rice et al. 2008) and thus impede the engineered tissue from integrating (Rice et al. 2008). Disrupting certain matrix molecules via enzymatic treatment can enhance integration; for example, collagenase and hyaluronidase applied to the wound site increased chondrocyte density and improved cartilage integration (van de Breevaart Bravenboer et al. 2004). Furthermore, β-aminopropionitrile (BAPN) may enhanced integration or “priming” the implant with exogenous lysyl oxidase homolog (LOXL)2

(McGowan and Sah 2005; Makris et al. 2014a). As mentioned above, using c-ABC or trypsin was shown to be promising, too (Obradovic et al. 2001; Hunziker and Kapfinger 1998). These enzymes can further be combined with anabolic factors to counteract their potential catabolic effects (Responte et al. 2012) or with factors disrupting ECM formation, like IGF-1, BAPN, and para-nitrophenyl-β-D-xyloside (Bastiaansen-Jenniskens et al. 2009). Although the presence of matrix components, such as GAG and collagens, in engineered cartilage is necessary to withstand stresses in vivo, only their temporal absence may allow for robust integration.

Conclusions

Current articular cartilage TE is mainly inspired by phenomena occurring during cartilage development and homeostasis. Most recent advances in cartilage biofunctionality arose from our understanding of these aspects. Still, engineering articular cartilage with native biochemical and biomechanical properties is challenging. Existing bioreactors designed for mechanical stimulation mostly do not enable non-invasive analysis of the engineered construct. Integration of imaging modalities allowing non-invasive assessment of load-induced tissue changes is currently being developed and may apply for cartilage TE in the future (Nebelung et al. 2016). The design of multimodal high-throughput stimulation platforms might further help speeding up this research (Moraes et al. 2010). Applying sequential stimuli of different natures synergistically, rather than trying to combine them all in one bioreactor design (Brady et al. 2015a), should also be considered. However, since the physiological conditions mimicked in bioreactors have not yet been shown to result in cartilage repair in vivo, even the act of mimicking these conditions in vitro may be questioned.

Normally, moderate joint loading serves to maintain cartilage homeostasis, but tissue composition, histomorphology, and structural properties continually change during lifetime. To better understand which load-induced biophysical changes are the most important in cartilage, research should attempt to define the cellular pathways that are responsible for generating an appropriate biosynthetic response.

Adequate biophysical stimulation seems to enhance the synthesis of GFs through natural pathways and could offer an alternative cheap, simple, and flexible way to deliver cytokines (Balint et al. 2013). Interestingly, this seems to hold for a broad spectrum of stimuli like mechanical, osmotic, and electrical cues.

The responsiveness of chondrocytes to a vast number of experimental perturbations was rewarding for experimentalists, but a bane to achieving consensus in the field (Wong and Carter 2003). Standardization is urgently needed. Currently, material properties of the engineered cartilage replacement constructs are reported in less than 30% of cases (Lujan et al. 2011). The cartilage tissue engineer should further be aware of site-specific mechanical properties and should rigorously confirm and report relevant mechanical values. Altogether, the achievement of cartilage TE strategies that fulfil all required conditions will still need significant work to realize suitable next-generation articular cartilage repair constructs.

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Animal Models in Cartilage Repair

11

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Abstract

Animal models play an important role to test novel experimental strategies and reconstructive surgical treatments of focal articular cartilage defects. Such animal models need to reflect the different appearances and aetiologies of cartilage defects, e.g. caused by trauma or osteoarthritis. Depth of articular cartilage defects plays an important role. Full-thickness chondral defects do not extend into the subchondral bone, while osteochondral defects penetrate the cement line and extend to the subchondral bone, thereby changing its structural integrity. Mice, rats, rabbits, goat, sheep, minipigs and horses are representing good models, bridging the gap between in vitro studies and clinical experiments in human. Each of them has benefits and limitations. Evaluation of cartilage repair may be performed using a large variety of methods, among which non-destructive evaluations and histological scoring, the latter being considered as the gold standard. As the available reconstructive surgical approaches for articular cartilage repair become increasingly complex, precise animal models to test and to translate new surgical techniques into appropriate clinical treatments are required.

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11.1 Introduction

Animal models play an important role to test novel experimental strategies and reconstructive surgical treatments of focal articular cartilage defects (Hunziker 2009; Blaney Davidson et al. 2014). Such experimental strategies include, but are not limited to, cell-, scaffold- and gene-based approaches (Cucchiarini et al. 2014). Reconstructive surgical treatments include marrow stimulation techniques, autologous or allogeneic chondrocyte implantation (ACI), osteochondral transplants and the refixation of chondral or osteochondral fragments, among others (Hunziker et al. 2015). When such novel treatments are introduced into the clinical practice or when established techniques are refined, studies in animal models are indispensable to closing the gap between in vitro experiments and the clinical reality in a human joint. Moreover, clinical data on the macroscopic and histological aspects and the biochemical composition of the cartilaginous repair tissue are nearly never available, mainly due to ethical concerns of performing a second-look operation with the sole purpose of evaluating articular cartilage repair.

This chapter will focus on the different animal models reflecting focal cartilage defects. It will first give an overview of the different aspects that need to be addressed when choosing the right animal model. Next, the benefits and limitations of each major animal model currently used in articular cartilage research are given. Finally, a discussion of the evaluation of articular cartilage repair is included.

11.2 Classification of Articular Cartilage Defects

Articular cartilage defects can be clinically classified based on their nature and their depth.

11.2.1 Nature of Articular Cartilage Defects

Animal models for cartilage repair need first to reflect the different appearances and aetiologies of cartilage defects, which may be caused by trauma, osteochondritis dissecans but also osteoarthritis (OA) and osteonecrosis (Madry et al. 2010). It is of key importance to understand the difference between focal, non-OA cartilage defects and the often ill-defined OA lesions. Focal defects are usually surrounded by a normal adjacent cartilage. In contrast, OA lesions are often larger in size, may affect the entire joint surface and are of different depths (Madry et al. 2011; Pritzker et al. 2006). OA models differ from focal articular cartilage defect models (Little and Zaki 2012), as the method for OA induction needs to reflect the clinical entity which caused the disease and therefore has to be carefully chosen (Cook et al. 2010; Gerwin et al. 2010; Glasson et al. 2010; Kraus et al. 2010;

Laverty et al. 2010; Little et al. 2010; McIlwraith et al. 2010). Moreover, the time of therapeutic intervention has to be selected following or during OA induction. As the Osteoarthritis Research Society International (OARSI) devoted an entire issue of their journal *Osteoarthritis and Cartilage* for highly detailed recommendations for animal models to study osteoarthritis, such models are not discussed within the context of this chapter.

11.2.2 Articular Cartilage Repair and Regeneration

Articular cartilage repair is different from articular cartilage regeneration, the latter referring to a complete restoration to its original architecture, indistinguishable from normal hyaline articular cartilage. Damaged articular cartilage, for example, resulting from a traumatic event, does not regenerate (Hunziker 2002). Often, lesions are termed "critical size", referring to the fact that they encounter a limited repair process if they are larger than a (species-specific) certain defect size, although in most adult animals even smaller lesions do not regenerate (Hunziker 2009).

11.2.3 Depth of Articular Cartilage Defects

When performing studies on articular cartilage repair, a focus should be laid on the entire osteochondral unit which consists of the articular cartilage and the subchondral bone. The articular cartilage may be separated in the superficial, intermediate and deep layer of the hyaline cartilage, which is separated by the tidemark from the calcified cartilage. The cement line is considered as border to the subchondral bone, which itself is discriminated into the subchondral bone plate and the subarticular spongiosa (Fig. 11.1) (Madry et al. 2010). Accordingly, defects may be graded as partial- or full-thickness chondral or as osteochondral defects (Fig. 11.2).

A full-thickness chondral defect does, by definition, not extend into the subchondral bone. It ends at the intersection of the calcified cartilage with the subchondral bone plate without penetrating the cement line (Frisbie et al. 2006b; Hunziker 1999b; Madry et al. 2010). Coherently, a partial-thickness articular cartilage defect does not affect all cartilage layers. The spontaneous repair of these chondral defects is very limited, as it depends on cells which migrate from the synovial membrane. However, here the integrity of the subchondral bone remains untouched.

In contrast, osteochondral defects penetrate the cement line and extend to the subchondral bone alternating the structural integrity of the subchondral bone plate and spongiosa. Nevertheless, the spontaneous repair of these defects is usually much more effective, as they access bone marrow with cells from a pluripotent progenitor pool migrating into the defect (Hunziker and Rosenberg 1996).

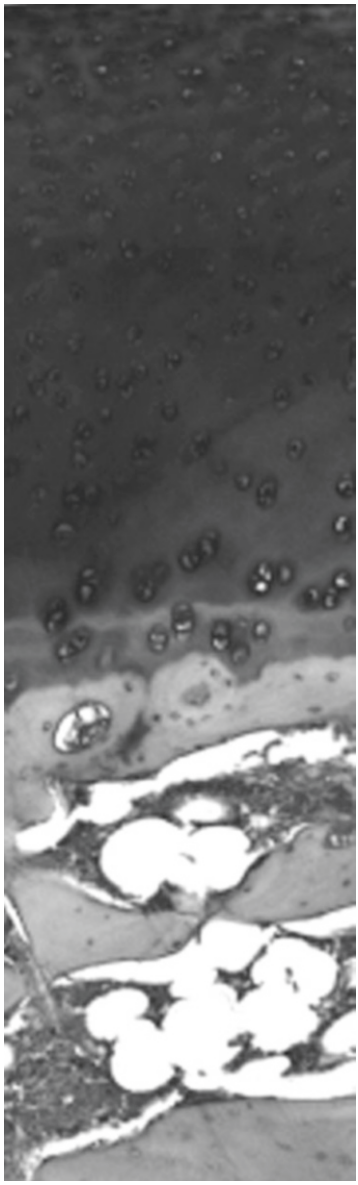

Fig. 11.1 The osteochondral unit is composed of the articular cartilage and the subchondral bone. The hyaline articular cartilage can be separated in a superficial, intermediate and deep layer, separated from the calcified cartilage by the tidemark. The cement line is the border to the subchondral bone, which itself is categorized into the subchondral bone plate and the subarticular spongiosa

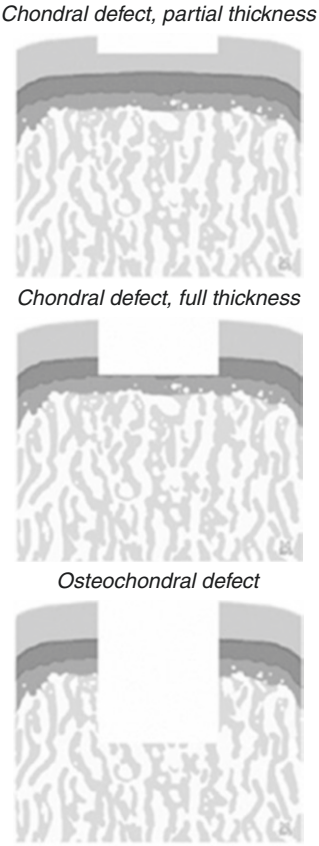

Fig. 11.2 Classification of articular cartilage defects. Partial- and full-thickness chondral defects involve only the articular cartilage layers. In contrast to chondral defects, osteochondral defects penetrate the subchondral bone plate and often reach into the subarticular spongiosa. Modified from (Madry et al. 2015), with permission

11.3 Common Requirements for Animal Models of Focal Articular Cartilage Repair

Common requirements for animal models of focal articular cartilage repair are diverse:

1. The model needs to reflect the precise clinical nature of the defect (e.g. a traumatic lesion).
2. The defect size and depth should also be comparable to the human situation.
3. The articular cartilage defect should not regenerate, but merely repair at different levels. This begs for a good knowledge of the age of the animals, including knowledge of the time of skeletal maturity and of growth place fusion (when applicable).
4. The results must be applicable to the specific clinical situation.
5. The biomechanical properties of the joint cartilage should be close to the human knee cartilage, as these are species specific (Simon 1971) and show different mechanical properties (Athanasiou et al. 1991).

6. Not only the cartilage thickness is important but also its microstructure such as cartilage cellularity (Aigner et al. 2010; Poole et al. 2010) and the thickness of the subchondral bone plate in relation to the articular cartilage thickness (Chevrier et al. 2015).
7. The size of the animal joint needs to be comparable to the human (knee) joint.
8. The usual quadruped pattern, joint range of motion and its resting position need to be similar to the bipedal locomotion of humans, as they result in different biomechanics (Rudert et al. 2000).
9. The reconstructive surgery for articular cartilage repair must be technically feasible and reflect the clinical situation.
10. The rehabilitation of the selected limb may need to be as close as possible to the clinical regimen in patients, including a phase of protected partial weight-bearing.

Taking these considerations into account, all models differ in many key structural and functional aspects compared to the human situation (Poole et al. 2010). Therefore, no animal model exists that perfectly replicates the human knee joint as a gold standard (Aigner et al. 2010).

11.4 Topographic Considerations of Defect Location

Articular cartilage defects may occur in any human joint. However, most clinical studies on cartilage repair have been performed in the knee joint, followed by the ankle and hip joints. This fact is also reflected in animal models, as most studies have been performed in the knee (stifle) joint, comparably less in the ankle joints and only a few in the hip joints.

Within a joint, topographic location of a defect also is of importance. Clinical work indicates that articular cartilage defects are often located in the femoral condyles and the trochlear groove (Hjelle et al. 2002; Curl et al. 1997). Interestingly, chondral lesions in the human femoral condyles do heal better than when located in the trochlea (Kreuz et al. 2006b). In most animal models, the medial and also lateral femoral condyles, the trochlear groove and the patella are the joint regions most often used to introduce articular cartilage defects. However, it is long since known that the histological (Armstrong et al. 1995), biomechanical (Appleyard et al. 2003) and biochemical (Little et al. 1996) characteristics of articular cartilage show a discrepancy between such separate topographical regions of the knee joint. Of note, these divergences do also have the potential to bias comparative analyses of experimental cartilage repair. It has been shown in a large animal model that the topographic location also dictates the structural patterns and biochemical composition of the repair tissue in the sheep model. For instance, cartilage repair in the sheep model is improved when the defect has been created in the trochlea and not in the condyle. Here, the repair pattern of the sheep trochlea may

be more reflective of the human femoral condyle (Orth et al. 2013c). Thus, the different repair characteristics of cartilage defects at different anatomical sites need to be respected, and defects in such regions need to be assessed independently.

11.5 Defect Geometry and Surgical Technique of Defect Creation

Deciding on the geometry of the articular cartilage defect is important when contemplating about its standardized evaluation. Articular cartilage defects in animal models are usually created applying either circular or rectangular patterns. Circular defects can be outlined using commercial or custom-made punches, e.g. dermal biopsy punches. Rectangular defects are outlined using custom-made punches rather than a scalpel blade to allow for a standardized volume. The articular cartilage within these defects is meticulously removed using curettes or spoons.

Particular attention has to be paid to a meticulous surgical technique when creating chondral defect models. Here, the defect must not extend into the subchondral bone (Frisbie et al. 2006a, b; Drobnic et al. 2010), as this would allow for an influx of marrow elements that may interfere with the different spontaneous repair patterns of a purely chondral lesion (Shapiro et al. 1993). Spontaneous repair of chondral defects is always incomplete, as only some cells originating from the synovial membrane do migrate into the defect over time (Hunziker and Rosenberg 1996).

In contrast, an access to the subchondral bone marrow is granted, e.g. by performing a marrow stimulation at the bottom of the defects using subchondral drilling (Smillie 1957; Pridie 1959), microfracture (Steadman et al. 2001) or abrasion arthroplasty (Johnson 2001); pluripotent progenitor cells from the subchondral bone marrow region below the defect migrate into the lesion, differentiate into chondrocytes and form a repair tissue (Frisbie et al. 1999, 2003; Shapiro et al. 1993).

11.6 Animal Models of Focal Articular Cartilage Repair

Mice, rats, rabbits, goat, sheep, minipigs and horses are representing good models to induce articular cartilage defects (Hunziker 1999a, b, 2000, 2009). These animal models bridge the gap between in vitro studies and clinical experiments in human. Depending on the scope of the study, different animal models are more suitable for specific research questions. In general, joint size and cartilage thickness correspond to the animal size, while articular conditions comparable to human joints may only be found in large animal models (Ahern et al. 2009; Osterhoff et al. 2011). The major considerations of focal articular cartilage defect models are summarized in Table 11.1.

Table 11.1 Considerations for focal articular cartilage defect models

Factor	Comments
Cartilage thickness	Cartilage thickness usually increases with the size of the animals. Depends on the anatomic location within the joint
Subchondral bone plate thickness	Subchondral bone plate thickness is not always reflective of the size of the animals. Minipigs, for example, have a thin subchondral bone plate, while sheep have a thick subchondral bone plate
Age of animals	Adult animals are preferred as juvenile animals have a higher degree of spontaneous repair
Defect size	Can be given as the area of the defect and placed in relation with the condylar width
Defect depth	Needs to be adapted to the osteochondral anatomy to reflect the desired defect type, e.g. chondral or osteochondral
Defect anatomy	Circular or rectangular patterns are commonly used
Defect location	Topographic differences within a joint exist for cartilage thickness, biochemical composition and repair potential
Knee resting position	Differs among animals, often lack of full extension as in humans
Gait patterns	Differs among animals, the sheep/goat/horse usually considered to best resemble the situation in humans

Modified from Madry et al. (2015) with permission

11.6.1 Mice

Mice are suitable to serve as proof-of-concept models. Here, large animal numbers are possible, and usage of animals from identical strains allows for very homogenous study populations. Additionally, for specific research questions, athymic, transgenic or knockout strains as well as mice with a compromised immune system are available. These mice develop, for example, osteoarthritis early and are ideal candidates for allogeneic or xenogenic in vivo studies of novel cartilage repair protocols. For example, Matsuoka et al. described an osteochondral cartilage repair mouse model (Matsuoka et al. 2015). Like other rodents, mice are in general inexpensive to house and breed, while the development and maintenance of specific knockout mice may be more challenging and expensive.

Mice joints are small and the articular cartilage layer is thin, only consisting of a few cell layers. This makes it more or less impossible to study the effects of solid scaffolds for articular cartilage repair in partial- or full-thickness cartilage defects. In contrast to humans, their growth plates remain open, increasing the intrinsic healing potential of cartilage repair. For outcome assessment besides macroscopic, histological and biochemical evaluation, high-field magnetic resonance imaging (μMRI) or micro-computed tomography (μCT) can be considered (Mak et al. 2015), but biomechanical testing may also be performed.

11.6.2 Rats

Compared to mice, rat joints are much larger, while the cartilage layer still remains very thin. In the rat knee joint, chondral or even osteochondral defects with a diameter up to 3 mm may be created in the femoral condyle or the trochlea. The critical size of a defect is unknown. Choi and coworkers described in a rat model the intra-articular application of transforming growth factor β1 in modular chitosan hydrogels for the treatment of articular cartilage defects (Choi et al. 2015). Also, xenogenic cells may be implanted in (osteo-)chondral defects to examine their repair potential in vivo (Pagnotto et al. 2007). Rats are utilized for in vivo testing of new solid and hydrogel scaffolds. Similar to mice, they are economical to house but face analogous problems, such as improved intrinsic repair and persisting open growth plates. The outcome assessment recommendations are the same as for mice, but the translational potential remains very limited.

11.6.3 Rabbits

The lapine model is commonly used in articular cartilage research (Laverty et al. 2010). Rabbits serve to bridge the gap between small and larger animal models and require relatively simple husbandry. The condyles and the trochlea are large enough to create osteo- or chondral defects with a maximal size of 3–4 mm. Distal femoral growth plates close at 6–8 months of age (Kaweblum et al. 1994; Hunziker et al. 2007). In rabbits, the degree of knee flexion is much higher compared with humans, resulting in higher forces in the trochlea than in humans. The relatively good endogenous healing potential has led to the assumption that cartilage defects in the rabbit do regenerate. However, even osteochondral defects of 2 mm in diameter in adult rabbits only repair and not regenerate. As in the rat, there is a cartilaginous patella located proximally from the bony patella.

11.6.4 Dogs

Accumulating ethical concerns for the continuous use of dogs in articular cartilage research have been arisen in the past years. Of note, however, dogs suffer from very similar diseases as humans, e.g. osteochondritis dissecans or osteoarthritis, and have a similarly poor intrinsic healing potential for cartilage damages, mimicking more closely the situation in human than other animal models. While defect sizes up to 12 mm are described in the literature, most defects have a diameter of 4 mm (Ahern et al. 2009). Cartilage thickness is thinner compared to humans but allows creation of partial-thickness cartilage defects as well. Canines reach skeletal maturity between 12 and 24 months.

In spite of the ethical concerns, some specific points may advocate the use of dogs for (osteo-) chondral repair studies: The stifle joint is amendable to arthroscopic examination, if longitudinal observations or in vivo studies are warranted.

Additionally, dogs may be trained to perform specific rehabilitation programmes or use a treadmill and also tolerate braces or slings, for example, if partial weight-bearing is necessary. For canine, beside standardized cartilage evaluation protocols, pain level, quality of life or gait analyses may be performed.

11.6.5 Goat

The articular cartilage of goat has a poor intrinsic capability to heal. Jackson et al. reported the critical size of an osteochondral defect in the medial femoral condyle of the stifle joint to be 6 mm in diameter (Jackson et al. 2001). Compared to smaller animal models, the joint size is larger, and the ratio between articular cartilage and subchondral bone structures is comparable to humans, while in total the thickness of the articular cartilage layer remains thinner with respect to humans (Jackson et al. 2001). In caprines, growth plates close after 36–48 months. Studies of partial- and full-thickness chondral, as well as osteochondral, defects are possible. Also, new resurfacing implants or scaffolds have been tested in goats. Custers et al. described the use of a metallic implant (Custers et al. 2009), and Kon et al. recently reported their results of a novel aragonite-hyaluronate biphasic scaffold for critical size osteochondral defects (Kon et al. 2015).

Goats are easy to house and, compared to other large animal models, relatively inexpensive. Here, if longitudinal assessments are warranted, arthroscopic examinations are possible, too, while complex rehabilitation programmes and partial weight-bearing are difficult to achieve.

11.6.6 Sheep

The sheep is an excellent large animal model for translational research of articular cartilage repair (Orth et al. 2015a; Eldracher et al. 2014; Goebel et al. 2015). Osterhoff et al. described common anatomical characteristics of human and ovine knee and concluded that the ovine stifle may be considered as scaled down model of a human knee (Osterhoff et al. 2011). Sheep are also simple to house, and the stifle joint can be assessed easily. Schinhan et al. described 7 mm cartilage defects to be critical to develop osteoarthritis in stable joints (Schinhan et al. 2012). Comparable to humans, the intrinsic repair potential is very limited. Chondral and osteochondral defect repair in the ovine trochlea is better compared with the medial condyle (Orth et al. 2013c), which is, however, contrary to the situation in human where defects of the medial femoral condyle perform in general better. Through a medial mini-arthrotomy, good exposure of the ovine femoral condyle and distal trochlea to create (osteo-) chondral defects, as well as assessment of the anterior cruciate ligament or medial meniscus, is possible. Here, outmost surgical caution must be taken to avoid a post-operative patellar luxation (Orth and Madry 2013). To evaluate the effect of pressure on cartilage repair, also performing of opening and closing wedge high tibial osteotomies is possible to mimic the clinical situation of patient with malalignment and cartilage pathology (Pape and Madry 2013).

Among gait analyses, macroscopic, histological, biochemical and biomechanical analyses, as well as evaluations using μMRI or μCT (Goebel et al. 2014; Orth et al. 2012a) or radiographs, can be performed. Arthroscopic follow-up surgery in longitudinal experimental settings is possible, too. Also, in this animal model, complex rehabilitation protocols are not practicable.

11.6.7 Minipigs

The stifle joint of the pig is closely similar to human condition in joint size, weight-bearing requirements and cartilage thickness (Jiang et al. 2007). However, conventional large size swines are difficult to handle, being sometimes aggressive and housing requirements are challenging. Here, minipigs offer some advantages while they are considerably expensive compared to goats or sheep. They become skeletally mature between 16 and 24 months.

The stifle joint of minipigs, notable smaller than the human knee, allows for (osteo-)chondral defect creation with a diameter of 6–8 mm or even larger either in the femoral condyles or the trochlear groove, while the thickness of articular cartilage and the subchondral bone plate is relatively thinner. Similar to human, the cartilage of adult minipigs also has limited capability for self-repair. The bone apposition rate, trabecular thickness, and collagen fibre arrangement in articular cartilage of the Göttingen minipigs are similar to those in humans (Kaab et al. 1998). Studies for both chondral and osteochondral defects can be performed with minipigs. Gotterbarm et al. proved the limited capability for endogenous repair of chondral and osteochondral defects and validated the utility of the Göttingen minipigs for articular cartilage repair research (Gotterbarm et al. 2008). Christensen et al. evaluated multiple cartilage repair strategies in the Göttingen minipig model and proved a clinical consistent repair process following different treatments in minipigs (Christensen et al. 2015). In an osteochondral defect repair study, the cartilage regeneration potential of human umbilical cord blood-derived mesenchymal stem cells with a hyaluronic acid hydrogel composite has been proven at 12 weeks postoperatively (Ha et al. 2015). These studies support the feasibility and practicability of the minipig model for cartilage repair studies with partial-thickness, full-thickness, chondral defects and osteochondral defects.

11.6.8 Horses

The horse is the largest animal model available for cartilage research. Large facilities for housing, special technical skill and equipment are required to manage horse studies. Furthermore, this animal model is expensive to purchase and ethical concerns may arise here as well. Compared with other animal models, the large joint dimension, articular cartilage and subchondral bone thickness and fully extended, upright stifle joints during gait reflect closely the human knee anatomy. Also, horses suffer from similar joint diseases as humans, e.g. osteochondritis dissecans, focal

cartilage lesions or osteoarthritis, and, due to horse racing environment, cartilage surgery techniques are established in this translational animal model (Nixon et al. 1999).

Similar to humans, horse articular cartilage shows little intrinsic capability for self-repair. Surgically created defects averagely measure between 6 and 20 mm, while a size above 9 mm is considered critical. The cartilage thickness allows for the production of partial- or full-thickness chondral or osteochondral defects. It also permits cartilage repair studies in defects approximately the same size and depth of lesions seen in humans (McIlwraith et al. 2011). Thus, the horse model highly benefits the preclinical evaluation of the efficacy of novel technologies. For example, Frisbie et al. examined chondroprogenitor cells derived from autologous and allogeneic articular cartilage to treat articular cartilage defects in horse (Frisbie et al. 2015). The relative long life and athletic property of the horse enable it to evaluate cartilage repair strategies in chronic defects. Moreover, the availability of post-operative exercises allows for evaluation of repair under various rehabilitation protocols.

Due to the body weight and joint loading biomechanics of the horse, the cartilage will be subjected to greater loading than in humans, and post-operative protected weight-bearing is commonly difficult to ensure (Murray et al. 2001). To avoid the overloading of the treated defects, the relatively unloaded lateral femoral trochlea has been frequently recommended for creation of cartilage defects in the horse model. The tibiotalar joint of horses is anatomically equivalent to the ankle joint of humans (McIlwraith et al. 2011). Specific rehabilitation programmes can be implemented in training, and therefore horses are suitable for studies requiring specific rehabilitation protocols. Second-look arthroscopies may be performed, if longitudinal data is warranted. Also, gait analyses may be performed. The suggested assessment methods of cartilage repair remains similar to other large animal models.

11.7 Evaluation of Articular Cartilage Repair

A large variety of methods may be applied for the evaluation of osteochondral repair (Orth et al. 2015b). After euthanization, high-resolution photographs of the articular cartilage defects should be taken under standardized conditions allowing for a non-destructive macroscopic evaluation of cartilage repair (Goebel et al. 2012). As next step, μMRI (Goebel et al. 2014) or μCT (Orth et al. 2012a; Eldracher et al. 2014) may be advocated to non-destructively and accurately evaluate structures of the entire osteochondral unit, e.g. articular cartilage and repair tissue, or microstructural indices of the subchondral bone. The histological analysis is considered the gold standard when experimental osteochondral repair needs to be graded (Getgood et al. 2014). However, it involves decalcification and the irreversible destruction of the samples, and, unfortunately, only single layers can be evaluated, while with μMRI or μCT, the entire sample can be assessed. For the histological analysis, several elementary and comprehensive scoring systems are validated and suit well to quantify the structure of cartilaginous repair tissue (Orth et al. 2012b). Of note, when

evaluating osteochondral repair, only the Sellers and the Pineda score allow for an assessment of the osteochondral junction to the subchondral bone (Sellers et al. 1997; Pineda et al. 1992). Also immunohistochemical examinations for collagens types I or II are mandatory. Similarly, an evaluation by polarized light microscopy which is uncomplicated and reveals the pattern of collagen fibril orientation has to be included (Madry et al. 2013). Biochemical (Kiss et al. 2014) and molecular biological examinations of the repair tissue (Cucchiarini and Madry 2014) may complete the analysis of the osteochondral samples.

11.8 Outlook and Conclusions

The simultaneous evaluation of the osteochondral unit in the context of articular cartilage repair has been a focus in the recent years (Chen et al. 2009, 2011; Hoemann et al. 2012; Madry 2010; Goebel et al. 2012; Orth et al. 2012a, 2013a, b, 2015a). Specific changes of the subchondral bone such as the upward migration of the subchondral bone plate, intralesional osteophytes, subchondral bone cysts and a generalized impairment of the osseous microarchitecture below the defect may affect articular cartilage repair both in animal models and in patients (Niemeyer et al. 2015; Bert 2015; Sansone et al. 2015; Orth et al. 2013a; Cole et al. 2011; Vasiliadis et al. 2010; Brown et al. 2004; Kreuz et al. 2006a; Mithoefer et al. 2005; Saris et al. 2009; Dhollander et al. 2011; Henderson and La Valette 2005).

Yet, the ever increasing complexity of available reconstructive surgical approaches for articular cartilage repair requires precise animal models to test and to translate new surgical techniques into appropriate clinical treatments. They will also provide better insights into the basic science of articular cartilage repair (Madry et al. 2014).

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