Articular Cartilage Regeneration in Veterinary Medicine

Metka Voga and Gregor Majdic

Abstract

Cartilage is an avascular tissue with a limited rate of oxygen and nutrient diffusion, resulting in its inability to heal spontaneously. Articular cartilage defects eventually lead to osteoarthritis (OA), the endpoint of progressive destruction of cartilage. In companion animals, OA is the most common joint disease, and many pain management and surgical attempts have been made to find an appropriate treatment. Pain management of OA is usually the first choice of OA therapy, which is often managed with nonsteroidal anti-inflammatory drugs (NSAIDs). To avoid known negative side effects of NSAIDs, other approaches are being considered, such as the use of anti-nerve growth factor monoclonal antibodies (anti-NGF mAB), hyaluronic acid (HA), platelet-rich plasma (PRP), and mesenchymal stem cells (MSCs). The latter is increasingly being recognized as effective in reducing or even eliminating pain and lameness associated with OA. However, the in vivo mechanisms of MSC action do not relate to their differentiation potential, but rather to their immunomodulatory functions.

Achieving actual regeneration of cartilage to prevent OA from developing or even revert already existing OA condition has not yet been achieved. Several techniques have been tried to overcome cartilage's inability to regenerate, from osteochondral transplantation, autologous chondrocyte implantation (ACI), and matrix-induced ACI (MACI). Combinatory use of MSCs unique features and biomaterials is also being investigated with the aim to as much as possible recapitulate the native microenvironment of the cartilage, yet so far none of the methods have produced reliable and truly effective results. Although OA, for now, remains an incurable disease, novel techniques are being developed, rendering hope for the future accomplishment of actual cartilage regeneration. The aim of this chapter is firstly to summarize known and developing pain management options for OA, secondly to present surgical attempts to regenerate articular cartilage, and finally to present the attempts to improve existing regenerative treatment options using mesenchymal stem cells, with the vision for the possible use of developing strategies in veterinary medicine.

M. Voga and G. Majdic (✉)
Institute for Preclinical Sciences, University of Ljubljana, Ljubljana, Slovenia
e-mail: gregor.majdic@vf.uni-lj.si

Keywords

Articular cartilage · Biomaterials · Mesenchymal stem cells · Osteoarthritis · Regeneration · Veterinary medicine

Abbreviations

ACI	Autologous chondrocyte implantation
Anti-NGF mAB	Anti-nerve growth factor monoclonal antibodies
BMP2	Bone morphogenetic protein-2
CD105	Cluster of differentiation 105
CD73	Cluster of differentiation 73
CD90	Cluster of differentiation 90
CD45	Cluster of differentiation 45
CD34	Cluster of differentiation 34
CD14	Cluster of differentiation 14
CD11b	Cluster of differentiation 11b
CD79a	Cluster of differentiation 79a
CD19	Cluster of differentiation 19
COMP	Cartilage oligomeric matrix protein
ECM	Extracellular matrix
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
GAGs	Glycosaminoglycans
HA	Hyaluronic acid
HIF-1α	Hypoxia-inducible factor-1alpha
HLA	Human leukocyte antigen
IGF	Insulin-like growth factor
MACI	Matrix-induced autologous chondrocyte implantation
MAT-3	Matrilin-3 protein
MMP13	Matrix metalloproteinase 13
MMP	Modified Maquet procedure
MSCs	Mesenchymal stem cells/ medicinal signaling cells
NGF	Nerve growth factor
NSAIDs	Nonsteroidal anti-inflammatory drugs
OA	Osteoarthritis
OCD	Osteochondritis dissecans
PDGF	Platelet-derived growth factor
PRP	Platelet-rich plasma
PCL	Polycaprolocone
PEG	Polyethylene glycol
PGA	Polyglycolic acid
PLA	Polylactic acid
PLGA	Polylactic-co-glycolic acid
PTHrP	Parathyroid hormone-related protein
ROS	Reactive oxygen species
RUNX2	Runt-related transcription factor 2
SOX9	SRY-box transcription factor 9
TGF-β	Transforming growth factor beta
TPLO	Tibial plateau leveling osteotomy
TSP-1	Thrombospondin-1
TTA	Tibial tuberosity advancement
VEGF	Vascular endothelial growth factor
2D	Two-dimensional
3D	Three-dimensional

1 Cartilage and Its (In)Ability to Heal

Cartilage is a connective tissue of mesodermal origin (Armiento et al. 2019). In the fetus, cartilage acts as a bone template and provides a structure for endochondral ossification (Chiara and Ranieri 2009). In the adult organism, cartilage remains in several areas in the body, such as joints, nose, ear, trachea, and intervertebral disks, playing a role of a supportive structure, shock absorber, flexibility, and movement (Hoshi et al. 2018). Four types of cartilaginous tissues are distinguished based on the cellularity, morphology, and extracellular matrix (ECM) composition: hyaline cartilage, fibrocartilage, and elastic and hypertrophic cartilage (Armiento et al. 2019). The development of certain cartilage type is dependent on the mechanical impact on the tissue. The most common is hyaline cartilage, the embryonic form of cartilage, present at the connection between the ribs and sternum, in the trachea, and on the joint surface where it resists compressive load and provides frictionless movement (Nürnberger et al. 2006). Major constituents of cartilage include a small number of cells, chondrocytes, and a large proportion of their product, ECM, embedded in an abundant interstitial fluid which represents the majority of tissue weight and is essential for joint lubrication and wear resistance (Bora Jr. and Miller 1987). In the vertebrate skeletal system, articular cartilage is highly organized (Nürnberger et al. 2006). Complex organization of articular cartilage rises from differentiation of the cartilage into four layers

(superficial, middle, deep, and calcified zone), ECM compartmentalization (collagen type I predominating in the uppermost part of the zone and collagen type II in the middle and deep zone), and orientation of collagen fibers (Nürnberger et al. 2006) (forming Benninghoff arcades, oriented mostly parallel to the articulating surface with average fibril rotating through the tissue until the orientation of collagen fibers in the middle and deep zones near the interface with bone is perpendicular to the joint surface) (Benninghoff 1925). Despite well-established cartilaginous tissue structure, there are considerable variations between the species. For example, small species such as mice have higher cellularity than larger animals (Stockwell 1971), whereas cartilage thickness is higher in smaller animals (Stockwell 1971; Frisbie et al. 2006).

2 Osteoarthritis in Companion Animals

In the adult organism, cartilage lacks blood and lymph vessels, nerves, and perichondrium. Chondrocytes are thus sustained by nutrients, gases, and cytokines delivered by the synovial fluid (Stockwell 1978). Cartilage metabolism is relatively slow. Low rate of tissue turnover, ascribed to cartilage avascularity and limited rate of oxygen and nutrient diffusion from synovial fluid, results in cartilage inability to heal spontaneously (Hayes Jr. et al. 2001). Intrinsic repair mechanisms, even in minor cartilage defects, are insufficient for the regeneration of cartilage ad integrum (Nürnberger et al. 2006). Natural repairing process of hyaline cartilage results in mechanically inferior fibrocartilage that in comparison to hyaline cartilage contains high levels of type I collagen and only a small portion of glycosaminoglycans (GAGs) and collagen type II, making it less resilient to wear, with higher-friction motion between bones (Armiento et al. 2019). Cartilage injuries may often appear asymptomatic but symptoms appear with progressive cartilage destruction (Mehana et al. 2019; Janakiramanan et al. 2006). The loss and dysfunction of articular cartilage eventually lead to

osteoarthritis (OA), a clinical and pathological endpoint of progressive cartilage destruction, affecting both humans and animals worldwide. OA is a slowly progressing degenerative joint disease characterized by whole joint structural changes including varying degrees of osteophyte formation, subchondral bone change, and synovitis, leading to pain and loss of joint function (Dieppe and Lohmander 2005; Enomoto et al. 2019). OA is the most common joint disease in companion animals, especially dogs and horses (Gencoglu et al. 2020) and also geriatric cats (Clarke et al. 2005). Risk factors for OA in dogs are associated with genetics, breed and conformational predispositions, body weight, age, and neuter status (Anderson et al. 2020). In horses, changes in composition and structure properties of cartilage result from cartilage damage due to trauma, impact injuries, abnormal joint loading, excessive wear, or aging process (Gencoglu et al. 2020). In cats, idiopathic OA mediated by congenital, traumatic, infectious, nutritional, and immune-mediated causes is prevailing (Enomoto et al. 2019). The prevalence of OA is higher in older animals, but can also occur in young animals (Gencoglu et al. 2020; Anderson et al. 2020). Although the exact etiology of OA has yet to be identified, the environmental stress followed by metabolic changes in chondrocytes may play a key role in cartilage degeneration (Zheng et al. 2021): Adverse microenvironmental conditions lead to a switch in chondrocyte metabolism from a resting regulatory state in which oxidative phosphorylation is a leading metabolic process to highly metabolically active glycolysis (Zheng et al. 2021). The consequential increase in biosynthesis of inflammatory and degrative mediators and exposure of chondrocytes to proinflammatory cytokines, hypoxia, and nutrient stress are promoting signaling pathways of catabolism. Enhanced catabolism is followed by mitochondrial dysfunction, resulting in excessive production of reactive oxygen species (ROS) and oxidative damage, a hallmark of OA (Zheng et al. 2021; Mobasheri et al. 2017). The important consequence of ROS is the activation of AMP-activated protein kinase (AMPK) and consequential upregulation of the expression of

collagen type I, proinflammatory cytokines, and matrix metalloproteinases (MMP) (Zheng et al. 2021). In particular, MMP13 is known to break down collagen type II, a key structural component of cartilage ECM. Matrix degradation products further promote inflammation and prevent the cycle of degeneration to break (Bedingfield et al. 2020).

3 Pain Management of OA

3.1 Conservative Treatment

OA is currently an incurable disease (Enomoto et al. 2019) and pain management is usually the first step in cartilage therapy. In veterinary medicine, nonsteroidal anti-inflammatory drugs NSAIDs are often the first choice for the treatment of OA and can be used for long-term management of the inflammatory component of OA pain. In addition to NSAIDs, gabapentin, amantadine, and tramadol can be administered when treatment with NSAIDs is not an option. Conservative treatment of OA also relies on the use of weight management, nutritional joint support, and physical rehabilitation including laser therapy, magnetic field therapy, shock wave therapy, massage, and balneotherapy (Zylinska et al. 2018; Rychel 2010). Unfortunately, existing therapies are often associated with severe side effects, such as potential renal, gastrointestinal, or hepatic adverse reactions, and are also often not sufficiently effective (Rychel 2010).

Additional conservative treatment option for treating OA is arthrocentesis or articular puncture, performed to inject supplements such as GAGs or HA to improve the natural qualities of HA, present in the articular fluid, and to increase the mobility of the joint (Zylinska et al. 2018). IM injections of polysulfated GAGs to dogs with OA resulted in improved lameness scores in 12 out of 16 dogs. Reduced lameness was ascribed to GAGs inhibition of cartilage oligomeric matrix protein (COMP) degradation seen as a decrease in serum COMP concentration (Fujiki et al. 2007). However, these results were short-lived similar to the HA treatment. Single intraarticular injection

of HA alone in dogs with naturally occurring hip OA also had only a temporary amelioration of the symptoms as measured by Canine Brief Pain Inventory. However, intraarticular injection of HA combined with corticosteroids appeared superior in positive effects compared to HA alone (Alves et al. 2020). Although intraarticular injection of HA and corticosteroids might prove useful for patients that cannot tolerate NSAIDs (Franklin and Cook 2013), based on the retrospective studies in dogs, there was weak or no evidence to support the use of HA for OA (Sanderson et al. 2009; Aragon et al. 2007). Evidence for the efficacy of HA is relatively weak due to the lack of control groups, and the limited numbers of controlled clinical studies make it difficult to suggest the superior effect of HA over the use of NSAID (Aragon et al. 2007).

3.2 Novel Pain Management Treatment Options

3.2.1 Platelet-Rich Plasma

In comparison to intraarticular injection of HA combined with corticosteroids, patient-based assessment scores in lameness and pain were better with intraarticular injection of autologous conditioned platelet-rich plasma (PRP) (Franklin and Cook 2013). PRP is an autologous product, containing an increased concentration of growth factors and bioactive proteins that may enhance the healing process on a cellular level. Besides bioactive factors such as serotonin, histamine, dopamine, calcium, and adenosine that have fundamental effects on the biological aspect of wound healing, PRP contains cytokines and growth factors, including transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) that play an important role in cell chemotaxis, proliferation, differentiation, and angiogenesis and therefore represent a potential to enhance healing of tendon, ligament, muscle, and bone (Foster et al. 2009). The advantage of PRP is primarily that it is a simple, rapid, cost-

effective, and safe way to obtain a clinical improvement of animals affected by OA (Catarino et al. 2020), although diverse methods and devices used to evaluate pain and lameness among different studies make the results difficult to compare (Vilar et al. 2018). Several studies have shown beneficial, albeit temporary, results of intraarticular injection of PRP in the treatment of canine OA. A single injection of PRP into OA joints of dogs was shown to have a positive effect estimated by the lameness grades (Catarino et al. 2020) or force platform gait analysis (Vilar et al. 2018; Venator et al. 2020), but these effects only lasted for 3 to 6 months. Prolonging management of pain was achieved by combining PRP treatment with physical therapy (Cuervo et al. 2020). In comparison to dogs, intraarticular administration of PRP in horses with naturally occurring OA indicates variable changes in kinetic gait parameters (Mirza et al. 2016). Due to differences in PRP concentrations used in different studies, optimization of number of enriched platelets, the volume applied, and concentration of growth factors used for clinical application is needed. Furthermore, characteristics of PRP products differ considerably in the amount of blood processed, method of PRP preparation, and the amount of PRP produced (Franklin et al. 2015). Despite mentioned promising results, there is a lack of data supporting the use of a particular PRP for a specific medical condition, and a consensus on the actual benefits of PRP has not yet been established.

3.2.2 Anti-nerve Growth Factor Monoclonal Antibodies Therapy

A potential alternative to pharmacological pain management in dogs and cats is analgesia using anti-nerve growth factor monoclonal antibodies (anti-NGF mAB) therapy. NGF is a soluble signaling protein, belonging to a family of neurotrophin molecules. During development, NGF has an essential role in the development of sensory and sympathetic neurons, whereas in the adult organism, NGF takes an important part in the sensitization of nociceptors after tissue injury (Mantyh et al. 2011). NGF is produced and released by peripheral tissues such as

chondrocytes (Enomoto et al. 2019) and white adipose tissue depots (Ryan et al. 2008). NGF serum level was shown to be associated with stress-related conditions, for example, during transportation (Kawamoto et al. 1996) or exercise load (Matsuda et al. 1991; Ando et al. 2016), and was thus recognized as an important factor to evaluate stress status in an animal (Ando et al. 2020). Besides psychological stress, NGF was correlated also with the mechanical stress associated with OA. Isola et al. (Isola et al. 2011) reported that the concentration of NGF in synovial fluid in dogs with OA was significantly higher in comparison to healthy dogs, suggesting the involvement of NGF in OA inflammation. Similarly, as in dogs, NGF concentration in horses was also higher in synovial fluid from acutely inflamed joints and joints with chronic OA in comparison to healthy joints (Kendall et al. 2021). Some recent clinical studies used anti-NGF mAB to alleviate OA pain in animal patients and are limited to a few studies conducted on dogs and cats. For the treatment of inflammatory pain in dogs, rat anti-NGF mAB were fully caninized (Gearing et al. 2013). Canine-specific anti-NGF mAB were used intravenously in pilot, masked, placebo-controlled clinical studies to alleviate pain in dogs with degenerative joint disease (Lascelles et al. 2015). With 25 dogs included in the study, a positive analgesic effect, similar to that expected with NSAIDs, was recognized based on significantly improved patient-specific outcomes of pain and mobility and significantly increased objectively measured activity. Positive effects of the treatment were observed over 4 weeks after a single treatment with anti-NGF mAB (Lascelles et al. 2015). Similar observations were made in another study conducted by Webster et al. (Webster et al. 2014) where OA-associated pain was alleviated in dogs up to 4 weeks after IV treatment with anti-NGF mAB. Similarly, as in dogs, species-specific anti-NGF mAB were developed for pain treatment in cats (Gearing et al. 2016). In a study with 34 cats, feline-specific anti-NGF mAB were used subcutaneously to treat degenerative joint disease-associated pain. A positive analgesic effect was observed for 6 weeks

during the study with significantly increased objectively measured activity (Gruen et al. 2016). Current evidence suggests that anti-NGF mAB therapy of OA in dogs and cats and possibly in horses could be an alternative to NSAIDs and other pharmacological drugs. The efficiency of a single injection of anti-NGF mAB seems to last 4–6 weeks, but further studies are needed to better understand the level of analgesia and to determine possible adverse side effects and the long-term safety of NGF use.

3.2.3 Mesenchymal Stem Cells/Medicinal Signaling Cells

Longer-lasting pain management effects of treating OA were accomplished using adult multipotent mesenchymal stem cells (MSCs). Stem cells are undifferentiated cells with the capability of self-renewal and differentiation into different specialized cells (Morrison et al. 1997). Compared to other stem cell types such as embryonic stem cells and induced pluripotent stem cells, MSCs were recognized as the most promising type of stem cells for therapy because of the relatively simple harvest techniques, isolation, and the absence of greater ethical concerns associated with their use (Sasaki et al. 2018). For both laboratory-based scientific investigations and preclinical studies, a set of standards to define human MSCs was proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) (Dominici et al. 2006). In essence, (1) MSC must be plastic-adherent when maintained in standard culture conditions using tissue culture flasks; (2) 95% of the MSC population must express CD105, CD73, and CD90 and lack the expression of CD45, CD34, CD14, or CD11b, CD79a or CD19, and HLA class II; and (3) MSCs must be able to differentiate into osteoblasts, adipocytes, and chondroblasts under standardized *in vitro* differentiating conditions. For the identification of animal MSCs, minimal criteria are yet to be defined. MSCs are found in numerous tissues, which, when endogenously activated, act to replace dead, injured, or diseased tissue cells (Caplan 1991). MSCs of common veterinary patients, e.g., dogs, horses, and cats,

have been isolated from several tissues including the bone marrow and adipose tissue (Sasaki et al. 2018; Arevalo-Turrubiarte et al. 2019; Webb et al. 2012), umbilical cord (Zhang et al. 2018; Denys et al. 2020), umbilical cord blood (Kang et al. 2012; Koch et al. 2007), muscle and periosteum (Radtke et al. 2013; Kisiel et al. 2012), gingiva and periodontal ligament (Mensing et al. 2011), peripheral blood (Sato et al. 2016; Longhini et al. 2019), endometrium (Rink et al. 2017), and placenta (Carrade et al. 2011). MSCs have also been described in several joint tissues such as synovium (Sasaki et al. 2018), synovial fluid, and synovial membranes (Arevalo-Turrubiarte et al. 2019; Prado et al. 2015) and inside the infrapatellar fat pad. One of the important aspects of the therapeutic potential of MSCs is their ability to migrate into the damaged tissue and secrete immunomodulatory and trophic bioactive factors (Caplan 2017). Therapeutic properties of MSCs, ascribed to their immunomodulatory functions, are exhibited by paracrine action, secretion of extracellular vesicles, immunomodulation mediated by apoptosis, and mitochondrial transfer (Voga et al. 2020). In veterinary medicine, the therapeutic potential of MSCs is being exploited for the treatment of various organ systems. Musculoskeletal diseases have especially been proven indicative for MSC therapy, as was shown in horses with tendon injuries (Pacini et al. 2007; Godwin et al. 2012; Dyson 2004; Smith et al. 2013; Muir et al. 2016), bone spavin (Nicpon et al. 2013), and meniscal damages (Ferris et al. 2014). Notably, as recently reviewed by our group (Voga et al. 2020), remarkable clinical outcomes of MSC treatment have also been shown in dogs (Mohoric et al. 2016; Black et al. 2007; Vilar et al. 2013; Shah et al. 2018; Harman et al. 2016; Maki 2020; Kriston-Pal et al. 2020) and horses (Magri et al. 2019; Marinas-Pardo et al. 2018) with osteoarthritic conditions, showing as significant longer-termed reduction or even elimination of pain and lameness. Based on the results of these studies, MSC treatment for OA appears safe with promising clinical outcomes, showing reduced lameness and pain associated with OA, decreasing the need for use of anti-inflammatory drugs

with their known side effects. However, in comparison to clinical evaluation, the long-term follow-ups with radiographic and CT imaging are scarce and often do not report improvements following MSC therapy, as recently reviewed by Brondeel et al. (2021). Some reduction in progression of OA, demonstrated with radiographic images, was shown in an equine model of OA in fetlock joints (Bertoni et al. 2021), but there is a need for the long-term follow-up imaging performed on actual patients where the progression of the disease is often very different from the experimentally induced pathologies. Demonstrated ability of MSCs to slow down or even stop OA progression is indicative of their well-established immunomodulatory function. One of the important features of MSCs is their tendency to home to injured or inflammation sites when administrated in vivo. However, in contrast to the initial belief that MSCs differentiate and replace damaged tissue, evidence from recent years suggest that MSCs in vivo rarely or never differentiate into the tissue at the site (Guimaraes-Camboa et al. 2017; Meirelles Lda et al. 2009) but secrete bioactive factors. The in vitro multipotency of MSCs thus cannot be directly related to their mechanisms of action in vivo. To avoid the confusion originating from the discrepancy between the name and therapeutic potential of MSCs, it was proposed by Caplan that the term "mesenchymal stem cells" should be changed into "medicinal signaling cells" (MSCs) (Caplan 2017). The actual regeneration of cartilage to prevent OA from developing or even revert already existing OA condition, therefore, remains the topic of research, which is in recent years focusing on exploiting the in vitro differentiation capabilities of MSCs as a basis for finding novel potential solutions to address this issue.

The following part of this chapter focuses on the regenerative surgical attempts to treat cartilage defects, starting with the initial MSC-free attempts to regenerate cartilage, followed by the presentation of the studies exploiting the in vitro differentiation potential of MSCs for cartilage regeneration.

4 Surgical Treatment of OA and Cartilage Defects

4.1 Conventional Treatment Options

Conventional surgical treatment of OA is indicated when conservative therapy fails or is inadequate in alleviating pain and maintaining the function of the joint (Cook and Payne 1997). In dogs, several surgical techniques for OA have been developed. Surgeries may offer treatment of the primary cause, such as cranial cruciate ligament rupture, where tibial plateau leveling osteotomy (TPLO) (Slocum and Slocum 1993), tibial tuberosity advancement (TTA) (Lafaver et al. 2007), or modified Maquet procedure (MMP) (Ness 2016) is indicated. In cases where providing pain relief and lessening the progression of future OA is needed, salvage procedures are performed, such as femoral head and neck excision (indicated in coxofemoral luxation; severe coxofemoral OA; comminuted or complicated fractures of the femoral head, neck, or acetabulum; avascular necrosis of the femoral head; or failed total hip replacement) (Harper 2017a), arthrodesis (indicated for intractable articular fractures, luxations, subluxations, or failed total joint replacement) (McCarthy et al. 2020), and total joint replacement (indicated for patients with debilitating OA secondary to trauma or joint dysplasia) (Harper 2017b).

4.2 Reparative Treatment Techniques

In contrast to salvage surgical interventions used to treat irrevocably damaged articular cartilage by removal or replacement, reparative bone marrow stimulation techniques are used to expose the subchondral bone to stimulate bone marrow and improve cartilage vascularization, enabling the diffusion of nutrients from the subchondral bone into the cartilage and stimulating bone marrow cells to reach the avascular cartilage lesion and initiate a healing response (Stupina et al. 2015). In

humans, the method of bone marrow stimulation is one of the most recommended reparative surgical techniques to treat OA (Gill and Steadman 2004). It can be achieved via drilling, chondral abrasion, or microfractures. The latter are of special interest as it can be performed arthroscopically. Light scraping, but not complete removal of calcified cartilage, is indicated to facilitate attachment of the reparative tissue to exposed calcified cartilage (Breinan et al. 2000). In veterinary medicine, objective evidence documenting the efficiency of bone marrow stimulation is not available. In a canine model of OA, chondral abrasion resulted in a fibrocartilage (Altman et al. 1992). In another study using a dog model of OA, subchondral tunneling of subchondral bone together with the injection of autologous bone marrow into the canals resulted in improved cartilage vascularization and consequently improved chondrocyte metabolism and functionality of cartilage (Stupina et al. 2012). Neither method of bone marrow stimulation resulted in hyaline cartilage formation, but rather in reparation of cartilage with the formation of fibrocartilaginous tissue. Similar was shown in horses. While microfractures increased the tissue volume in the defects (Frisbie et al. 1999) and did not cause any negative effects, this technique did not seem to have clinical effects in horses with stifle lameness diagnosed with naturally occurring OA (Cohen et al. 2009). Bone marrow stimulation results in the formation of fibrocartilage, with poor structural and mechanical properties that do not provide long-term efficacy of reparative surgical treatment techniques (Zylinska et al. 2018). Moreover, poor long-term wear characteristics of fibrocartilage do not prevent the progression of OA (Lane et al. 2004). Since the prevention of degenerative joint changes over time is one of the ultimate goals in the treatment of cartilage lesions (Burks et al. 2006), the limited intrinsic ability of cartilage to heal is proposed to alter with the regenerative treatment options that are therefore at the forefront of the cartilage treatment research.

4.3 Regenerative Treatment Options

A common feature of OA is cartilage defects that may either be associated with pain and decreased function or may appear asymptomatically (Janakiramanan et al. 2006). Either way, without treatment, cartilage defects may lead to progressive joint disease (Mehana et al. 2019; Burks et al. 2006). Treatment of cartilage defects is thus directed toward the regeneration of the defective cartilage and prevention of progression of the disease. Cartilage regeneration methods include osteochondral grafting, autologous chondrocyte implantation (ACI), matrix-induced ACI (MACI), and combinatory use of MSCs and biomaterials, aiming to replace the damaged cells and extracellular matrix while preserving the microarchitecture and biomechanical functions of the cartilage (Zylinska et al. 2018).

4.3.1 Osteochondral Transplantation

Osteochondral grafting is an attractive option for cartilage reconstruction because live homologous tissue is used. In humans, osteochondral and meniscal allograft transplantation in the knee has been performed for over 40 years (Rucinski et al. 2019; Familiari et al. 2018; De Armond et al. 2021). In animals, the majority of the studies are performed on animal models. One of the indications for using osteochondral grafts as a means for cartilage reconstruction in dogs is osteochondritis dissecans (OCD). OCD is an inflammatory condition that occurs when the diseased cartilage separates from the underlying bone. The disease can increase the risk of developing OA and it is an important cause of lameness in dogs (Schreiner et al. 2020). It was previously reported that no differences were detected between the surgical and medical treatment of OCD in 19 dogs. Medical treatment resulted in an even more rapid return to normal weight-bearing. Despite some clinical improvement, in most dogs, lameness continued and the disease progressed (Bouck et al. 1995). Albeit demonstrated to be technically feasible in canine

caudocentral humeral head, medial humeral, and medial femoral condyle, positive clinical outcomes of osteochondral autograft transfer in dogs with OCD were short-termed, with minimal donor site morbidity (Fitzpatrick et al. 2010; Fitzpatrick et al. 2009; Fitzpatrick et al. 2012). The osteochondral graft may not even render clinical changes, as was shown in a canine model of full-thickness cartilage defect, where phalangeal osteochondral graft did not result in significant functional difference compared to the nongrafted group of dogs 6, 12, or 20 weeks after surgery (Dew and Martin 1992). In comparison to OCD in dogs, osteochondral grafts in the case of subchondral bone cysts in horses that can also lead to osteochondrosis (Bodo et al. 2004) resulted in the reconstruction of the articular surface, subchondral decompression, and a renewed cartilage gliding surface. Promising clinical outcomes demand further investigation of the suitability of treatment of subchondral bone cysts with osteochondral grafts (Bodo et al. 2004).

Even though studies on animals are for the most part conducted on animal models and not the actual patients, up to 20% of procedures are unsatisfactory (Huang et al. 2004). The clinical success of the grafts is dependent on the viability of cartilage cells, the capacity of host bone to join graft cartilage, and the host's immunologic tolerance. Integration of donor allograft into recipient's bone can thus be incomplete and can cause failure (Pritzker et al. 1977). Although function and quality of life, based on owner perception, seem to improve after osteochondral grafting (Cook et al. 2008), donor site morbidity is considered a major ethical concern albeit donor sites from canine stifle are currently the only reliable available source of canine donor osteochondral autograft material (Fitzpatrick et al. 2009). Morbidity associated with autografted tissue for treating osteochondral defects could be avoided using fresh allograft tissues. In a canine model of knee cartilage defect, allografts were shown to be similar to autografts regarding bone incorporation, articular cartilage composition, and biomechanical properties (Glenn Jr et al. 2006). Despite being a promising

solution for mismatch of transplanted cartilage, allografts may be immunogenic; hence the cartilage becomes vulnerable to direct injury by cytotoxic antibodies or lymphocytes or to indirect injury by inflammatory mediators and enzymes induced by the immune response. However, the literature on the immunogenicity of allografts is contradictory. In some studies, the severe immune response was demonstrated upon allograft transplantation, as shown by an induced inflammatory response, thinned, dull, and roughened cartilage of allografts, with the severely fibrotic and hyperplastic synovial membrane of the joints in dog models (Stevenson et al. 1989). In other studies, no immune response was detected (Glenn Jr et al. 2006; McCarty et al. 2016), or immune response was dependent on whether or not allografts were previously frozen or were vascularized (Stevenson et al. 1996). Freezing was reported to cause harm to the cartilage and thus lower the success rate of osteochondral transplantation (Stevenson et al. 1989). As it was demonstrated in a canine model, viable chondrocytes in osteochondral allografts at the time of transplantation are primarily responsible for the maintenance of donor articular cartilage health in the long term, confirming that not only storage but also procurement, processing, transportation, and clinical implantation are of great importance for allograft clinical use (Cook et al. 2016).

Novel systems for preserving osteochondral allografts, such as MOPS (Missouri Osteochondral Allograft Preservation System) (Cook et al. 2014), and novel methods for enhancing graft integration are being developed. A lack of osteochondral graft integration is one of the important problems in transplanting osteochondral grafts that can cause a treatment failure, especially since there is often a mismatch of transplanted cartilage regarding the contour and thickness of the injured surface (Huang et al. 2004; Hurtig et al. 2001). Also, transplantation of osteochondral grafts involves manual precise preparation of the donor graft and recipient bed. The process is user-dependent, not standardized, and subject to human error. A possible solution for bypassing the issue of

insufficient supply of available donor tissue with accurate anatomical features is a fabrication of osteochondral constructs with the use of 3D printing techniques, improving the accuracy of anatomical architecture and topology, suggesting clinical relevance for large area cartilage repair (De Armond et al. 2021; Roach et al. 2015). Additionally, enhancing graft integration was attempted by using saturating grafts with bone marrow aspirate concentrate (Schreiner et al. 2020; Stoker et al. 2018) or PRP (Stoker et al. 2018), with the assumption that growth factors, cytokines, and other proteins contained in bone marrow aspirate concentrate may enhance osteoinductive, chemotactic, and neovascular signals needed for better graft integration. For example, in an in vitro study, bone marrow aspirate concentration was shown to be superior to PRP in enhancing integration potential for canine osteochondral allografts (Stoker et al. 2018). A combination of novel graft preservation and implantation techniques may therefore result in more satisfying clinical outcomes, as was demonstrated in a study where osteochondral allograft transplantation technique using fresh unicompartmental bipolar osteochondral and meniscal osteochondral allografts and application of bone marrow aspirate concentrate were used to treat medial compartment gonarthrosis in a canine model. Clinical, radiographic, and arthroscopic assessment of the graft and joint demonstrated the maintenance of the integrity of transplants and integration into the host tissue, leading to superior outcomes without early OA progression compared to NSAID controls (Schreiner et al. 2020).

While animal models provide crucial information about disease mechanisms, the artificially induced disease cannot recreate the natural in vivo environment (Cope et al. 2019). Studies conducted on actual veterinary patients are scarce, and extensive research is still needed to prove the efficacy and usefulness of osteochondral graft transplantation on actual patients. However, advancement in allograft transplantation in animal models suggests that osteochondral grafting is worthy of further investigation also in actual veterinary patients.

4.3.2 Autologous Chondrocyte Implantation

The lack of significant cellular activity in chondral defects was indicative for the researchers that chondrocytes are needed for articular cartilage regeneration (Shortkroff et al. 1996). Autologous chondrocyte implantation (ACI) was thus developed as an alternative for treating defects of articular cartilage. In humans with full-thickness cartilage defects, the procedure was described in 1994 by Peterson et al. (Brittberg et al. 1994): Cartilage slices were obtained from an uninvolved area of the injured knee during arthroscopy. Chondrocytes were then isolated and cultured for 14 to 21 days in the laboratory and then injected into the injured area under a periosteal flap taken from the proximal medial tibia. ACI seems to be advantageous over bone marrow stimulation techniques in that the cartilage that is formed is predominantly hyaline-like, containing collagen type II (Brittberg et al. 1994; Min et al. 2007; Cherubino et al. 2003). It was demonstrated by Min et al. that cartilage regeneration after ACI is correlated with at least 4-week-long survival of transplanted chondrocytes (Min et al. 2007). Fluorescently labeled chondrocytes implanted in the goat model were shown to integrate into the surrounding tissue and become a structural part of repaired tissue, rich in collagen type II and proteoglycans (Dell'Accio et al. 2003). In the canine model, ACI was shown to be superior to bone marrow stimulation techniques based on morphology, histology, and serum marker levels, with smooth surface, less fissure, and good border integration (Nganvongpanit et al. 2009). Similar as in dogs, in three horse models of cartilage lesions of fetlock joints in the forelimb, hyaline-like cartilage was formed after ACI treatment (Barnewitz et al. 2003). In the majority of animal models, ACI is investigated in full-thickness cartilage lesions. Partial-thickness cartilage lesions represent a more hostile environment for regeneration due to avascularity, poor cellularity, and smoothness of calcified cartilage. However, in patellofemoral joints in equine models, partial-thickness defects with intact calcified cartilage were proven to be a good indication for treatment

with ACI. ACI improved cartilage healing (although less obviously as in full cartilage defects), as seen with improved histological, immunohistological, and biochemical scores, including defect filling with collagen type II and attachment to the surrounding cartilage (Nixon et al. 2011).

Although ACI has produced promising results, it was indicated in previous studies that the degree to which hyaline-like cartilage fills a defect is insufficient to integrate with surrounding tissue (Breinan et al. 1997). Significant effects after ACI treatment in dog models seem to be short-termed and degenerative changes are not prevented (Nixon et al. 2011). In attempts to enhance the filling of cartilage defects with the functional tissue, biomaterials were developed to serve as carriers of cells.

4.3.3 Matrix-Induced ACI (MACI)

In the original ACI technique, the periosteal cover was used since it was thought to have the chondrogenic potential (O'Driscoll and Fitzsimmons 2001) and stimulate subchondral bone remodeling (Russlies et al. 2005). However, with ACI, there are damage associated with periosteal harvest (Ueno et al. 2001), damage associated with the suturing of articular cartilage (Hunziker and Stahli 2008), and hypertrophy observed after periosteal grafting (Ueno et al. 2001). The downside of this method is also a non-homogenous distribution of chondrocytes due to the use of cellular suspension, together with the risk of leaking out in case of inadequate sealing (Haddo et al. 2004). These limitations were improved by using the matrix-induced ACI (MACI), where alternative covers, such as porcine-derived type I/III collagen membrane, are used. The bilayered structure of a membrane is cell occlusive at the compact side, protecting cells from diffusion and mechanical impact, and the porous side consists of collagen fibers, allowing for cell invasion and attachment (Haddo et al. 2004). Autologous chondrocytes are seeded onto the membrane, enabling the membrane to be attached to the defect with the fibrin glue eliminating periosteal harvest, and procedure is faster and with less extensive exposure, as surgical implantation could be achieved via arthroscopy or mini-arthrotomy (Cherubino et al. 2003). Besides facilitating the handling of the cells, scaffolds are also useful for immobilization and broader distribution of the cells (Nuernberger et al. 2011). The procedure is traditionally performed by arthrotomy (Cherubino et al. 2003), but arthroscopy was also shown to be possible, as was shown in some studies with equine models that underwent arthroscopic implantation of cell-polymer (Ibarra et al. 2006; Masri et al. 2007) or cell-collagen membrane constructs (Frisbie et al. 2008; Nixon et al. 2017). In several studies of equine joint defect models, treatment with MACI resulted in significantly improved cartilage compared to spontaneously healing empty controls, as shown by arthroscopy, gross healing, histology scores, and mechanical analysis (Nixon et al. 2017; Nixon et al. 2015; Griffin et al. 2015). Materials other than collagenous membranes were also used for MACI, for example, PGLA, used in eight horse models and were shown to efficiently contain a large number of chondrocytes without the risk of cell loss when implanted arthroscopically with the use of a fluid pump (Masri et al. 2007). Although ACI and MACI have produced promising results and MACI treatment indeed improved cartilage healing, characterization of MACI graft implant in animal models showed that formed tissue has inferior shear properties to native cartilage (Nixon et al. 2015; Griffin et al. 2015; Lee et al. 2003). The loss of chondrocyte capacity to produce hyaline cartilage might be associated with the cell dedifferentiation occurring during chondrocyte culturing (Rakic et al. 2017).

Although increasing the dose of articular chondrocytes was shown to improve articular cartilage repair in a sheep model (Guillen-Garcia et al. 2014), chondrocytes cultured in vitro are prone to spontaneous dedifferentiation, albeit less so when cultured in a 3D environment. It was shown by Sanz-Ramos et al. (2014) that chondrocytes cultured in a 3D collagen environment possessed a better chondrogenic capacity in vitro and in vivo than the cells expanded on a plastic surface (Sanz-Ramos et al. 2014).

Interestingly, the extent of dedifferentiation seems to vary between species. For example, sheep chondrocytes were shown to be able of spontaneous redifferentiation into hyaline-like cartilage, whereas human chondrocytes were able to redifferentiate only when stimulated by chondrogenic inducers (Giannoni et al. 2005). In the equine model, chondrocyte redifferentiation was shown to be possible under the influence of 3D collagenous microenvironment, hypoxia, and BMP2 (bone morphogenetic protein-2) and RNA interference (Rakic et al. 2017). In comparison to human and equine chondrocytes, dog chondrocytes showed no capacity to redifferentiate regardless of the inducers present (Giannoni et al. 2005). The interspecies differences in chondrocyte characteristics in culture indicate that species should be considered when extrapolating data from one species to another and that differences between species in terms of chondrocyte phenotype stability during expansion might also result in different clinical outcomes when used in ACI. In addition to interspecies differences, chondrogenic differentiation of chondrocytes was dependent also on the number of passages and aging (De Angelis et al. 2020; Acosta et al. 2006; Veilleux et al. 2004), as well as whether the cells were osteoarthritic or not (Acosta et al. 2006). While, interestingly, adult donors showed a more stable expression of some chondrogenic markers, chondrocytes from elderly animals dedifferentiated at earlier passages, associated with a reduced proliferative capacity (De Angelis et al. 2020). Chondrocyte dedifferentiation could therefore be controlled from different aspects of donor and culture factors.

Another hurdle in using ACI/MACI for the treatment of chondral defects is a need for a two-step surgery. In 2006 the evidence that ACI could be delivered without cell expansion was presented. It was proposed that mechanical fragmentation of cartilage was sufficient to mobilize embedded chondrocytes through the increased surface of tissue area. In goats, cartilage fragments were placed on resorbable scaffold hyaline-like tissue (Lu et al. 2006). The procedure was adopted also in horse models with autologous cartilage fragments on a polymer scaffold implanted in a defect within the equine femoral trochlea. Compared to two-step ACI treatment, one-step treatment with minced cartilage achieved an even higher score in arthroscopic, histologic, and immunohistochemistry evaluation and prompted a phase 1 clinical study in humans (Frisbie et al. 2009). In a study performed in dogs, it was demonstrated that 100-µm-sized cartilage particles yielded the highest number of cells and provided the most optimal cartilage regeneration, based on the autologous intrafacial implantation of the microcartilage together with the absorbable scaffold and the slow release system of the basic fibroblast growth factor (Nishiwaki et al. 2017). Another possibility to overcome the need for two-step surgery was proposed by Bekkers et al. who showed that a one-stage procedure could be achieved by combining chondrocytes or chondrons with bone marrow mononuclear cells or MSCs. In a goat model, such implantation outperformed microfracture (Bekkers et al. 2013a, b).

Despite promising results associated with ACI/MACI for treatment of chondral defects, there are still many challenges that have not yet been overcome, such as insufficient integration of implanted chondrocytes, insufficient capacity of chondrocytes to produce hyaline cartilage, dedifferentiation of cultured chondrocytes, the need for two-step surgery, and the harvesting procedure that may result in changes in the articular cartilage that potentially represent a risk of becoming clinically relevant (Lee et al. 2000). This is why in recent years other treatment options for cartilage defects are increasingly being investigated. MSCs as possible substitute cells for chondrocytes are the focus of the most recent research. MSCs seem promising candidates for replacing chondrocytes because of their immunomodulatory properties and their ability to differentiate into several specialized cells, including chondrocytes. At the same time, many novel biomaterials are at the forefront of cartilage regeneration research, aiming to (i) resemble native cartilage tissue to provide the most optimal environment for chondrogenic differentiation of MSCs and (ii) simultaneously develop clinically relevant biocompatible material for in vivo implantation.

5 Attempts to Improve Existing Regenerative Treatment Options with the Use of Mesenchymal Stem Cells

5.1 Chondrogenic Differentiation of MSCs

MSCs have in recent years received significant interest in veterinary and human medicine due to their immunomodulatory and multilineage differentiation properties. Under appropriate culture conditions, MSCs can be induced toward differentiation into different lineages such as adipocyte, osteocyte, and chondrocyte lineages (Dennis et al. 1999). Although there are some reports on spontaneous chondrogenic differentiation of MSC ascribed to either high cell density (Bosnakovski et al. 2004; Dudakovic et al. 2014), presence (Fortier et al. 1998) or absence (Cho et al. 2018) of fetal bovine serum (FBS) in cell culture media, early passages (De Bari et al. 2001), or tissue source (Naruse et al. 2004), chondrogenesis on a standard 2D polystyrene surface is commonly induced with specific culture conditions such as chondrogenic differentiation media, high cell density, and highly humid atmosphere. Chondrogenic differentiation of MSCs is commonly performed in two ways. One technique is a pellet culture – a scaffold-free three-dimensional (3D) culture with high cellular density, where cells are grown in polystyrene conical tubes to form a spherical aggregate at the bottom of a tube (Johnstone et al. 1998). Another method is a micromass culture system where cells are placed in the microwell cell culture plate as droplets of cells with high density that become coalesced to form micromasses of cartilaginous tissue (Mello and Tuan 1999). During early chondrogenesis progenitor cells condense and express collagen type I. By the 5th day, collagen type II is detected and type X collagen is detected by the 14th day. The presence of aggrecan and link protein in the cell aggregates demonstrate that aggregating proteoglycans of the cartilaginous tissue are synthesized by the newly differentiating cells (Yoo et al. 1998). Commonly recognized markers of chondrogenesis in MSCs are SOX9, collagen type II, aggrecan, GAG, and COMP (De Angelis et al. 2020). In chondrogenic differentiating media, growth factors and hormones, namely, TGF-β and dexamethasone (Li and Pei 2018; Mwale et al. 2006), are often used to induce chondrogenesis. TGF-β upregulates chondrogenesis by enhancing SOX9 expression and inhibiting osteoblast differentiation by repressing expression of RUNX2 (Pei et al. 2009), while dexamethasone potentiates the growth factor-induced chondrogenesis of MSCs in vitro, although its influence is not indispensable for chondrogenic differentiation of MSCs as it is dependable on tissue source and microenvironment of MSCs (Shintani and Hunziker 2011). Besides TGF-β, other growth factors, namely, IHH and BMP2 (Steinert et al. 2012; An et al. 2010), FGF (Handorf and Li 2011), and IGF (An et al. 2010; Patil et al. 2012), were also shown to be inducers of chondrogenesis of human MSCs. However, the molecular mechanisms of chondrogenesis are not yet fully understood.

5.2 Hypertrophy Associated with Chondrogenic Differentiation of MSCs

Due to their rapid expansion in culture, trilineage differentiation potential, and easier retrieval that is not associated with articular cartilage damage as opposed to chondrocytes, using MSCs over articular chondrocytes is thought to be advantageous, especially since chondrogenesis of MSCs can be achieved with relatively simple procedures on a standard polystyrene surface. However, the undesirable effect of differentiating MSCs toward chondrogenic lineage is the constitutive expression of hypertrophic markers in MSCs. Hypertrophic markers include collagen type X, MMP13, VEGF (Chen et al. 2019), and a novel biomarker, thrombospondin-1 (TSP-1), known by its antiangiogenic properties and recently described

as an antihypertrophic protein (Cortes et al. 2021; Gelse et al. 2011). The chondrocyte hypertrophy stage can ultimately lead to apoptosis, vascular invasion, and ossification, similarly as in the growing cartilage (Bruderer et al. 2014; Mueller and Tuan 2008). Notably, hypertrophy-related changes can also be related to pathological conditions such as OA (Tchetina et al. 2005; Walker et al. 1995; Nakase et al. 2002). Importantly, it was shown that chondrogenically differentiated MSCs with expressed hypertrophy-associated genes result in mineralization, related to endochondral ossification when transplanted to ectopic sites in severe combined immunodeficient mice (Pelttari et al. 2006). The main hesitation associated with the clinical use of MSCs is therefore their inability to recapitulate stable articular chondrocyte phenotype. Indeed, the extent of the expression of hypertrophic factors might be dependent on the protocol for induction of chondrogenesis. Micromass culture was shown to be superior to pellet culture in that induced cartilaginous tissue was larger, more homogenous, and enriched in collagen type II, while the expression of hypertrophic markers was lower than in a pellet culture (Zhang et al. 2010). Yet, MSCs cultured under either of the two chondrogenic conditions are prone to hypertrophy and matrix calcification, unlike articular chondrocytes that under the same conditions maintain a non-hypertrophy phenotype (Pelttari et al. 2006). Hypertrophy correlated with both techniques is therefore undesirable as it may cause endochondral ossification in vivo.

Reduction of chondrocyte hypertrophy is extensively being investigated by using different techniques, such as co-culturing MSCs with chondrocytes; culturing MSCs in the hypoxic atmosphere; adding hormones, proteins, or other components to the culture media; silencing hypertrophic genes; or using biomaterials to imitate the natural cell environment. Some of these techniques offer promising results, although to date none have shown clinically relevant reduction, let alone complete prevention of hypertrophic differentiation.

5.3 Attempts at Reduction of MSC Hypertrophy

5.3.1 Co-culture

Chondrogenesis of MSCs greatly depends on the microenvironment, as soluble factors from surrounding tissue/cells or direct cell-cell contact can alter gene and protein expression profiles (Grassel and Ahmed 2007). The accurate regulation of key factors involved in chondrocyte hypertrophy might enable guidance of MSCs between chondral and endochondral pathways (Dreher et al. 2020). One of the ways to reduce hypertrophic differentiation of MSCs is thus co-culturing MSCs with chondrocytes, as it was previously shown that chondrocytes provide chondrogenic signals to MSCs via paracrine secretion of soluble factors including TGF-β1, IGF-1, and BMP2 (Liu et al. 2010). Inversely, chondrocytes were also shown to be affected by paracrine secretion of MSCs, as was shown by co-culturing human adipose or bone marrow-derived MSCs, leading to reduction of hypertrophy and dedifferentiation of chondrocytes, which was partially ascribed to HGF secretion by MSCs (Maumus et al. 2013). In rats, reduced hypertrophy by MSC and chondrocyte co-culture was demonstrated by increased expression of aggrecan and collagen type II together with a reduction of collagen type X and MMP13 formation (Ahmed et al. 2014). Similarly, hypertrophy reduction was shown in 3D in vitro environment with co-cultures of bovine MSCs and ACs (Meretoja et al. 2013). Effects of hypertrophy suppression were demonstrated in several other studies where MSCs were co-cultured with chondrocytes (Fischer et al. 2010; Ramezanifard et al. 2017; Amann et al. 2017). Since there is a lack of proper chondrogenic niche, it is a great challenge to stabilize ectopic chondrogenic differentiated MSC phenotype not only in vitro but also in vivo, e.g., in subcutaneous tissue. It was previously shown that the differentiation potential of MSCs is different in vitro when compared to implantation in vivo. Yang et al. (2009) demonstrated that the proliferation rate of bone

marrow-derived rat MSCs cultured in vitro in a 3D environment was similar to self-renewal capacity during in vivo implantation (Yang et al. 2009), whereas trilineage differentiation potential was suppressed in vivo in comparison to in vitro conditions. However, it was shown by Liu et al. (2010) that chondrogenic niche within subcutaneous environment could be created by co-transplantation of MSCs and articular chondrocytes, as was shown with bone marrow-derived porcine MSCs and articular chondrocytes. Chondrogenic signals were provided by the secretion of soluble factors by chondrocytes, including TGF-β1, IGF-1, and BMP2, and not by cell-cell interactions (Liu et al. 2010). Interestingly, there are some reports about the inability of articular chondrocytes to prevent hypertrophy of MSCs in pellet cultures (Giovannini et al. 2010). Similarly, nasal chondrocytes were not able to prevent MSC hypertrophy and calcification in vivo unless parathyroid hormone-related protein (PTHrP) was added to the culture (Anderson-Baron et al. 2020).

5.3.2   PTHrP

PTHrP along with its receptors is generally accepted as an inhibitor of chondrocyte development during chondrogenesis of the growth plate (Kronenberg 2003) and is a commonly reported factor to reduce hypertrophy. Fischer et al. showed that when cultured in a chondrocyte-conditioned medium together with PTHrP, expression of collagen type X, the activity of alkaline phosphatase, and matrix calcification in human MSCs were reduced. Pulsed rather than constant application of PTHrP was shown to be even more effective in the reduction of endochondral differentiation (Fischer et al. 2014). PTHrP was shown to be effective in the reduction of endochondral ossification in several other studies investigating the effect of PTHrP on human MSCs (Mwale et al. 2010; Weiss et al. 2010; Mueller et al. 2013). However, although PTHrP was shown to reduce hypertrophy, it was also reported to simultaneously reduce GAG synthesis and thus have a negative effect on chondrogenesis in human MSCs (Browe et al.

2019). Therefore, further research is needed to better understand the role of PTHrP in the chondrogenesis of MSCs.

5.3.3   Matrilin-3

Besides PTHrP, a non-collagenous ECM protein matrilin-3 (MAT3) was reported to play a regulatory role in cartilage homeostasis. It was previously shown that mutation or deletion of human MAT3 is associated with the early onset of cartilage degenerative diseases (Stefansson et al. 2003; Borochowitz et al. 2004). Indicative chondroprotective properties of MAT3 were supported in a study conducted on human and mice chondrocytes, where it was shown that MAT3 was responsible for the upregulation of cartilage matrix components such as collagen type II and aggrecan. Moreover, it was shown to slow down cartilage degeneration by downregulation of matrix-degrading enzymes, namely, collagenase MMP13 and aggrecanase ADAMTS-4 and ADAMTS-5 (Jayasuriya et al. 2012). The role of MAT3 in slowing cartilage degeneration was shown also in vivo, where MAT3 -primed MSCs suspension slowed the progression of cartilage degeneration in the medial meniscus OA mouse model (Muttigi et al. 2020). In addition to its chondroprotective role, MAT3 was also shown to significantly reduce hypertrophy in chondrocytes and MSCs. In hypertrophic chondrocytes, MAT3 acts as a BP-2 antagonist as it was shown to inhibit BMP/SMAD 1 activity leading to downregulation of collagen X expression and thus inhibition of premature chondrocyte hypertrophy (Yang et al. 2014). In hypertrophic human adipose-derived MSCs, MAT3 significantly reduced the expression of hypertrophic markers such as collagen type X, RUNX2, and ALP (Muttigi et al. 2020). In a study conducted by Liu et al. (2018) where the chondroprotective role of MAT3 was demonstrated in vivo as well as in vitro, the role of MAT3 was ascribed to its function in promoting the expression of HIF1-α. Hypoxia-inducible factor-1alpha (HIF-1α) was shown to be a key mediator in the cellular response to hypoxia (Kanichai et al. 2008) and vital in articular cartilage homeostasis (Liu et al. 2018).

5.3.4   Hypoxia

Since the articular cartilage microenvironment is relatively low in partial oxygen pressure (~ 1–5% O_2) (Gale et al. 2019; Brighton and Heppenstall 1971), a low-oxygen environment for cell chondrogenic differentiation culture conditions was proposed as opposed to standard incubator culture conditions ($\sim 21\%$ O_2). In fetal mice forelimb organ culture, HIF-1α was shown to regulate chondrocyte differentiation and function during endochondral ossification through triggering BMP2 activation and suppressing the activity of alkaline phosphatase and suppressing collagen type X expression (Hirao et al. 2006). When combined with BMP2, hypoxia and BMP2 synergistically promote the expansion of proliferating chondrocyte zone and inhibit chondrocyte hypertrophy and ossification (Zhou et al. 2015). In chondrocytes, hypoxia promoted chondrocyte rather than osteoblast commitment by suppressing collagen type X mediated by downregulation of RUNX2 activity (Hirao et al. 2006). Interestingly, in chondrocytes, hypoxic culture conditions were shown to induce the expression of PTHrP in a HIF-1alpha-dependent manner (Pelosi et al. 2013). Combining hypoxia and exogenous PTHrP may therefore result in an additive effect in maintaining high levels of GAGs while reducing ALP activity (Browe et al. 2019). Similar effects of hypoxia that were shown with chondrocytes were also shown with MSCs. Kanichai et al. demonstrated that a hypoxic cell environment together with chondrogenic culture conditions significantly enhances collagen II expression and proteoglycan deposition in rat MSCs (Kanichai et al. 2008). HIF-1α in human and murine MSCs, similarly as in chondrocytes, potentiated the expression of BMP2-induced chondrogenic markers and inhibited expression of RUNX2 and osteogenic markers in vitro (Zhou et al. 2015). As in chondrocytes, where hypoxia was shown to induce the expression of PTHrP, hypoxia was also shown to induce PTHrP and reduce MEF2C expression in human MSCs, demonstrating a pathway by which hypoxia attenuates hypertrophy (Browe et al. 2019). Based on the published results from human and

murine stem cells, hypoxia seems to enhance chondrogenesis while suppressing hypertrophy. In addition, hypoxia was shown to enhance chondrogenesis also in canine and equine MSCs (Lee et al. 2016; Ranera et al. 2013). Interestingly, in another study investigating the effect of hypoxia on chondrogenesis of equine MSCs, hypoxia did not significantly increase the chondrogenesis of either synovium or bone marrow-derived MSCs, but it did downregulate the expression of hypertrophic marker collagen type X (Gale et al. 2019). Moreover, when studying hypertrophy of bovine MSCs and ACs cultured in a 3D microenvironment under different atmospheric conditions, hypertrophy was reduced in co-cultures of MSCs and ACs in both normoxic and hypoxic conditions, whereas culturing MSCs alone even increases hypertrophic differentiation in hypoxia compared to normoxic conditions (Meretoja et al. 2013). These studies indicate the possibility that there is a difference in susceptibility of MSC to hypoxic conditions between species. The effect of hypoxic culture conditions on suppressing hypertrophy in MSC chondrogenic differentiation might also be dependent on the tissue source of MSCs (Gale et al. 2019). Further studies are therefore needed to more accurately establish the role of hypoxia in MSC chondrogenesis.

Silencing genes associated with hypertrophy is another possible approach in stabilizing chondrogenic phenotype, as was demonstrated in a study conducted on equine bone marrow-derived MSCs, where it was shown that silencing the hypertrophic genes might prevent the persistence of collagen I expression and increase the collagen type II/collagen type I ratio. Introducing siRNA to cells targeting col1a1 resulted in 50% inhibition of col1 expression, suggesting the need for further exploration of the knockout strategy to limit hypertrophic differentiation of MSCs (Branly et al. 2018).

Besides abovementioned attempts to revert hypertrophy, there are also some reports of other possible ways to reduce chondrogenic differentiation-related hypertrophy. For example, it was previously shown that TGF-β and high

doses of steroid hormones together with the absence of thyroid hormones inhibit the induction of hypertrophy (Mueller and Tuan 2008; Karl et al. 2014). Pei et al. showed that TGF–βinduced chondrogenesis was enhanced when synovium-derived MSCs were transfected with histone deacetylase 4, while type X collagen expression was simultaneously reduced (Pei et al. 2009). One of the reported agents to suppress the expression of hypertrophic genes is XAT (xanthotoxin), a furanocoumarin, also named methoxsalen, otherwise used in treating various skin diseases in humans such as vitiligo and psoriasis. It was previously shown to be able to prevent bone loss in ovariectomized mice through inhibition of RANKL-induced osteoclastogenesis (Dou et al. 2016). In the following study examining the effect of XAT on chondrocyte hypertrophic differentiation, it was shown that XAT inactivates the p38-MAPK/HDAC4 signaling pathway leading to reduced degradation of HDAC4 and inhibition of RUNX2 and thus participates in maintaining chondrocyte phenotype in regenerated cartilage (Cao et al. 2017). Hypertrophy of IPSC during chondrogenesis was also reduced using lithium-containing bioceramics with bioactive ionic components (Hu et al. 2020).

Studies investigating different options to revert hypertrophy provide promising results and offer the potential for new ways of maintaining chondrogenic differentiation by suppressing endochondral ossification. However, in most of these studies, MSCs were cultured in a standard 2D environment, which is fundamentally different from their natural environment, and none of the methods described above have provided satisfactory results, preventing the application of differentiated cells in clinical use for cartilage regeneration. To further address this issue, other approaches in the induction of chondrogenic differentiation of MSCs and cartilage regeneration are being investigated, with the focus on recapitulating MSCs native environment.

5.4 Biomaterials for Mimicking Native Cartilage Tissue

5.4.1 The Influence of the 3D Structure on MSCs

The importance of mimicking cellular natural microenvironment lies in spatially and temporally complex signaling that directs the cellular phenotype. The cell, together with the ECM, growth factors, hormones, and other molecules, is connected into an entity, which guides the functioning of individual organs and the whole organism (Tibbitt and Anseth 2009). The interaction of stem cells and their niches creates a dynamic system that is being imitated by in vitro niche models to move closer to the possibility of the therapeutic use of chondrogenic differentiated MSCs. 3D cell culture mimics mechanical and biochemical properties of the natural cellular environment and consequently provides a better insight into the physiological function of MSCs (Jensen and Teng 2020), which is especially important from the therapeutic aspect of using MSCs (Egger et al. 2019). Studies investigating the influence of the 3D environment on MSCs have shown that the 3D environment provides better conditions for expressing biological mechanisms, including cell number, vitality, morphology, proliferation, differentiation, response to environmental signals, intercellular communication, migration, angiogenesis stimulation, immune system avoidance, gene expression, and protein synthesis. 3D cell environment has thus been shown to be more suitable for cell culture than 2D (Antoni et al. 2015). In 3D cultures using carriers or biomaterials, four basic groups of materials are used – polymeric, ceramic, metallic, and composite materials (Kapusetti et al. 2019) – among which the most commonly used are hydrogels, polymeric materials, hydrophilic glass fibers, and organoids (Jensen and Teng 2020).

5.4.2 Influence of Biomaterial Properties on MSCS

The mechanical, surface, and chemical properties of the biomaterial are recognized as crucial in controlling cell fate (Martino et al. 2012). Stem cells are known to be sensitive to the mechanical properties of biomaterials and can recognize a solid substrate even when they are not in direct contact with it (Schaap-Oziemlak et al. 2014). Their adhesion to the substrate depends on the elasticity of the biomaterial, suggesting that even the smallest changes in the mechanical properties of the biomaterial can affect stem cell differentiation. Thus, the different elasticities of the biomaterial have different effects on cell adhesion, proliferation, and differentiation potential. For example, higher biomaterial strength leads to greater potential for osteogenic differentiation due to increased integrin activation, and softer biomaterials increase expression of II type collagen and lipoprotein lipase, markers for adipogenic and chondrogenic differentiation, respectively (Xu et al. 2013). In addition to the mechanical properties of the biomaterial, the surface properties also play an important role in the fate of MSCs. Stem cells do not bind directly to the surface of the biomaterial. In proteinaceous solution, e.g., in cell culture medium, stem cells bind indirectly to the surface of the biomaterial by binding to pre-bound proteins because of their slower movement compared to proteins (Tamada and Ikada 1993). The binding of cells to proteins depends on the distribution and conformation of the proteins, the latter of which depends on the wettability and chemical composition of the biomaterial (Schaap-Oziemlak et al. 2014). Therefore, the manipulation of proteins bound to the surface of the biomaterial is of particular importance in controlling cell adhesion (Schaap-Oziemlak et al. 2014). The results of several studies also indicate the influence of the chemical properties of the biomaterial surface on the direction of cell differentiation (Ren et al. 2009; Curran et al. 2006; Benoit et al. 2008). The surface treatment of biomaterials with different chemical groups, e.g., methyl (-CH3), amino (-NH2), thiol (-SH), hydroxyl (-OH), or carboxyl (-COOH) groups, can have different effects on cell fate and lead MSCs to adipogenic, osteogenic, or chondrogenic differentiation (Curran et al. 2006; Benoit et al. 2008). However, the direction of cell differentiation in a 2D or 3D environment may differ with the addition of the same chemical group (Schaap-Oziemlak et al. 2014). Therefore, the 2D or 3D environment may affect the fate of MSCs differently depending on the functional chemical group.

5.4.3 General Structure of Biomaterials for Cell Encapsulation

In addition to the mechanical, surface, and chemical properties, the scaffold structure itself also importantly affects stem cells. 3D biomaterials can be microporous, nanofibrous, or composed as hydrogels. Microporous structure supports the encapsulation of cells, but due to the pore size (100 μm) being larger than the average cell diameter (10 μm), they represent a curved 2D microenvironment. Nanofibrous structures containing fibrillar ECM proteins provide a better approximation of the natural cellular environment, but their mechanical properties are too weak to handle the stress required for mechanotransduction. Hydrogels do not have these limitations, making them a suitable biomaterial for the development of an ECM-like environment. The network structure of interconnected polymer chains allows for high water content and transport of oxygen, nutrients, waste, and other soluble molecules. Hydrogels can be composed from a range of natural or synthetic materials that exhibit a wide range of different mechanical and chemical properties (Tibbitt and Anseth 2009). Compared to synthetic hydrogels, natural hydrogels not only enable but also promote their cell activities. Natural hydrogels are usually composed of ECM proteins such as collagen, fibrin, hyaluronic acid, or components from other biological sources such as chitosan (Ribeiro et al. 2017), alginate (Sun and Tan 2013), and silk (Kundu et al. 2013).

5.4.4 Natural Biomaterials to Promote MSC Chondrogenesis

For cartilage regeneration, various scaffold materials have been developed. Most commonly used biomaterials for cartilage tissue regeneration are of natural origin, which are biocompatible, contain bioactive molecules such as RGD tripeptides that enable cell adhesion, but have in most cases poor mechanical properties and high degradation rate. Natural biomaterials are composed either of polymers, for example, agarose, alginate, chitosan, and hyaluronate, or of proteins, such as collagen, gelatin, fibrin, and silk (Ge et al. 2012). On the other hand, synthetic polymers such as polyglycolic acid (PGA), polylactic acid (PLA), poly(lactic-co-glycolic acid (PLGA), or poly(ethylene glycol) (PEG) lack the binding sites for adhesion molecules and have been shown to promote the undesirable endochondral ossification (Salonius et al. 2020), but usually provide with controllable degradation rate, high reproducibility, and easy manipulation to form specific shapes (Ahmed and Hincke 2010). Due to the advantages and disadvantages of either natural or synthetic materials, hybrid materials are also thought of as promising materials for providing microenvironment resembling cartilage tissue that is suitable for induction of stem cell chondrogenesis. Below, the commonly used biomaterials for induction of chondrogenesis are described.

Collagen

One of the most extensively used biomaterials in tissue engineering is collagen as it is a key component of cartilage ECM. It is also biocompatible and easy to manipulate with. Bioactive domains in its structure allow for good adhesion of cells. Type I/III collagen membrane has been frequently used in MACI therapy (Haddo et al. 2004). However, there are several disadvantages associated with the use of collagen as a scaffold. Firstly, the use of collagen is associated with the risk of immunogenicity (Kim et al. 2020a, b). Secondly, there is also a possibility of prion transmission (Raftery et al. 2016). Thirdly, collagen does not possess suitable mechanical strength to withstand the in vivo forces (Ahmed and Hincke 2010; Raftery et al. 2016), and lastly, culturing MSCs on collagen does not prevent hypertrophic differentiation of MSCs, as shown by human bone marrow-derived MSCs cultured either on commercial type I/III membrane or collagen/polylactide composite scaffolds, both resulting in a hypertrophic state of the cells (Salonius et al. 2020).

Regarding the immunogenicity of collagen, atelocollagen – telopeptides-free collagen – provides a biomaterial with no immunogenic activity. For treatment of chondral defects in human medicine, atelocollagen combined with microdrilling is used as an enhancement of traditional microfracture technique using the off-the-shelf product (Kim et al. 2020a). Atelocollagen, obtained by salt precipitation, was also tested for chondrogenesis of MSCs. Compared to type I collagen, type I atelocollagen enhanced chondrogenic markers' expression of human adipose-derived MSCs. Moreover, reduction of chondrogenic markers' expression RUNX2, osterix, and MMP13 was observed in cells cultured on atelocollagen, indicating better suitability of atelocollagen compared to collagen for in vitro cartilage engineering applications (Kim et al. 2020b). As a less immunogenic alternative to collagen, gelatin is also used. It is produced from processed bovine or porcine bones and skin and is usually used in combination with other materials to combine positive properties of both (Ahmed and Hincke 2010). For example, the gelatin-alginate scaffold was used to demonstrate that the proliferation rate of bone marrow-derived rat MSCs cultured in vitro on the scaffold was similar to self-renewal capacity during in vivo implantation (Yang et al. 2009).

To avoid the risk of prion transmission, other sources of collagen, besides mammal, are being investigated, such as salmon skin. However, it was shown that salmon skin-derived collagen is inferior to bovine-derived collagen in several terms such as porosity, pore size, architecture, compressive modulus, capacity for water uptake, and rat MSC proliferation and differentiation (Raftery et al. 2016).

In structural and load-bearing performance, collagen plays a pivotal role, while surrounding polysaccharides are needed for internal stress management and elastic reinforcement of collagen and absorption of fluids due to their hydrophilic nature. A protein-polysaccharide scaffold was therefore thought of as a promising material for induction of stem cell chondrogenesis. When used either alone or cross-linked with dextran or chitosan, the PEG-chitosan construct was determined as the most appropriate in inducing chondrogenesis as well as in reducing hypertrophy in human bone marrow-derived MSCs (Sartore et al. 2021). To improve the mechanical strength of the scaffold, chitosan is also increasingly studied and often used in combination with collagen. The addition of chitosan to collagen not only improved the mechanical strength of collagen but also increased compressive strength and swelling ratio and prolonged the degradation rate (Raftery et al. 2016).

Hyaluronic Acid

In addition to collagen, hyaluronic acid (HA) is one of the promising biomaterials in use for chondrogenic induction of stem cells. Hyaluronic acid is a natural component of the cartilage ECM. However, HA is highly degradable in vivo and cannot bind proteins with high affinity because of the lack of negatively charged sulfate groups. Sulfated HA was therefore fabricated to encapsulate human MSCs. The sulfated HA exhibited slower degradation, improved protein sequestration, and promoted chondrogenesis. Furthermore, it suppressed hypertrophy in vitro and in vivo in the OA rat model, due to improved growth factor retention (Feng et al. 2017). When HA was added as a supplementation to a collagen hydrogel, it was shown to stimulate chondrogenic differentiation of adipose-derived human MSCs in a dose-dependent manner. Among different concentrations from 0 to 5%, 1% HA showed the best overall results in terms of SOX and Coll type II expression. Furthermore, exchanging 25% of human articular chondrocytes with 75% of adipose-derived human MSCs didn't change the chondrogenic potential of MSCs, but reduced

hypertrophy and improved biomechanical properties (Amann et al. 2017).

Silk Fibroin

One of the promising biomaterials for use in tissue engineering is silk fibroin, derived from the silkworm *Bombyx mori*. It is biocompatible, has suitable mechanical properties, and is produced in bulk in the textile industry (Kundu et al. 2013). In comparison to other natural biomaterials used for tissue engineering, SF provides a remarkable combination of strength, toughness, and elasticity that are ascribed to its crystallinity, hydrogen bonding, and numerous small β-sheet crystals (Altman et al. 1992). Another advantage of SF is its ability to take the form of different shapes such as hydrogels, tubes, sponges, composites, fibers, microspheres, and films that could be used in tissue engineering (Rockwood et al. 2011). It was previously reported that silk fibroin can aid in MSC differentiation when combined with different components. It was previously shown that silk fibroin with incorporated L-ascorbic acid 2-phosphate significantly promoted collagen type I in mouse fibroblast L929 cells (Fan et al. 2012). It was shown to promote osteogenic differentiation and mineralization of human ADMSCs (Gandhimathi 2015), and in another study, it was shown that silk fibroin scaffold combined with PRP effectively induced chondrogenesis of human ADMSCs (Rosadi et al. 2019). Interestingly, it was shown by Barlian et al. that silk fibroin combined with silk spidroin promoted better chondrogenesis of human Wharton jelly's MSCs than silk fibroin alone and that cell culture medium supplemented with PRP promoted higher GAG accumulation in comparison with medium supplemented with ascorbic acid (Barlian et al. 2018). Contrary to mentioned studies where combining silk fibroin with other components was needed to induce chondrogenesis in MSCs, we have shown in our previous research that SF alone could also induce chondrogenesis in canine adipose-derived MSCs, possibly as a species-specific effect.

Decellularized Cartilage Matrix

Besides natural biomaterials such as collagen, hyaluronic acid, gelatin, chitosan, or silk fibroin, which have provided some promising results regarding chondrogenic differentiation of MSCs and reducing their hypertrophy phenotype, other ways for more accurate recapitulation of the cartilage microenvironment are being exploited. Among them, decellularized cartilage scaffolds have shown promise in providing the structural integrity of engineered tissues, better load-bearing ability, and functioning as a reservoir of signaling molecules, e.g., cytokines and growth factors, providing a specific microenvironment similar to native tissue. A hybrid natural ECM scaffold/artificial polymer polycaprolactone (PCL) was developed by combining ECM produced by bovine chondrocytes co-cultured with rabbit MSCs on electrospun microfibrous PCL. This hybrid scaffold was shown to have a positive effect on rabbit MSCs on aggrecan, collagen II, and collagen II/I expression compared to PCL controls (Levorson et al. 2014). Further, Yang et al. developed a cartilage ECM-derived acellular matrix by physically shattering human cartilage, followed by decellularization, freeze drying, and cross-linking techniques. They showed that ECM enabled attachment, proliferation, and chondrogenic differentiation of canine bone marrow-derived MSCs (Yang et al. 2008). ECM scaffold was also shown to be beneficial in reducing loss of chondrogenic phenotype as shown by using ECM scaffold derived from porcine chondrocytes seeded with rabbit MSCs in vivo compared with PGA scaffold (Choi et al. 2010).

In comparison to other mentioned biomaterials, decellularized cartilage ECM is advantageous in that it importantly recapitulates the native cartilage structure. However, achieving the complexity of articular cartilage structure regarding the mechanical stimulation to which the articular cartilage is constantly subjected and related orientation of collagen fibrils is especially challenging. The effect of mechanical loading and orientation of collagen fibrils on cartilage regeneration potential has been investigated in several studies.

5.4.5 Role of Mechanical Stimulation in Cartilage Regeneration

Since articular cartilage is subjected to constant movement and mechanical load, mechanical stimulation was proposed as a factor to affect ECM development. For example, it was shown in chicken micromass cultures that mechanical loading significantly augmented cartilage matrix production and upregulated expression of collagen type III, aggrecan, and hyaluronan synthases through enhanced expression of SOX9 and protein kinase A activity (Juhasz et al. 2014). Improvement of cartilage formation with reduction of hypertrophy was demonstrated to depend on several parameters, such as loading intensity, duration, and frequency of mechanical stimulation (Thorpe et al. 2012; Haugh et al. 2011; Zhang et al. 2015; O'Conor et al. 2013; Li et al. 2010; Bian et al. 2012). Optimal mechanical load, therefore, plays a crucial role during in vitro chondrogenesis of MSCs. Although mechanical forces importantly regulate MSC chondrogenic gene expression, sustained TGF-β exposure is usually also necessary for mechanically based chondrogenic improvement (Zhang et al. 2015; Huang et al. 2010; Goldman and Barabino 2016). Also, the dosage of growth factor was shown to importantly affect hypertrophy, in that only high levels of TGF-β stabilized chondrogenic phenotype (Zhang et al. 2015; Bian et al. 2012). There are, however, reports on mechanically induced proteoglycan synthesis in the absence of chondrogenic cytokines (Kisiday et al. 2009). In a study investigating the influence of mechanical load on porcine bone marrow-derived MSCs cultured on agarose or fibrin scaffolds, the mechanical load was even shown to override the influence of specific substrates, scaffolds, or hydrogels that have been shown to regulate MSC fate (Thorpe et al. 2012). In contrast to studies supporting the effectiveness of mechanical load in MSC chondrogenesis, it was shown that in the initiation stage of cartilage repair, the mechanical load may not necessarily positively affect the cell fate. In a study investigating the effect of chondrogenic priming of equine peripheral blood MSCs on adhesion and incorporation

into cartilage explants, it was shown that mechanical loading reduced the adhesion of cells and altered integration of MSCs into isolated cartilage explants (Spaas et al. 2015). These results are consistent with other studies investigating the effect of biomaterial properties on cell chondrogenesis, showing that mechanical properties can influence cells in terms of their spreading, migration, and differentiation (Toh et al. 2012; Vainieri et al. 2020). This indicates that adjusting biomaterial properties to match mechanical properties, alongside composition and architecture of cartilage, may prevent the incorporation of cells into the cartilage and consequently alter initiation steps of tissue repair (Vainieri et al. 2020). In support of these data, it was also previously demonstrated that mechanical load was associated with bone formation. Mechanical load led to the expression of NGF in mice osteoblasts, followed by the activation of NGF-receptor-positive sensory neurons, resulting in osteogenic cues and bone mass formation (Tomlinson et al. 2017). The data indicate that removing the mechanical load could have a positive effect on MSC in enabling them to reestablish joint homeostasis. Due to the contradictory results from different studies investigating mechanical load on MSCs, further research of the biomechanics, especially early in the disease course, will be needed to provide the data on which MSC repair strategies are needed for optimal cartilage regeneration (McGonagle et al. 2017).

5.4.6 Importance of Biomaterial Architecture

The mechanical performance of articular cartilage directly correlates with the complexity of its structure. Scaffold geometry, recapitulating native orientation of collagen fibrils forming Benninghoff arcades (Benninghoff 1925), thus also seems to play an important role in regulating the cartilage-like activity of cells. For example, bone marrow-derived porcine MSCs expressed collagen type II and synthesized GAGs to a greater extent when cultured on aligned polycaprolactone (PCL) microfibers than on randomly oriented scaffold that was more supportive of an endochondral phenotype as indicated by

higher expression of bone morphogenetic protein-2 (BMP2) and type I collagen gene (Olvera et al. 2017). Similarly, mimicking aligned structures of ECM fibrils in cartilage tissue led to better chondrogenesis of human BM-MSC in a nanofibrous scaffold compared to a scaffold with randomly aligned nanofibers (Zamanlui et al. 2018). Furthermore, it was shown that chondrocytes respond differently to geometrically different scaffolds, for example, nanofibrous poly (L-lactide) scaffold more efficiently promotes the cartilage-like activity of bovine chondrocytes than microfibrous scaffolds (Li et al. 2006). A similar tendency of cells toward favoring nanoultrastructure of the scaffold was shown for MSCs. Culturing human MSCs on nanofibrous polycaprolactone resulted in an increased expression of aggrecan compared to MSCs cultured on a microfibrous scaffold (Schagemann et al. 2013). These studies indicate that nano-topographical geometry with aligned structures is favored by cell types such as chondrocytes and MSCs.

To further improve the imitation of the complex structure of cartilage tissue, Nurnberger et al. (2021) have fabricated decellularized articular cartilage scaffold treated for GAG removal and engraved with a CO_2 laser to create the well-defined structure of native cartilage. With the laser, lines and crossed lines were created allowing enough space for homogenous distribution and for the new matrix to be generated. Interestingly, it was shown that new collagen fibers perpendicularly aligned to the cartilage superficial zone, corresponding to the natural alignment of the collagen fibers, deeming superior over scaffolds that promote random matrix deposition (Nurnberger et al. 2021).

One of the novel techniques used for creating complex 3D scaffold structures is 3D bioprinting, as was shown by printing decellularized ECM cross-linked with gelatin methacrylate. Bioactive factors and cells were quantitatively and accurately placed within to form a bionic multifunctional scaffold to recognize, bind, and recruit endogenous stem cells to the site. Scaffold with implanted aptamers for specifically recognizing and recruiting adipose-derived stem cells, together with TGF-β for stem cell

chondrogenesis, resulted in a great improvement of in vivo cartilage full-thickness defects in rabbit models (Yang et al. 2021). Similarly, as in rabbit models, pig models of cartilage defects were used for testing 3D-printed hybrid scaffolds made of gelatine and hydroxyapatite. Gelatine-hydroxyapatite scaffolds, compared to gelatine scaffolds or blank controls, were shown to be the best in reducing hypertrophic markers and repairing cartilage injuries (Huang et al. 2021). 3D bioprinting allows for the fabrication of complicated yet stable structures of tissue analogs and is thus considered a very promising technology, holding considerable potential for articular cartilage repair.

The architectural complexity of cartilage tissue and its constant subjection to mechanical forces demands an understanding of complex mechanisms required for induction of stable chondrogenic phenotype with minimizing the upregulation of hypertrophic genes. Challenges faced in scaffold fabrication are achieving a layered structure mimicking highly specific hierarchical ultrastructure arrangement of ECM of cartilage, mechanical environment for cells resembling native cartilage, and providing physical and biochemical cues to control the biological environment of cells. Mimicking native mechanotransduction pathways may thus be a promising way in creating the desired environment for controlled and stable chondrogenesis. Although cartilaginous tissue structure is well established, its simulation in vitro has proven very challenging, yet novel technologies and increasing acquisition of comprehensive knowledge in regenerative medicine and tissue engineering are encouraging for future cartilage treatment options in both veterinary and human medicine.

6   Summary

Cartilage's unique characteristics encourage scientist to develop methods to overcome its inability to heal. So far, medication-mediated treatment is often the first choice of therapy; however, the therapy is focused on relieving the symptoms but cannot induce repair or regeneration and is often associated with severe side effects. Due to cartilage avascularity, bone marrow stimulation techniques were developed, which have shown some short-term beneficial effects but resulted in a formation of fibrocartilage, which is mechanically insufficient to bear loading stress. Further attempts at repairing cartilage were focused on using native tissue to produce osteochondral grafts. The main disadvantages of this method are the limited amount of donor cartilage availability, donor site morbidity, and the lack of osteochondral graft integration. To overcome the lack of significant cellular activity with osteochondral grafts, ACI was proposed. ACI seemed to be advantageous over other techniques in that the cartilage that formed was predominantly hyaline-like, containing collagen type II. However, there was an issue with the non-homogenous distribution of chondrocytes and the consequential need for periosteal coverage, resulting in damage associated with periosteal harvest. The latter was overcome with the use of MACI. Although MACI treatment improved cartilage healing, the tissue formed was still inferior to the native hyaline cartilage. Moreover, cultivating chondrocytes is associated with chondrocyte dedifferentiation and thus potentially variable treatment results. Although this was shown as possible to overcome with one-step surgery where minced cartilage instead of isolated chondrocytes were used, novel methods to substitute the use of chondrocytes are being developed. MSCs' immunomodulatory properties and multilineage differentiation ability make them attractive candidates as an alternative to chondrocytes. However, the generation of cartilage tissue from MSC is challenging as in vitro chondrogenic differentiation of MSC reflects endochondral ossification unable to maintain a stable hyaline stage. Hypertrophic development of MSCs leads to the bone formation on ectopic sites and is thus unsuitable for cartilage therapy in vivo. Other approaches in the induction of stable chondrogenic phenotype of MSCs are being investigated, with the focus on recapitulating MSCs native environment and providing MSCs the best options to express their

biological function. Many novel biomaterials are thus at the forefront of cartilage regeneration research, from standard collagen-based matrices to novel decellularized ECM cell carriers. Recapitulating the exact architecture of cartilage tissue has proven challenging yet of great importance for cartilage tissue engineering. Despite advances made in biomaterial-based stem cells therapies, each scaffold material currently used in tissue engineering approaches is still limited in possessing all the requirements needed for cartilage regeneration. Moreover, the knowledge of stem cell mechanisms of action is still elusive. A more detailed comprehensive understanding of the MSC mechanisms of action and their responses to complex structural, architectural, and geometrical properties of biomaterials is therefore needed to find the most appropriate way of delivering stable cartilage tissue formation. Combining technologies and knowledge of different scientific fields is essential for engineering a biomaterial that would fundamentally contribute to cartilage regeneration. The collaboration of scientists from interdisciplinary fields is thus of key importance for the further development of advanced cartilage therapies. Looking forward, one can be hopeful that, based on the novel cutting-edge technologies being available and progressive knowledge acquisition, we are on the verge of future developmental breakthroughs in the field of cartilage regeneration.

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