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REVIEW ARTICLE

State of the art and future perspectives of articular cartilage regeneration: a focus on adipose-derived stem cells and platelet-derived products

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Abstract

Trauma, malposition and age-related degeneration of articular cartilage often result in severe lesions that do not heal spontaneously. Many efforts over the last centuries have been undertaken to support cartilage healing, with approaches ranging from symptomatic treatment to structural cartilage regeneration. Microfracture and matrix-associated autologous chondrocyte transplantation (MACT) can be regarded as one of the most effective techniques available today to treat traumatic cartilage defects. Research is focused on the development of new biomaterials, which are intended to provide optimized physical and biochemical conditions for cell proliferation and cartilage synthesis. New attempts have also been undertaken to replace chondrocytes with cells that are more easily available and cause less donor site morbidity, e.g. adipose derived stem cells (ASC). The number of *in vitro* studies on adult stem cells has rapidly increased during the last decade, indicating that many variables have yet to be optimized to direct stem cells towards the desired lineage. The present review gives an overview of the difficulties of cartilage repair and current cartilage repair techniques. Moreover, it reviews new fields of cartilage tissue engineering, including stem cells, co-cultures and platelet-rich plasma (PRP).

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1. The difficulty of treating articular cartilage lesions

Trauma, erroneous biomechanically defective positioning and age-related degeneration often result in chondral lesions. Clinically, cartilage defects are accompanied by persistent pain and functional limitations of the joint and are therefore considered a severe medical and therapeutic problem. In many cases minor lesions can dramatically influence structure and function of articular cartilage (Alford and Cole, 2005). In addition, the potential of cartilage for self-regeneration is very limited. Since vascularization is absent in articular cartilage, neither inflammation nor the formation of a fibrin clot can contribute to the healing of defects. Only cells present in the surrounding tissue, including chondrocytes or synoviocytes, may contribute to the filling of defects by increasing proliferation and matrix synthesis. However, even in very small defects this is not sufficient to regenerate the surface of the injured cartilage (Mankin, 1982). In the case of deep cartilage defects down to the subchondral bone, mesenchymal stem cells (MSC) can contribute to filling by migration, proliferation, differentiation and matrix synthesis (Furukawa *et al*., 1980). The synthesized matrix, however, usually resembles fibrous cartilage and has poor stiffness and resistance capabilities (Nehrer *et al*., 1999). Due to the poor self-repair potential of articular

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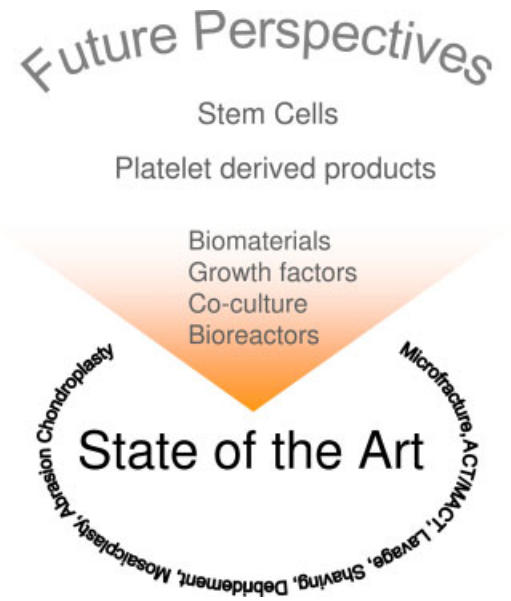

Figure 1. Treatment strategies for articular cartilage defects: state of the art and possible future applications

cartilage, there is a need for techniques to achieve regeneration of cartilage defects. The present review gives a short overview on the state of the art in the clinics (for further reading, see Bedi *et al*., 2010) and focuses on possible future perspectives, including the use of MSC and platelet-derived products (Figure 1).

2. State-of-the-art procedures in cartilage repair and regeneration

2.1. Techniques developed prior to autologous chondrocyte transplantation

Treatment of cartilage defects ranges from simple pain-relieving techniques to sophisticated tissue-engineering approaches. A gold standard of cartilage regeneration, however, has not yet been found.

Allogenic material derived from cadaveric donors has been widely used, particularly for the treatment of large osteochondral defects, with varying degrees of success. Best clinical results were achieved using small-fragmented osteochondral allografts when transplanted into traumatic knee joints (Czitrom *et al*., 1986). Although articular cartilage as a non-vascularized tissue is immunologically privileged, immune responses originating from the transplanted subchondral bone still constitute a potential problem (Stevenson, 1987). Therefore, studies performed during the last 20 years have mainly focused on the use of autologous material and marrow stimulation techniques. Transplantation of autologous cylindrical osteochondral grafts is most commonly referred to as mosaicplasty and can be carried out by an arthroscopic procedure or as an open joint approach (Hangody *et al*., 1997; Szerb *et al*., 2005). Grafts of variable diameters in the range 2.7–8.5 mm are harvested, allowing filling rates of more than 90% (Szerb *et al*., 2005). Depending on the defect location, good to excellent clinical results were demonstrated in 79–94% of 831 treated patients, based on clinical scores, imaging techniques, arthroscopy, histological examination of biopsy samples and cartilage stiffness measurements (Szerb *et al*., 2005). Nevertheless, the mosaicplasty only constitutes a symptomatic treatment method, does not fill the defect homogeneously and is associated with donor site morbidity.

Superficial cartilage defects and osteoarthritic joints can be treated with lavage and shaving procedures, aiming to relieve joint pain by removing intra-articular debris and smoothing the cartilage surface. Debridement is a more drastic version of shaving, which may also include meniscectomy, chondrectomy and removal of osteophytes (McLaren *et al*., 1991). Besides the effect of lavage to remove pain-signalling or pain-mediating molecules, it is assumed that proteoglycans are extracted from the superficial cartilage zone, which might promote adhesion of synovium-derived repair cells (Hunziker and Kapfinger, 1998). The success rates of the described treatments are highly variable and a lack of prospective randomized clinical trials makes it impossible to measure their usefulness (Hunziker, 2002).

Abrasion chondroplasty and the microfracture technique induce spontaneous repair/regeneration by providing access to the bone or bone marrow space. Success rates for abrasion chondroplasty are controversial and depend on multiple factors, including age of the patient, degree of arthritis, activity level and length of follow-up (Bert, 1993). In the microfracture technique, improved joint functionality and relief from pain in 75% of cases is reported when applied in young patients, especially in young athletes (Sledge, 2001). Today athletes often still prefer microfracture to more recent techniques, such as matrix-associated autologous chondrocyte transplantation (MACT), aiming to be back in competition after a shorter period of rehabilitation.

2.2. Autologous chondrocyte transplantation and matrix-associated autologous chondrocyte transplantation

The autologous chondrocyte transplantation (ACT) was primarily described by Brittberg *et al*. (1994). The principle of ACT is to implant autologous chondrocytes into the cartilage defect in order to fill it with newly synthesized cartilage matrix. The procedure involves arthroscopic excision of a biopsy (about 200 mg) from a non-loadbearing area of healthy articular cartilage. Chondrocytes are then isolated by collagenase treatment and expanded *in vitro*. During a second procedure, injured cartilage is debrided up to the healthy borders and the defect is covered with a periosteal flap, which is taken from the medial tibia. Finally, 50–100 µl chondrocyte cell suspension containing 2.6–5 million cells is injected under the periosteal graft (Brittberg *et al*., 1994), which has been sutured in the beginning but has later also

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been sealed with fibrin sealant. With a follow-up period of 2–10 years, results of clinical studies revealed well-integrated repair tissue in 90% of treated patients (Peterson *et al.*, 2003). One of the drawbacks of this method is the symptomatic hypertrophy of the periosteal flap in 5–25% of cases (Horas *et al.*, 2000). As an alternative to periosteal flaps, collagen membranes, e.g. Chondro-Gide™ (Geistlich Biomaterials, Wolhusen, Switzerland), are used during the second generation of ACT, resulting in satisfactory repair without hypertrophic development at a 1 year post-surgery arthroscopy (Haddo *et al.*, 2004).

MACT represents the latest in the regeneration of articular cartilage (Marlovits and Trattnig, 2006; Resinger *et al.*, 2004). Today it is widely considered to be one of the best techniques to repair traumatic cartilage defects. In contrast to ACT, cells are seeded to a scaffold prior to intra-articular implantation. The following biomaterials represent the state of the art of currently applied scaffolds in the clinics: collagen type I/III fleece (Chondro-Gide™), hyaluronan fleece (Hyalograft® C, Fidia Advanced Biopolymers, Abano Terme, Italy), collagen I gel (CaReS™, Ars Arthro, Krems, Austria), collagen matrix (Novocart® 3D, TeTeC, Reutlingen, Germany). These have recently been compared regarding morphological and seeding differences (Nuernberger *et al.*, 2010). As limitations of both ACT and MACT, it should be mentioned that only traumatic cartilage lesions of patients up to the age of 50 years can be treated. Moreover, ACT and MACT represent a highly cost-intensive two-step procedure including the drawback of chondrocyte dedifferentiation during cell expansion. This is typically accompanied by decreased collagen type II and increased collagen type I expression (Diaz-Romero *et al.*, 2005). Finally, biopsies must be taken from healthy cartilage for cell isolation, which implies additional donor site morbidity for the patient.

Although it is more than 20 years ago that the ACT was primarily applied, previously established techniques are still of importance. A randomized clinical trial demonstrated that there is no difference in the clinical and radiographic results between MACT and microfracture at 5 years post-surgery (Knutsen *et al.*, 2007). As concluded by the authors, further long-term follow-up is needed to find out which method is the best for the patient. Additionally it is important for future evaluations to take into account factors such as cost and time for cell isolation and expansion.

3. The potential of MSC to improve the state of the art

Based on the limitations of MACT as described in the previous section, cell types of higher availability, such as MSC, have also been investigated to treat cartilage defects.

Stem cells are defined as undifferentiated cells possessing the ability to self-renew as well as to convert to specialized cells (Smith, 2001; Weissman *et al.*, 2001). They can be classified by their potency, which gives information about the ability to differentiate towards one or multiple lineages.

Adult tissues such as bone marrow or adipose tissue contain multipotent cells which are able to turn into cell types of the mesenchymal lineage. Under appropriate culture conditions, MSC are capable of differentiating towards the osteogenic, chondrogenic, myogenic and adipogenic lineages (Barry *et al.*, 2001; Franchini, 2003; Krampera *et al.*, 2006; Pittenger *et al.*, 1999). Bone marrow-derived MSC (BMSC) represent the most intensively investigated MSC type in cartilage tissue engineering. They can be isolated from bone marrow aspirates, expanded in monolayer and induced for chondrogenic differentiation (Johnstone *et al.*, 1998; Mackay *et al.*, 1998). Zuk *et al.* (2001) found that adipose-derived stem cells (ASC) also show an ability to form a cartilage-like matrix, as indicated by alcian blue and collagen type II staining. Major advantages for this cell source are abundant availability accompanied by minimal donor site morbidity. The simple surgical procedure and the uncomplicated enzyme-based isolation procedure make adipose tissue an attractive source for MSC. In contrast to bone marrow, adipose tissue can be harvested in large amounts with low donor site morbidity. Therefore it would be advantageous if the same or higher cartilage quality could be attained with ASC. A considerable number of papers have been published since 2003, addressing the question of which cell type, ASC or BMSC, possesses a higher chondrogenic potential (Table 1). A variety of authors concluded that BMSC can be more easily differentiated towards the chondrogenic lineage than ASC (Afizah *et al.*, 2007; Danisovic *et al.*, 2009; Huang *et al.*, 2005; Im *et al.*, 2005; Jakobsen *et al.*, 2009; Kisiday *et al.*, 2008; Koga *et al.*, 2008; Liu *et al.*, 2007; Vidal *et al.*, 2008). However, Diekman *et al.* (2010) pointed out that ASC and BMSC require unequal growth factor treatment for chondrogenic induction. While aggrecan was upregulated in ASC when treated with bone morphogenetic protein 6 (BMP-6) during differentiation, transforming growth factor-β_3 (TGFβ_3) led to aggrecan upregulation in BMSC. An explanation for this has been given by Hennig *et al.* (2007), who reported that ASC possess a distinct TGFβ receptor repertoire. Apart from that, the cell surface phenotype of ASC is quite similar to BMSC. Both cell types express CD105, CD73, CD90 and lack the haematopoietic lineage markers c-kit, CD14, CD11b, CD34, CD45, CD79, CD19 and HLA-DR. These markers are considered the minimal prerequisite to describe MSC (Katz *et al.*, 2005; Schaffler and Buchler, 2007). In 2006, MSC were defined by the International Society for Cellular Therapy as a plastic-adherent cell population with the following surface marker profile: $CD13^+$, $CD44^+$, $CD90^+$, $CD73^+$ and $CD105^+$, $CD14^-$, $CD11b^-$, $CD79^-$, $CD34^-$, $CD45^-$ and $HLA\text{-}DR^-$ (Dominici *et al.*, 2006). Analysis of ASC and BMSC revealed differences in only four (CD49d, CD105, CD106, NGFR) of 50 markers (Table 2) (De la Fuente *et al.*, 2004). Rider *et al.* (2008) found higher expression

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Table 1. The chondrogenic potential of ASC and BMSC

Reference	Cell source	Outcome
(Lee *et al*., 2004)	Human	No difference between ASC and BMSC found by toluidine blue staining
(Danisovic *et al*., 2009)	Human	ASC possess slightly decreased chondrogenic potential
(Diekman *et al*., 2010)	Human	Collagen type II is higher expressed by BMSC. Regarding aggrecan expression, BMSC respond better to TGFβ, while ASC require BMP-6
(Jakobsen *et al*., 2009)	Human	In hyaluronic acid scaffolds chondrogenesis of BMSC was higher compared to ASC
(Liu *et al*., 2007)	Human	BMSC differentiate better into osteoblasts and chondrocytes, while ASC have higher adipogenic differentiation potential
(Afizah *et al*., 2007)	Human	BMSC and ASC from the same donor: BMSC are more suitable for cartilage tissue engineering than ASC
(Huang *et al*., 2005)	Human	A patient-matched study: under the described conditions, BMSC demonstrated a higher chondrogenic potential
(Im *et al*., 2005)	Human	Results of this study suggest that ASC have inferior capacity for chondrogenic differentiation
(Winter *et al*., 2003)	Human	There are no differences in the expression of chondrogenic marker genes in 2D cultures between ASC and BMSC. In 3D cultures BMSC expressed a gene profile similar to that of osteoarthritic cartilage
(Koga *et al*., 2008)	Rabbit	BMSC have higher chondrogenic potential compared to ASC *in vitro* and *in vivo*
(Peng *et al*., 2008)	Rat	There are no major differences between ASC and BMSC regarding the chondrogenic differentiation potential
(Kisiday *et al*., 2008)	Equine	Superior chondrogenesis of BMSC was demonstrated in comparison to ASC
(Vidal *et al*., 2008)	Equine	Superior chondrogenesis of BMSC was demonstrated in comparison to ASC

Table 2. Differences in surface marker expression

	BMSC	ASC
CD49d	−	±
CD105	++	+++
CD106	++	−
NGFR	±	−

+++, Markers staining positive in more than 85% of cells; ++, markers positive in 41–85% of cells; +, markers positive in 11–40% of cells; ±, markers positive in 2–10% of cells; −, markers expressed in <2% of cells (De la Fuente *et al*., 2004).

of HLA-ABC in BMSC, making ASC more suitable for allogenic transplantations.

MSC can also be easily extracted from the umbilical cord matrix, the so-called 'Wharton's jelly' (Lund *et al*., 2007; Wang *et al*., 2004; Weiss *et al*., 2006). The main role of Wharton's jelly is to prevent overly intense mechanical forces on the vessels of the umbilical cord. The Wharton's jelly matrix consists of glycosaminoglycans (GAGs) and collagen fibrils (Meyer *et al*., 1983). Hyaluronic acid represents the most abundant GAG (Sobolewski *et al*., 1997), which provides a gel-like environment for embedded immature progenitor cells and differentiated myofibroblast-like cells (McElreavey *et al*., 1991; Nanaev *et al*., 1997). Termed 'human umbilical cord matrix cells' (HUCM), the immature progenitor cells have been found to possess multipotent differentiation capacity, as indicated by successful transformation into adipocytes, chondrocytes, osteoblasts and myoblasts (Can and Karahuseyinoglu, 2007). A few studies focused on chondrogenic differentiation of HUCM in 3D culture, during which GAGs as well as collagen I and II were detected histologically (Bailey *et al*., 2007; Karahuseyinoglu *et al*., 2007; Wang *et al*., 2004, 2008). In contrast to BMSC and ASC, which have been demonstrated to possess the ability to synthesize hyaline-like cartilage, the expression of collagen type II and aggrecan is decreased in HUCM, indicating the synthesis of fibrous tissue (Hildner *et al*., 2010b; Wang *et al*., 2009). Although it appears that HUCM possess inferior chondrogenic potential compared to ASC and BMSC, it is important to note that the difference could also be due to the supplementation of specific growth factors, which might be more beneficial for one cell type than for the other. This could be the case for the addition of BMP-6, which has been shown to induce chondrogenesis in ASC, while its effect on HUCM is unknown (Hennig *et al*., 2007). A screening for a large panel of growth factors would be advantageous to elucidate the optimal growth factor combination for directing HUCM towards the chondrogenic lineage.

Regarding the clinical application of MSC in cartilage regeneration, the question arises whether the cells can be applied in a simple and cost-effective one-stage procedure, or have to be expanded prior to implantation. To answer this question, it is important to address not only the cell quantity but also the differentiation potential of primary versus expanded cells. For porcine BMSC it is reported that the chondrogenic potential of expanded cells is strongly attenuated (Vacanti *et al*., 2005). In contrast, expanded human ASC possess an increased chondrogenic potential compared to unpassaged ASC (Estes *et al*., 2006b). ASC from the infrapatellar fat pad it is reported that unpassaged cells differentiate towards the chondrogenic lineage as well as expanded cells (Jurgens *et al*., 2009), which opens both ways, one- and two-stage procedures. A prerequisite for performing one-stage procedures is the availability of rapid cell isolation techniques that can be applied in the operating theatre. Lysis of red blood cells

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can be performed to enhance the concentration of MSC in porcine bone marrow, which yields a cell population capable of chondrogenic differentiation (Peterbauer-Scherb *et al.*, 2010). In an equine model, application of concentrated bone marrow aspirates in combination with microfracture clearly improved cartilage repair in a one-step procedure (Fortier *et al.*, 2010).

In conclusion, different types of adult stem cells should be considered for cartilage tissue engineering, also taking into account factors such as autologous/allogenic use, donor site morbidity and availability. By comparing different cell types for their chondrogenic potential, it is important to note that monolayer expansion conditions, three-dimensional (3D) culture conditions and the time point of analysis also influence the outcome (Diekman *et al.*, 2010).

3.1. The ability of biomaterials to improve chondrogenic differentiation of MSC

3.1.1. 3D cultivation of cells

During chondrogenic differentiation, cells prefer a 3D environment, provided in the simplest strategy by the cells themselves. This system is referred to as 'micromass pellet culture' or 'aggregate culture'. Constructs are generated by centrifugation of cell suspensions (Johnstone *et al.*, 1998) or by culturing high-density cell droplets (Dragoo *et al.*, 2003). The formation of micromass pellets is based on cell condensation (mediated by *N*-cadherin) (Hellingman *et al.*, 2010), which represents the onset of chondrogenic differentiation (DeLise and Tuan, 2002a, 2002b; Denker *et al.*, 1999). Because it is an easy and effective way to study chondrogenesis, this system is frequently used for *in vitro* cartilage tissue-engineering approaches (Chiou *et al.*, 2006; De Ugarte *et al.*, 2003; Dragoo *et al.*, 2003; Hildner *et al.*, 2010a; Malladi *et al.*, 2006; Mochizuki *et al.*, 2006; Zheng *et al.*, 2006; Zuk *et al.*, 2002).

In vivo implantation of cells requires suitable biomaterials that support cell attachment, proliferation and differentiation. Considerations for scaffolds include porosity, bioactivity, mechanical integrity and integration as well as easy handling. Moreover, biodegradability is an important factor in facilitating replacement of the material by newly synthesized cartilage matrix. Products manufactured from collagen and hyaluronan fulfil these requirements to a certain degree and are therefore used in clinical MACT (see sections on ACT and MACT, above).

3.1.2. State of the art in the laboratory

Alginate, agarose, fibrin and gelatin have been extensively investigated in order to evaluate their ability to support chondrogenic differentiation of MSC *in vitro* and *in vivo* (Awad *et al.*, 2003, 2004; Dragoo *et al.*, 2003, 2007; Erickson *et al.*, 2002; Estes *et al.*, 2006a). Alginate is a material which is produced from brown algae and consists of α-L-guluronic acid and β-D-mannuronic acid. Cells can be entrapped by inducing gelation with calcium. Cartilage-like matrix production of ASC entrapped in alginate was demonstrated after induction with TGFβ_1 (Awad *et al.*, 2003; Erickson *et al.*, 2002) or BMP-6 (Estes *et al.*, 2006a). A comparative study revealed that cells in gelatin, which is collagen-based, produce higher amounts of sulphated GAGs (sGAG) and hydroxyproline than cells in agarose and alginate (Awad *et al.*, 2004). As one of the most abundant molecules in cartilage, collagen is of special interest for use as a biomaterial. Nevertheless, Awad *et al.* (2004) also reported that the cell morphology was more spherical and similar to chondrocytes when ASC were cultured in alginate and agarose compared to gelatin, in which the cells appeared fibroblastic. Poor biodegradability of agarose (Rahfoth *et al.*, 1998) and extensive immune reactions after implantation of alginate have been reported (Hunziker, 2002), limiting the applicability of these materials for cartilage regeneration *in vivo*. Fibrin, a blood-derived biomaterial, is the polymerized form of fibrinogen. Cells can be enclosed by suspending them in fibrinogen, followed by mixing the suspension with thrombin, which induces polymerization (Hildner *et al.*, 2009; Ho *et al.*, 2010). Encapsulated in fibrin, ASC from the infrapatellar fat pad demonstrated collagen type II and aggrecan expression and the sGAG content reached 50% of native cartilage (Dragoo *et al.*, 2003). *In vivo*, fibrin was demonstrated to be a suitable material for healing full-thickness cartilage defects in rabbits (Dragoo *et al.*, 2007). By mixing chondroitin sulphate with fibrin sealant, cell *proliferation*, sGAG content and type II collagen expression could be significantly enhanced compared to fibrin matrices without chondroitin sulphate (Wei *et al.*, 2007).

3.1.3. Future perspectives

There is a remarkable emerging field of functional/instructive scaffolds, including natural and synthetic biomaterials. They include peptide gels, combinations of different biomaterials as well as the combination of scaffolds, nanoparticles and growth factors. Betre *et al.* (2006) synthesized a polypeptide gel consisting of Val–Pro–Gly–Xaa–Gly with incorporation of Val, Gly or Ala at the Xaa residue. Surprisingly, this elastin-like polypeptide demonstrated chondrogenic induction potential for ASC without the addition of exogenous factors such as TGFβ. A dual growth factor-releasing scaffold is described by Im and Lee (2010), who incorporated TGFβ_2 and BMP-7 into a porous polycaprolactone (PCL)/F127 scaffold. In contrast to the latter, chondrogenic differentiation of ASC could also be achieved when seeded on nanoparticles composed of poly(lactide-co-glycolide) (PLGA), Pluronic F127 and heparin loaded with TGFβ_1 (Jung *et al.*, 2009).

While one part of the scientific community relies more and more on the use of synthetic and functional biomaterials to improve chondrogenic differentiation, some researchers hypothesize that the natural environment of chondrocytes – cartilage matrix itself – has the potential

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to induce chondrogenic differentiation of MSC or redifferentiation of chondrocytes (Cheng *et al*., 2009; Diekman *et al*., 2010; Peretti *et al*., 2000; Yang *et al*., 2008). The underlying mechanism is potentially based on the establishment of interactions between cell surface receptors and extracellular matrix ligands. ASC and BMSC cultivated on scaffolds derived from lyophilized articular cartilage demonstrated chondrogenic differentiation by cartilage-specific gene and protein expression (Diekman *et al*., 2010). However, these researchers reported that the scaffolds alone are not responsible for initiating the differentiation. In fact, addition of growth factors such as BMP-6 and TGFβ is necessary to achieve chondrogenic induction of ASC and BMSC. In contrast to these findings, Cheng *et al*. (2009) reported that the cartilage scaffold by itself has inductive capacity, most likely through direct cell–matrix interactions. Moreover, the release of signalling factors from the cartilage matrix, as well as 'matrikines' (partially broken-down matrix molecules), may play a role (Cheng *et al*., 2009). However, it is also important to note that, differently to Diekman *et al*. (2010), the culture conditions included the use of fetal calf serum (FCS) during differentiation, which is also a source of growth factors such as TGFβ (Cheng *et al*., 2009).

Obviously, many studies have demonstrated that the 3D environment has a profound effect on chondrogenic differentiation of MSC. However, in order to elucidate the best culture conditions, further investigations require defined standards to be able to compare the properties of different biomaterials.

3.2. The impact of growth factors

As already indicated in the previous section, not only the physical environment but also the action of either endogenous or supplemented growth factors is important for the differentiation of MSC. Studies performed with BMSC and chondrocytes demonstrated that TGFβ is one of the most important growth factors for the induction of cartilage formation (Johnstone *et al*., 1998; Mackay *et al*., 1998; Yaeger *et al*., 1997). Three TGFβ isoforms are known. It is reported that TGFβ_3 and TGFβ_2 lead to significantly higher collagen type II and proteoglycan expression of BMSC than TGFβ_1 (Barry *et al*., 2001). TGFβ can act via three different TGFβ receptors (Dore *et al*., 1998; Johnson *et al*., 1995). However, TGFβ receptor I is weakly expressed by ASC and BMPs are necessary to enhance its expression, which results in better chondrogenic differentiation (Hennig *et al*., 2007).

To date, approximately 20 BMPs have been identified. BMPs play an important role in a wide range of biological processes, from tissue differentiation during early embryogenesis to maintenance of the postnatal tissue homeostasis (Xiao *et al*., 2007). In 1965 Urist found that ectopic bone formation could be induced by implantation of demineralized bone (Urist, 1965). However, the proteins responsible for the induction remained unknown until the late 1980s, when BMP-2, BMP-3 (osteogenin) and BMP-4 were isolated and cloned (Luyten *et al*., 1989; Wozney *et al*., 1988; Wozney, 1992).

Knippenberg *et al*. (2006) investigated the ability of BMP-2 and BMP-7 to induce differentiation and found that a short treatment for only 15 min with BMP-2 is capable of directing ASC towards the osteogenic lineage, while BMP-7 stimulates ASC to differentiate towards a chondrogenic phenotype. The addition of 500 ng/ml BMP-6 to the differentiation medium increased aggrecan gene expression by 200-fold and collagen II gene expression by 38-fold compared to the untreated control (Estes *et al*., 2006a). Hennig *et al*. (2007) found that, in combination with TGFβ_3 10 ng/ml BMP-6 was sufficient for chondrogenic induction, while the effects of BMP-2, BMP-4 and BMP-7 were less pronounced. In contrast, Kim and Im (2009) reported that BMP-7 is the most promising candidate for chondrogenic induction of human ASC in combination with 5 ng/ml TGFβ_2. The discrepancy between these results and those of Hennig *et al*. (2007) might be due to higher concentration of BMP-2, BMP-6 and BMP-7 (100 ng/ml) and the use of TGFβ_2 in contrast to TGFβ_3 (Kim and Im, 2009). Although both studies conclude that the combination of TGFβ and BMPs enhances the chondrogenesis of ASC, they also admit that this results in hypertrophic development, as indicated by enhanced collagen type X expression (Hennig *et al*., 2007; Kim and Im, 2009) (Figure 2). This problem can possibly be overcome with parathyroid hormone-related protein (PTHrP), which is reported to downregulate collagen type I and collagen type X protein but upregulates a wide range of chondrogenic markers (Kim *et al*., 2008).

Growth and differentiation factor 5 (GDF-5) is a synonym for BMP-14, which also belongs to the BMP family. It was first cloned in 1994 and plays an important role in skeletal development (Buxton *et al*., 2001; Hotten *et al*., 1994). It is mainly expressed during early cartilage condensation and in the interzone as well as the perichondrium at later stages (Buxton *et al*., 2001). Feng *et al*. (2008) investigated the chondrogenic potential of rat ASC transfected with Ad-GDF-5, which was comparable to induction using exogenous GDF-5 (100 ng/ml) and TGFβ (10 ng/ml).

Basic fibroblast growth factor (FGF-2) represents another widely used growth factor in cartilage tissue engineering, demonstrating enhanced chondrogenesis when applied to equine cartilage explant cultures (Henson *et al*., 2005), rabbit cartilage defects (Ishii *et al*., 2007), periosteal explant cultures (Stevens *et al*., 2004) or canine chondrocytes (Veilleux and Spector, 2005). It is reported that ligand binding to FGF receptors 1–4 leads to activation of mitogen-activated protein kinase/ERK kinase (MEK), which then phosphorylates mitogen-activated protein kinase (MAPK) (Murakami *et al*., 2000). In turn, activation of MAPK modulates a cascade of kinases and transcription factors, which finally upregulates SOX9 [SRY (sex-determining region Y)-box 9], a key regulator in chondrogenesis (Murakami *et al*., 2000).

Interestingly, information about the chondrogenic induction potential of FGF-2 is controversial when applied

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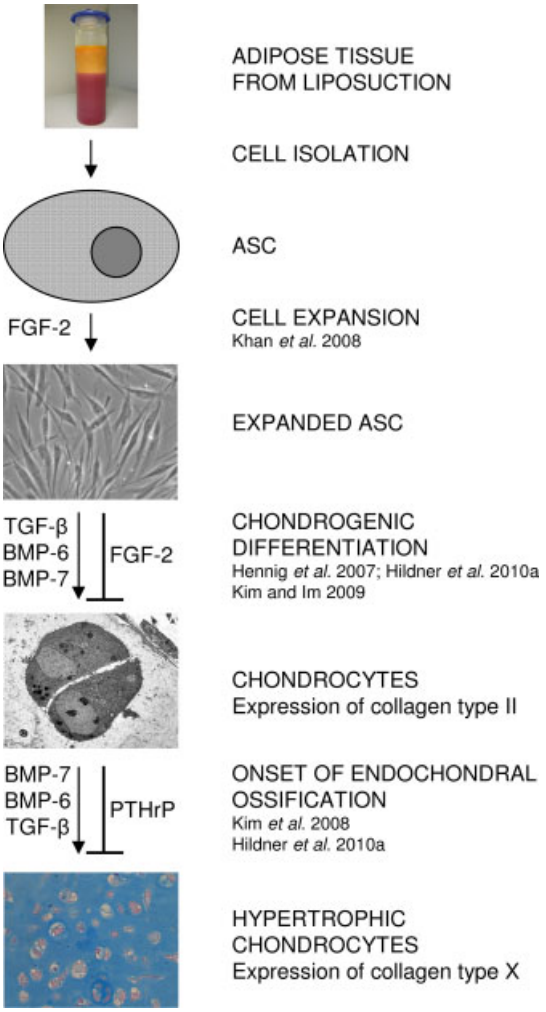

Figure 2. Important factors for expansion and chondrogenic differentiation of ASC. FGF-2 promotes proliferation of ASC and retains their chondrogenic differentiation potential. BMP-6 and TGFβ induce the chondrogenic differentiation but lose their potential in the presence of FGF-2. Final differentiation towards hypertrophic chondrocytes is attenuated by PTHrP

to ASC. Chiou *et al.* (2006) reported that FGF-2 supplemented to the differentiation medium promotes sGAG synthesis and collagen type II expression. In contrast, Hennig *et al.* (2007) could not confirm these findings and our own results demonstrate that FGF-2 even counteracts BMP-6/TGFβ_3-induced chondrogenesis (Figure 3) (Hildner *et al.*, 2010a). The controversial effects of FGF-2 on chondrogenesis have also been addressed by Khan *et al.* (2008), who found that FGF-2 downregulates chondrogenic markers during cell expansion. Nevertheless, ASC expanded in the presence of FGF-2, demonstrating stronger upregulation of chondrogenic genes and greater cartilage matrix production during differentiation in 3D micromass pellets compared to cells expanded without FGF-2 (Khan *et al.*, 2008). Therefore, it is important to note that FGF-2 is essential for cell propagation but negatively influences cartilage formation at the differentiation stage. This is also consistent with Hellingman *et al.* (2010), who report that FGF-2 has a strong binding affinity to FGF receptor 1, which is strongly expressed in expanding MSC but is downregulated during condensation; when FGF-2 was added afterwards (days 3–14), chondrogenic differentiation was inhibited.

Many studies published within recent years have investigated the function of growth factors and provide broad knowledge on the induction and inhibition of stem cell chondrogenic differentiation. Now it is important to utilize this knowledge to further optimize the quality of tissue-engineered cartilage towards hyaline cartilage. However, to get the approval for clinical use of a growth factor may take decades. As described in the next section, another way that stem cells can be induced for chondrogenic differentiation is by the presence of mature cells, directing them towards the desired lineage.

3.3. Co-culture in cartilage tissue engineering: being supported by teachers

It is well accepted that different cell types communicate via soluble factors (paracrine signalling) or direct cell–cell contact (reviewed in Hendriks *et al.*, 2007). In the latter, two possible signalling mechanisms are known: (a) gap junctions, which are able to build a direct connection between the cytoplasm of interacting cells; and (b) what is referred to as juxtacrine communication, when exposed signals of one cell are bound by the membrane receptors of another cell. Most co-culture studies performed in the field of osteochondral tissue engineering focused on the regeneration of nucleus pulposis tissue (Li *et al.*, 2005; Lu *et al.*, 2007, 2008; Richardson *et al.*, 2006; Yamamoto *et al.*, 2004; Zhang *et al.*, 2005). ASC are accessible to paracrine factors released by disc cells, as demonstrated by upregulated cartilage-specific gene expression collagen type II and aggrecan (Li *et al.*, 2005; Lu *et al.*, 2007, 2008). The release of soluble factors has also been shown to support chondrogenesis in an indirect co-culture model of human embryonic stem cells (hESCs) and primary chondrocytes by significantly elevated expression of GAGs, type II but also type I collagen (Vats *et al.*, 2006). In contrast, in co-cultured BMSC and nucleus pulposis cells, a direct cell–cell contact is required to achieve differentiation (Richardson *et al.*, 2006; Yamamoto *et al.*, 2004). One of our recent studies shows that ASC contribute to fibrocartilage formation when co-cultured with human expanded chondrocytes (Hildner *et al.*, 2009). Fibrocartilage is required for the regeneration of meniscal tissue and contains a mixture of type II and type I collagen. Co-culture of fibrochondrocytes and articular chondrocytes also resulted in the formation of a matrix very similar to native knee meniscus (Aufderheide and Athanasiou, 2007; Hoben *et al.*, 2007; Hoben and Athanasiou, 2008). Gan and Kandel (2007) investigated the influence of unpassaged chondrocytes on passaged, dedifferentiated chondrocytes, aiming to induce redifferentiation. Passaged, dedifferentiated chondrocytes were directly co-cultured with 5–40% primary chondrocytes. Indeed, induction of redifferentiation, as indicated by upregulated collagen II and downregulated collagen I expression, could be shown.

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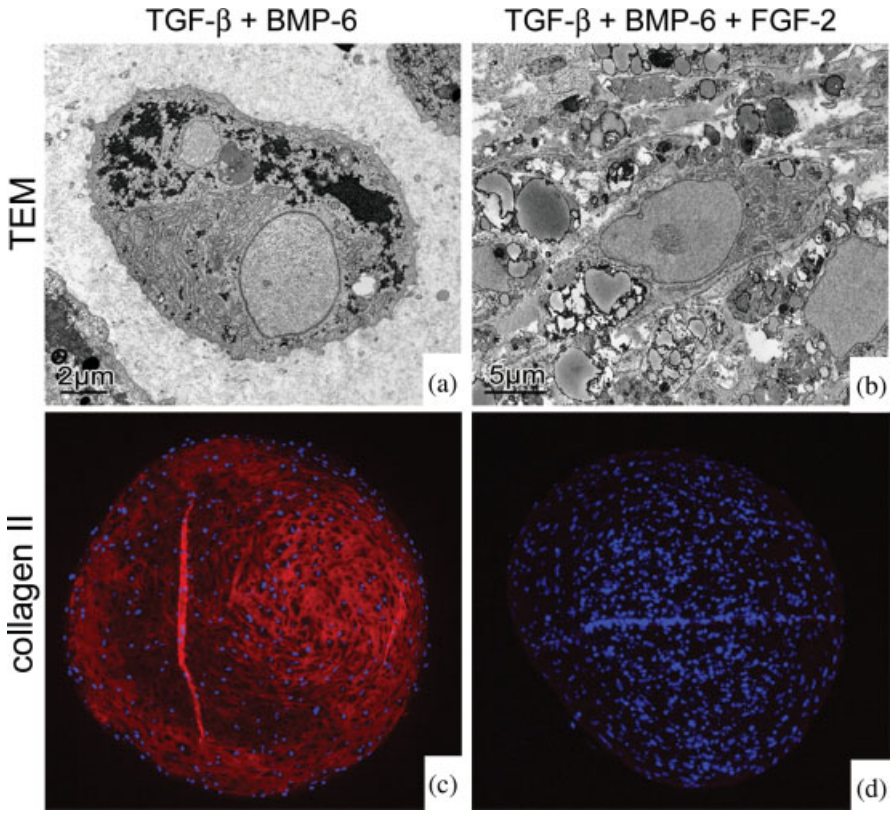

Figure 3. Micromass pellet of ASC treated with TGFβ_3 and BMP-6 (a, c); transmission electron microscopy demonstrates a spherical morphology of the cells which include dense glycogen inclusions in the cytoplasm, which is typical for chondrocytes (a). In the presence of TGFβ_3, BMP-6 and FGF-2, the synthesized tissue appears heterogeneous and includes undifferentiated cells (b). Collagen type II staining was only positive in the presence of TGFβ and BMP-6 in the differentiation medium (c). Additionally supplemented FGF-2 inhibited collagen II expression (Hildner *et al*., 2010a)

The difficulty in direct co-culture is to prove which cell type is responsible for the chondro-inducing effect. Recently, Bigdeli *et al*. (2009) presented a technique that addresses this question (Figure 4). In order to distinguish between hESCs and human chondrocytes, donors of different gender were used for direct co-culture. Prior to the formation of micromass pellets, human chondrocytes were irradiated to prevent proliferation of this cell type. On day 14 micromass pellets were treated with collagenase to remove synthesized matrix and the released cells were further subcultured in 2D, whereby only hESCs were able to proliferate. After monolayer expansion, the absence of chondrocytes was proven by FISH. Subsequent 3D culture of co-cultured hESCs demonstrated significantly higher cartilage formation potential compared to the control group, which was not co-cultured with human chondrocytes.

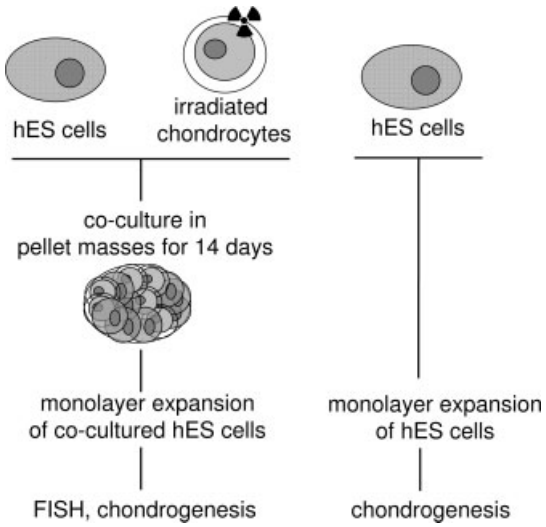

Figure 4. Schematic drawing of the co-culture model described by Bigdeli *et al*., (2009)

The idea of indirect co-culture is that paracrine communication functions via soluble factors. However, only few studies have analysed the release of these substances. A latent form of TGFβ_1 and TGFβ_2 is reported to be expressed by growth plate chondrocytes, which becomes activated by matrix metalloproteinase 3 (MMP-3). This enzyme is stored in matrix vesicles and is regulated by lysophospholipids (Boyan *et al*., 1994; Gay *et al*., 2004). Ahmed and colleagues (2007) analysed the conditioned medium of cartilage samples and found secreted vascular endothelial growth factor (VEGF), tissue inhibitor of metalloproteinases 1 (TIMP-1), TIMP-2 and MMP-13; paracrine signalling of these factors resulted in upregulated Sox9 and collagen II expression of rat BMSC and downregulated collagen type X expression. This demonstrates that a co-culture of MSC and chondrocytes

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not only results in enhanced matrix accumulation but also reduces the risk of hypertrophic development.

3.4. Bioreactors

The previous sections described the potential of growth factors in combination with adult stem cells and scaffolds to improve cartilage regeneration. The 'tissue-engineering triangle' can be extended by a fourth component, bioreactors. Bioreactors are used for different purposes, including cell seeding, cell proliferation and the generation of 3D tissue constructs. The overall aim of using a bioreactor is to mimic physiological conditions, including nutrients, oxygen, temperature, pH, carbon dioxide, humidity and mechanical stress. In addition, automated processing reduces the risk of contaminations and increases reproducibility (Nagel-Heyer *et al*., 2005; Portner *et al*., 2005). One of the most important prerequisites for generating tissue-engineered cartilage is the homogeneous distribution of cells in the scaffold. In contrast to static loading of cells in a scaffold, which often does not result in uniform cell distribution (Wendt, *et al*., 2005), high efficiencies and uniformities can be obtained by using stirred-flask bioreactors (Vunjak-Novakovic *et al*., 1996) and direct perfusion bioreactors (Wendt *et al*., 2003).

Once seeded on the scaffold, mechanical forces, such as hydrostatic pressure, direct compression or shear stress, stimulate the cells to synthesize more extracellular matrix compared to static cultures (Gooch *et al*., 2001; Wu *et al*., 1999). All three types of mechanical force are at work hundreds of times daily in our knee joints when we walk, run or jump. Less than 10% of the mechanical load is absorbed by direct compression of cartilage. The majority is absorbed by hydrostatic pressure, which uniformly stimulates chondrocytes from all directions (Elder and Athanasiou, 2009; Schulz and Bader, 2007).

In vitro, there are two ways that hydrostatic pressure can be applied. The load can be transmitted to the medium by compression of the gas phase or by direct compression of the fluid phase. The main advantage of hydrostatic pressure chambers using both gas and liquid phases is the ability to control the oxygen tension by variation of the partial pressure (Darling and Athanasiou, 2003). Several studies have demonstrated the positive influence of hydrostatic pressure on the differentiation of BMSC by increased GAG and collagen content (Angele *et al*., 2003; Luo and Seedhom, 2007; Miyanishi *et al*., 2006a, 2006b; Schulz and Bader, 2007; Wagner *et al*., 2008). In contrast, only two studies describe the use of bioreactors to stimulate chondrogenesis of ASC. Increased GAG and type II collagen expression compared to static cultures could be achieved by applying hydrodynamic shear levels with a perfusion-type recirculation bioreactor (Mahmoudifar and Doran, 2010). Ogawa *et al*. (2009) applied hydrostatic pressure at 0–0.5 MPa, 0.5 Hz, and also found increased chondrogenic differentiation of ASC at both the mRNA and the protein level.

Obviously, mechanical stimulation positively influences the chondrogenic differentiation of MSC. With regard to future perspectives, the question arises which role bioreactors will play in cartilage tissue engineering. Are bioreactors tools for basic research to gain knowledge about the behaviour of cells under controlled conditions, or will it really be possible to grow cartilage in bioreactors suitable for transplantation into large chondral or osteochondral defects? Focusing on the latter, we have to keep in mind that engineered cartilage constructs lose their ability to integrate into the host tissue at a higher developmental stage (Obradovic *et al*., 2001). Moreover, predifferentiation of BMSC *in vitro* is reported to be associated with hypertrophic development when implanted into minipig cartilage defects. In contrast, no signs of endochondral differentiation were observed when BMSC were directly implanted without predifferentiation (Steck *et al*., 2009). Thus, it will be important to use bioreactors for the optimization of *in vitro* conditions to generate well-integrating, non-hypertrophic tissue rather than fully differentiated, mature cartilage constructs. In conclusion, regarding availability and donor site morbidity, ASC are especially promising for use in cartilage tissue engineering. However, in order to attain equal or superior cartilage matrix synthesis compared to the use of chondrocytes, they need further support. It has been shown that growth factors, biomaterials and mechanical stimulation, but also the presence of 'teacher cells' in co-cultures, can support chondrogenic differentiation and matrix accumulation. In order to transfer the use of ASC from bench to bedside, optimization and combination of the techniques is required and finally a comparison to the clinical state of the art.

4. Improvement of cartilage regeneration with human platelet-derived products

4.1. Platelet-derived products: a substitute for fetal calf serum?

Fetal calf serum (FCS) can be regarded as the 'gold standard' medium supplement in cell culture. Besides the fact that the production of FCS is accompanied by ethical concerns, it is reported that MSC cultured in the presence of FCS internalize xenogeneic proteins (Spees *et al*., 2004). This implies the risk of virus and prion transmission and the use of FCS for human products is therefore not recommended by European legislation (Doucet *et al*., 2005). Furthermore, the use of FCS for cell expansion implies immunological risks in cell therapy (Kadri *et al*., 2007; Tuschong *et al*., 2002). FCS has been replaced by autologous human serum (HS) in order to achieve safe expansion of chondrocytes for the use in ACT (Brittberg *et al*., 1994). Nevertheless, donation of autologous HS implies extra effort for the patient. Therefore, and also with respect to using standardized growth supplements

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in cell culture, application of standardized, serum-free, platelet lysates (PL) might be a suitable allogenic alternative to HS.

4.2. Platelet-derived products

Platelets contain a wide range of cytokines [FGF-2, insulin-like growth factor 1 (IGF-1), platelet-derived growth factor (PDGF), TGFβ, VEGF, epidermal growth factor (EGF)], which are known to increase the proliferation rate of various cell types (Anitua *et al*., 2007; Kandler *et al*., 2004; Okuda *et al*., 2003). Moreover, they play an important role in tissue synthesis and remodelling (Anitua *et al*., 2006; Werner and Grose, 2003). The cytokines are stored in the α-granules of the platelets and are released upon activation with thrombin, collagen or by mechanical destruction (e.g. freeze–thaw cycles). Platelet-derived products are termed platelet lysate (PL), platelet-rich plasma (PRP), platelet-rich plasma releasates (PRPr) or plasma rich in growth factors (PRGF) and have been studied and utilized since the 1970s in the field of bone, ligament and tendon healing. By definition, PRP is a volume of plasma fraction of autologous blood having a platelet concentration above baseline (Marx, 2001; Pietrzak and Eppley, 2005). In order to achieve clinical efficacy, enrichment of four- to six-fold to 1 millon/µl is recommended (Nikolidakis and Jansen, 2008; Sampson *et al*., 2008).

4.3. Clinical use of PRP

A large number of studies exist which demonstrate promising results for treating chronic non-healing tendon injuries by PRP injections into elbow, ankle and knee tendon structures. However, it is important to note that most studies are pilot studies with small sample sizes (Sampson *et al*., 2008). Although many controversial results are reported, PRP is also widely used in the field of bone tissue engineering. Clinical studies by Marx *et al*. (1998) and Oyama *et al*. (2004) in the field of oral–maxillofacial reconstructions showed a strong effect of PRP on bone formation (88 and 12 patients, respectively), while there was weak to absent effect on bone formation in other studies investigating 26 and 10 patients (Dori *et al*., 2008; Kassolis and Reynolds, 2005).

Aside from bone tissue-engineering approaches, PRP was also suggested to possess impact to improve articular cartilage disorders by direct injection into the patient's knee. This issue has been poorly investigated thus far, but preliminary results are promising. Kon *et al*. (2010) reported on 91 patients (115 knees) treated with PRP. The health status was evaluated by IKDC- and EQ-VAS scores, which showed that PRP treatment is safe, reduces pain and improves knee function, especially in younger patients (Kon *et al*., 2010). Nevertheless, this study is limited by the lack of control groups and short follow-up periods up to 12 months. Very recently, the 24-month follow-up results of this study were published. Unfortunately the outcome (IKDC score) worsened from 67% to 59% of normal or nearly normal knees between the 12- and 24-months evaluations (Filardo *et al*., 2010). A similar study was presented by Kon *et al*. at the Annual Meeting of the American Academy of Orthopaedic Surgeons in March 2010, which included viscosupplementation (injection of hyaluronic acid) as a control group. Compared to hyaluronic acid, injection of autologous PRP demonstrated significant improvement regarding pain and articular function.

A consistent nomenclature, as well as standardized protocols to produce PRP, are still missing and would highly improve the comparability of studies.

4.4. The underlying effect of platelet-derived products: knowledge from *in vitro* studies

In vitro studies showed that PRP activates proliferation and migration of osteoprogenitor cells (Gruber *et al*., 2003, 2004) but decreases osteogenic differentiation of BMSC (Gruber *et al*., 2004). This suggests that the osteogenic effect of PRP mainly relies on proliferation and migration, rather than on induction of differentiation.

Different authors agree that PL also stimulate the cell proliferation of chondrocytes (Akeda *et al*., 2006; Drengk *et al*., 2009; Gaissmaier *et al*., 2005; Kaps *et al*., 2002; Spreafico *et al*., 2009). However, controversial results are published on the influence of chondrogenic differentiation and cartilage matrix accumulation. Akeda *et al*. (2006) used directly isolated porcine chondrocytes entrapped in alginate, which were stimulated with FCS for 7 days followed by 24 h in serum-free medium and finally 72 h with FCS, platelet-poor plasma (PPP) or PRP. Compared to PPP and FCS, stimulation with PRP resulted in highest proteoglycan and collagen synthesis, whereas most of the collagen was identified as collagen type II. Spreafico *et al*. (2009) expanded human chondrocytes with FCS, PPP or PRP and subsequently cultivated the cells in a PRP/fibrin gel, again with FCS, PPP or PRP. Less dedifferentiation and increased matrix synthesis was obtained for cells treated with PRP. Different results were achieved by Kaps *et al*. (2002) (bovine chondrocytes), Gaissmair *et al*. (2005) (human chondrocytes) and Drengk *et al*. (2009) (sheep chondrocytes), who reported that PRP leads to enhanced dedifferentiation of chondrocytes and does not contribute to cartilage matrix synthesis when applied during *in vitro* 3D culture. The contradictory results might be due to different culture conditions and different preparations of PRP. One key factor of PRP preparation is the type of activation. Han *et al*. (2009) showed that thrombin activation eliminates the chondro- and osteoinductive potential of PRP. Two other studies (Doucet *et al*., 2005; Zaky *et al*., 2008) strengthen these results, reporting successful chondrogenic induction of BMSC; in fact, they did not apply thrombin but used several freeze–thaw cycles to activate PRP. Based on the available data, growth factors derived from platelets are promising as a substitute for HS for the expansion of

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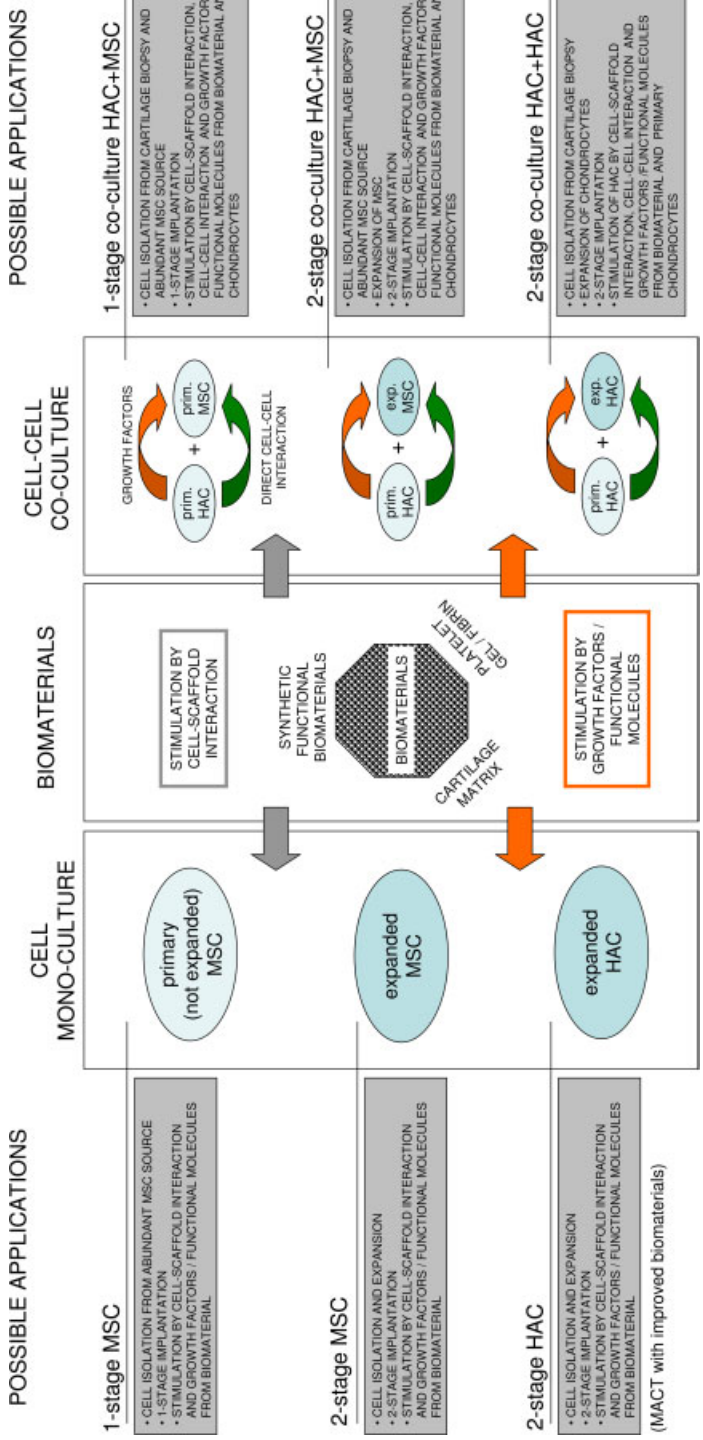

Figure 5. Possible future applications: one- and two-stage procedures, cells induced by biomaterials and released bioactive factors from biomaterials and cell–cell co-cultures of ASC and human articular chondrocytes (HAC)

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chondrocytes in MACT. However, for an allogenic application a serum-free, specifically defined PL product has yet to be established.

In conclusion, platelet-derived products show promise in the field of cartilage regeneration. However, implementation of a consistent nomenclature and standardized production protocols would be highly beneficial for future studies to prevent divergent results based on different preparation methods.

5. Summary

Compared to other tissues, the composition of cartilage is rather simple. It contains only one cell type and is not vascularized. Nevertheless, at the ultrustructural level articular cartilage possesses a unique anisotropic structure which is able to tolerate tremendous amounts of physical stress. Unfortunately, the self-repair potential of injured articular cartilage is very limited and functional regeneration is challenging. Cartilage regeneration according to tissue engineering principles requires the joint expertise of biologists, chemists and engineers to provide highly potent cells, specific growth factors and optimized physical environments.

Although the use of autologous chondrocytes in MACT is accompanied by concerns such as harvesting site morbidity and dedifferentiation, chondrocytes are currently still the cells of choice in the clinic. Future perspectives include the use of freshly isolated stem cells applied in a one-stage procedure or expanded stem cells in a two-stage procedure. Various types of adult stem cells have been investigated *in vitro* and *in vivo* for their potential to synthesize cartilage matrix (Hildner *et al*., 2010b; Johnstone *et al*., 1998; Mackay *et al*., 1998; Zuk *et al*., 2001). Their potential strongly depends on induction by growth factors, type of scaffold and age of the patient. Growth factors from the TGFβ superfamily, such as BMPs and GDF, have demonstrated effective induction potential, especially on BMSC and ASC (Barry *et al*., 2001; Feng *et al*., 2008; Hennig *et al*., 2007; Hildner *et al*., 2010a; Knippenberg *et al*., 2006). However, it may take decades from the discovery of a growth factor to its clinical approval, as was the case for BMP-2 and BMP-7 in bone regeneration. Therefore, intra-operative use of autologous material, which does not require marketing authorization, has been investigated to support cartilage matrix synthesis. These materials include cartilage matrix and freshly isolated primary autologous chondrocytes, which are reported to improve chondrogenic differentiation by either direct or indirect signalling (Figure 5) (Bigdeli *et al*., 2009; Cheng *et al*., 2009; Diekman *et al*., 2010; Gan and Kandel, 2007; Peretti *et al*., 2000; Vats *et al*., 2006; Yang *et al*., 2008). PRP, another autologous material that has been clinically applied since the 1970s to heal bone fractures as well as ligament and tendon injuries, has recently gained special interest for use in cartilage regeneration. It is reported that PRP can be applied for the expansion of chondrocytes but also has potential to act as a scaffold when gelled with calcium gluconate (Spreafico *et al*., 2009). Serum-free, specifically defined PL may probably also be applied as an allogenic product for propagating cells to be used in cartilage regeneration. The effect of intra-articular PRP injection on injured or degenerated cartilage is promising but has been poorly investigated to date.

In summary, many efforts to improve cartilage regeneration have been undertaken during recent decades. ACT has been improved by MACT and a series of new scaffolds have been introduced. Research on stem cells and various autologous materials has shown a wide range of prospects and shows promise in supporting cartilage regeneration in the future.

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