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Clinical and experimental approaches to knee cartilage lesion repair and mesenchymal stem cell chondrocyte differentiation

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ABSTRACT

Cartilage has poor regeneration capacity due to the scarcity of endogenous stem cells, its low metabolic activity and the avascular environment. Repair strategies vary widely, including microfracture, autologous or allogenic tissue implantation, and *in vitro* engineered tissues of autologous origin. However, unlike the advances that have been made over more than two decades with more complex organs, including vascular, cardiac or bone tissues, similar advances in tissue engineering for cartilage repair are lacking. Although the inherent characteristics of cartilage tissue, such as the lack of vascularity and low cellular diversity, suggest that it would be one of the more simple tissues to be engineered, its functional weight-bearing role and implant viability and adaptation make this type of repair more complex. Over the last decade several therapeutic approaches and innovative techniques show promise for lasting and functional regeneration of hyaline cartilage. Here we will analyze the main strategies for cartilage regeneration and discuss our experience.

Key words: Cartilage; lesion; microfracture; stem cells

1. INTRODUCTION

Traumatic and chronic focal injuries to articular cartilage are frequent, especially among young athletes; the incidence of chondral lesions in arthroscopies ranges from 60% to 66% (Aroen et al., 2004; Curl et al., 1997; Widuchowski et al., 2007). Symptoms associated with these lesions include pain, stiffness, swelling and a clicking sound, among others, which can affect patients' work, physical activity and activities of daily living. Although the etiology is not well understood, they have the potential to progress to osteoarthritis (OA) of the joint along with all of the associated social and personal costs if left untreated.

Highly organized articular cartilage (Figure 1) is avascular, aneural and has little regeneration potential upon damage, which results in functional loss. Currently there are two major treatments for focal defects of the knee joint. The first approach involves stimulation of the bone marrow by inducing perforations (microfracture) or abrasions of the subchondral plate with the aim of generating scar tissue, which induces deposition of fibrocartilage derived from mesenchymal stem cells (MSCs) that is mechanically different from the hyaline cartilage (Steadman et al., 2003b). The second approach involves generation or transplantation of tissue with similar features, such as native hyaline cartilage. In this approach, autogenic and allogenic mature cartilage blocks or autogenous and allogenous chondrocytes are transplanted with the goal of generating hyaline-like tissue (Hangody et al., 1997).

Brittberg et al. (1994) initially reported the "first generation" of autologous chondrocyte implantation (ACI) in which a solution of *in vitro* cultured and expanded autologous chondrocytes were injected over the lesion under the periosteal flap. In the last 10 years this technique has been improved, using scaffolds as carriers for cells, *in vitro* cartilage-tissue constructions and using MSCs as a source to generate hyaline cartilage (Russlies et al., 2002; Trattnig et al., 2005). In addition, long-term follow-up studies reporting the outcomes of knee chondral lesions treated with the primary ACI and more recent techniques to transplant cells have been published, with adequate results (Beris et al., 2012; Filardo et al., 2012a; Pelissier et al., 2013; Rogers et al., 2010). However, questions regarding the structural quality of the repaired tissue, complications (e.g., graft hypertrophy) and the high cost of the procedure has motivated further research in this area (Brittberg, 2010).

We will review the techniques used to treat articular cartilage defects of the knee, as well as the indications and associated complications of these approaches. In addition, we will review the mid- to long-term follow-up clinical studies, with a focus on the cell-based therapies.

2. REPAIR TECHNIQUES

The most common surgical option to treat focal defects of knee cartilage includes bone marrow stimulation techniques that perforate the subchondral plate, creating small fractures with the aim of stimulating MSCs to generate a scar over the lesion. Among its advantages, bone marrow stimulation is a low-cost and relatively simple procedure (Steadman et al., 2003a; Steadman et al., 2003b). Current indications for microfracture are small full-thickness chondral defects (up to 4 cm^2) in a stable, aligned knee.

The outcomes of microfracture are correlated with different factors, including size of the defect and patient characteristics. For example, traumatic lesions, lesions smaller than 4 cm^2 and those with shorter onset of symptoms at the time of treatment

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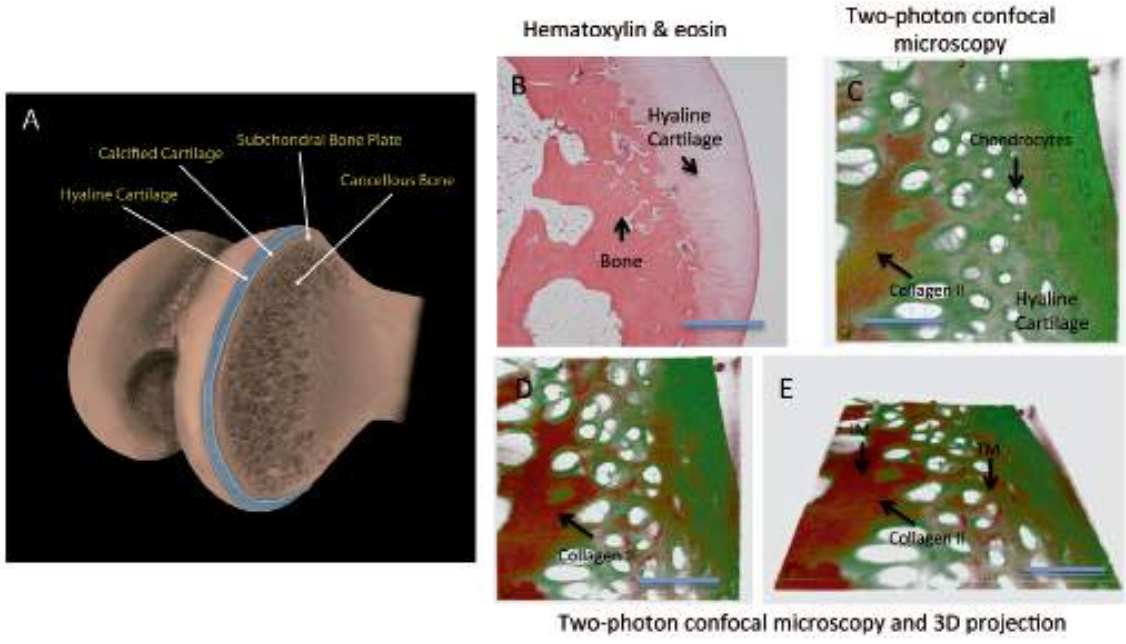

Figure 1. Normal histology of hyaline cartilage using two-photon microscopy.
A. Schematic representation of hyaline cartilage of the knee. B. Hyaline cartilage observed with hematoxylin and eosin staining. A homogenous surface was observed over the articular bone. C. Hyaline cartilage analyzed using two-photon confocal microscopy, showing chondrocytes and the cartilage matrix. D, E. Hyaline cartilage analyzed using two-photon confocal microscopy and 3D projection. Second harmonic generation showing type II collagen in red and the cartilage matrix in green. Several lacunae are visible. The territorial matrix (TM) and interterritorial matrix (IM) may also be discerned. Scale bars in B, 500 mm; in C-E, 30 □m.

respond better to microfracture (Steadman et al., 2003a; Steadman et al., 2003b). In addition, females, older patients and patients with greater body mass index present with poorer outcomes (Mithoefer et al., 2005; Steadman et al., 2003a; Steadman et al., 2003b).

In an 11 year follow-up study of the outcomes of microfracture for traumatic full-thickness chondral defects in 71 knees, Steadman et al. (2003a) reported good and excellent clinical outcomes as measured with the Lysholm and Tegner scores. In a prospective follow-up study that included 48 patients with isolated full-thickness chondral defects of the condyles in stable knees, Mithoefer et al. (2005) found significant improvement in functional and subjective scores after a minimum follow-up of 2 years. The best results were observed in patients with better fill of the lesion as evaluated with magnetic resonance imaging (MRI), lower body mass index and shorter onset of symptoms (Mithoefer et al., 2005). In a recent retrospective case series study that evaluated 145 patients with a single chondral lesion of the knee joint, Salzmann et al. (2013) reported improvements in the clinical outcomes (International Knee Documentation Committee [IKDC], Lysholm and Tegner scores and the numeric analogue scale) following single lesion microfracture after a minimum follow-up of 2 years. Significantly better outcomes were observed in male patients and in those patients with a shorter duration of symptoms. Thus although satisfactory outcomes for microfracture have been reported for this cost-effective and relatively simple procedure, full recovery is not acheived in all patients (Salzmann et al., 2013). Moreover, the achieved clinical and structural outcomes may decrease over time (Kon et al., 2012).

2.1. Experimental approach to evaluate microfracture for knee cartilage repair

Hyaline cartilage is the most common and widely distributed type of cartilage; it is found in the tracheal rings, articular surfaces of synovial joints and developing bones in endochondral ossification. With the exception of articular cartilage, hyaline cartilage is enclosed by a perichondrium. The territorial matrix, which surrounds chondrocytes or chondrocyte clusters, is an intensely staining, basophilic matrix. The staining reaction is likely due to a higher concentration of stainable sulfated glycosaminoglycans or other ground substance macromolecules near the chondrocytes. In our analysis, the territorial matrix was Bismarck brown-positive and showed very low immunoreaction for type II collagen (Figure 2A-C). In contrast, most type II collagen immunoreaction was detected in the interterritorial matrix, which is relatively distant from the chondrocytes (Figure 2A), and collagen fibrils in the interterritorial matrix were larger than those fibrils closer to the chondrocytes. However, this matrix is less intensely stained using histochemical techniques (Figure 2B).

Knee arthrotomy and microfracture require drilling into the subchondral bone to release bone marrow that forms a stem cell-rich clot at the site of the wound, which spontaneously differentiates into fibrocartilage. We have analyzed knee lesions treated with microfracture in rabbits to evaluate the scar formed after treatment (Figure 4). The histochemical techniques of safranin, Bismarck brown and alcian blue staining were used to evaluate the lesion (Figure 4A-F). Additionally, immunohistochemical analysis for types

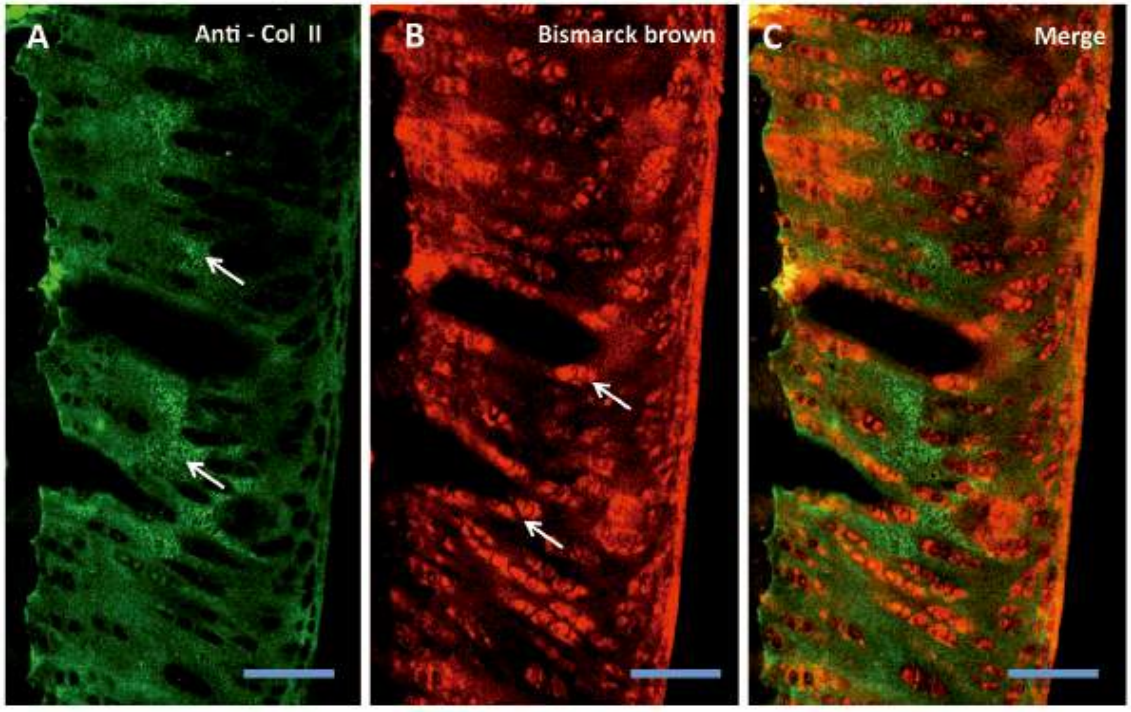

Figure 2. Histochemical and immunohistochemical analysis of cartilage.
A-C. Confocal microscopy analysis of different hyaline cartilage components after immunohistochemistry and fluorescence analysis for type II collagen (cy2 in green, 488 nm laser excitation) (A) and histochemical and auto-fluorescence detection after Bismarck brown staining (633 nm laser excitation) (B). A, B. Similar to the imaging generated with two-photon and second harmonic generation, type II collagen distribution is mainly observed in the inner part of the hyaline cartilage, forming the interterritorial matrix (A, arrows). Bismarck brown staining is observed mainly in the territorial matrix (B, arrows). C. A low co-localization is observed between the stains. Scale bars in A-C, 50 □m.

I and II collagen and aggrecan was performed (Figure 4G-L). After microfracture, the scar tissue was negative for safranin and Bismarck brown staining and positive for type I collagen (Figure 3 and Figure 4B, D and H). The scar tissue was also negative for type II collagen and aggrecan (Figure 4J, L).

As described in previous reports, the experiments performed in this study allow us to conclude that the microfracture procedures are adequate to induce repair of the damaged articular cartilage. However, the neo-synthesized tissue in the lesions does not correspond to articular cartilage, which is mainly composed of type II collagen. The fundamental findings in the damaged tissue were the increased cellular proliferation and the deposition of type I collagen as identified with a collagen I-specific antibody. The cells formed a compact tissue with no apparent formation of territorial/inter-territorial matrices or isogenous groups. Furthermore, there was no deposition of other cartilage extracellular matrix components, including glycosaminoglycans, as was shown by the lack of safranin, Bismarck brown and alcian blue staining. Moreover, the neo-synthesized tissue did not show a positive reaction with an anti-aggrecan antibody. Thus the methods used for cartilage repair will have to incorporate new strategies to stimulate the synthesis of an articular matrix similar to that found in normal tissue.

2.2. Osteochondral autograft and allograft transplantation

In 1997, Hangody et al. (1997) first described mosaicplasty, in which autologous osteochondral plugs were harvested from a non-weight bearing area to treat osteochondral lesions of the knee joint in humans. Osteochondral cylinders were harvested using a tubular chisel, mostly from the superolateral side of the femoral trochlea to fill osteochondral lesions of weight-bearing areas of the same knee. Usually multiple cylinders are required to fill the lesion completely and to establish a congruent hyaline surface. Correct placement of the graft is crucial in order to prevent extensive pressure to the graft and avoid chondrocyte death; therefore grafts should be placed at the same level as the surrounding healthy cartilage, or slightly impacted (Duchow et al., 2000; Hangody et al., 2004; Whiteside et al., 2005).

This one-step procedure corrects the lesion with native hyaline cartilage from the same patient. Indications for this technique include Outerbridge grade III and IV cartilage loss and full-thickness chondral defects located in weight-bearing areas of the knee condyles and in the patellofemoral joint. The size of the defect is limited to 4 cm^2 due to limitations in the donor area. As older patients present limited repair capacity, this procedure is indicated in patients younger than 45-50 years. If malalignment, meniscal and/or ligament lesions are present, these concomitant lesions should be addressed in order to permit adequate repair of the damaged cartilage. For larger lesions, the transference of an osteochondral allograft is an option; however, the lack of donors, difficulties in managing fresh allografts and the possibility of disease transmission make this a less suitable alternative (Bugbee et al., 2012; Demange and Gomoll, 2012; Gross et al., 2005).

Long-term outcomes of mosaicplasty were reported by Hangody et al. (2008). Of a total of 1097 mosaicplasties,

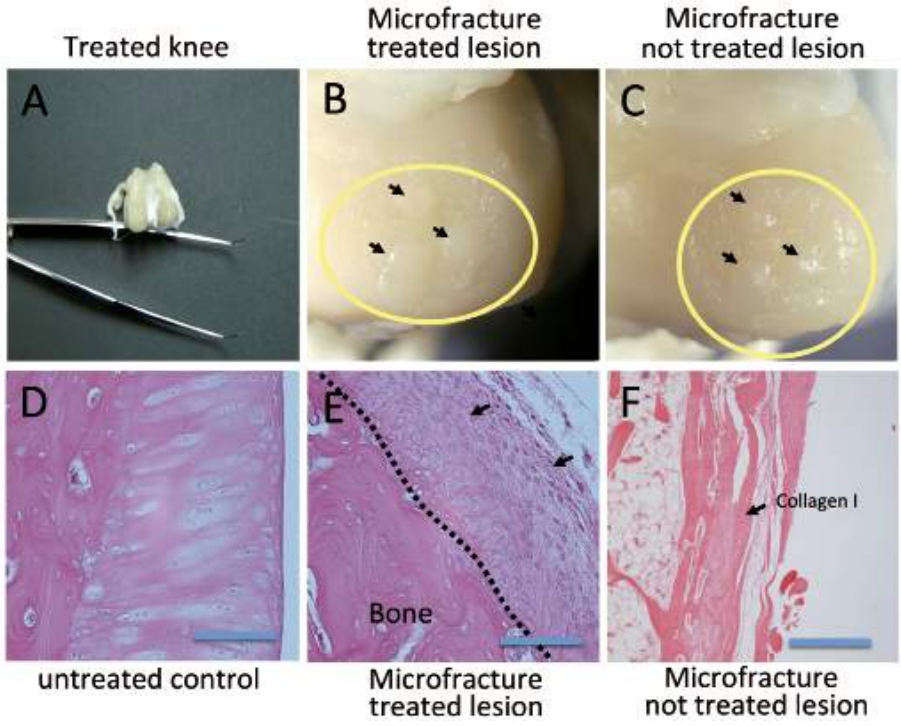

Figure 3. Hyaline cartilage repair of knee arthrotomy using microfracture.
After induction of knee arthrotomy in rabbits, microfracture repair technique was undertaken in left knees; untreated right knees were used as controls. A. After microfracture, the knee appeared normal. B, C. Regenerated tissue was observed around the treated and untreated lesions (arrows). Histological analysis using hematoxylin and eosin staining showed different type of tissues in the lesion areas. D. Normal hyaline cartilage. E. Microfracture-treated lesion in which the scar is composed mostly of type I collagen and fibroblasts (arrows). F. The scar of the untreated lesion is composed mostly of type I collagen (arrows). Scale bars in D-F, 150 μm.

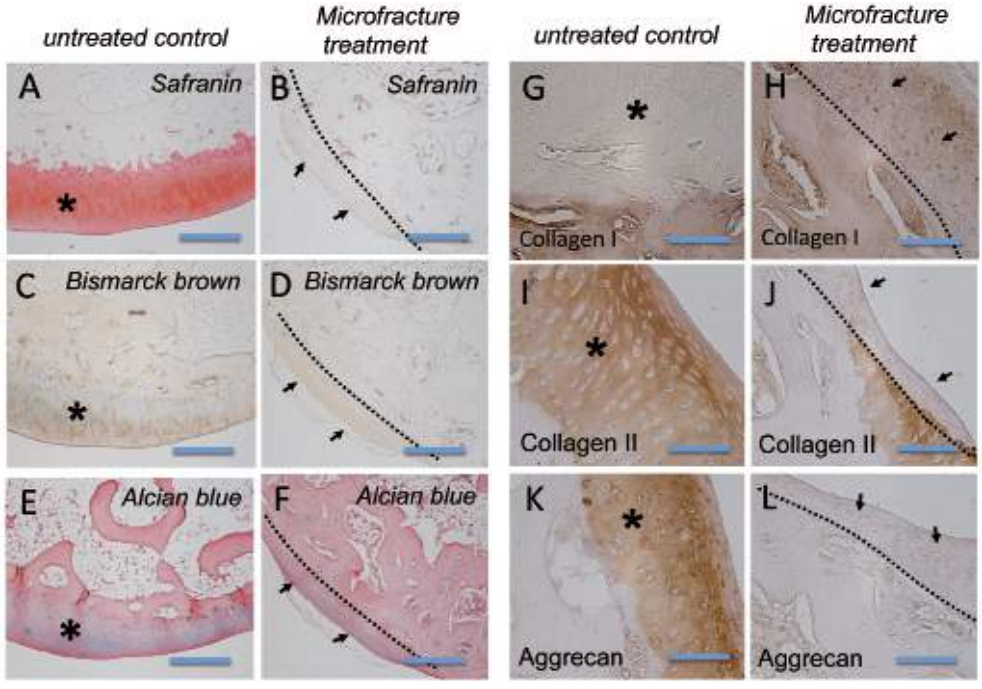

Figure 4. Immunohistochemical analysis of hyaline cartilage repair after knee arthrotomy and microfracture.
Knee arthrotomy and microfracture repair was undertaken in rabbits. A-F. After microfracture or not treated lesion, the scar was negative for safranin (B) and Bismarck brown (D) staining. Low reaction was observed with alcian blue staining (E-F). G, H. Anti-collagen type I immunoreaction was detected after microfracture. I-L. A negative immunoreaction was observed for anti-type II collagen and aggrecan (J and L). Asterisks, normal cartilage. Arrows, Treated lesion and scar. Scale bars in A-L, 100 μm.

789 were in femoral condyles, 147 in the patellofemoral joint and 31 in tibial condyles. Concomitant reconstructive procedures, including anterior cruciate ligament (ACL) reconstructions, realignment osteotomies, meniscus surgeries and patellofemoral realignment procedures were performed. At last follow-up, good or excellent results were encountered in 92% of patients with femoral condyles defects, 87% with tibial defects and 74% of patients with patellar or trochlear defects. In a recent long-term follow-up analysis of 73 patients (mean age of 34 years) with focal full-thickness chondral lesions of the knee, Solheim et al. (2013) reported significant improvement in mean Lysholm score and visual analogue scale for pain after 10-14 years of follow-up. However, up to 40% of the patients had a poor Lysholm score (< 64 points); poorer scores were observed in older patients (> 40 years), women, and in those with defects larger than 3 cm^2 (Solheim et al., 2013).

Reported limitations of mosaicplasty include morbidity in the donor area, lesion size limitation (< 4 cm^2), and the eventual mortality of the chondrocytes from the cylinder edges, which can be associated with poor incorporation of the plug into the surrounding healthy cartilage.

2.3. Autologous chondrocyte transplantation

In 1994, Brittberg et al. reported the results of a novel technique used to treat full-thickness cartilage defects of the knee, in which they arthroscopically harvested a sample of healthy hyaline cartilage from the damaged knee. Chondrocytes were subsequently isolated, cultured and expanded for 14 to 21 days, after which a solution of the cultured cells was injected over the damaged area, which was covered previously with a flap of periosteum. After 2 years of follow-up, 14 of 16 patients with condylar lesions and 2 of 7 patients with patellar transplants presented with good to excellent outcomes. Biopsies of 11 femoral transplants and one patellar transplant revealed the appearance of hyaline cartilage (Brittberg et al., 1994).

Advantages of this procedure include the capacity to treat lesions greater than 4 cm^2 and the eventual repair of the lesion with hyaline cartilage. In addition, this procedure does not require the harvesting of osteochondral plugs from donor areas of the knee. Current indications for this procedure are chronic and traumatic full-thickness chondral lesions of the knee in patients younger than 50 years and those with failed repairs using other techniques, including microfracturing and mosaicplasty. As with the other techniques, a higher body-mass index, older age at the time of surgery and the duration of the symptoms correlated with poorer outcomes (Jaiswal et al., 2012).

Some problems associated with this first generation technique included the eventual *in vitro* dedifferentiation of the chondrocytes into fibroblast-like cells in response to monolayer culturing, as well as graft hypertrophy, difficulties suturing the periosteal flap and the resultant leakage of the cells and non-homogenous accumulation of the suspended cells. As a result, subsequent studies developed different scaffolds composed of animal types I and III collagen, polyglycolic/polylactic acid, hyaluronic acid, fibrin gel and alginate to secure the cells within the lesion (i.e., matrix-assisted ACI, MACI; matrix-associated chondrocyte transplantation, MACT; and matrix-based ACI), as well as construction of engineered cartilage to achieve a more uniform repair of the lesion (Behrens et al., 2006; Beris et al., 2012; Filardo et al., 2012b; Pelissier et al., 2013; Rogers et al., 2010). The parameters and outcomes of mid- and long-term studies of patients treated with ACI, ACI seeded in scaffolds and other tissue-engineered cartilage techniques are listed in Table 1.

Postoperative evaluation of patients receiving matrix-induced procedures using MRI have shown adequate quality of the tissue, with almost complete filling of the lesion, restoration of the articular surface and integration of the tissue (Cherubino et al., 2003; Trattnig et al., 2005). Second-look arthroscopies have shown incorporation of the graft into the knee and a macroscopic appearance similar to the surrounding healthy cartilage. At short- to mid-term follow-up, histological evaluation of the repaired tissue showed good integration and characteristics of hyaline-like cartilage, including columnar distribution of the cells and the presence of hyaline-like cartilage extracellular matrix (ECM) consisting of type II collagen, proteoglycans, chondroitin sulfate and S-100 protein (Bartlett et al., 2005; D'Anchise et al., 2005; Zheng et al., 2007).

Despite the adequate clinical outcomes reported for ACI and its subsequent derivative procedures, the structural properties of native hyaline cartilage have still not been achieved. The reported problems and complications, including the *in vitro* dedifferentiation of mature chondrocytes, the presence of graft hypertrophy even in matrix-induced procedures, lack of incorporation, loss of the implant and the requirement for a previous arthroscopy to obtain a biopsy of healthy chondrocytes, have motivated further research to investigate the therapeutic potential of other scaffolds and cellular sources such as heterologous chondrocytes and MSCs (Hwang et al., 2009; Kon et al., 2012; Niethammer et al., 2013).

3. STEM CELL THERAPY

Given the difficulty in isolating endogenous cartilage stem cells, studies have focused on identifying a cell type that is easily and rapidly expandable and capable of differentiating into chondrocytes. Recently, MSCs have received much attention due to their multipotency, wide histological distribution and capability to differentiate into cells of the chondrocyte lineage (Gardner et al., 2013). For example, bone marrow-derived and umbilical cord MSCs (BM-MSCs and UC-MSCs, respectively) have been widely used *in vitro* to recreate hyaline cartilage; however, generation of long-standing implants that meet the mechanical and functional cues necessary has yet to be shown (Wang et al., 2009). Therefore, current strategies aim to improve graft integration and the differentiation of the expanded cells towards a true chondrocyte lineage, which should secrete the proper ECM molecules, including types II, X and XI collagen, aggrecan, SOX9 and lubricin, that give cartilage its fundamental mechanical characteristics.

Adding different organic scaffolds to concentrate and stimulate stem cell differentiation can augment the microfracture procedure or promote the *in vitro* or *in situ* differentiation of BMSC-engineered tissue, thereby improving hyaline quality and increasing fill percentage (Gomoll, 2012; Siclari et al., 2012). However, although many scaffold strategies have good *in vitro* or *in vivo* outcomes in animal models, their use for cartilage repair in humans has been discouraged

TABLE 1

Mid- and long-term studies of patient outcomes after ACI, ACI seeded in scaffolds and other tissue-engineered cartilage

Study	Type	Surgical technique	Indications	N	Age (y)	Follow-up (mean)	Clinical results	Histological results	Complications
Beris et al.	Level IV, Therapeutic case series	ACI-P	Traumatic lesions and OCD	45	28	96 months	56 to 89 (Lysholm), $P<0.055$ to 69 (IKDC), $P<0.055.5$ to 6.5 (Tegner), $P<0.053.8$ to 2.8 ICRS, $P<0.05$	Not reported	2 graft failures
Filardo et al.	Prospective	Bioengineered tissue Hyaluronic acid membrane, Hyalograft-C (Fidia Advanced Biopolymers Laboratories, Padova, Italy	OCD grade III or IV (ICRS)	34	21	6 y	38 to 81 (IKDC subjective score), $P<0.0005$ 15% to 85% (IKDC objective score), $P<0.0005$ 2 to 5 (Tegner), $P<0.0005$	Not reported	11% failure rate
Rogers et al.	Prospective	ACI-C (type I/III collagen porcine membrane)	Osteochondral defects (traumatic, OCD, patella chondromalacia) >1.0 cm^2	56	31	6 y	60.1 to 72.1 (Modified Cincinnati rating score) 6.5 to 1 (VAS), $P<0.001$ 3.3 to 0.8 (Bentley), $P<0.001$ Sequential improvement in Lysholm (coefficient of determination of >0.87)	24 second looks, with fair to excellent grade of repair (ICRS) 3 graft hypertrophies	3 manipulation under anesthesia
Pellisier et al.	Retrospective case series	ACI-P	Focal cartilage lesions in femoral condyles	12	29	10 y	39 to 78 (IKDC), $P=0.004$ 41 to 88 (Lysholm score), $P=0.002$ 7 to 4 (Tegner), $P=0.003$	Not reported	Not reported
Filardo et al.	Prospective case series	Hyalograft C (Fidia Advanced Biopolymers Laboratories, Padova, Italy)	Focal grade III to IV (ICRS), chondral defects involving femoral condyles	62	28	7 y	VAS and Tegner significant improvement 39.6 to 77.3 (IKDC subjective, $P<0.0005$ 21% to 90% (IKDC objective), $P<0.0005$		11% failure rate
Takazawa et al.	Retrospective	Tissue engineered cartilage based on autologous chondrocytes (Japan Tissue Engineering Co., Ltd, Gamagori, Japan)	Full-thickness defects (traumatic, OCD, osteoarthritis)	14	33	6 y	63 to 86 (Lysholm scores), $P<0.05$)	Not reported	Not reported
Moradi et al.	Level IV, therapeutic case series	ACI-P	Full-thickness cartilage lesion (2 to 12 cm^2) in femoral condyles (65% OCD)	23	30	9.9 y	3.0 to 4.6 (TAS), $P<0.0039$ 65.1 to 78.4 (Lysholm), $P<0.05$ 40.9 to 69.1 (IKDC subjective), $P=0.009$		

because of several safety concerns regarding possible side effects (van der Kraan et al., 2002).

Another line of investigation with successful preclinical development explores the implantation of scaffoldless engineered cartilage, exploiting the self-organization and self-assembling characteristics of cells cultured in non-adherent conditions. This technique involves the generation of small spherical aggregates of cartilage from a critical mass of cells. In the absence of adhesive substrates these aggregates self-assemble, develop rounded cell morphology typical of chondrogenic phenotype and form spheres reminiscent of native cartilage formation (Handschel et al., 2007). This alternative has several advantages over the use of scaffolds; it avoids the unsynchronized degradation of ECM in neotissue formation that is important for remodeling and integration as well as critical for its functional properties. Additionally, it avoids scaffold-induced toxicity and immunogenicity (Liu and Ma, 2004), phenotype alteration (Levy-Mishali et al., 2009) and pressure-tension force-induced differentiation (Bryant et al., 2004). Studies using this strategy report tissues with higher ECM content and mechanical properties (Aufderheide and Athanasiou, 2007; Dehne et al., 2009). Additionally, type VI collagen generation in the perichondrial matrix (PCM), a functional structure important for the development of cartilage characteristics, has been shown, along with the secretion of types II and VI collagen and GAG (CS-4, CS-6) that induce native cartilage development (Ofek et al., 2008). In general terms, this technique better represents the sequential phases of development seen in native cartilage formation (Hu and Athanasiou, 2006; Ofek et al., 2008) and overcomes the clinical concerns regarding the use of scaffolds. On the other hand, certain drawbacks remain. For example, reported studies have mainly relied on animal non-expanded autologous chondrocytes to avoid dedifferentiation (Ofek et al., 2008). However, it is not possible to harvest similar quantities of human chondrocytes.

The main challenge today is the correct differentiation of highly expandable cells, such as MSCs and induced pluripotent stem cells (iPSCs). As the latter is still in its infancy, most studies have analyzed BMSCs. BMSCs have recently been used as a viable cellular substrate in combination with the micromass technique. This technique involves the reaggregation of a critical mass of initially dispersed BMSCs into small spheres, which grow in a three-dimensionaal (3D) suspension culture for several weeks. The neocartilage formed this way can then be transplanted alone into the injured site to complete differentiation or in combination with microfracture or the other aforementioned techniques. Furthermore, this method is very useful to assess the effect of different molecules on the cartilage differentiation process. However, the hurdles of this approach include the generation of tissues with multiple cellular phenotypes and a much wider genotypic background, such as fibrochondrocytes and hypertrophic chondrocytes that secrete molecules which are not typically found in native cartilage (Johnstone et al., 1998; Sekiya et al., 2005). For example, these cells express not only hyaline cartilage, but also the hypertrophy markers (e.g., type X collagen and matrix metalloproteinase 13 [MMP13]) and bone markers (e.g., type I collagen, osteopontin and bone sialoprotein) (Pelttari et al., 2008; Steck et al., 2005). Therefore, they generate an ECM that does not fulfill the mechanical properties compatible with physical integration. Thus obtaining a cell-homogeneous tissue seems to be one of the critical points for implant viability.

Cartilage spheres can be generated using a self-assembling process, pellet culture or aggregate culture. The basic protocol to obtain cartilage using the pellet protocol involves BM-MSCs placed in conical propylene tubes and then cultured in serum-free chondrogenic medium, typically containing DMEM supplemented with proline, pyruvate, ITS (insulin, transferrin and selenium), bovine serum albumin (BSA), linoleic acid and 2-phosphate ascorbic acid (AA-2P). Currently, researchers use agarose, 2-hydroxyethyl methacrylate (Novotny et al., 2006) or semi-permeable membranes (Brehm et al., 2006) as non-adherent substrates to coat culture wells.

Similar approaches have been used with human UC-MSCs (Liu et al., 2013; Pelttari et al., 2008). Compared to human BMSCs, human UC-MSCs have the advantages of abundant supply, painless collection and faster and longer self-renewal *in vitro*. Recently, Wang et al. (2009) demonstrated that hUC-MSCs produce more glycosaminoglycans and collagen than hBM-MSC. Similarly, they produce types I and II collagen and aggrecan, suggesting that hUC-MSCs are the optimal cells for fibrocartilage tissue engineering.

Several molecules that regulate cellular differentiation have been used in the generation of cartilage spheres, and several signaling pathways are known to induce the chondrogenic phenotype. For example, studies have reported that this differentiation protocol can be enhanced by adding tumor growth factor (TGF)-ß3, 10^7 M dexamethasone (DEX), and bone morphogenic proteins (BMPs) (Johnstone and Yoo, 2001; Sekiya et al., 2005). Microarray analysis of approximately 12000 genes in BMSCs has shown that TGF-ß3 and DEX in combination with BMP-4, BMP-6, and especially BMP-2 increase the cartilage weight and the expression of the critical genes for cartilage synthesis in the expected time sequence (Sekiya et al., 2005). These included cartilage-specific genes such as types II, IX, and XI collagen, chondroadherin, aggrecan and SOX-9. Moreover, the SOX-9 transcription factor has been described as promoting the expression of types II, IX, and XI collagen as well as aggrecan (Lefebvre et al., 2001). However, in this report, upregulation of non-specific genes, including hypertrophic chondrocyte markers (e.g., type X collagen, parathyroid hormone-related protein receptor [PTHrP-R], integrin-binding sialoprotein [IBSP] and osteomodulin) raise the possibility that the implanted cells become calcifying after *in vivo* implantation, indicating that this protocol needs perfection. In addition, Weiss and colleagues (2010) have described protocols in which acidic fibroblast growth factor (aFGF/FGF1) and insulin growth factor (IGF)-1 exert an inducing capacity that was similar to the aforementioned molecules. Additionally, they reported PTHrP and bFGF as early and late inhibitors of chondrogenesis necessary for inhibition of chondrocyte hypertrophy *in vitro*. However, they were not able to inhibit type X collagen expression or *in vivo* calcification, indicating that terminal chondrocyte differentiation was not achieved as the inhibiting molecules were not produced by the implanted cells (Zhang et al., 2012).

Recently, new molecules that regulate chondrocyte differentiation, including kartogenin (KGN) (Johnson et al., 2012), chondroitinase-ABC (C-ABC) and TGF-β1 (Responte et al., 2012), have been explored. Specifically, KGN was used in a chondrogenic model using human BMSCs, achieving promotion of chondrocyte differentiation (median effective

concentration = 100 nM) and chondroprotective effects *in vitro*; it was also shown to be efficacious in alleviating the symptoms of two OA animal models. Most interestingly, KGN binds filamin A, disrupting its interaction with the transcription factor, core-binding factor b subunit (CBFb) and induces chondrogenesis by regulating the CBFb-RUNX1 transcriptional program. In this respect, the interplay between RUNX1, RUNX2 and RUNX3 modulates the differentiation of chondrocytes, where RUNX1 is important for the initial growing and secreting phase, and RUNX2 and 3 predominate in the final differentiation phase. On the other hand, Responte et al. (2012) used immature bovine chondrocytes to develop a novel self-assembly process for articular chondrocytes, which was improved using a combination of catabolic and anabolic agents. Specifically, TGF-β1 in conjunction with C-ABC additively increased the tensile properties and synergistically enhanced the collagen content; microarray analysis indicated that TGF-β1 upregulated MAPK signaling while C-ABC increased collagen fibril diameter and density. At four weeks post-transplantation in nude mice, mature neocartilage expressing type II collagen and GAGs was observed.

3.1. Engineering of heterologous MSCs into cartilage

Although autologous transplantation using MSCs is currently the most commonly used cell for cartilage repair, the use of heterologous transplantation presents many advantages that cannot be matched by allogenic or autologous strategies, including the availability of a relatively abundant supply without the need for additional surgical interventions, as is the case for autologous transplantation. However, contagious diseases and a shorter life span of implanted animal cells are some of the concerns that discourage this option (Kaminski et al., 1980; Ksiazek and Moskalewski, 1983; Malejczyk et al., 1991; Revell and Athanasiou, 2009). Most of these problems can be overcome, as diseases can be limited by controlled breading, and implant viability of only a few years is currently acceptable. Finally, several mechanisms that promise to overcome immunogenicity towards the xeno-implanted cells have been reported.

Although intramuscular xenografts of rat chondrocytes into a rabbit host readily generated an important humoral immune response due to the high vascularity of the tissue (Osiecka-Iwan et al., 2003), different results have been reported when the xenografts were implanted into a cartilage defect inside the joint, which is considered an immuno-privileged environment as it is avascular (Bolano and Kopta, 1991). One study addressing this used pig chondrocytes implanted into human cartilage defects in vitro and showed an important degree of wound filling and the synthesis of important levels of collagen fibers (types I and II) and proteoglycans (Fuentes-Boquete et al., 2004). Furthermore, varying degrees of responses have been described in different experimental settings when the grafts are implanted *in vivo* into joint defects, which was dependant on how the heterologous cells were implanted. For example, after rabbit articular cartilage defects were treated with pig chondrocytes and covered with a periosteal graft, significant tissue repair was observed after 24 weeks, showing a 90% defect fill and a good histological score; furthermore, neocartilage showed chondrocyte like cells, a smooth hyaline morphology and no tissue rejection was reported (Ramallal et al., 2004). In another report, rabbit chondrocytes suspended in fibrin glue were transplanted into goat full-thickness articular-cartilage defects. Macroscopic, histological and biochemical determinations showed that the xenografts produced matrix inside the fibrin glue and that rejection of transplanted cells did not occur. However, although histological analysis initially indicated that the defects treated with xenografts had a better regeneration tendency, this advantage was lost by the conclusion of a 52-week follow-up (van Susante et al., 1999). Additionally, a method called decellularization by photo-oxidation, which increases collagen fiber crosslinks by immersing the xenograft in a 0.01% methylene blue solution under specific conditions, showed no immunogenicity towards osteochondral xenotransplants from cattle shoulder joints into femoral condyles of sheep (von Rechenberg et al., 2003). This approach also reduced the factors that impair implant viability, such as resorption of subchondral bone and cyst lesions, thereby improving graft stability and cartilage survival (von Rechenberg et al., 2003). As this procedure kills the cells and destroys the intracellular antigenic proteins and DNA while preserving a functional ECM, it is believed to provide a viable option for tissue replacement. The use of MSCs in xenogeneic transplantation represents a step further as they have been reported to be immunoprivileged and do not generate an immune response when exposed to different types of immune cells *in vitro* (Aggarwal and Pittenger, 2005). An alternative method embraced this concept for osteochondral defect repair using a bone anchor to settle and graft the neocartilage better (Jang et al., 2013). hMSCs that were induced to differentiate *in vitro* (D-MSC) in conjunction with a biphasic composite construct consisting of fibrin glue and hydroxyapatite were implanted into osteochondral defects of adult New Zealand white rabbits. After 8 weeks the cartilage implants showed excellent macroscopic evaluation with a hyaline-like appearance and good defect filling. Furthermore, RT-PCR analysis showed increased expression of ECM proteins compared to non-differentiated MSCs (U-MSC). Histological evaluation showed good levels of safranin O staining and type II collagen that had a similar staining pattern to its surrounding tissue. Most importantly, no inflammation or immune cell infiltration was observed, indicating that xenogeneic transplantation using differentiated MSCs and a biphasic scaffold that anchors the neocartilage to the bone effectively repaired osteochondral defects. Although encouraging strategies have been reported, most of these studies need longer evaluation periods to establish the long-term functional viability of the xenotransplants that could enable them to scale towards clinical trials.

4. CONCLUSIONS

Cartilage has an extremely reduced regenerating capacity; therefore, multiple experimental approaches have been used to treat lesions in this tissue. The microfracture technique has been widely used in the clinic; however, the formation of cartilaginous tissue is scarce, yielding mainly a fibrocartilage. The isolation and application of MSCs together with collagen matrices is currently generating more effective repair models. Thus it is important to unveil the factors that stimulate the formation of articular cartilage to allow the *in vitro* synthesis of three-dimensional constructs for implantation in patients with pathologies of the knee cartilage. Finally, it is also

important to indicate that progress in intra-articular therapy is being achieved in new clinical trials using corticosteroid and hyaluronic acid preparations (Evans et al., 2013). The joint utilization of these trophic factors and stem cells will be the new challenge in the future treatment of arthropathies.

5. MATERIAL AND METHODS

*Knee arthrotomy and microfracture***.** Ten three-month-old female California rabbits weighing 3 kg were used to evaluate the effects of microfracture on the recovery from arthrotomy. Briefly, animals were initially anesthetized with 1 mg/kg tramadol, 1 mg/kg ketoprophene and antibiotics; animals subsequently received a subcutaneous injection of a 0.2 mL a mix containing Xylacine (8 mg/kg), Pacifor (1 mg/kg) and Ketamine (30 mg/kg) to maintain anesthesia. A longitudinal incision over the knee was made to expose the articulation followed by medial patella dislocation. Full-thickness osteochondral lesions of 5 mm in diameter were induced in the medial load-bearing condyles of both knees. Microfracture was performed in the left knee lesions with 1.5 mm interspace; right knees were used as untreated controls. After the wounds were sealed and sterilized, recovery from the anesthesia was induced. The histological analysis was performed after three months.

*Histologic evaluation of microfracture-treated and control animals***.** Samples were fixed in formalin for 1 week at room temperature, after which they were decalcified by incubating in 45% formic acid solution with 20% sodium citrate, which was changed every 2-3 days during 3 weeks as described by Ana Morse (Molina et al., 2001). After the area of interest was isolated and washed thoroughly in distilled water, it was dehydrated in ascending ethanol graded solutions and embedded in paraffin blocks.

Bismarck brown staining. Deparaffinized tissues were hydrated in descending ethanol solutions and incubated with 1% aqueous Bismarck brown solution for 5 min. After the tissues were washed in 95% ethanol, they were incubated with 0.5% methyl green solution until the slides turned dark green. Slides were then dehydrated in ascending ethanol solutions and xylol and mounted with Entellan medium.

Alcian blue staining. For intense blue cartilage staining, deparaffinized tissues were hydrated to distilled water in descending ethanol solutions and incubated for 30 min in recently filtered 0.5% Alcian-blue 8GX solution (pH 2.5), prepared with 3% acetic acid. Slides were then briefly washed with distilled water and counterstained for 5 min with 0.1% Kernechkrot solution for nuclear staining. Slides were next rapidly washed in distilled water, dehydrated in ascending ethanol solutions and xylol and then mounted with Entellan medium.

*Combined method for fast green and safranin staining***.** Tissues were deparaffinized and hydrated to distilled water in descending ethanol solutions and then incubated for 3 min in 0.01% Fast Green solution. Stained slides were then washed with 1% acetic acid and later stained for 5 min with 0.1% Safranin O solution. Slides were dehydrated in ascending ethanol solutions and xylol and then mounted with Entellan medium.

Immunohistohemical detection of extracellular molecules. Tissue sections of 7 μm thickness were deparaffinized in xylol and 100% ethanol and permeabilized for 15 min with methanol, followed by hydration to distilled water in descending ethanol solutions. Endogenous peroxidase activity was inhibited by incubation in 10% H_2O_2 for 15 min, after which slides were washed three times for 10 min each in Tris-phosphate buffer (TPB) (pH 7.8) at room temperature and incubated for 18 h with primary antibodies in a humid chamber. The following primary antibodies were used: anti-collagen type I (Sigma-Aldrich, Saint Louis, Missouri, USA); anti-collagen type II (Abcam, Cambridge, Massachusetts, USA); anti-aggrecan [or anti-cartilage proteoglycan] (Merk-Millipore, Darmstadt, Hesse, Germany). After slides were washed three times in TBP for 10 min each, they were incubated for 2 h with peroxidase-conjugated secondary antibodies, washed three times in TBP for 10 min each and developed using diaminobenzidine (DAB) substrate; slides were then washed with running tap water and then with distilled water. Additionally, contrast staining was performed with hematoxylin to reveal nuclei.

Second harmonic and multiphoton microscopy. Transplanted knee-joints were excised, fixed in Bouin's solution and prepared histologically as previously described. The 7-μm thick neocartilage sections were deparaffinized, hydrated to distilled water and then mounted with fluorescent mounting medium (DAKO). Collagen deposition was analyzed by second harmonic image generation. The images were acquired in a Zeiss 40X multiphoton microscope LSM 780-NLO. Both signals, two-photon fluorescence (TPF) and second harmonic generation (SHG), were produced by excitation with a Mai-Tai Ti:Sapphire laser at 940 nm. We used a LP490/SP485 NDD filter for SHG, and a BP565-610 NDD filter for fluorescence. In each case, ten images were acquired serially (512×512 μm) in Z-stack and joined together to one merged image. The acquired images were then processed by Imaris software (Bitplane v.7.3) to yield 3D reconstruction projections.

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