European Cells and Materials Vol. 26 2013 (pages 150-170) DOI: 10.22203/eCM.v026a11 ISSN 1473-2262

MENISCUS REPAIR AND REGENERATION: REVIEW ON CURRENT METHODS AND RESEARCH POTENTIAL

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Abstract

Meniscus regeneration is an unsolved clinical challenge. Despite the wide acceptance of the degenerative consequences of meniscectomy, no surgical procedure has succeeded to date in regenerating a functional and long-lasting meniscal fibrocartilage. Research proposed a number of experimental approaches encompassing all the typical strategies of regenerative medicine: cell-free scaffolds, gene therapy, intra-articular delivery of progenitor cells, biological glues for enhanced bonding of reparable tears, partial and total tissue engineered meniscus replacement. None of these approaches has been completely successful and can be considered suitable for all patients, as meniscal tears require specific and patient-related treatments depending on the size and type of lesion. Recent advances in cell biology, biomaterial science and bioengineering (e.g., bioreactors) have now the potential to drive meniscus regeneration into a series of clinically relevant strategies. In this tutorial paper, the clinical need for meniscus regeneration strategies will be explained, and past and current experimental studies on meniscus regeneration will be reported.

Keywords: Meniscus; meniscal tear; tissue engineering; scaffold; bioreactor.

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Introduction

Once described as "functionless remnants of leg muscle origin" (Bland Sutton, 1897), the menisci are now considered crucial structures for knee stability, shock absorption, and nutrient distribution to the articular cartilage (Ahmed and Burke, 1983; King, 1936; Krause *et al.*, 1976; Levy *et al.*, 1989; Seedhom and Hargreaves, 1979). However, their location and the extreme forces that the menisci can be subjected to make them frequently susceptible to injury, especially in contact-sport activities but also in sedentary young or elderly patients. Due in large part to the limited vascularity of the meniscus, which is restricted to the external third, this tissue has a poor healing potential (Arnoczky and Warren, 1982; Arnoczky and Warren, 1983). Although common in the past, total meniscectomy has been largely abandoned, for the direct relationship between meniscectomy and development of early osteoarthritis (Allen *et al.*, 1984; Appel, 1970; Berjon *et al.*, 1991; Ghosh *et al.*, 1990; Hoch *et al.*, 1983; Huckell, 1965; Jackson, 1968; Lufti, 1975; Northmore-Ball and Dandy, 1982; Voloshin and Wosk, 1983). Partial meniscectomy is still indicated if the lesion cannot be satisfactorily sutured (Sommerlath, 1991; Shelbourne and Carr, 2003). Regenerative medicine holds a great potential for restoring form and function of meniscal fibrocartilage. Specific considerations must be given to the type of cells necessary, the scaffold, and the physical forces within the microenvironment in which the meniscus is located. For these reasons, new strategies involving the use of bioreactors seem to be promising for the development of engineered meniscus tissue.

In this review, we will give an overview on the potential of research for meniscus regeneration, and provide a perspective, from the clinician's standpoint, for designing future regenerative strategies.

Current perspectives on meniscus structure, lesions and treatment

Anatomy, composition and function

The menisci are two semilunar or C-shaped fibrocartilage structures located medially and laterally between the femoral condyles and tibial plateau (King, 1936). They are crucial structures for knee joint homeostasis. Their function is to deepen the articular surfaces of the tibial plateau to better accommodate the condyles of the femur, providing knee stability, shock absorption, lubrication, proprioception and load distribution during motion. In

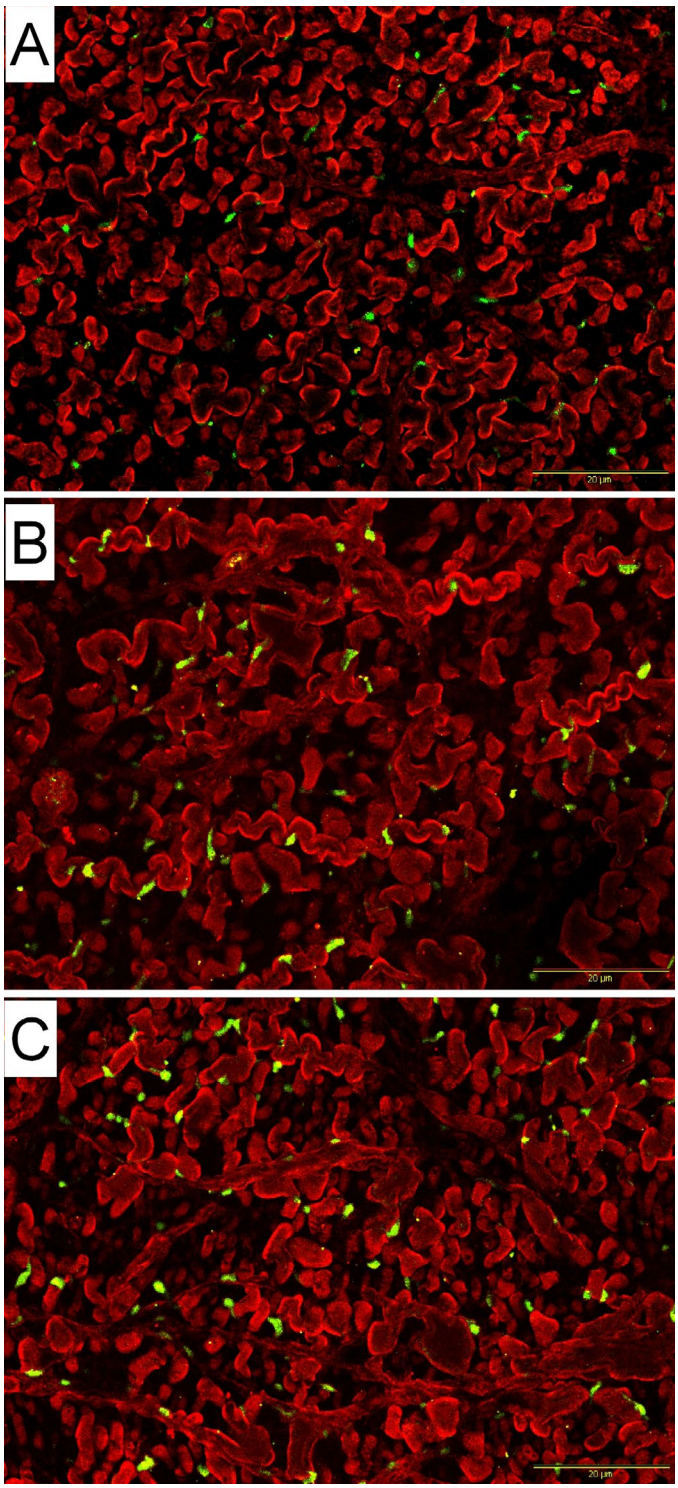

Fig. 1. Anterior horn, young pig, double immunofluorescence of proliferating cell nuclear antigen (PCNA) and collagen type I staining: in the inner **(A)**, middle **(B)** and outer **(C)** zone. A strong immunopositivity of collagen type I fibres (red colour) is evident with no differences in the three zones, while the proliferating cells (PCNA, green colour) revealed a higher degree in the outer zone **(C)**, followed by the middle **(B)** and then by the inner one **(A)**. Scale bar 20 μm.

fact, in cross section, they appear as wedge-shaped and slightly concave on the femoral surface and have a thick external region. Together, the two menisci cover about two-thirds of the tibial plateau. The menisci are held in position through the attachments to the joint capsule and ligaments surrounding the knee (Bland-Sutton, 1897; King, 1936; Setton *et al.*, 1999; Renstrom and Johnson, 1990). These attachments maintain correct positioning of the meniscus during radial and anteroposterior displacement of the knee and provide crucial blood supply to the peripheral regions of the meniscus.

Unlike the articular cartilage, the menisci possess an intrinsic innervation, which is related to joint proprioception (O'Connor and McConnaughey, 1978; Wilson *et al.*, 1969). It is scarce in the body region but well represented in the anterior and posterior horns. It has been postulated that they are activated during flexion and extension of the knee, providing the central nervous system with information regarding joint position thus contributing to a reflex arc that stimulates protective or postural muscular reflexes (O'Connor and McConnaughey, 1978; O'Connor, 1984). Regarding vascularisation, the human medial meniscus is vascularised in the outer 10-30 %, while the lateral meniscus is vascularised in only the outer 10-25 % of its width (Arnoczky and Warren, 1982). These vascularised outer regions have greater propensity

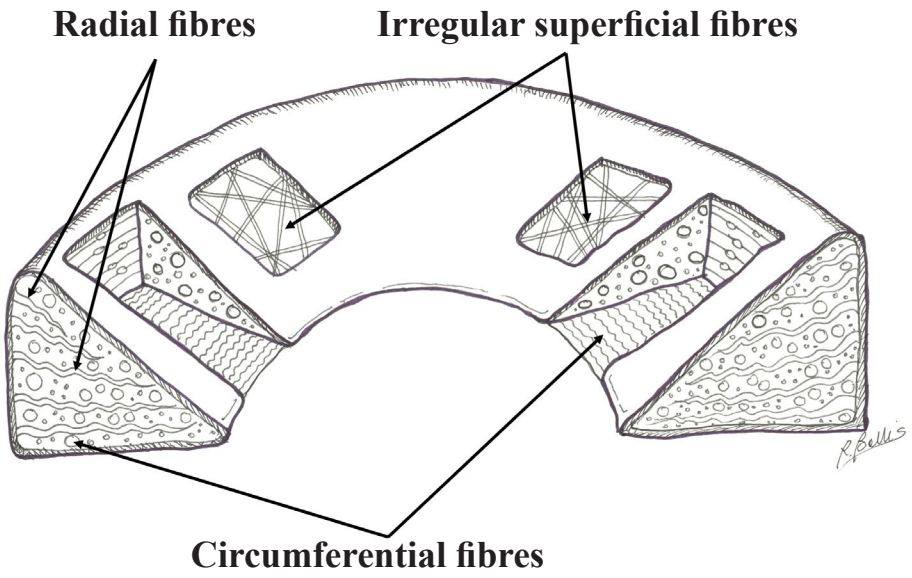

Fig. 2. Schematic diagram of the ultrastructure of collagen fibres of the meniscus. The large collagen fibres on the interior of the tissue have a predominant circumferential orientation. The fine fibres of the surface layer have no preferred orientation. Within the interior of the meniscus are radially oriented collagen "tie" fibres.

to heal when stabilised with sutures or anchors than the inner avascular regions. At the microvascular level, a capillary plexus that originates in the joint capsule and synovial tissues surrounding the joint supplies the menisci (Arnoczky and Warren 1982; Renstrom and Johnson, 1990; Cooper *et al.*, 1990; Cooper *et al.*, 1991). Additionally, the limited vascularity of the meniscus probably has a negative effect on the ability to recruit cells to a lesion for normal wound repair. This may be especially true for the nonvascular inner regions. Regarding biochemical composition, about 70-75 % of the wet weight of the meniscus consists of water (Arnoczky *et al.*, 1988; Adams and Hukins, 1992; Ghosh and Taylor, 1987; McDevitt and Weber, 1990). The dry weight is comprised of 60-70 % collagen, 1 % proteoglycans, and 8-13 % non-collagenous proteins such as elastin. Collagens are primarily type I (90 %) (Fig. 1) with smaller amounts of II, III, V, and VI (Adams and Hukins, 1992; McDevitt and Weber, 1990). The collagen fibres are predominantly oriented circumferentially, with some radially oriented fibres (Fig. 2). This may be related to the mechanical forces which act on the menisci. At the meniscal surface, a collagen fibrillar network, woven into a mesh-like matrix, has been identified. The meniscus has elastin fibres throughout that bridge the collagen fibres. There are also inhomogeneities in tissue composition from the peripheral vascular regions to the inner avascular regions. This is appreciated by the histological appearance of the inner portion that resembles articular cartilage, whereas the outer portions are more fibrocartilage-like. Similarly, the superficial portion of the meniscus has an appearance similar to fibrous tissues. Regarding cell types, cells in the superficial layers of the tissue are fusiform, whereas those located in the deeper zones are more polygonal. Cell morphology also changes from the inner avascular region, where the cells are nearly indistinguishable from articular chondrocytes, to the outer vascular regions, where the cells are more fibroblast-like. Although the cells share many morphologic similarities to articular chondrocytes, they predominantly produce type I collagen. Thus, the cells are most often referred to as fibrochondrocytes and have a complex developmental origin (McDevitt *et al.*, 1992; Hyde *et al.*, 2008).

In all, the material properties of the various regions of the meniscus are determined largely by the composition and microstructure of the tissue. These properties are highly anisotropic and different in compression and tension. They also vary considerably, depending on the depth and circumferential location of the force in the tissue (Mow *et al.*, 1992). Approximately 50 % of the compressive load is transmitted through the menisci as the knee goes to extension, while the load can increase to as much as 85 % in flexion (Ahmed and Burke, 1983). In humans, the medial meniscus bears about 50 % of load whereas the lateral meniscus bears as much as 70 % (Walker and Erkman, 1975). Under normal physiologic loading, the meniscus experiences large tensile and shear stresses as well as compressive stress. Under loaded conditions the meniscus is not only subjected to a vertical force to the inner portions, but there is also a radially directed force applied to the concave surface pushing the meniscus outward (King, 1936, Shrive *et al.*, 1978). These high mechanical requirements make the meniscus a crucial player in knee homeostasis and represent the rationale for a regenerative approach to meniscal lesions.

Meniscal lesions

In the early years of meniscal surgery, it was the gold standard to entirely excise the injured meniscus. Since then, after recognising that a total or subtotal meniscectomy inevitably leads to development of osteoarthritis within 5-10 years after surgery, it was advocated that as much meniscus tissue as possible should be preserved (McGinity *et al.*, 1977). Only the meniscus tissue which is identified as unrepairable should be excised (McGinity *et al.*, 1977). As a matter of fact, during the last decades, profound knowledge of the meniscus function has been established (Wojtys and Chan, 2005) and the importance of the integrity of the menisci for the homeostasis of the knee joint has been recognised and highlighted by a number of clinicians and researchers (Dye, 1996; Arnold *et al.*, 2012).

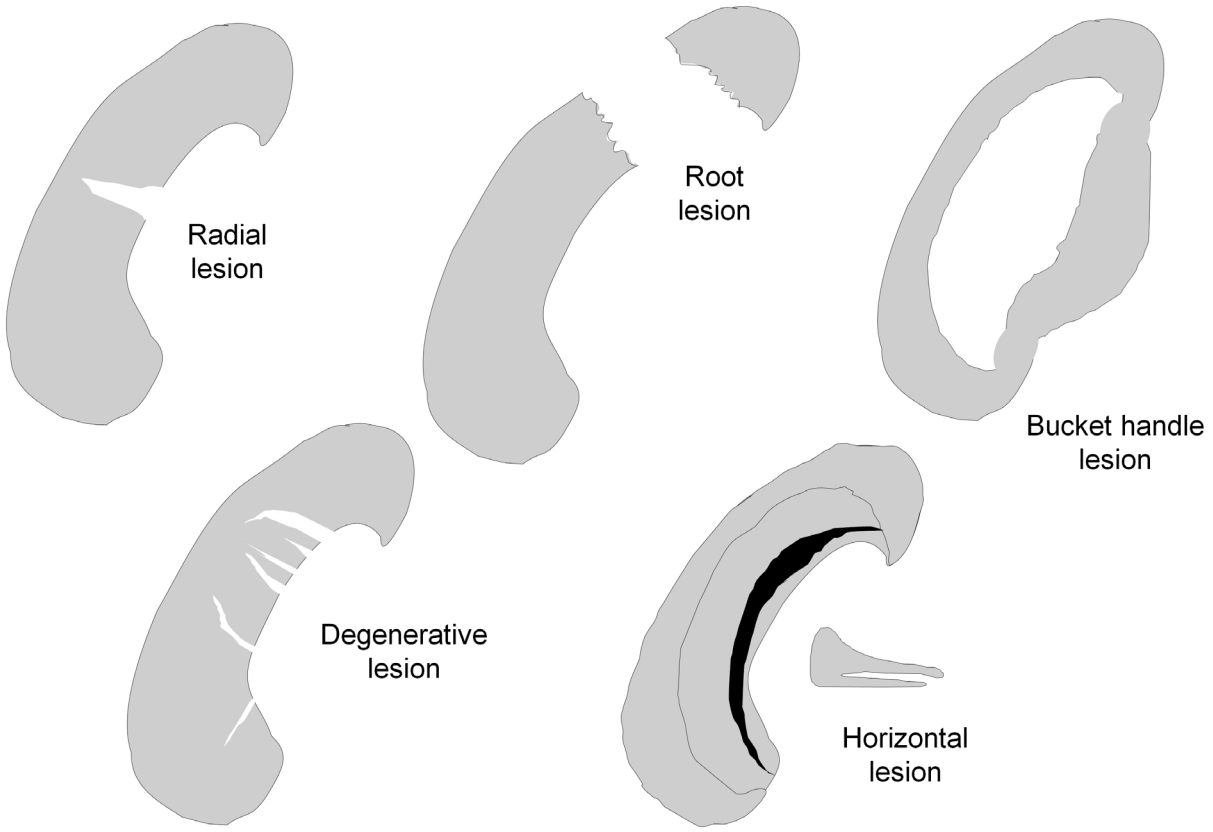

Fig. 3. Schematic diagram of different types of meniscal lesions.

In children, meniscal lesions are typically due to trauma or more frequently due to congenital meniscal variants such as a discoid meniscus or meniscal cysts (Hirschmann and Friederich, 2009). In adults, meniscal lesions are due to trauma, degenerative disease or a combination of both (Pujol and Boisrenoult, 2009; Verdonk and Verfevre, 2009). In contrast to meniscal lesions in children, in adults the meniscal injuries, which predominantly involve the medial meniscus, are often associated with concomitant ligament or cartilage lesions (Pujol and Boisrenoult, 2009; Verdonk and Verfevre, 2009). In addition, the lesion itself is more complex in adults, as the meniscus undergoes a significant degeneration in the course of a lifetime (Pujol and Boisrenoult, 2009). Not surprisingly, there is an increase of the incidence of meniscal lesions with increasing age (Pujol and Boisrenoult, 2009).

The meniscal injuries can be classified with regards to the tear pattern in radial, longitudinal, horizontal, circumferential, and root lesions (Fig. 3) (Hirschmann and Friederich, 2009). It is believed that the central ⅓ of the meniscus (white zone) has less healing potential than the middle (red-white zone) and the peripheral ⅓ (red-red zone) (Hirschmann and Friederich, 2009).

Current surgical treatments

The treatment of meniscal lesions has evolved tremendously during the last decades. Numerous techniques and methods have been established in patients with meniscal lesions (DeHaven, 1990). A considerable number of patients with traumatic or degenerative meniscal lesions can be treated non-operatively. In fact, this is true for all patients who do not present with (i) blocking of the knee joint, (ii) pain non-responsive to pharmacological treatment, and (iii) meniscal lesions that appear to be biomechanically unstable. In this situation, the meniscus lesions could be masterly neglected. Typically, these lesions include partial thickness tears (< 5 mm), short radial tears (< 5 mm) and short full thickness vertical or oblique tears (< 5 mm). Some authors also prefer non-surgical treatment for most of the lateral meniscal lesions.

Nowadays, a subtotal or total meniscectomy, whether open or arthroscopically, is only rarely performed, which can be attributed to the increasing awareness of the deleterious effect of meniscus removal (Fairbank, 1948; DeHaven, 1985; Noble and Turner, 1986). Thus, it is spared for patients with unrepairable, complex, mostly degenerative meniscus lesions (Noble and Turner, 1986). A partial medial or lateral meniscectomy often becomes necessary in symptomatic patients. Along with the increasing interest in meniscus preserving techniques and unconvincing results in partially meniscectomised patients, numerous meniscal repair procedures have been advocated (Rodkey *et al.*, 2008; Han *et al.*, 2010). Generally, these can be differentiated as inside-out, outside-in and all-inside repairs (Steenbrugge *et al.*, 2004; Majewski *et al.*, 2009; Hoffelner *et al.*, 2011). A young patient age and recent injury are common indications for meniscal repair, but only lesions located in the vascular zone of the meniscus can be repaired. However, even in older patients and older meniscal lesions, many surgeons believe that a repair of the meniscus is of clinical benefit and thus should be attempted.

To date, all-inside fixation devices can be considered as a valuable option for most of the patients with repairable meniscal lesions (Hoffelner *et al.*, 2011). Inside-out and

outside-in repair are good treatment alternatives for selected patients, in particular meniscal lesions in the anterior horn or corpus (Steenbrugge *et al.*, 2004; Majewski *et al.*, 2009). Importantly, although these repair devices have evolved, failure still does occur (Katabi *et al.*, 2009).

Aiming for improved healing of the meniscus, several different methods, including very basic ones, such as needling, abrasion, trephination and gluing, or more complicated ones, such as synovial flaps, meniscal wrapping or the application of fibrin clots, have been proposed (Jacobi and Jakob, 2009; Longo *et al.*, 2012; Scordino and Deberardino, 2012).

Clinical need for regenerative strategies

Generally, a more active lifestyle in the younger but also older age group puts knees at a higher risk for sustaining a meniscal injury (Hirschmann and Friederich, 2009). All age groups and in particular children increasingly participate in more extreme, competitive or even professional sports (Hirschmann and Friederich, 2009). The consequence of this fact is that meniscal surgery is performed at a younger age, and less meniscus tissue is preserved for a longer lifetime period. In young, active patients a partial medial meniscectomy may be the starting point for a disturbed homeostasis of the knee, even if the mechanical axis is only slightly varus aligned (Arnold *et al.*, 2012). This altered knee homeostasis and increased loading then inevitably leads to the development of osteoarthritis, which should be prevented under all circumstances. There is therefore a clear need for regenerative strategies in these young and middle aged patients (Arnold *et al.*, 2012). This is where orthobiologic treatments come into play. The orthobiologic treatment algorithm is defined by a distinct hierarchy: (i) neutral leg alignment, (ii) ligamentous stability, (iii) meniscus integrity, and (iv) cartilage restoration. Each of these concepts is crucial to restore knee homeostasis (Arnold *et al.*, 2012).

In the last decade, striving for optimal restoration of meniscal tissue, the orthopaedic surgeon`s armamentarium has been enriched by the use of biocompatible meniscus scaffold and meniscal allograft transplantation (Efe *et al.*, 2012; Monllau *et al.*, 2011; Zaffagnini *et al.*, 2011; Harston *et al.*, 2012). Some authors also even recommend a meniscal substitution for young athletes after meniscectomy, independent of their symptoms (Efe *et al.*, 2011; Monllau *et al.*, 2011; Zaffagnini *et al.*, 2011; Harston *et al.*, 2012). However, despite promising short-term results, none of the current strategies have demonstrated regeneration of a functional, long-lasting meniscal tissue and re-establishment of a proper knee homeostasis in the meniscectomised knee.

Cell-free techniques

The rationale for using a cell-free biomaterial to replace part of the meniscus is based on repopulation of the scaffold by the host cells recruited from the synovium and the meniscal remnants, and subsequent tissue ingrowth which renders this approach cell-based after implantation. A mandatory prerequisite is the absence of both knee instability and malalignment. In addition, meniscal substitutes are not indicated in case of radial tears since their implant would require extensive tissue resection.

The collagen meniscus implant (CMI) (ReGen Biologics, Franklin Lakes, NJ) is the first regenerative technique applied to meniscal tissue in clinical practice (Stone *et al.*, 1992; Stone *et al.*, 1997; Steadman and Rodkey, 2005; Zaffagnini *et al.*, 2007). Since an outer rim of meniscal tissue is needed for CMI implantation, it is indicated only for partial and not total meniscus regeneration. Satisfactory clinical results have been reported (Rodkey *et al.*, 1999), while MRI and histological results are controversial: (i) the CMI shrinks over time; (ii) it showed no histological remnants 5 to 6 years after implantation (Steadman and Rodkey, 2005); and (iii) it predominantly generates a scar tissue instead of fibrocartilage (Martinek *et al.*, 2006). In a recent medium-term follow-up, non-controlled case series involving 34 patients, Bulgheroni *et al*. (2010) showed good to excellent clinical results after 5 years from a CMI implantation for a symptomatic deficiency of medial meniscal tissue. In particular, chondral surfaces had not further degenerated after placement of the CMI, and MRI signal had indicated a progressive maturation between 2 and 5 years after implantation, progressively resembling the low signal of a normal meniscus. Authors confirmed the tendency of the CMI-new tissue complex of undergoing shrinking, but with no generally negative effects on the clinical outcome. Another non-controlled case series reported significant pain relief and functional improvement at a minimum 10-year follow-up (Monllau *et al.*, 2011). However, no negative controls were included in these two studies, making it difficult to ultimately assess the value of this procedure. Overall, despite the wide clinical use, no randomised, high-quality controlled trial supports the use of this implant.

Following several experimental studies in animal models for total meniscus replacement (van Tienen *et al.*, 2009; Welsing *et al.*, 2008; Hannink *et al.*, 2011), polyurethanes are now being assessed as alternative biomaterials for partial meniscus replacement. The Actifit™ (Actifit, Orteq Ltd, London, United Kingdom) meniscus implant is a polyurethane-polycaprolactone (PU-PCL)-based synthetic meniscal substitute intended for use in the irreparable, partial meniscal defects (Verdonk *et al.*, 2009; Verdonk *et al.*, 2011). Because of its polymeric nature, Actifit™ has a higher mechanical strength and ease of handling compared to CMI. On the other hand, degradation is expected to occur in 4 to 6 years, thus being much slower than that of CMI. In particular, polycaprolactone is degraded by hydrolysis while polyurethane is slowly degraded by macrophages and giant cells. The clinical application of this meniscal implant started recently and medium to long-term evidence on chondroprotection is not yet available. The first 12-month report of a multi-centre, prospective clinical trial involving 52 patients with irreparable meniscal tear or partial meniscal loss has been recently published, showing tissue ingrowth into the scaffold at 3 months and further tissue ingrowth at 12 months, with consistent MRI and histology data (Verdonk *et al.*, 2011). Another recently published study, involving 10 patients with a 12-month follow-up, showed the safety

of this scaffold for the treatment of symptomatic patients with segmental medial meniscus defects. (Efe *et al.*, 2012). However, only when more medium-to-long term evidence about its safety and effectiveness will be available, could Actifit™ be considered a valid alternative for partial meniscal replacement.

In conclusion, meniscal implants demonstrated good short- to medium-term clinical results, but no evidence supports their use routinely in clinical practice and no study reported convincing long-term protection from joint degeneration to date.

Meniscal transplantation

Meniscus represents the ideal tissue for transplantation: vascular anastomosis is not needed and its cells are immunoprivileged because of the avascular environment. As a consequence, surgery is less demanding and immunosuppressive therapy is not needed (Jackson *et al.*, 1992). Meniscal transplantation is actually the only biologic option available for the symptomatic, totally meniscectomised, non-osteoarthritic, stable and well-aligned knee (Verdonk *et al.*, 2007; Lubowitz *et al.*, 2007). On the other hand, although this technique is not new (Milachowski *et al.*, 1989), the long-term effects, especially in terms of chondroprotection and prevention of osteoarthritis, still remain to be proven (Wirth *et al.*, 2002). Limitations of this procedure include tissue availability, risk of immune reaction, risk of disease transmission, and graft sizing (Lubowitz *et al.*, 2007).

Regarding the type of allograft, four have been used: cryopreserved, deep-frozen (fresh-frozen), fresh, and lyophilised (freeze-dried) (Cole *et al.*, 2003; Lubowitz *et al.*, 2007). Fresh grafts present the advantage of being rich in viable cells and this has been demonstrated to improve mechanical integrity following transplantation, determining a lower failure rate of the procedure compared to those of deep-frozen and lyophilised grafts (Siegel and Roberts, 1993; Verdonk *et al.*, 2005). Despite this advantage, no evidence supports the increased expense related to these procedures (Verdonk *et al.*, 2005; Lubowitz *et al.*, 2007). Moreover, an animal study demonstrated that donor cells are replaced by host cells, so that the need of a viable graft has been questioned (Jackson *et al.*, 1993). However, donor DNA remains detectable in human patients for a longer period than in the animal models (Verdonk *et al.*, 2005).

According to current literature, indications to this technique are not yet defined; however, it seems to be indicated in two clinical scenarios (Verdonk *et al.*, 2007; Lubowitz *et al.*, 2007): (i) young patients (<50 years of age) with a symptomatic, meniscus-deficient compartment in a stable joint, without malalignment and with only minor chondral lesion (no more than grade 3 according to ICRS score); and (ii) patients with an ACL-deficient knee which sustained a medial meniscectomy. In the second group of patients, meniscal transplantation is performed together with ACL reconstruction as it grants an improved stability compared to that obtained with ACL reconstruction alone (Barber, 1994). A further clinical scenario has been advocated by some authors (Johnson and Bealle, 1999): prophylactic transplantation in young, sportive patients who had complete meniscectomy, before the onset of symptoms. However, such an aggressive approach is not routinely recommended to date (Lubowitz *et al.*, 2007).

A recently published meta-analysis, analysing 44 published clinical trials with at least 6 months follow-up in the last 26 years, concluded that this procedure should not be considered experimental surgery anymore, but, instead, it is a safe and effective technique allowing patients to resume high levels of activity and work, at least, as a long-term ‘‘bridging’’ procedure before arthroplasty (Elattar *et al*., 2011). Actually, a number of orthopaedic surgeons have reported clinical and radiological mid-term results (Kim *et al.*, 2012; Lee and Caborn, 2012). However, fixation of the allograft, whether soft-tissue or bone plug associated, remains a significant source of failure (Hommen *et al.*, 2007). Additional problems of meniscal allograft transplantation are the limited availability, the technically demanding surgical procedure, and the frequent mismatch of graft and host tissue (Shaffer *et al*., 2000). Xenografts have only been used in animal or cadaver studies till now, but might become an interesting treatment alternative (Jiang *et al.*, 2012).

In conclusion, meniscal transplantation allows for good short-to-intermediate term results in selected patients, while it is not yet demonstrated whether this technique provides long term protection from joint degeneration (Wirth *et al.*, 2002).

Research potential for meniscus repair and regeneration: from cell therapy to tissue therapy

Currently, a number of experimental approaches are addressing the issue of meniscal regeneration. In particular, researchers work on the development of different potential regenerative solutions for different clinical scenarios requiring different surgical treatments. These can be summarised as follows: (i) improved biological bonding in case of reparable tears; (ii) partial meniscus regeneration, to restore the tissue removed after meniscectomy; and (iii) total meniscus regeneration, when sub- or total meniscectomy is performed. In the following sections the mainstays of tissue engineering (cells, growth factors and scaffolds), with respect to meniscus regeneration, and the experimental strategies combining them will be reported.

Cell sources

Several adult differentiated cells from various tissues have been used in tissue engineering studies. In this chapter, we will discuss the potential for meniscus regeneration of meniscus cells (MC), articular (AC), costal (CC) and nasal chondrocytes (NC), bone marrow-derived mesenchymal stem cells (MSC), synovial membrane-derived MSC and embryonic stem cells (ESC).

From a clinical standpoint, the ideal tissue for cell harvesting is the meniscectomised meniscus itself, as no additional morbidity is produced. Nakata *et al*. (2001) investigated the potential for tissue engineering approaches of MC isolated from meniscectomised menisci. In this study, authors expanded *in vitro* human MC and seeded them on a collagen scaffold with success, demonstrating

the feasibility of obtaining an adequate number of cells from small meniscal specimens and of seeding them on biologic scaffolds. In a more recent study, Baker *et al*. (2009) demonstrated that expansion and seeding of meniscal debris-derived cells onto nanofibrous scaffolds led to engineered constructs with mechanical properties in tension approaching native tissue levels. However, many patients candidate to meniscus allotransplantation have already undergone total meniscectomy and an alternative cell source is, therefore, needed. This feature limits the use of this cell type only to meniscus repair and regeneration approaches.

Articular cartilage is an attractive cell source also for meniscus tissue engineering since it can be easily harvested from non-weight bearing areas and AC can be enzymatically isolated and used for tissue engineering strategies. Marsano *et al*. (2007) investigated and compared the potential for meniscus tissue engineering of inner meniscus cells, fat pad cells, synovial membrane cells and AC in pellet culture, in scaffold, and in nude mice culture. The authors reported the formation of meniscus-resembling tissue by AC only, while in the samples obtained with the other cell types, the glycosaminoglycan (GAG) content was negligible and no collagen type II was detected. This is consistent with all the previous works reporting the AC capability of accumulating large quantities of cartilaginous matrix. Interestingly, AC deposited also collagen type I and IV, the latter typically present in the outer vascularised area of the meniscus. In addition, the phenotype of AC may adapt upon exposure to specific regimens of physical forces in order to generate a tissue structure resembling that of the human meniscus (Marsano *et al.*, 2006), making them an attractive cell type for meniscus tissue engineering.

Interest has recently focused on the potential of CC for knee and temporomandibular joint meniscus tissue engineering (Johns and Athanasiou, 2008; Anderson and Athanasiou, 2009). Importantly, costal cartilage represents a clinically-compliant cell source since the harvest can be abundant while leaving little donor site morbidity. These cells display also a high synthetic activity after *in vitro* expansion, being able to deposit large quantities of fibrocartilaginous matrix with GAGs, collagen type II and type I (Johns and Athanasiou, 2008). This feature can be further manipulated by co-culturing CC with other cell types in order to create a spectrum of fibrocartilaginous engineered tissues. In particular, the collagen I/II ratio could be modified by cell types used in co-culture and serum presence in order to span the whole native range (Hoben and Athanasiou, 2008).

Thanks to their high chondrogenic potential, ease of harvesting, lower inter-individual variability, faster proliferation and positive response to load, NC have been extensively studied for cartilage tissue engineering purposes (Scotti *et al.*, 2012; Candrian *et al.*, 2008; Tay *et al.*, 2004). In particular, their capability to adapt to a joint-like environment (low-oxygen tension, inflammatory environment and load) makes them a suitable cell source for joint regeneration (Scotti *et al.*, 2012; Candrian *et al.*, 2008). However, to date there is no report in the literature of NC-based approaches for meniscus regeneration.

Stem cells are characterised by self-renewal capacity and multilineage differentiation potential to distinctive end-stage cell types of mesenchymal tissues, such as bone, cartilage, muscle, tendon/ligaments and fat, in response to environmental cues (Caplan, 1991; De Bari *et al.*, 2007). They can be extensively expanded *in vitro* while maintaining their differentiation potential and subsequently cryopreserved. In addition, MSC have a natural capacity to home to injured tissues and to participate to tissue healing. This second feature seems particularly interesting as MSC not only provide a substrate for regeneration but also secrete paracrine factors that enhance the potential for tissue repair, acting as "trophic mediators" (Caplan, 2007). MSC have been isolated from several tissues, including bone marrow (Pittenger *et al.*, 1999), periosteum (De Bari *et al.*, 2001a), synovial membrane (De Bari *et al.*, 2001b) and adipose tissue (Zuk *et al.*, 2002).

Bone marrow is the main cell source for adult MSC because of its ease of harvest with limited morbidity and, therefore, MSC have been used in a myriad of experimental studies and also in some clinical trials. Adult bone marrow contains both haematopoietic stem cells (HSC) and MSC (Caplan, 1991; Herzog *et al.*, 2003). HSC and MSC can be distinguished by the expression of cell surface antigens, as MSC typically lack antigens such as CD45 and CD34 that usually identify HSC (Herzog *et al.*, 2003). However, the number of progenitor cells present in human fresh bone marrow biopsies is very low (about 0.01 % of the total mononucleated cells), further decreasing with donor age and thus requiring *in vitro* cell expansion to reach an adequate cell number for regenerative purposes (Muschler *et al.*, 2001). Another possible drawback of bone marrow-derived MSC, limited to articular cartilage and meniscus regenerative strategies, is that they seem to retain osteogenic propensities, through an endochondral pathway, as the default differentiation route (Muraglia *et al.*, 2000; Scotti *et al.*, 2010). As a consequence, chondrocyte hypertrophy and subsequent vascularisation and mineralisation can impair the final quality of the newly formed tissue. However, bone marrow-derived MSC still remain the mainstay of stem cell-based approaches in orthopaedic clinical practice.

Another attractive cell source for the orthopaedic surgeon is represented by the synovial membrane, since it can be easily harvested in both open and arthroscopic surgery. Synovial membrane-derived MSC have been described by De Bari *et al*. (2001b) reporting a progenitor nature with remarkable self-renewal capacity. Recently, work has described the superior capacity of synovial membrane-derived MSC to generate cartilaginous tissues compared to MSC obtained from other tissues (Sakaguchi *et al.*, 2005). On the other hand, a following report of De Bari *et al*. (2004) showed that these cells failed to produce *in vivo* a stable and differentiated cartilaginous tissue, undergoing cell death and neoangiogenesis. This suggests that *in vitro* pre-differentiation is not sufficient to guarantee stable lineage commitment and restriction of differentiation. However, synovial membrane-derived MSC are still a valuable and promising cell type for tissue engineering, and different approaches, such as intra-articular delivery, are currently being investigated.

The use of ESC is also advocated for meniscus tissue engineering strategies (Koay and Athanasiou, 2009; Hoben *et al.*, 2008). Despite the concerns raised about ethical issues, their safety, since they tend to form tumours once implanted unless they are pre-differentiated, and since they are immunogenic, their highly pluripotency makes them an attractive cell type for the regeneration of various tissues (De Bari *et al.*, 2007). While the issue of the oncogenic potential is currently being addressed by improving *in vitro* ESC commitment and culture, the immunogenicity could be overcome by implementing large cell banks with genotyped ESC cell lines (De Bari *et al.*, 2007). This would theoretically allow transplanting a large number of HLA-matched recipients with these cells. However, a possible clinical application of ESC for non-lethal diseases is still to be planned.

Intra-articular delivery of MSC

An attractive strategy for joint regeneration is intra-articular injection of progenitor cells that can participate to and enhance tissue regeneration. This approach presents several advantages: (i) it is easy to be performed in an outpatient setting with minimum morbidity; (ii) it can be used to deal with either single lesions or degenerative diseases; (iii) it works in two directions, delivering cells that can both actively regenerate the damaged tissues and secrete anti-inflammatory and trophic factors that can re-establish joint homeostasis according to Caplan (Caplan, 2007; Caplan and Correa, 2011); (iv) it can be repeated; and (v) it minimises systemic diffusion of the implanted cells (Horie *et al.*, 2009).

A first report of this strategy has been performed by Murphy *et al*. (2003) by injecting bone marrow-derived MSC in suspension with sodium hyaluronan in an osteoarthritis (OA) goat model. In this work, the authors showed successful cell survival and engraftment in the regenerated medial meniscus which was completely excised, together with the ACL, to determine OA. Importantly, this involvement of the injected MSC in the regenerated meniscus resulted in protection from OA progression at the latest experimental time point. However, the number of injected cells in the regenerated tissue was too low for being responsible of the massive tissue regeneration, while, on the other hand, MSC had more likely trophically enhanced the regeneration of the meniscus.

In a more recent work, Horie *et al*. (2009) investigated the potential for meniscus regeneration of intra-articularly injected synovial membrane-derived MSC in a rat massive meniscal defect model. The authors not only demonstrated active participation of the injected MSC in the regeneration process, adhering to the injured sites and synthesising new tissue, but also showed with *in vivo* imaging analysis that the injected cells do not mobilise out of the injected joint. This is a crucial safety issue with respect to a possible clinical application of this approach. Another interesting finding in this work is that the authors could detect MSC activity in the knee joint up to 28 d after the injection but not at a longer time point. This can be explained by the fact that, after triggering tissue regeneration, most cells die while only a few remain in the newly formed tissue. This is further evidence supporting the hypothesis of MSC as trophic factor releaser for tissue repair. However, stronger evidence, possibly obtained with more relevant large animal models, should be provided prior to considering this approach as an option with clinical relevance.

Growth factors and gene therapy

To date, the effect of many growth factors (GF) on meniscal fibrochondrocytes or meniscal explants has been investigated (Table 1), since matrix synthesis enhancement and metalloproteinase inhibition are crucial mechanism to be addressed for meniscus repair and tissue engineering (Buma *et al.*, 2004). In particular, transforming growth factor β (TGF-β) demonstrated a promising effect in term of stimulation of GAGs and biglycan production by MC *in vitro* (Collier and Gosh, 1995). However, it remains to be demonstrated whether TGF-β stimulation may lead to a tissue engineered meniscus more similar to native meniscus or to a more efficient repair, because collagen, rather than GAGs, seems to be important for meniscus function (Buma *et al.*, 2004). Other GF demonstrated to have a trophic effect on meniscal fibrochondrocytes are: hepatocyte growth factor (HGF); insulin-like growth

Table 1. Growth factors of relevance for meniscus tissue engineering.

Growth factor	Effect on meniscal cells or tissue	References
FGF-2	Enhance cell proliferation in monolayer cultures Enhance collagen synthesis in cell-seeded scaffold	(Marsano *et al*., 2007) (Pangborn and Athanasiou, 2005)
TGF-β1	Enhance cell proliferation in monolayer culture Enhance SMA expression Enhance collagen and proteoglycan synthesis Enhance cell proliferation on a PU scaffold	(Marsano *et al*., 2007) (Zaleskas *et al*., 2001) (Collier and Ghosh, 1995, Pangborn and Athanasiou, 2005) (de Mulder *et al*., 2011)
IGF-1	Main anabolic factor of cartilage Enhance collagen synthesis in cell-seeded scaffold Stimulate cell migration	(Buma *et al*., 2004) (Pangborn, 2005) (Bhargava *et al*., 1999)
HGF	Improve vascularisation of engineered tissue	(Hidaka *et al*., 2002)
PDGF-BB	Enhance cell proliferation *in vitro* Decrease SMA expression	(Marsano *et al*., 2007) (Zaleskas *et al*., 2001)
BMP-2 BMP-7 (OP-1)	Enhance cell proliferation Stimulate cell migration Enhance proteoglycan synthesis	(Bhargava *et al*., 1999) (Bhargava *et al*., 1999) (Lietman *et al*., 2003)

factor 1 (IGF-1); fibroblast growth factor 2 (FGF-2); platelet-derived growth factor (PDGF); and certain bone morphogenetic proteins (BMPs) (Buma *et al.*, 2004, Pangborn and Athanasiou, 2005). In particular, HGF was demonstrated to enhance vascularisation of engineered meniscal fibrochondrcyte-PGA constructs, but without improving mechanical properties (Hidaka *et al.*, 2002), and IGF-1 is one of the main anabolic factors of articular cartilage (Schmidt *et al.*, 2006). As the outer part of the meniscus is vascularised (Clark and Ogden, 1983) and the inner region is characterised by an ECM partially similar to that of the articular cartilage (Chevrier *et al*., 2009), the use of these two GF could be a promising combination to be evaluated. Another interesting finding was the modulation of smooth muscle actin (SMA) expression by TGF-β1 and PDGF-BB (Buma *et al.*, 2004). A study demonstrated that TGF-β1 increases SMA expression and cell contraction while PDGF-BB has the opposite effect: investigation of the mechanisms underlying SMA-enabled contraction may be crucial for cell expansion and differentiation (Zaleskas, 2001).

Recent advances in gene therapy techniques have demonstrated the feasibility of gene transfer to musculoskeletal tissues (Evans and Robbins, 1999; Madry *et al.*, 2003; Madry *et al.*, 2004). GF gene transfer is an attractive option to enhance meniscal repair, and this has been demonstrated both *in vivo* and *in vitro* with different approaches (Goto *et al.*, 1999; Hidaka *et al.*, 2002; Martinek *et al.*, 2002; Nakata *et al.*, 2001). Several vectors have been used: retroviral, adenoviral and adeno-associated; each of them presenting peculiar characteristics (Madry *et al.*, 2004; Goto *et al.*, 1999; Hidaka *et al.*, 2002). Retroviral vectors have been widely used in gene therapy studies, nevertheless they need actively replicating cells as target, while fibrochondrocytes do not duplicate *in vivo*, and they can theoretically cause cancer, through insertional mutagenesis. Although the first limitation can be overcome by the use of lentiviral vectors, that can transduce also non-dividing cells, safety concerns make them unattractive to orthopaedic researchers who deal with non-lethal disorders (Madry *et al.*, 2004; Daniel and Smith, 2008). Adenoviral vectors are immunogenic and do not integrate into the host cell genome; as a consequence they prevent the risk of cancer but they do not grant long-term transgene expression (Madry *et al.*, 2004; Steinert *et al.*, 2007). Adeno-associated vectors (AAV) can carry only a small amount of DNA; on the other hand, they are not immunogenic and not pathogenic, and they can transduce non-dividing cells: these features make them very attractive for orthopaedic use, making them a promising option for the treatment of musculoskeletal diseases (Madry *et al.*, 2004; Cucchiarini *et al.*, 2009).

Following the first studies with marker genes (Madry *et al.*, 2004; Goto *et al.*, 1999), the main GF used for gene transfer to the meniscus to date are HGF (Hidaka *et al.*, 2002), TGF-β1 (Steinert *et al.*, 2007), and FGF-2 (Cucchiarini *et al.*, 2009). HGF induced blood vessel formation in an engineered construct made of meniscal fibrochondrocytes seeded onto a PGA scaffold, improving the potential for integration and metabolic exchanges of the engineered implant (Hidaka *et al.*, 2002). TGF-β1 gene transfer enhanced the cellularity and the deposition of proteoglycans and collagen type 2 both in monolayer and in 3D cultures (Steinert *et al.*, 2007; Goto *et al.*, 2000). On the other hand, of importance are the consequences of ubiquitous overexpression of TGF-β1, as it determines severe joint fibrosis and detrimental systemic effects (Mi *et al.*, 2003; Steinert *et al.*, 2007). Consequently, a localised delivery of TGF-β1 to the site of lesion is mandatory. FGF-2 transfer through an AAV resulted in increased proliferation, survival and metabolic activity of human MC *in vitro*, in a three-dimensional *in vitro* culture system, and *in situ* in a human meniscal defect model (Cucchiarini *et al.*, 2009). This study proved that direct application of AAV vectors has the potential to induce healing in the injured meniscus. However, since no complete healing was achieved and since proliferation is just one player of tissue regeneration, additional studies are required to assess the optimal gene delivery strategy for meniscus regeneration.

Overall, the application of gene transfer techniques to meniscus regeneration holds some potential. However, several variables have to be still assessed, such as i) the modality of transduction (modification of many cell types, transduction of whole tissues, intra-articular injection); ii) the ideal combination of genes to be transferred; or iii) the most efficient cell type, when transfected cells are used.

Scaffolds

A biomaterial used as scaffold for meniscus tissue engineering purposes should present many features. In particular, the ideal meniscal scaffold should be (i) "cell-instructive", promoting cell differentiation and proliferation if cell-seeded, or cell migration if cell-free; (ii) "biomimetic", mimicking architecture, tribology and mechanical features of the native meniscus; (iii) resilient and resistant to withstand mechanical forces acting in the joint while cells produce ECM; (iv) biocompatible, not evoking any foreign-body reaction also with its degradation products; (v) slowly biodegradable allowing to be gradually replaced by biologic tissue; (vi) open, with high porosity, allowing diffusion of nutrients and catabolic substances; and (vii) easy to handle, to be sutured and to be implanted by the surgeon (Arnoczky, 1999; van Tienen *et al.*, 2009).

With the ultimate goal of designing the ideal scaffold for meniscus tissue engineering, many biomaterials have been evaluated both natural and synthetic (Buma *et al.*, 2004) (Table 2). Natural materials used to date are: periosteal tissue (Walsh *et al.*, 1999); perichondral tissue (Bruns *et al.*, 1998); small intestine submucosa (SIS) (Cook *et al.*, 1999); acellular porcine meniscal tissue (Stapleton *et al.*, 2008); and bacterial cellulose (Bodin *et al.*, 2007). While these tissues have high biocompatibility, some of them cannot be employed for tissue engineering techniques as they do not allow varying structure geometry and initial mechanical properties (Buma *et al.*, 2004). A more attractive strategy is represented by isolated tissue components as collagens and proteoglycans (Mueller *et al.*, 1999; Pabbruwe *et al.*, 2010). They maintain the high biocompatibility of the whole tissues while allowing to create custom-made scaffold with definite pore dimension and geometry and, consequently, biomechanical features.

Table 2. Biomaterials of relevance for meniscus tissue engineering.

Biomaterial	Summary of results	References
Collagen	CMI scaffolds seeded with autologous fibrochondrocytes. Macroscopic and histological improvement of the transplants compared to cell-free CMI.	Martinek *et al*., 2006
SIS	A cell-free SIS implant demonstrated better results than meniscectomy with less cartilage damage. Another study reported the potential of SIS as a scaffold to support co-culture of synovial membrane-derived MSC and meniscal cells.	Cook *et al*., 2006 Tan *et al*., 2010
PCL	PCL scaffold with defined nanofibres alignment determines better neotissue organisation and mechanical properties.	Baker *et al*., 2007 Baker *et al*., 2010 Baker *et al*., 2012
Hyaluronan-PCL	Partial and total meniscus replacement, both cell-free and cell-seeded, in ovine models. 4 months follow up. Good tissue ingrowth/formation. Better results for cell-seeded scaffolds also in terms of chondroprotection.	Chiari *et al*., 2006 Kon *et al*., 2008
Polyurethane-PCL	Cell-free total meniscus implant demonstrated good integration and fibrovascular tissue ingrowth in a 2-year follow-up study in dogs. Mild foreign body reaction was noticed. Not better chondroprotection compared to meniscectomised knees.	Hannink *et al*., 2011 Welsing *et al*., 2008 Heijkants *et al*., 2005 Heijkants *et al*., 2004 van Tienen *et al*., 2009
PGA-PLGA	Total meniscus PLGA implant seeded with allogeneic meniscal cells in a rabbit model. Positive histological results at 36 weeks. Biochemical and biomechanical improvement of the neotissue over time.	Kang *et al*., 2006
Silk	Tri-layered silk fibrous protein scaffold seeded with human fibroblasts and chondrocytes or MSC showed cell growth with aligned ECM deposition.	Mandal *et al*., 2011a Mandal *et al*., 2011b
Carbon	Replacement of the whole meniscus with a polyester-carbon cell-free implant in a rabbit model. Better results than meniscectomised, untreated knees.	Wood *et al*., 1990
Hyaluronan-gelatin	Partial meniscus regeneration with a 70 % HA-30 % gelatin scaffold seeded with autologous MSC in a rabbit model. Better results than with cell-free scaffold. A second study showed better results for unpassaged MSC.	Angele *et al*., 2008 Zellner *et al*., 2010
PVA	Total meniscus replacement with cell-free implant in rabbit model with 2-year follow up. Successful chondroprotection. No regeneration of meniscal tissue. Concerns about long-term durability, safety and fixation method.	Kobayashi *et al*., 2003 Kobayashi *et al*., 2005
Agarose	*In vitro* engineering of critically sized meniscal constructs with bovine and ovine fibrochondrocytes in a mixing bioreactor.	Ballyns *et al*., 2008 Ballyns *et al*., 2010
Scaffold-free	Self-assembled engineered meniscal tissues, obtained with co-cultures of fibrochondrocytes and MSC in ring-shaped moulds, displayed better morphological and mechanical features than cell-loaded PGA scaffolds.	Aufderheide and Athanasiou, 2007 Huey and Athanasiou, 2011

However, these scaffolds have usually low biomechanical properties and are characterised by rapid biodegradation, thus not long enough to be completely replaced by the newly formed tissue (Buma *et al.*, 2004).

On the other hand, polymer materials can be manufactured in custom-made shapes of any geometrical structure, porosity and biomechanical properties, according to the characteristics of the host tissue and the seeded cells. In particular, it has been shown that for optimal ingrowth and incorporation of a meniscal scaffold, macropore sizes must be in the range of 150-500 μm (Klompmaker *et al.*, 1993). The biodegradation rate can be also modulated by acting on polymer composition. To date, the most used synthetic polymers are: polyglycolic acid (PGA) (Vacanti *et al.*, 1991); poly(L)lactic acid (PLLA) (Freed *et al.*, 1993); poly-(lactic-co-glycolic acid) (PLGA) (Kang *et al.*, 2006); polyurethane (Heijkants *et al.*, 2004; van Tienen *et al.*, 2002); polyester carbon (Wood *et al.*, 1990); polytetrafluoroethylene (Toyonaga *et al.*, 1983); and polycaprolactone (PCL) (Lebourg *et al.*, 2008). Possible drawbacks of the use of synthetic polymers for tissue engineering purposes are the low cell-adhesive properties, since they lack the cell-adhesion domains normally present on natural macromolecules, and the even mild foreign-body reaction occurring after implantation (Cao *et al.*, 1998; Welsing *et al.*, 2008). In order to improve biocompatibility and biodegradability of polymer scaffolds, the use of a biopolymer, such as silk fibrous protein, has been proposed (Mandal *et al.*, 2011a; Mandal *et al.*, 2011b).

An alternative attractive strategy is represented by the use of hydrogel materials. Their semi-liquid nature allows engineering anatomic geometries derived from medical imaging techniques, such as computed tomography or magnetic resonance, by the use of custom-printed moulds (Ballyns *et al.*, 2008). Promising results were reported with alginate (Ballyns *et al.*, 2010; Ballyns *et al.*, 2008) and polyvinyl alcohol (PVA) (Kobayashi *et al.*, 2003; Kobayashi *et al.*, 2005). However, despite their wide implementation in cartilage tissue engineering they have been hardly utilised for meniscus engineering.

Briefly, both natural materials and synthetic polymers present peculiar advantages and disadvantages. Most importantly, no biomaterial demonstrated to be superior to the others in terms of supporting cell proliferation and

tissue growth. No clear advantage was also evident in term of biomechanical properties suitable for implantation in the knee joint. A possible solution is represented by combining them, in order to couple the high cell-affinity and biocompatibility of natural polymers with the superior mechanical strength and ease of being tailored of synthetic polymers. This strategy has been recently evaluated in two large animal studies on partial and total meniscus tissue engineering with a hybrid material composed of PCL and hyaluronic acid (HA) with promising results (Chiari *et al.*, 2006; Kon *et al.*, 2008).

In the following sections we will discuss different experimental studies for partial and total meniscus engineering performed to date and having a potential clinical exploitation.

Cell-based bonding studies

As long as lesions occurring in the inner "red-white zone" and "white-white zone" do not allow a biological repair to be achieved, even after stabilisation, cell-based strategies have been evaluated in order to enhance the bonding of a torn meniscus. From a clinical point of view, a tool which can improve the results of meniscal sutures is valuable. The rationale of this approach has been validated in studies demonstrating that isolated chondrocytes, either seeded onto cartilaginous (Peretti *et al.*, 1998; Peretti *et al.*, 1999) and meniscal matrices (Peretti *et al.*, 2001), PLGA scaffold using dynamic conditions (Yoo *et al.*, 2011; or embedded in fibrin glue (Peretti *et al.*, 2008; Scotti *et al.*, 2009), were able to bond separate pieces together. These studies were performed ectopically, in a subcutaneous environment in a nude mouse model, which is vascularised and not subjected to weight bearing, and therefore not suitable to ultimately evaluate the value of a regenerative strategy for the knee joint. In order to test the potential of transplanted chondrocytes for use as a reparative technique in lesions involving the human knee, further studies were performed *in situ* in a pig model (Peretti *et al.*, 2004; Weinand *et al.*, 2006a; Weinand *et al.*, 2006b). Among the animal models for meniscus regeneration studies (Deponti *et al.,* 2013), the pig is very valuable as it has been demonstrated that the vascularisation of the porcine meniscus remains confined in the outer part of the menisci and never extends into the inner third of its structure (Peretti *et al.*, 2004).

In the first study, an allogeneic devitalised meniscal scaffold was chosen as a carrier for autologous chondrocytes in an orthotopic pig model (Peretti *et al.*, 2004). A one-centimetre longitudinal tear was created in the avascular portion of the medial meniscus at the junction of its inner third and outer two-thirds and the chondrocyte-seeded meniscal scaffold was inserted inside the lesion and secured by two vertical sutures. Histological evaluation demonstrated bonding tissue, resembling cartilaginous and fibrocartilaginous matrix, synthesised by the transplanted chondrocytes in samples from the experimental group. Although the repair was not uniformly complete in this study, the results were considered encouraging as good integration of the scaffold material with the native meniscus appeared to be present in numerous areas by the formation of new cartilage matrix.

According to these results, in subsequent studies the potential of different allogeneic cell sources and of Vicryl meshes as a scaffold was evaluated, in the same orthotopic model as the previous study (Weinand *et al.*, 2006a; Weinand *et al.*, 2006b). The rationale of using allogeneic cells of different sources is that it is unlikely to obtain a sufficient number of healthy meniscal fibrochondrocytes from an injured meniscus. Additionally, Johnson *et al*. (2004) showed the healing potential of auricular and costal chondrocytes suspended in fibrin polymer and placed between articular cartilage discs, demonstrating the feasibility for alternative cell types to heal cartilage. Moreover, the harvesting of autologous cells can add additional trauma to the patient, while large quantities of cartilage cells may be obtained using allogeneic chondrocytes from cadaveric sources. Regarding the results of these studies, some degree of new tissue formation was found in all experimental samples, whereas none was found in any of the control groups. The newly formed tissue was uniform in all samples, having a characteristic fibrous tissue-like appearance. Interestingly, the length of the repaired lesion was highest in the auricular based specimens, which also demonstrated a higher overall rate of repair: more than 65 % of samples were repaired compared to more than 55 % of the articular specimens. This histological finding is consistent with higher biomechanical testing values in fracture energy, stiffness and extension of the tested samples. These results may also be due to the fact that auricular chondrocytes produce a higher amount of elastin than articular chondrocytes. Regarding the issue of using allogeneic cells, they showed a slightly lower complete healing rate than the use of autologous cells. Although cellular rejection might be expected when using allogeneic cells, none of the menisci repaired with allogeneic articular or auricular cells had evidence of immune rejection. Only few histiocytes were observed within the newly formed tissue, and these may be related to a foreign body reaction to the Vicryl scaffold. Possible explanations for the absence of an inflammatory reaction include the avascular environment into which the implant is placed and questionable expression of MHC antigens on the surface of the chondrocytes (Bujia *et al.*, 1994). In all, these studies suggested that allogeneic cell populations have a potential to repair tears in the meniscus, used in combination with biodegradable scaffolds, and that auricular chondrocytes could provide complete healing. Moreover, the use of allogeneic cells could overcome the potential clinical limitations of donor site morbidity and inadequate cell numbers, which represent the main bottleneck to the clinical application of every single cell-based strategy.

From a surgical point of view, a suture capable to release in a controlled manner trophic GF or a cellular gel acting as a biological glue could be more manageable tools than a solid matrix to improve the bonding obtained with meniscal sutures. The first approach has been investigated by Kopf *et al*. (2010), reporting the results of a VEGF-coated non-absorbable suture for meniscal repair in a sheep orthotopic model. Although very interesting, this approach did not improve meniscal healing, probably because of the

inappropriate kinetics of VEGF release. In this regard, an ectopic nude mouse study was performed, consisting of the evaluation of the potential of bonding meniscal slices by cellular fibrin glue (Peretti *et al.*, 2008; Scotti *et al.*, 2009). In this study, the firm gross bonding seen macroscopically was confirmed by the histology, as the cellular fibrin glue provided a microscopic bonding between the two meniscal slices through a fibrocartilaginous tissue. Moreover, penetration buds from the cellular fibrin glue to the native meniscal tissue were evident. This represents a positive finding as we had previously demonstrated that this characteristic is associated with an improved bonding (Peretti *et al.*, 1998; Peretti *et al.*, 1999). Additionally, these features were not found in the control samples, confirming the need of cells to grant bonding between tissues. However, further *in vivo* studies in an orthotopic model are needed to evaluate the feasibility of this approach in a weight-bearing environment.

Partial meniscus engineering

From a clinical standpoint, partial meniscus regeneration represents a critical topic since the treatment of irreparable lesions of the avascular zone of the meniscus is still an open issue.

Following the large clinical experience with CMI, Martinek *et al*. (2006) reported an interesting study on an autologous fibrochondrocytes-loaded CMI implanted in an ovine orthotopic model with a short-term follow up (3 months). The implant was pre-cultured *in vitro* for 3 weeks to allow for cell adhesion and ECM deposition, and then implanted orthotopically. Cell-seeding was demonstrated to improve the mechanical properties and histological results. However, the tissue-engineered meniscus was biomechanically unstable and the implant size reduced during the three-month observation period. Therefore, the authors suggested that an improvement in scaffold and cell seeding procedure was required before human application.

SIS has been applied to menisci regeneration in an orthotopic dog model in a 12 months follow-up study (Cook *et al.*, 2006). In this study, partially meniscectomised menisci receiving cell-free SIS in a posterior vascular lesion had more tissue filling in the defects, with a meniscus-like tissue, and significantly less cartilage damage than menisci receiving no implants. Authors concluded that SIS implantation had better results than meniscectomy. Despite these positive experimental findings, it has been demonstrated that SIS evokes a TH-2 lymphocytes mediated immune response (Ansaloni *et al.*, 2007), probably dependent on the presence of porcine DNA. However, this response usually does not determine graft rejection and it is used in clinical practice in inguinal hernioplasty (Ansaloni *et al.*, 2007).

MSC seeded onto a hyaluronan-collagen-based scaffold were recently used to repair a critical-size defect in an orthotopic rabbit model, with a tissue engineering approach (Angele *et al.*, 2008). In this study, the authors removed the pars intermedia of the medial meniscus and replaced the resected section with the biocompatible scaffold only, or with the scaffold loaded with MSC and previously cultured *in vitro* for 14 d. Menisci repaired with the cell-free scaffold showed only a fibrous, scar-like repair tissue, while those repaired with the engineered tissue demonstrated a significantly better filling and meniscal regeneration. However, the cell-loaded and precultured implants did not completely restore the surface area and the tissue quality of normal meniscus. The authors concluded that, even if further studies are needed to optimise this approach, MSC demonstrated a potential for the regeneration of meniscal defects (Angele *et al.*, 2008). In a more recent orthotopic study, the same authors evaluated the regeneration potential of hyaluronan-collagen matrices without cells, loaded with platelet-rich plasma, autologous bone marrow, or autologous MSC in a 2 mm punch defect model (Zellner *et al.*, 2010). This study presented several interesting findings. First, neither bone marrow nor platelet-rich plasma loaded onto the matrices determined an improved healing compared with cell-free implants. Second, the implantation of 14 d precultured MSC-matrix constructs in chondrogenic medium resulted only in fibrocartilage-like repair tissue, displaying incomplete integration with the host meniscus. Third, non-precultured MSC in hyaluronan-collagen composite matrices stimulated the development of completely integrated meniscus-like repair tissue. The latter suggests the potential for an intra-operative, one-step approach for partial meniscus tissue engineering with autologous MSC and a proper biomaterial. However, the defect model used in this study does not reflect the typical meniscal lesion, and therefore the translation of this approach to clinical practice may require further studies.

Total meniscus engineering

Total meniscus tissue engineering may be considered as a potential alternative to allografts, in order to overcome by means of an autologous tissue the problems of availability, immune reaction and sizing related to allotransplantation. However, tissue engineering techniques have been applied to meniscal regeneration with controversial results, and only a few studies investigated in orthotopic models the feasibility of engineering total meniscus substitutes (Kon *et al.*, 2008; Kang *et al.*, 2006).

Kang *et al*. (2006) described a PGA-PLGA scaffold seeded with allogeneic meniscal cells in a rabbit total meniscectomy model. The authors reported positive results at 36 weeks, with a neotissue resembling the normal meniscus by histology. Both the overall histological appearance and mechanical properties improved over the experimental times. Importantly, the seeded scaffolds showed no shrinkage or shape alterations at 36 weeks, while the unseeded scaffold showed failure of maintenance of shape and size. Less severe cartilage degeneration was observed in the rabbits treated with the seeded scaffolds. No or minor immune response was observed. In conclusion, this study demonstrated the feasibility of total meniscus substitution with a tissue engineering approach using a polymer scaffold and allogeneic fibrochondrocytes, highlighting the role of seeded cells in maintaining shape and size and for chondroprotection.

A crucial aspect of scaffold design for meniscus tissue engineering was highlighted by Baker and Mauck (2007) reporting that nanofibres alignment served as a micro-pattern for directed tissue ingrowth and that, when cell-seeded with bovine meniscal cells or MSC, resulted

in better tissue properties in a PCL meniscal scaffold. An important finding of this study was that the improvement in mechanical properties depended on the overall better organisation of the tissue and not on a higher neotissue deposition. Considering the fine organisation of the native meniscal collagen fibres, these results seem particularly relevant to meniscal scaffold design. In a more recent study by the same authors with human cells harvested by adult human donors, they reported better results with fibrochondrocytes compared to MSC (Baker *et al.*, 2010). This concept has been further optimised, reaching near-native properties in tension, and showed multi-scale collagen organisation in the scaffolds (Baker *et al.*, 2012). A further fibre-reinforced degradable scaffold for meniscus tissue engineering was developed by Balint *et al*. (2012) with the hypothesis that the fibre network design shares part of the compressive loads *via* the generation of circumferential tensile loads, resulting in tensile properties similar to those of the meniscus.

Chiari *et al*. (2006) described the early results of a cell-free hyaluronan-PCL scaffold in an orthotopic large animal model. Their study was aimed to evaluate the biocompatibility, tissue ingrowth and neovascularisation properties of this material and demonstrated tissue formation and bonding to the capsule with an overall satisfactory integration in the host joint. Additionally, abundant blood vessels were found in the scaffold after 6 weeks. According to these favourable preliminary results, Kon *et al*. (2008) investigated the use of this biomaterial seeded with expanded autologous articular chondrocytes in the same orthotopic model in order to improve the biological response and the remodelling processes. At the end of the experimental time (16 weeks) no significant macroscopic difference was evident between the cell-seeded and the cell-free implant. However, histological analysis demonstrated deposition of cartilaginous matrix only in the cell-seeded scaffold. Moreover, the authors reported that the cartilaginous distribution was mainly located at the edges and the tip of the implants, consistent with the distribution of cells at the time of grafting, thus confirming that matrix deposition depended on or at least was enhanced by the previously seeded cells. Additionally, the implants were well tolerated immunologically, with only a mild foreign body giant-cell reaction. Limited peripheral implant extrusion was frequently observed. The authors concluded that the hyaluronan-PCL scaffold had a potential for total meniscus regeneration and that the seeding of the scaffold provided some benefits at 4 months follow-up allowing for a larger amount of fibrocartilaginous tissue formation. A following study with 12-month evaluation confirmed the improvement in tissue formation within the tissue engineered menisci, but showed no significant differences in protection from osteoarthritic degeneration between cell-seeded and cell-free scaffolds (Kon *et al.*, 2012). However, both cell-seeded and cell-free implants resulted in better chondroprotection compared to meniscectomised knees.

The results of an orthotopic study in dogs with a 6-month and 2-year follow up have been reported (Welsing *et al.*, 2008; Hannink *et al.*, 2011). In this study, they compared the outcome of total meniscectomy with that of the implant of a PU-PCL total meniscal implant. This work followed a large series of *in vitro* and *in vivo* experimental studies on the development of this scaffold (Heijkants *et al.*, 2005; Heijkants *et al.*, 2004; van Tienen *et al.*, 2009). The authors described satisfactory integration and fibrovascular tissue ingrowth into the implant with mild foreign body reaction. However, specific structural organisation and a fibrocartilage phenotype was lacking after both 6 and 24 months. The porous polymer scaffold was histologically not noticeably degraded after 24 months, thus confirming the expected slow degradation rate of this implant. Surprisingly, chondroprotection was not superior compared to that of knees that underwent meniscectomy only. The authors concluded that further improvements in the implant model and surgical technique were needed for making the implant suitable for clinical application in totally meniscectomised patients. In fact, the clinical scenario in which a PU-PCL cell-free scaffold is currently used (Actifit™, Orteq Ltd, London, UK) is represented by irreparable meniscal tears or partial meniscal defects. In addition, *in vitro* studies are currently being performed to evaluate the feasibility of a cell-based approach using this scaffold (de Mulder *et al.*, 2013). In particular, addition of TGF-β seems to be mandatory in order to promote MC proliferation and distribution throughout the construct (de Mulder *et al.*, 2013).

Kobayashi and co-workers described a cell-free total meniscus replacement model based on high water content PVA hydrogel in a rabbit model (Kobayashi *et al.*, 2003; Kobayashi *et al.*, 2005). Authors reported successful results in terms of chondroprotection. In particular, they proposed this strategy as salvage procedure for young athletes since it could allow for early return to athletic activity. However, some concerns have been raised regarding implant long-term durability, safety and also on the fixation method. Another interesting cell-free hydrogel approach has been reported by Kelly *et al*. (2007), consisting of the use of a hydrogel-based implant that was secured and reinforced by sutures running circumferentially through it. However, significant cartilage degeneration and implant failure were seen at 1 year, and overall performance was worse than with allograft transplantation. The authors hypothesised that the source of graft failure arose from the size mismatch, the inadequate peripheral fixation of the hydrogel implant, or the structural composition of these particular implants.

Mandal *et al*. (2011a) described the development of a tissue engineered meniscus based on a tri-layered silk fibrous protein scaffold and human expanded fibroblasts, seeded in the outer part, and human expanded chondrocytes, seeded in the inner part. According to the authors, its versatile processability, outstanding mechanical properties and biocompatibility make silk fibrous protein an ideal biopolymer for meniscus regeneration purposes. The rationale of using a tri-layered scaffold was to duplicate the native meniscus pores architecture. Fibroblasts and chondrocytes were seeded in each scaffold layer separately and cultured for 28 d. The resulting tissues displayed biochemical and biomechanical properties consistent with those of the native human meniscus. In a second study, the same authors reported the use of human MSC in such tri-layered scaffold showing native-like

compressive properties and tissue structure (Mandal *et al.*, 2011b). However, the three layers should be combined firmly before an *in vivo* application is planned: the authors hypothesised that cell-deposited ECM and cell migration, eventually supplemented with stitches or a rivet approach (e.g., using silk cylindrical pieces pushed into holes through the layers) can help to keep together the three layers.

An interesting and clinically-relevant approach is represented by image-guided tissue engineering of critically-sized whole meniscus constructs. In particular, because of its complex geometry, meniscus engineering can benefit from computer-aided design and tissue injection moulding techniques. Ballyns *et al*. (2008) demonstrated the feasibility of this approach by engineering a critically sized meniscus with bovine and ovine fibrochondrocytes suspended in agarose hydrogel. Even if the cells were not of human origin, this is an important proof of principle, opening new possibilities in meniscus tissue engineering.

A further innovative strategy is represented by the development of a self-assembled, scaffold-free engineered meniscal tissue (Aufderheide and Athanasiou, 2007). According to this strategy, high-density co-cultures of MSC and fibrochondrocytes have been performed in ring-shaped moulds. This approach maximises cell-to-cell contact and interaction between differentiated fibrochondrocytes and undifferentiated MSC. An interesting finding of this study was that the tensile modulus was proportional to the percentage of fibrochondrocytes employed. However, a 50 % ratio of cells displayed overall better results. Scaffold-free constructs were also compared to cell-loaded PGA scaffold and were stiffer and stronger in tension with circumferential fibres similar to those of native tissue. On the other hand, cell-loaded PGA scaffold did not present a defined fibre direction. The authors suggested that the geometric constraint imposed by the ring-shaped, non-adhesive mould guided collagen fibril directionality and, thus, influenced mechanical properties. In another work, authors showed that the mechanical properties of a scaffoldless, self-assembled meniscus tissue, obtained by culturing bovine articular chondrocytes and meniscal cells in elliptical agarose wells, can be improved by addition of TGF-β1 and chondroitinase-ABC (Huey and Athanasiou, 2011). In particular, in this study 20 million cells were used to engineer a rabbit-sized meniscus tissue. Although this method is very promising, the large number of cells needed represents a limitation to the clinical application of this approach, which, anyway, does not exclude the use of a biomaterial as augmentation to reach a clinically-relevant size with a reasonable number of cells (Makris *et al.*, 2011).

Although promising, results of recent experimental studies prompt for new strategies for total meniscus engineering as mere cell seeding does not result typically in an improved outcome and chondroprotection (Kon *et al.*, 2012). In addition, the ideal biomaterial for meniscus tissue engineering has still to be developed. For these reasons, alternative and more effective methods are desirable. In this regard, a critical tool to develop more functional engineered tissues, possibly leading to improved outcomes, is represented by bioreactors.

Bioreactors for meniscus engineering

Engineering of critically sized cellular grafts using standard static culture conditions is typically challenged by inefficient mass transport to the internal regions of the construct, ultimately resulting in cell death and necrosis in the tissue core (Martin *et al.*, 2004). A possible solution to this issue is offered by bioreactor-based dynamic culture techniques, allowing for medium perfusion through the pores of the scaffold or convective media flow around the construct, thus overcoming diffusional transport limitations (Wendt *et al.*, 2009). In the context of meniscus engineering, it was reported that hydrodynamic forces generated by a spinning impeller could result in the formation of bizonal tissues, resembling the structure and function of native meniscus (Marsano *et al.*, 2006). In this study, medium mixing around the constructs imparted orientation of the extracellular matrix molecules in an outer zone and at the same time an enhanced mass transport in the inner zone. This resulted in the formation of circumferential collagen fibres in the peripheral region, associated with higher stiffness in tension, and in increased amounts of GAGs in the central region, associated with higher stiffness in compression. The range of effective mixing intensities, generated by different impeller speeds and quantified by the corresponding Reynolds numbers, was further investigated using anatomically shaped engineered constructs (Ballyns *et al.*, 2010). The findings indicated that fluid mixing can be optimised to modulate the spatial heterogeneity of engineered menisci, as well as the correlated mechanical properties.

Compressive deformation or hydrostatic pressure have alternatively been used to enhance the structure and function of engineered meniscus tissues. Dynamic compression of constructs based on micro-channelled scaffolds resulted in aligned cell layers and collagen fibres (Martinez *et al.*, 2012), while hydrostatic pressure combined with TGF-β1 increased collagen and GAG deposition by meniscus cells, ultimately leading to enhanced compressive properties (Gunja and Athanasiou, 2010). Cyclic tension specifically stimulated collagen I mRNA expression and protein synthesis, but had no effect on collagen II, aggrecan, or osteocalcin mRNA levels resulting in enhanced fibrochondrocyte-like differentiation of bone marrow-derived MSC (Connelly *et al.*, 2010). Combinatorial modes of mechanical stimulation, including tension-compression loading (Huey and Athanasiou, 2011) or perfusion and cyclic compression (Petri *et al.*, 2012), were also reported to additively increase matrix production and tissue mechanical properties.

The availability of dynamic culture systems allowing generation of tissue grafts with superior mechanical properties raises the question of "how good is good enough?", and thus what is the target level of functionality which is required to support a superior clinical outcome. Indeed, an increased level of maturation of engineered meniscus grafts would require not only more complex culture modalities, but also likely longer culture durations, which in view of a clinical translation would be reflected in higher manufacturing costs. Addressing this critical issue requires one to identify a match between the properties of

a meniscus graft and a suitable regime of post-operative rehabilitation. In this regard, the use of bioreactors applying regimes of forces mimicking those at the site of implantation offers the opportunity to investigate which structural and functional properties are sufficient to tolerate certain loading regimes that would be experienced by the graft upon implantation, or *vice versa* which loading regimes are compatible for grafts of a defined functionality (Dèmarteau *et al.*, 2003).

The introduction of bioreactor systems in the manufacturing of cellular grafts can not only be exploited to reach higher levels of tissue organisation and mechanical functionality, but also to reduce operator handling, automate processes and ultimately standardise the quality of the product (Martin *et al.*, 2009). A bioreactor-based manufacturing would thus be critical to make tissue engineered products available at an industrial scale and quality, similar to what has been achieved in other better established biotechnological sectors (e.g., for production of antibodies, vaccines, and recombinant proteins).

Finally, the use of state-of-the-art monitoring and control systems for relevant culture parameters, which is a standard feature of classical 'fermenters', would introduce the important advantage of a well-defined environment in the engineering of meniscus tissues. The availability of controlled culture conditions is crucial to test the effect of specific factors on the development of a meniscus tissue and would thus support a better understanding of regenerative processes at a cellular and molecular level (Rouwkema *et al.*, 2011). Ultimately, this knowledge will be critical to identify factors that may induce *in vivo* regeneration and thus instrumental to operate the expected transition from the classical tissue-engineering approaches to the more modern concepts of regenerative medicine, relying on our body as the "*in vivo* bioreactor" for *in situ* tissue production.

Conclusion

Meniscus tissue is a crucial player in knee homeostasis. Re-establishing its integrity after injury is now considered a mandatory approach in knee surgery. However, current procedures result in variable outcomes while experimental strategies hold great potential to address this relevant clinical challenge. Cell-based biological bonding studies showed the possibility to improve the healing of meniscus tears treated with sutures, which would normally heal only in the red-red zone. However, the lack of efficient intra-operative systems for the isolation of autologous cells impedes its clinical application. Tissue engineering strategies showed several biological implants trying to address the regeneration of a part or the whole meniscus tissue when meniscectomy has to be performed. None of these cell-based strategies has entered clinical practice to date, since results on large animal studies have been controversial with no clear benefits. Functional engineered tissue has not been demonstrated to date upon orthotopic implantation. In fact, the relevance of engineered tissue generated ectopically (e.g., in subcutaneous pouches of nude mice) is limited and more pre-clinical evidence in large animals orthotopic models is desirable. In addition, the ideal biomaterial characterised by appropriate mechanical properties and providing adequate environment to cells for tissue regeneration has still to be described. However, new technologies and advancements in molecular biology, genetics and bioengineering research have the potential to foster meniscus research towards the solution of this clinical challenge.

Acknowledgments

The authors thank Dr. Alessia Di Giancamillo for the expert help in preparing Fig. 1 and Dr. Rosa Ballis for drawing Fig. 2. We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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