Engineering anisotropic meniscus: zonal functionality and spatio-temporal drug delivery

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Abstract

Human meniscus is a fibrocartilaginous structure that is crucial for an adequate performance of the human knee joint. Degeneration of the meniscus is often followed by partial or total meniscectomy, which enhances the risk of developing knee osteoarthritis. The lack of a satisfactory treatment for this condition has triggered a major interest in drug delivery and tissue engineering strategies intended to restore a bioactive and fully functional meniscal tissue. The aim of this review is to critically discuss the most relevant studies on spatio-temporal drug delivery and tissue engineering, aiming for a multi-zonal meniscal reconstruction. Indeed, the development of meniscal tissue implants should involve a provision for adequate active molecules and scaffold features that take into account the anisotropic ultrastructure of human meniscus. This zonal differentiation is reflected in the meniscus biochemical composition, collagen fiber arrangement and cell distribution. In this sense, it is expected that a proper combination of advanced drug delivery and zonal tissue engineering strategies will play a key-role in the future trends in meniscus regeneration.

Keywords

Meniscus, drug delivery, growth factors, zonal reconstruction, tissue engineering, anisotropic tissue regeneration

Impact Statement

Meniscus degeneration is one of the main causes of knee pain, inflammation and reduced mobility. Currently used suturing procedures and meniscectomy are far from being ideal solutions to the loss of meniscal function. Therefore, drug delivery (DD) and tissue engineering (TE) strategies are currently under investigation. DD systems aim at an *in-situ* controlled release of growth factors, whereas TE strategies aim at mimicking the anisotropy of native meniscus. The goal of this review is to discuss these two main approaches, as well as synergies between them that are expected to lead to a real breakthrough in the field.

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1. **Introduction**

The human knee meniscus is a C-shaped anatomical structure made of fibrocartilage. It absorbs the shock generated during motion and distributes the compressive load derived from the femur to the underlying hyaline cartilage and bone. The meniscus also contributes to joint stability, lubrication and proprioception, i.e. the perception of the body position and movement[1]. Due to its limited vascularization and poor cellularity, the self-healing capacity of the meniscus following an acute injury or a chronic degenerative process is minimal. Therefore, surgical intervention using sutures or (partial or total) meniscal removal is often needed. Although these procedures are currently part of the clinical routine, the limited success rate of suturing and the increased risk of osteoarthritis upon meniscectomy, have triggered a growing interest in the **tissue engineering** (TE) field. A typical TE approach involves the use of (i) a biomaterial, capable to provide a transitory mechanical support and a cell-friendly three-dimensional (3D) environment, (ii) cells (seeded or recruited from the surrounding healthy tissue), and/or (iii) active factors that can augment the cell-mediated tissue repair.

An emerging concept in meniscal TE is the bio-mimicry of meniscal anisotropy. Indeed in human meniscus, the extra-cellular matrix (ECM) composition, the cells distribution and the collagen fiber arrangement vary in a spatially-relevant manner, being these architectural features crucial for the meniscus function[2]. The three main strategies currently exploited to promote anisotropic and functional meniscal regeneration are: (i) controlled delivery of growth factors via drug delivery (DD) systems, (ii) mimicry of meniscal ultrastructural features via modern TE technologies, and (iii) a combination of the previous two. The aim of this review is to provide a full and critical picture on this topic. This, to the best of our knowledge, has not been done before.

The active factors used in meniscus regeneration are mainly **growth factors** (GFs) that are meant to promote tissue repair by different mechanisms, e.g. by stimulating cell migration, proliferation and tissue ingrowth. However, one of the main challenges for the clinical application of GFs is their rapid clearance after *in vivo* injection. To circumvent this

limitation, long-lasting controlled **DD strategies** are being investigated. In this context, research has focused on the *in-situ* controlled delivery of GFs and polynucleotides. The first part of this review presents the approaches that have been reported for the controlled delivery of GFs. Other approaches based on the use of polynucleotides have already been described in recent reviews[3-5].

The second part of this review focuses on modern **TE technologies** that allow a spatially-controlled scaffold architecture and distribution of different cell types. These TE strategies involve the use of (i) advanced manufacturing technologies (3D printing and electrospinning) for the fabrication of zonally-variant architectures, (ii) post-fabrication zone-dependent mechanical stimulation, able to mimic the complex mechanics of the *in vivo* osteoarticular environment, and (iii) spatially-selective seeding of different cell types in the scaffold, inspired by the cellular variation of the different meniscal zones.

The fabrication of scaffolds that resemble zonal tissue variations is a recent trend in the field of meniscus TE, and the majority of the reported strategies lack a biological augmentation via GF delivery systems. Notwithstanding, a new generation of scaffolds is starting to combine biological augmentation via GF delivery systems with zonal TE approaches. In these scaffolds, discussed in the third part of this review, the DD system is part of a TE construct that attempts to recapitulate the native tissue architecture. In this context, modern TE technologies may allow a spatially-controlled distribution of DD systems within a scaffold displaying a zonally-variant architecture. We envisage that such approaches have a much higher potential to reconstruct a fully functional, anisotropic meniscus and will lead to a real breakthrough as the field evolves.

2. Human knee meniscus

2.1. Biochemistry and composition

As stated above, human meniscus has an anisotropic structure, which presents zonal variations in terms of vascularization, cell type distribution, ECM composition and collagen

fiber arrangement (Figure 1). Three main zones have been identified, i.e. an innervated and vascularized outer region (also called red region), a non-innervated and avascular inner region (white region) and an intermediate region (red-white region).

The meniscal ECM is composed of water (72%), collagen (22%), glycoproteins (3.5%), and a low percentage of proteoglycans (0.5%), although their content may vary based on the age and pathological conditions[1, 2]. As depicted in figure 1, collagen fibers have a zone-dependent organization, crucial for the load transmission, which consists of a circumferential fiber arrangement more pronounced in the deeper layers and a radial assembly more pronounced in the superficial ones[1]. Collagen type I is the most abundant type of collagen in the outer region. In contrast, in the inner region, both collagen type I (typical collagen of scar tissue, ligaments and tendons) and type II (typical collagen of hyaline cartilage) are present in relatively high percentages (40 and 60%, respectively)[1]. Regarding the cell types, elongated fibroblast-like cells are present in the outer zone, whereas rounded chondrocyte-like cells, referred to as fibrochondrocytes, are embedded in the ECM of the inner zone. In the superficial layers, the presence of progenitor cells with a possible role during healing processes has been reported[2].

Given that the human temporomandibular joint disk (TMJD) and the anulus fibrosus of the intervertebral disk (IVD) are also fibrocartilaginous structures, we have included in this review the relevant studies based on DD strategies for TMJD and IVD tissue engineering that could, potentially, be applied also to meniscus regeneration. More details about the anatomy, biochemistry and functions of meniscus, TMJD and IVD, can be found in previous articles[1, 2, 6-8].

2.2. Pathology and treatments

Meniscal tissue degeneration typically occurs after an acute injury due to excessive axial and rotational load applied on the knee joint (traumatic or acute tears), or upon repetitive motion cycles during normal physical activity (degenerative tears)[1, 9]. The self-healing capacity of damaged meniscus is very limited, due to the scarce cell population and the low degree of

vascularization. Moreover, cell migration to the damaged tissue, which is an essential step for tissue healing, may be hindered by the stiffness and the high density of the meniscal tissue[10].

The treatment of the meniscal tears involves surgical suturing, and partial or total meniscectomy[11, 12]. Unfortunately, it has been estimated that 25% of the surgical interventions leads to an incomplete healing and 15% fails completely[11]. For lesions that cannot be repaired by suturing, such as complex degenerative tears, the treatment is based on partial or total meniscectomy.

Nowadays, after decades performing meniscectomies, clinicians have become aware of the risk of post-meniscectomy osteoarthritic changes and reduced physical capability of the knee joint. Although these side effects depend on the general physio-pathological condition of the joint[11], the current trend is toward minimizing the use of meniscectomy. DD and TE approaches may enable tissue regeneration of meniscal tears and may offer a potential treatment even after a partial or total meniscectomy, as discussed in the following sections.

3. In situ delivery of growth factors for meniscus regeneration

3.1. Growth factors for meniscus regeneration

The active factors used in meniscus regeneration, for the re-establishment of a healthy and functional tissue after an acute or chronic degenerative process, are mainly GFs (i.e. proteins or their encoding polynucleotides). GFs are intended to promote meniscus regeneration by: (i) stimulating cell migration, especially needed for acellular scaffolds to recruit endogenous cells towards the defect area[3, 13]; (ii) promoting cell proliferation and chondrogenic differentiation[3, 13, 14], (iii) activating anabolic pathways involved in meniscal ECM synthesis[3], (iv) supporting angiogenesis[15], and/or (v) stimulating a controlled tissue degradation to enhance cell penetration[10].

Of all the GFs, the transforming growth factor-β (TGF-β) superfamily (particularly TFG-β1, TFG-β3, bone morphogenic protein-2 (BMP-2) and bone morphogenic protein-7 (BMP-7)), the basic fibroblast growth factor (bFGF), and the insulin-like growth factor-1 (IGF-1) are

the ones that have been most thoroughly investigated for meniscal regeneration. For an extensive reading about the GFs employed in meniscal TE we refer to a recent review on the topic[14].

Considering the heterogeneous structure of the meniscus and the different cell types involved in its formation, achieving a successful meniscal regeneration poses a big challenge, that may require the delivery of multiple GFs with different release kinetics. In fact, examples of synergistic effects between GFs, such as bFGF and TGF-β3; IGF-1 and TGF-β1; connective tissue growth factor (CTGF) and TGF-β3 have been reported[14], and the exploitation of these synergies seems to be the direction this field will go as it evolves.

3.2. Drug delivery systems

Given the limited stability of the GFs, their application in an *in vivo* setting for meniscus regeneration would require multiple knee injections, a process whose translation into the clinical practice is far from ideal. To overcome this, DD systems have been investigated for the *in-situ* controlled release of GFs.

When developing a novel DD system, commonly encountered challenges involve loading efficiency, adequate release kinetics and maintenance of the **functionality of the cargo** during the release time frame[16-18]. The latter aspect is of great importance especially for proteins, such as GFs, that can lose their activity under certain environmental conditions (pH, temperature, radiations, mechanical stress, organic solvents, etc.)[19-21]. Therefore, in the case of protein-loaded delivery systems, mild fabrication techniques and post-fabrication protein loading are often preferred[22, 23].

Another challenge that arise when developing DD systems for TE applications is to adapt the **prolonged drug release** profile to the slow process of neo-tissue formation, maturation and remodeling; a process that usually takes from weeks to months. In the case of certain biomaterials, the release kinetics of GFs can be controlled by adjusting material properties (e.g. particle size and lactide/glycolide ratio for poly-(lactic-co-glycolic acid) (PLGA)

particles)[24, 25]. Alternatively, the payload can be covalently bound to the biomaterial to achieve a prolonged release[22].

An emerging strategy in DD for TE is the spatio-temporal release of active molecules, which enables to precisely control the location and the time-frame of the drug release[26]. Due to the anisotropic structure of the meniscus, a **region-dependent release** of GFs could be crucial to accomplish the zonal tissue variation present in the native meniscus. Spatially-controlled positioning of active factors can be achieved using 3D building technologies, such as 3D printing (discussed in section 5.1). Moreover, the temporal control over the release of one or more factors is crucial as synergistic effects can be obtained by a dual or multiple release of GFs[27].

The following sections describe GF delivery strategies that attempt to heal mainly meniscal tears and other small fibrocartilaginous defects. Studies aiming at the regeneration of meniscus, TMJD and IVD, grouped according to the nature of the DD system are discussed below.

3.2.1. Scaffolds as DD systems

Scaffolds in TE can be defined as 3D porous structures capable of harboring living cells and promoting new tissue formation. For this purpose, scaffolds must fulfill certain requirements in terms of mechanical and bioactive properties. Often, scaffolds with adequate mechanical properties do not represent an ideal milieu for cell proliferation and differentiation, whereas those showing a cell-friendly microenvironment may not withstand the high load the meniscus undergoes *in vivo*[28]. Finding an adequate balance between these two requirements has been one of the biggest challenges for researchers in the field. Besides, bio-functionalization of scaffolds via GF incorporation appears to be a promising strategy to modulate tissue healing and regeneration. Among the different scaffolds that have been investigated in TE for fibrocartilage regeneration[29], only those dealing with GF delivery are discussed herein. As described in the following sections, GF-loaded scaffolds have been developed by

incorporating the GF directly in the scaffold matrix or by engineering the GF in a multi-component delivery system (i.e. microparticle-loaded scaffolds).

3.2.1.1. Scaffolds based on extracellular matrix (ECM) components

In an attempt to mimic the natural microenvironment of meniscal tissue, researchers have employed different macromolecules present in the ECM, such as collagen, hyaluronan, and fibrin, to develop scaffolds with biomimetic properties. These biomaterials form 3D hydrogel networks that are particularly suitable to carry GFs and to support cellular growth and proliferation. However, due to their poor mechanical properties, the use of these hydrogels as carriers for GFs has been restricted to the regeneration of small meniscal and fibrocartilaginous defects. The results obtained with specific materials are described below.

Collagen

Collagen, the most abundant protein in the ECM, has cell-binding sites that makes it an interesting material for use in the fabrication of scaffolds[30]. In fact, a meniscus scaffold based on collagen, namely Collagen Meniscus Implant (CMI®), is available on the market since 2016. In order to improve the capacity to attract and differentiate cells, collagen scaffolds have been loaded with GFs for the treatment of fibrocartilage defects (Table 1). For example, Suzuki *et al*. tested the effect of BMP-2-loaded collagen type I scaffolds in a leporine TMJ injury model and observed a dose-dependent tissue healing[31]. However, scaffolds prepared only with collagen tend to show poor mechanical properties, for this reason this material is usually used in combination with other components, as discussed in section 3.2.1.4.

Gelatin

Gelatin, obtained by the basic or acidic hydrolysis of collagen, also possesses cell-binding sites and it is currently investigated as a natural ECM-mimicking building block[32-34]. In general,

gelatin-based biomaterials can be made from gelatin of different isoelectric points, which can load cationic or anionic GFs via electrostatic interactions[30, 35].

Gelatin has also the capacity to form thermosensitive hydrogels[30], however, these hydrogels have to be crosslinked in order to improve their mechanical properties. For GF delivery, gelatin hydrogels have been crosslinked mainly via the chemical reaction of methacrylated gelatin (gelMA) in the presence of UV light and a photo-initiator[36, 37], or via glutaraldehyde[38, 39] (Table 1). For example, TGF-β3-loaded hydrogels based on gelMA showed a significant chondrogenic differentiation of adipose-derived stem cells during 21 days. About 80% of the GF was released during this period[36]. Glutaraldehyde-crosslinked gelatin hydrogels have also been loaded with single GFs, such as FGF-2[39], or with GFs cocktails, such as platelet-rich plasma (PRP)[38], an endogenous cocktail of GFs and platelets widely studied for musculoskeletal tissue regeneration. In both cases, positive outcomes were reported in terms of tissue repair in rabbits[38, 39]. In the studies describing glutaraldehyde-crosslinked gelatin matrices, anionic gelatin (pI=5.0) was used, and loading of the cationic GFs was performed onto the pre-formed matrices. This post-fabrication loading technique avoided GF deactivation and chemical immobilization[35].

Only one of the studies dealing with gelatin-based hydrogels described, reports the release kinetic analysis of the loaded factor[36]. All other studies assumed a sustained release of the loaded factor, an assumption based on the results previously reported that indicated a degradation-based release mechanism[35]. In our opinion, although the *in vitro/in vivo* correlation is far from ideal, the *in vitro* release studies should be a must when using GFs, since their kinetics are crucial for a successful outcome of the biomolecule.

In general, gelatin scaffolds seem to be preferred to collagen scaffolds in meniscal TE, likely due to the higher versatility and easier chemical modification of gelatin over collagen.

Fibrin

Fibrin is a protein involved in blood clotting that is being currently investigated for biomedical applications[40, 41], meniscus regeneration among others (Table 1). Fibrin is prepared from fibrinogen and thrombin, and it has the advantages of being biodegradable and of having both a rapid *in situ* gelation and the capacity to stimulate the endogenous healing response[42]. Moreover, because of its GFs selective binding, it is considered an appealing biomaterial for DD in TE. For example, He *et al*. demonstrated that CTGF-loaded fibrin hydrogel supported the tissue repair of the avascular meniscus in rabbits[43]. The structural properties of the fibrin systems can be tuned by changing, for example, the concentration of its precursors, which will also affect the release kinetics of the entrapped GFs[42]. Nevertheless, the main drawback of using fibrin is its poor mechanical stability. To overcome this limitation, platelet-rich fibrin (PRF) can be used. Unlike fibrin clots, PRF is a gelatinous material that can be produced as mechanically stable membranes, as reported by Kemmochi *et al*., who found that PRF promoted meniscal healing in a clinical trial[44]. Remarkably, PRF is the only autologous blood-derived material that is a source of GFs (e.g. PDGF-BB, TGF-β1, IGF-1) and that has, at the same time, scaffolding properties. Moreover, PRF releases GFs in a more sustained way (up to 10 days) than PRP[45].

3.2.1.2. Scaffolds based on other natural polymers

Apart from the ECM-derived macromolecules, other natural polymers, such as chitosan (CS) and silk fibroin, have been employed for GF delivery in meniscus TE (Table 2). CS is a biodegradable and positively charged polymer with mucoadhesive, hemostatic and antimicrobial activity[46]. The group of Buschmann described chitosan freeze-dried implants for the sustained release of PRP[47, 48]. The implants promoted platelet activation, granule secretion, and a sustained release of epidermal growth factor (EGF) over a 7 day period[48]. *In vivo* assays after subcutaneous implantation of CS/PRP scaffolds in rabbits revealed the capability of these implants to persist for at least 6 weeks *in situ* and to evoke cell migration, as well as the formation of a collagen-rich tissue[48]. Positive outcomes in terms of scaffold

integration and tissue healing were also observed in an ovine model after orthotopic implantation[47].

Silk fibroin is an attractive biomaterial due to its biocompatibility, mechanical and degradative properties. Silk fibroin sponges have been loaded with platelet-rich gel (PRG), a PRP-derived gel, and supported a sustained release of TGF-β1 over a 20 day period[49]. Orthotopic implantation of the sponges in a total meniscectomy model revealed cell infiltration, tissue matrix synthesis and reduction of cartilage degeneration at 3 months.

3.2.1.3. Scaffolds based on polyesters

Due to their biodegradability and biocompatibility, synthetic polyesters have been widely used to prepare 3D porous constructs in TE. Scaffolds made of polyesters generally offer reproducibility, versatility and adequate mechanical properties[29]. Moreover, the most commonly used polyesters have a thermoplastic behavior, which enables their processing via 3D printing[50, 51]. However, the main limitation of synthetic polyesters for TE application is their lack of domains that can be recognized by cells, and therefore, unlike most of the natural polymers, they have poor cell-instructive properties[52].

PLGA-based scaffolds offer the possibility to tune their hydrolytic degradation profile and the release kinetics of the entrapped proteins by varying the polymer characteristics (e.g. molecular weight, lactide/glycolide ratio), as well as the morphological properties of the system (e.g. porosity, scaffold density)[53, 54]. In fibrocartilage TE, PLGA scaffolds have been used to deliver PRP-derived GFs[55]. Kwak *et al*. demonstrated that PRP adsorbed onto PLGA meshes favored cell attachment of human articular chondrocytes and supported meniscal healing in a subcutaneous murine model[55] (Table 3). Other studies described scaffolds fabricated by ethanol-sintering PLGA microspheres, previously loaded with TGF-β1 and BMP-2[56, 57]. Unfortunately, the percentage of GF released was lower than 20%, suggesting its inactivation within the polymer matrix. This could be attributed to the well-known phenomenon of protein inactivation during the polymer degradation process[53].

Polycaprolactone (PCL) has a slow degradation rate (from months to years), excellent mechanical properties and an easy-to-handle thermoplastic behavior[58]. In fact, 3D printing of PCL has been used to fabricate scaffolds that host GF-releasing microparticles for meniscus and TMJ disc regeneration[25, 59, 60], as further described in section 5.1.

3.2.1.4. Scaffolds based on hybrid materials

The success achieved by combining multiple components is underlined by the fact that the two meniscal implants available in the clinics, i.e. CMI® and Actifit®, are indeed hybrid materials. Collagen Meniscus Implant (CMI®, Ivy Sports Medicine, Germany), commercialized in Europe since 2016, is based on purified bovine collagen I and glycosaminoglycans; whereas Actifit® (Orteq Ltd., UK) contains polycaprolactone and polyurethane[61-63]. Moreover, NUsurface® (Active Implants Israel Ltd., Israel), made of polyethylene and polycarbonate-urethane, is in the process of FDA approval for total meniscus replacement[61]. Although none of these scaffolds has been loaded with GFs, several GF-loaded hybrid scaffolds are currently under investigation.

Hybrid scaffolds for meniscal GF delivery have been made by combining different natural polymers, or natural and synthetic polymers (Table 4). The combination of cell-binding macromolecules (e.g. collagen) with highly charged natural polymers (e.g. negatively charged polysaccharides), which support electrostatic interactions with GFs, has been investigated. For example, scaffolds based on collagen and chondroitin sulfate have been designed for the sustained release (up to 21 days) of PRP GFs (IGF-1, PDGF-AB and TGF-β1)[64]. Moreover, Matrigel, a gelatinous mixture of proteins secreted by Engelbreth-Holm-Swarm mouse sarcoma cells, has been mixed with hyaluronic acid for the delivery of VEGF[65]. Furthermore, scaffolds based on bovine meniscus-derived decellularized matrix have been chemically functionalized with heparin to efficiently entrap PDGF-BB[66]. In the latter study, the sustained release of PDGF-BB over the course of two weeks promoted cell migration and scaffold integration within the avascular meniscus, in an *in vitro* bovine meniscus organ culture.

Another trend is the use of hydrogels based on chemically cross-linked natural derivatives and synthetic polymers. This strategy exploits the biocompatibility and bioactive properties of natural polymers, the reproducibility, versatility and non-immunogenicity of synthetic polymers, and the mechanical stability given by the chemical cross-linking. In these systems, polyethylene glycol seems to be the synthetic polymer most commonly used. For example, a system composed of thiol-modified hyaluronic acid (HA-SH) crosslinked with polyethylene glycol diacrylate (PEG-DA) was used to load PDGF-BB[67]. An *in vivo* study performed in rabbits concluded that this system decreased disc degeneration and cell apoptosis[68]. Details about PDGF-BB release kinetics from HA-SH/PEG-DA hydrogels, absent in this study, can be found in the work of Peattie *et al.*[69]. In this article, a first-order release kinetics was observed for all GFs in the study (VEGF, bFGF, TGF-β, keratinocyte GF (KGF), Angiopoietin-1 (Ang1), PDGF). Moreover, thermosensitive, injectable hydrogels based on glycol CS and multialdehyde-functionalized PEG have been loaded with TGF-β1, and supported fibrochondrogenic differentiation of bone mesenchymal stromal cells *in vitro* and fibrocartilaginous tissue growth *in vivo*[70].

3.2.1.5. Microparticle-loaded scaffolds

Aiming at the regeneration of small defects, injectable microparticles have also been proposed to deliver *in situ* GFs for meniscus regeneration. To reduce the clearance of the microparticles at the injection site and to further modulate GF release, microparticles are often loaded into hydrogels[71]. For example, PLGA particles embedded into fibrin hydrogels have been investigated for GF delivery into meniscal tears. A sequential release of CTGF and TGF-β3 was achieved by entrapping CTGF, as such, in the fibrin gel and TGF-β3 in the polymeric particles[72, 73]. This differentiated delivery of GFs supported the healing of avascular meniscal tears in an *in vitro* bovine explant model and in an *in vivo* leporine model.

GF-loaded PLGA particles have been also included in more sophisticated 3D printed meniscal implants with zonal distribution, as discussed in section 5.1.

3.2.2. Film-coated suturing wires

Another possibility for the local delivery of GFs is to impregnate the wires used in meniscus surgery with these bioactive molecules. For example, poly-(lactic acid) (PLA)-coated suturing wires has been reported for the local administration of pro-angiogenic vascular endothelial growth factor (VEGF) (Table 5)[15, 74]. Petersen *et al*. investigated the potential of this approach to promote healing of meniscal tears in the avascular zone, where the poor spontaneous healing is often attributed to its lack of vascularization[15]. Results show that VEGF/PLA-coated sutures induced accumulation of endothelial cells in the tissue, but did not promote vascularization nor meniscal healing in an ovine *in vivo* model[15]. Similar outcomes were reported by Kopf *et al*. using (PLA)-coated suturing wires in a similar *in vivo* setting[74]. Lack of vascularization may be explained by the low amount of VEGF used and/or by its fast release kinetics, mainly governed by a pore diffusion-based mechanism[74, 75]. Moreover, it has been reported that local administration of VEGF up-regulates degradative enzymes, i.e. metalloproteinases (MMPs), and reduces the levels of MMPs-inhibitors, which in turn may hamper the VEGF-mediated tissue regeneration[15, 74]. Early work using suture coatings based on glutaraldehyde-crosslinked gelatin for sealing meniscal tears has also been reported. For example, a positive effect on meniscal cell proliferation was observed when FGF-2 was delivered using this strategy in an *in vitro* organ culture[76].

To sum up this section, scaffolds have been the GF delivery system for meniscal regeneration most frequently used. The most promising scaffolds are hydrogels based on ECM derivatives (e.g. collagen and gelatin) because they offer a biomimetic microenvironment that supports cell infiltration and differentiation, and their mechanical properties can be improved thanks to specific cross-linking reactions. On the other hand, scaffolds based on polyesters, despite providing adequate mechanical properties, have been scarcely employed so far. This is likely due to their poor cell-material interactions and the possibility of GF inactivation during polymer hydrolytic degradation. Another path also receiving a lot of attention is the development of hybrid scaffolds that aim at synergizing the advantages of two

or more materials. Finally, the use of microparticle-loaded scaffolds, hardly explored so far, represents a promising strategy that combines the benefits of the scaffolding material with the flexibility that microparticles offer in terms of GFs controlled delivery.

It is important to note that the aim of the majority of the reported studies is to deliver GFs in order to heal meniscal tears and other small fibrocartilaginous defects. For this reason, the described biomaterials are mainly designed as DD systems (as opposed to TE constructs), and limited emphasis is given to the scaffold architectural and mechanical requirements. On the contrary, when aiming at a biofunctional meniscus regeneration, especially after total or subtotal meniscectomy, scaffold requirements are much more demanding and they have to take into account the complexity of the native tissue (regionally-variant biochemical and cellular composition) and the mechanically challenging location. To this purpose, advanced manufacturing technologies and synergies between these technologies and DD strategies are being explored, as discussed in the following sections.

4. Recent advances in meniscal zonal reconstruction

The intensive research on meniscus anisotropy of the last decades has boosted technological approaches that exploit this knowledge to develop novel therapeutic solutions[77]. In this context, modern techniques capable of designing scaffolds at the micro- and nanoscale are expected to make a huge impact in the field. This is the case of **3D fabrication technologies**, such as 3D (bio)printing and electrospinning, that can generate zone-dependent structural features. 3D printing is based on the layer-by-layer deposition of a biomaterial and allows a precise construct shaping in terms of overall morphology and internal structure. This means that 3D printed TE constructs not only can fit the size and the shape of the tissue defect precisely, but can also reproduce zonal variations of the native tissue[78-81].

3D fabrication technologies for zonal fibrocartilage TE currently under investigation rely on (i) mimicry of the arrangement of collagen fibers and bundles[25, 59, 60, 82-90], (ii) zone-dependent biomaterial composition[91-93] and (iii) different degree of porosity[91, 92, 94].

Besides these manufacturing technologies, the **mechanical stimulation** of pre-formed, cell-seeded TE constructs in bioreactors is another strategy to obtain zonal fibrocartilage differentiation[89, 93, 95-97]. Modern bioreactors can produce mechanical loads in compression and tension that mimic the mechanically challenging environment of the articular joint. A zone-dependent mechanical stimulation, such as the one acting on the meniscus (tension-dominating in the outer zone and compression-dominating in the inner zone), can result in zonal cellular and tissue differentiation[97].

Furthermore, the region-specific use of **different cell types** has also been explored for zonal tissue regeneration. Different cell types are responsible for the synthesis of different ECM components and therefore of different types of tissue.

On the next section, we discuss relevant studies in which zonal meniscal reconstruction has been attempted by means of manufacturing technologies, mechanical stimulation, and cell seeding techniques.

4.1. Manufacturing technologies for zonally-variant meniscus

4.1.1. Conventional technologies for controlling porosity

Since the very beginning of the TE era, researchers realized that porosity was one of the key parameters that could determine the success of a scaffold. Indeed, **porosity and permeability** of TE constructs promote cell infiltration, tissue ingrowth, and transport of the nutrients and waste products generated during cellular metabolism. A typical porosity higher than 90% is often considered optimal, together with a pore size ranging between 10 and a few hundred micrometers, as a function of the targeted tissue. Moreover, interconnectivity between pores to form microchannels, is also a desirable feature of a TE scaffold[98].

Some of the most commonly used methods to prepare porous scaffolds from polymers include: (i) solvent casting/particle leaching, which relies on leaching out solid particles from the polymer solution; (ii) gas foaming, which involves the formation of gas bubbles within the melted polymer; (iii) thermally-induced phase-separation, based on a liquid-liquid phase separation of the polymer solution at temperatures lower than the freezing point of the solvent;

(iv) melt molding, which relies on the compression of the polymeric material and porogens at temperatures higher than the polymer glass transition temperature; and (v) cryostructuring procedures, such as cryotropic gelation and freeze-drying[98, 99].

Porosity can be controlled by properly adjusting the fabrication parameters. For example, in a study by Sarem *et al*., anatomically shaped multi-layered meniscal scaffolds made of crosslinked gelatin and CS were fabricated via the multi-step freeze-drying method (Table 6)[91]. Pore size, total porosity, as well as compressive and tensile moduli could be tuned over a broad range of values by varying the gelatin/CS ratio. Selected compositions were then used to develop laminated gradient scaffolds able to mimic the anisotropy of native meniscus[91].

Other studies highlighted that the **pore organization** (i.e. random *vs* ordered) plays a crucial role on the quality of the newly formed tissue and its remodeling[92, 94]. Formation of aligned micropores within meniscal scaffolds via cryostructuring has been reported by Stuckensen *et al.*[92]. In this study, the mimicry of inner and outer meniscus was achieved by controlling the porosity (through a zone-dependent pore size and pore alignment), and by using a zone-dependent hydrogel composition (different percentages of collagen type I, II, and chondroitin sulfate). In line with these findings, a beneficial effect of an ordered pore distribution in meniscal scaffolds was found *in vivo* in a subcutaneous model by de Mulder *et al*.[94]. Scaffolds made of PCL-PLA-polyurethane block-copolymer with different micro-pore organization (i.e. random *vs* channel-like), supported different assembly of the ingrown tissue.

No consensus has been established yet on what would be the ideal pore/channel diameter in an artificial meniscus. A study reported that the optimal pore size range for meniscal tissue ingrowth *in vivo* was between 150 and 500 μm[100]. Nevertheless, based on more recent studies, a broader range (20-550 μm) is currently being investigated for studying cell invasion and meniscal tissue ingrowth. In fact, a channel diameter <150 μm would be in line with that of collagen bundles in human meniscus (i.e. 88 μm)[94]. In general, porosity facilitates cell infiltration, mass influx/efflux, and tissue ingrowth, but the presence of big pores reduces the scaffold mechanical properties, which are crucial for the mechanically demanding

environment of the knee joint. The choice on pore size for meniscal scaffolds is currently a trade-off between different contrasting aspects, and future investigations may shed light upon this matter[101].

Apart from controlling the porosity of the scaffolds prepared by conventional methods, nowadays engineers count on technologies, such as 3D printing and electrospinning, that, not only offer the possibility of tuning the porosity, but also allow for a more accurate mimicry of the meniscus heterogeneous ultrastructure. In the next sections, we discuss relevant studies that use these technologies for meniscal TE.

4.1.2. 3D printing of zonally-variant meniscus

Meniscal scaffolds with controlled micro-architecture can be obtained by 3D printing. PCL is the most frequently used biomaterial because it can be easily processed by this method, and displays adequate mechanical properties[102]. Size and shape of the internal channels and mechanical properties of the scaffold can be modified by changing 3D printing parameters, such as fiber spacing, offset between layers, and fiber orientation (Table 7)[82]. Fiber spacing also affects the morphology of recruited cells and the organization of newly-deposited collagen. For example, according to Warren *et al*. the alignment of collagen *in vivo* (in an ectopic murine model) increased when they decreased the fiber spacing, probably because cell-scaffold interaction is facilitated in more narrow channels, and this better instructs the collagen deposition[83].

More importantly, grafts engineered by 3D printing can replicate the circumferential/radial collagen arrangement of native meniscus, and this can result in anisotropic mechanical properties, similar to those of the meniscus[60, 84, 85]. For example, scaffolds based on circumferentially/radially aligned multiwalled carbon nanotubes (CNT) were able to approximate circumferential, radial, and compressive moduli of native meniscus (120, 48 and 0.69 MPa, respectively)[84].

3D printing also allows scaffold fabrication based on two or multiple components. Therefore, meniscal constructs with a regionally-variant material composition can be fabricated. For example, in hydrogels based on reinforced alginate/acrylamide, region-specific compositions (different ratios between hydrogel and reinforcement) were used to obtain anisotropic mechanical properties[85]. Another possibility is to use dual 3D printing based on a thermoplastic material (e.g. PCL) and a hydrogel component, benefiting from the mechanical resistance of the former and the cell-friendly environment of the latter. To this purpose, constructs can be fabricated by the deposition of adjacent filaments of PCL alternating with cell-laden hydrogel[103]. Alternatively, pre-fabricated 3D printed constructs can be infused with cell-laden hydrogels. For example, Bahcecioglu *et al.* fabricated a 3D printed, anatomically-shaped meniscus implant and subsequently infused it with two different cell-laden hydrogels in the inner and outer part, to mimic the bizonal biochemical composition of meniscal ECM[90, 93].

3D printed scaffolds resembling collagen bundle organization of meniscus and other fibrocartilaginous structures have been also combined with spatio-temporal GF delivery[25, 59, 60], as further discussed in section 5.1.

Overall, the reported studies highlight the great potential of 3D printing technology to mimic the collagen bundles arrangement in fibrocartilage implants. A critical difference among the reported studies lies on the structural organization of the fibers. While some studies employed a 0/90° interlaying fiber orientation[83, 86, 93], others focused on a circumferential/radial fiber arrangement[25, 59, 60, 82, 84, 85, 89, 90]. Although the former approach can sufficiently mimic collagen bundle distribution of native fibrocartilage on a millimeter-size scale, it does not take into account the overall variation of fiber orientation on a bigger scale (Figure 2). In fact, for example, a change in fiber direction of ~160-200° over ~7-10 cm has been reported for the human meniscus, which results in circumferential fibers that run along the C-shaped meniscus structure[87]. For this reason, when aiming at the reconstruction of large meniscal defects or the whole meniscus, a circumferential/radial fiber organization that better mimics the macroscopic meniscal structure should to be adopted.

4.1.3. Electrospinning for zonally-variant meniscus

One of the main limitations of traditional 3D printing is that it does not allow the manufacturing of nano-scale fibers, like the ones present in tissue ECM[104] (Figure 3). As an alternative, the fabrication of nanofibers via electrospinning has attracted much interest[105, 106]. For example, Han *et al*. designed TE constructs made of aligned electrospun PCL nano-fibers, embedded with mesenchymal stem cells (MSCs) and meniscal fibrochondrocytes (Table 8)[107]. *In vitro* studies showed the formation of a heterogeneous neo-tissue with collagen type I and II zone-dependent deposition.

As mentioned in the previous section for 3D printed scaffolds, the orientation of the generated fibers is currently an important subject of study. For example, PLA nanofibrous scaffolds based on aligned fibers have a much higher tensile modulus compared to scaffolds based on randomly distributed fibers[108]. Moreover, the comparison of scaffolds made of PCL electrospun fibers with different orientation (0°, 90° or circumferential) revealed that, the direction of newly formed collagen and mechanical properties of the scaffolds were significantly affected by this parameter[88]. Remarkably, in scaffolds made of electrospun PCL nanofibers, circumferential alignment elicited regionally-variant mechanical properties that cannot be obtained with linearly aligned nanofibrous scaffolds[87].

Taken altogether, since bio-functional meniscal implants are likely to be achieved using a design that mimics the native tissue at nano-, micro- and macroscopic scales, cutting-edge technologies involving the combined use of electrospinning and 3D printing may represent a valid and novel strategy[104, 109].

To sum up this section, traditional porogenic technologies, 3D printing and electrospinning are currently being investigated with the aim to engineer zonally-variant meniscus. Although conventional porogenic technologies allow for the tuning of the total porosity, pore size and pore organization, 3D printing and electrospinning offer higher versatility in terms of macro-, micro- and nano-structuring. Overall, 3D printing is the most

used technique because it allows the modification of the channel size and shape by changing certain fabrication parameters (e.g. fiber spacing, layer offset, etc.), and this has a direct effect on the scaffold mechanical properties, cell morphology and organization of newly-formed collagen. Moreover, 3D printing of circumferential/radial fibers mimics the organization of collagen in native meniscus, and can result in anisotropic mechanical properties and zonally-variant tissue regeneration. Furthermore, 3D printing allows the fabrication of multi-component (e.g. gel and reinforcing material) scaffolds, which integrate a cell-friendly environment into a mechanically-resistant structure. Finally, 3D printing offers the highest versatility in terms of macroscopic scaffold shaping, which is crucial for customized therapies. Of all the materials currently employed, PCL is the most used in the 3D printing of meniscus scaffolds. The major disadvantage of 3D printing is the difficulty to fabricate nano-sized fibers, which would mimic better the endogenous collagen fibers. In this sense, electrospinning is the most suitable alternative. So far, the research on electrospun meniscal scaffolds is still limited, likely due to challenges in fabricating large scaffolds and controlling fiber orientation. Nevertheless, current research has demonstrated that linearly and circumferentially alignment can be achieved, which has a direct impact on the scaffold mechanical properties. 3D printing and electrospinning have been also used to fabricate protein-releasing meniscus scaffolds, that integrate a DD strategy into structurally-organized scaffolds (discussed in section 5).

4.2. Mechanical stimulation of pre-formed cell-seeded scaffolds for the fabrication of zonally-variant meniscus

Mechanical stimulation of pre-formed cell-seeded cartilage constructs in bioreactors, referred to as dynamic culture, is being currently under investigation with the aim to promote cell differentiation and maturation of neo-tissues *in vitro*[110-113]. In fact, cells respond to mechanical stimuli with morphological changes and production of specific signaling molecules[114]. Several studies have shown the superiority of the dynamic culture of fibrocartilage scaffolds over

traditional static culture, in terms of internal organization and mechanical properties of the newly formed tissue[93, 95-97, 114] (Table 9).

The methods to induce mechanical stimulation on meniscal implants rely on a bi-axial dynamic loading intended to simulate the compressive (generated by squeezing forces) and tensile (generated by stretching forces) stress generated in the joint during the movement. A mimicry of the complexity of meniscal mechanics has been obtained by gradually increasing both tensile and compressive stress forces from the outer to the inner zone, or by applying a tensile-dominating stimulation in the outer zone and a compressive-dominating stimulation in the inner zone[89, 93, 97]. The application of zonally variant stress forces was found beneficial for the zone-specific deposition of meniscal ECM components and the induction of cell differentiation[93, 97]. For example, in the study of Bahcecioglu *et al*., anatomically shaped 3D printed PCL scaffolds were impregnated with porcine fibrochondrocytes-laden agarose and gelatin hydrogels in the inner and the outer zone, respectively. The combination of zonally-variant mechanical stimulation and material composition resulted in the formation of a neo-tissue with zone-specific collagen type and GAG deposition, which resembles the biochemical composition of meniscal ECM[93]. Promising results were also reported in the study of Puetzer *et al*., where dual compressive-tensile mechanical stimulation of collagen gels loaded with bovine meniscal fibrochondrocytes promoted a high degree of collagen organization, a zone-dependent cell morphology and anisotropic mechanical properties[97]. Moreover, in the outer zone, the tensile-dominating stimulation favored a predominant collagen production, whereas in the inner zone, the compressive-dominating stimulation triggered a higher GAG production as observed in native meniscus.

Another way to promote native tissue organization in meniscal implants takes advantage of the mechanical clamping of the scaffolds at the horns, in order to mimic the physiological meniscal attachments to the tibia. To be able to use this approach, scaffolds with extensions at the horns have to be produced to allow the anchoring, as described by Puetzer *et al.*[115]. In this study, anatomically shaped collagen scaffolds loaded with fibrochondrocytes

and clamped at the horns, supported a circumferential/radial collagen organization and developed anisotropic mechanical properties.

Despite all the positive outcomes described above, matching the tensile mechanical properties of the native meniscus using mechanical stimulation remains an unmet challenge. Indeed, while the compressive moduli of these scaffolds are comparable, or even higher, than that of the human meniscus, the tensile moduli are significantly inferior[93, 97, 115]. This points out the importance in meniscal TE of aiming not only at a sufficient amount of deposited collagen, but also at a high level of its fiber organization into bundles, which is directly correlated with the tensile resistance.

The combined effect of mechanical and chemical stimulation on fibrocartilage scaffolds has been also investigated[89, 95, 96, 114]. In some of these studies, mechanical stimulation via rotary cell culture systems was combined with the use of TGF-β3 in the culture medium. Rotary cell culture systems are bioreactors in which the rotation of the vessel, where the construct is cultured, generates a dynamic laminar flow that promotes a better mass transport throughout the scaffold. Using this strategy, Marsano *et al*. engineered cell-seeded, hyaluronate-based scaffolds with a bi-zonal organization[95, 96]. The outer region of the scaffolds presented high content of collagen type I and contained elongated cells, whereas the inner region was richer in GAG, collagen type I and II, which resembles the composition of the native meniscus[95, 96]. Moreover, this zone-dependent composition had a direct impact on the mechanical properties of the scaffolds. Indeed, the outer region presented higher elastic modulus in tension as expected for a collagen-rich tissue, while the inner region displayed higher elastic modulus in compression as expected for a GAG-rich tissue. The synergistic effect of the mechanical and chemical stimulation has been also reported by Zhang *et al*.[89], who developed cell-seeded 3D printed meniscal scaffolds supporting zonally-variant matrix deposition *in vitro* and *in vivo*.

4.3. Cell type-dependent strategies for the fabrication of zonally-variant meniscus

Differences in the cell types present in the native meniscus play a pivotal role for the zonally-variant histological, biochemical, and mechanical profile of this tissue. The presence of fibroblast-like cells in the outer zone and chondrocyte-like cells in the inner zone of the meniscus is responsible for forming a more fibrocartilaginous tissue in the former and a more cartilaginous one in the latter. As a consequence, the outer zone is stiffer in tension and the inner zone in compression.

TE approaches aiming at reproducing the anisotropic meniscal architecture by using spatially-distributed different cell types have been reported (Table 10). A bio-inspired strategy in this respect is the use of meniscal cell sub-populations isolated from the outer and inner region, as described by Baek *et al.*[108]. In this study, human meniscal cells isolated from the vascular and avascular regions were seeded on PLA scaffolds made of electrospun fibers. Nevertheless, no significant differences in gene expression of relevant markers were seen between the two cell groups, likely because a fibroblast-like cellular phenotype persisted in both.

Conversely, the use of a different cell type combination involving articular chondrocytes and fibroblasts seems to be more promising. Mandal *et al*. reported the fabrication of silk scaffolds seeded with chondrocytes in the inner zone and human fibroblasts in the outer zone[116]. Results showed that chondrocytes maintained their native phenotype and were responsible for a high GAG, collagen type I and II production, which is desirable for the inner meniscal zone. In contrast, fibroblasts synthesized much less GAG, did not synthesize collagen type II, and supported the deposition of collagen type I, which mirrors the ECM composition of the outer meniscal zone.

As described by Higashioka *et al.*, a similar biomimetic tissue composition, in which GAG is more abundant in the inner zone and collagen in the outer zone, has been achieved also by seeding chondrocytes in the inner zone and a mixture of chondrocytes and meniscal cells in the outer zone in an *in vitro* scaffold-free approach[117]. Remarkably, in this study the differences in the biochemical composition of the two different regions had a direct impact on the mechanical properties of the newly formed tissues. Indeed, the inner zone displayed a

much higher compressive modulus compared to the outer zone, which was stiffer in (circumferential) tension.

The scarcity of studies performed employing spatially-variant cell seeding strategies in the field of meniscal TE indicates the complexity of the procedure, which involves several steps and challenges (isolation of different cell types, identification of the best cell cocktail for each zone, selective spatial localization of the cells, etc.). Nevertheless, when considered from another point of view, it might reflect the novelty of this approach and, from a more general perspective, it indicates that aiming at an anisotropic reconstruction of meniscus is still a cutting-edge research line.

5. Synergies between DD and TE technologies

Given the complex and anisotropic nature of the meniscus, novel approaches combine DD technologies for the controlled release of one or multiple GFs, with TE strategies that aim at resembling the native micro- and nanoarchitecture. In these biomaterials, the combination of a biochemical stimulation (via e.g. GFs) provided in a temporarily and/or spatially-controlled manner, together with the biomimicry of structural features may lead to a synergistic effect on zonal tissue differentiation.

5.1. Microparticle-loaded 3D printed scaffolds

Novel systems combine the mechanical and biomimetic properties of 3D printed scaffolds with the flexibility that microparticles offer in terms of GF encapsulation and controlled release. 3D printed scaffolds having fiber architecture resembling that of collagen bundles can be loaded with microparticles for the release of one or multiple GFs. In this context, particle properties can be tuned to have a sequential release of different GFs (temporal control), and a spatially-differentiated distribution of the particles can provide a zonal GF supply (spatial control). Using this approach, Lee and co-workers developed a meniscal implant with a spatio-temporal delivery of two different GFs, i.e. CTGF and TGF-β3, loaded into PLGA microparticles[25]. A

sequential and spatially-controlled release of the two GFs (faster CTGF release/outer zone and slower TGF-β3 release/inner zone obtained by using a different lactide/glycolide ratio), achieved over the course of 42 days, induced a zone-dependent collagen deposition *in vitro* (collagen type I in the outer zone and collagen type II in the inner zone) by human synovium mesenchymal stem/progenitor cells[25]. Moreover, zone-dependent fibrocartilaginous matrix deposition and mechanical properties were observed *in vivo* in an ovine model[25].

A similar system was used for TMJD regeneration, and *in vitro* and *in vivo* experiments confirmed a spatially-specific tissue regeneration that resembled that of native TMJD fibrocartilage[59, 60]. For such multi-component scaffolds, PLGA particles are a versatile tool that offers the possibility to control the release kinetics by adjusting the lactide/glycolide ratio and the molecular weight. These particles can be either infused within the channels of the 3D printed scaffold post-fabrication[25], or mixed in the PCL melt and subsequently printed[59, 60]. Alternatively, the particles can be embedded into a hydrogel that is 3D printed between adjacent PCL filaments[103]. In this case, the hydrogel can be also used as a cell carrier[103]. In the described 3D printed scaffolds, the native collagen organization of fibrocartilage is mimicked by printing adequate 3D patterns of mechanically resistant PCL (or particle-loaded PCL) fibers[25, 59, 60]. Such systems are promising for large fibrocartilaginous defects (e.g. in subtotal and total meniscectomy models) where anatomically-shaped scaffolds with specific internal organization are especially needed.

5.2. Protein-releasing nanofibrous scaffolds

Aligned ultrafine fibers produced via electrospinning may mimic the organization of native collagen and provide, at the same time, a tool to deliver active factors. For this double purpose, nanofibrous hybrid scaffolds for meniscal TE have been fabricated (i) by mixing fibers made of different materials[27, 118], (ii) by generating fibers from polymeric blends[119], or (iii) by producing core-shell nanofibers[120] (table 11).

Using different materials can help provide valid scaffolding properties and allow to tune the release kinetics of different active factors. For example, Mauck *et al.* reported nanofibrous

scaffolds composed of PCL fibers, mixed with collagenase-releasing poly-(ethylene oxide) (PEO) fibers, and PDGF-AB-releasing HA fibers[27, 118]. In this three-component nanofibrous scaffold, mechanical support was provided by resistant PCL fibers, and a sequential release of collagenase and PDGF-AB was promoted by fast-dissolving PEO and crosslinked-HA fibers, with different degradation profiles[27] (Figure 4). According to this study, a burst release of collagenase (80% in 5 h) is necessary to attain a localized matrix degradation of the tissue surrounding the defect to favor cell mobility and migration[10], which is further enhanced by the subsequent release of chemotactic PDGF-AB (over 5 weeks). Importantly, *in vivo* studies in a murine xenotransplant subcutaneous model showed that PEO/HA/PCL nanofibrous scaffolds elicited localized collagen degradation, cell migration-mediated tissue repair, deposition of new collagen type I and II, and scaffold integration[27].

A main concern regarding electrospun fibers for protein delivery is the potential damage to the protein caused by the high voltage applied during the fiber production[121]. To avoid this, hybrid materials containing stabilizers can be fabricated to protect the entrapped proteins. For example, in some studies on meniscal GF delivery, bovine serum albumin (BSA) has been included among the fiber components[119, 120]. Interestingly, Qu *et al.* found a dose-dependent protective effect of BSA on entrapped TGF-β3. For electrospun fibers, protein release kinetics generally consists on an initial burst release due to desorption of the GF from the fiber surface, followed by a sustained release due to polymer degradation[119].

The majority of the studies in the literature reported a predominant parallel alignment of the electrospun fibers, which could mimic the collagen arrangement for small tissue defects[118-120]. Nevertheless, achieving a highly defined circumferential/radial arrangement in anatomically-shaped scaffolds remains an unmet challenge for this technique.

6. Conclusions

DD and TE strategies are currently being investigated for the regeneration of human meniscus. DD focuses on the development of controlled release delivery systems for GFs,

which are key-players in cell recruitment and proliferation, extracellular matrix synthesis, and tissue remodeling. A large array of active factors (GFs and enzymes), mainly formulated into scaffolds, has been investigated. Nevertheless, the identification of the most suitable GF cocktail, GF ideal doses and release kinetics, as well as the determination of the most appropriate biomaterial(s) remain unmet challenges. On the other hand, recent advances in meniscal TE focus on the development of zonally-organized tissue constructs that attempt to mimic the anisotropic ultrastructure of the human meniscus. To this end, modern manufacturing technologies (e.g. 3D printing, electrospinning), dynamic cell culture techniques, and spatially-selective cell seeding have been explored with a certain degree of success. It is our understanding that these two strategies must be combined with the use of modern DD systems embedded in zonal TE constructs. This synergy has recently attracted much interest and may represent a key-point towards the achievement of a definitive breakthrough in this field. A scaffold with *zonal* GF supplementation, microarchitecture and/or cell disposition could have more chances of regenerating a fully active meniscus. Future work will clarify the suitability of this strategy taking also into account its bench-to-bedside translation and clinical relevance.

List of abbreviations

Ang1: Angiopoietin-1

BMP: bone morphogenetic protein

BSA: bovine serum albumin

CNT: carbon nanotubes

CS: chitosan

CTGF: connective tissue growth factor

DD: drug delivery

ECM: extracellular matrix

EDC/NHS: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide

EGF: epidermal growth factor

FGF: fibroblast growth factor

GAG: glycosaminoglycans

GelMA: methacrylated gelatin

GF(s): growth factor(s)

HA: hyaluronic acid

HA-SH: thiol-modified hyaluronic acid

IGF: insulin-like growth factor

IVD: intervertebral disk

IZ: inner zone

KGF: keratinocyte growth factor

MMPs: metalloproteinases

MSCs: mesenchymal stem cells

OC: osteochondral

OZ: outer zone

PCL: polycaprolactone

PDGF-AB: platelet-derived growth factor-AB

PDGF-BB: platelet-derived growth factor-BB

PEG: polyethylene glycol

PEG-DA: polyethylene glycol diacrylate

PEO: poly-(ethylene oxide)

PLA: poly-(lactic acid)

PLGA: poly-(lactic-co-glycolic acid)

PRF: platelet-rich fibrin

PRG: platelet-rich gel

PRP: platelet-rich plasma

TE: tissue engineering

TGF: transforming growth factor

TMJD: temporomandibular joint disk

UV: ultraviolet

VEGF: vascular endothelial growth factor

3D: three dimensional

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Figure captions

Figure 1. Schematic representation of human meniscus (horizontal cross-section, top, and vertical cross-section, bottom) highlighting zonal variations in vascularization, cell type localization, collagen type I and II distribution, and collagen fiber arrangement.

Figure 2. Graphical representation of collagen orientation in native meniscus (horizontal cross-section). Macroscopic circumferential/radial arrangement (left) and perpendicular arrangement at a much smaller scale (right).

Figure 3. Schematic representation of the use of 3D printing and electrospinning to engineer anisotropic meniscus constructs. 3D (bio)printing allows deposition of micro-sized, circumferentially/radially organized fibers, where a bi-zonal organization can be achieved by a spatially-selective deposition of different components (e.g. hydrogels, GFs-loaded particles and cells) (left). Electrospinning allows deposition of less ordered, but much thinner fibers that can mimic collagen in native meniscus (right).

Figure 4. Stimulation of meniscal cell migration and tissue repair via temporal delivery of collagenase and PDGF-AB. Initially, fast-dissolving PEO fibers trigger the release of matrix-degrading collagenase. Localized tissue degradation at the tissue-scaffold interface allows cell mobility through a less dense tissue matrix. Chemotactic activity of PDGF-AB further enhances cell migration into the scaffold, a crucial step for new ECM synthesis. Reproduced from[27].

Figures

Figure 1

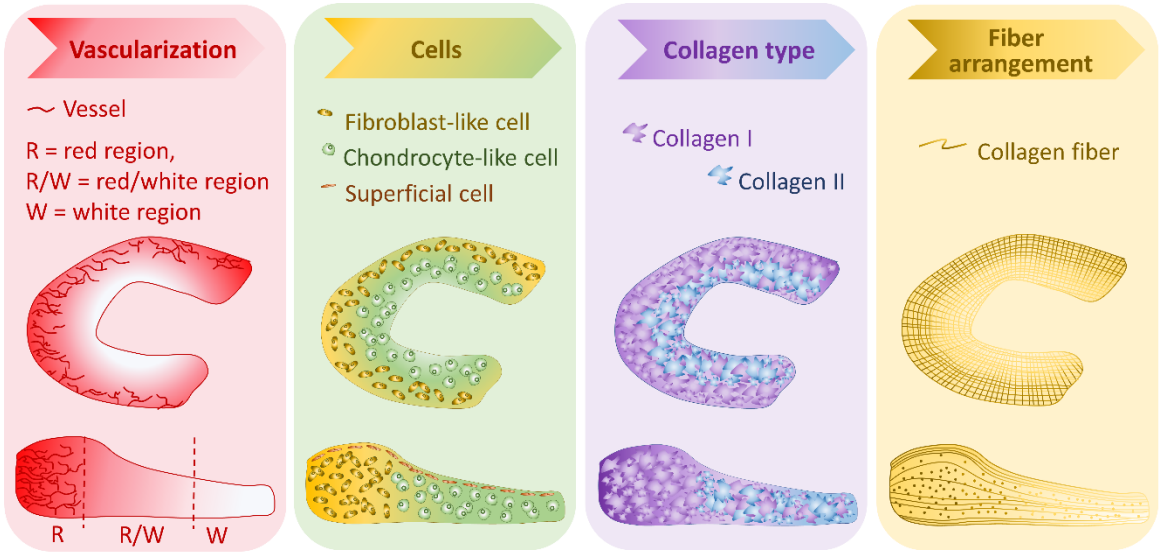


Figure 2

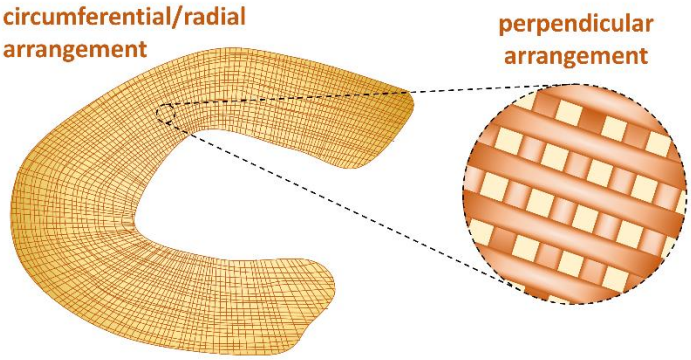


Figure 3

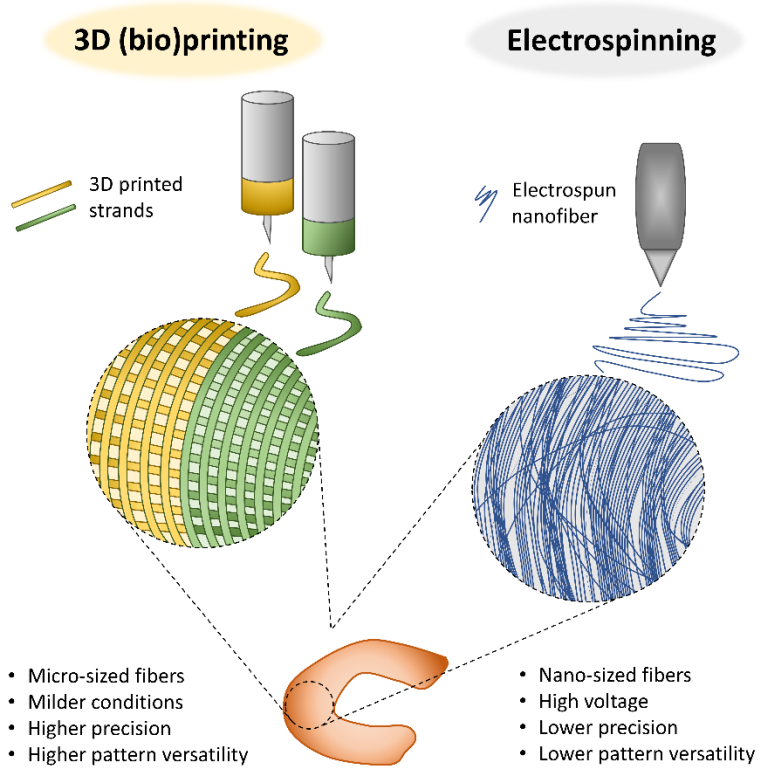
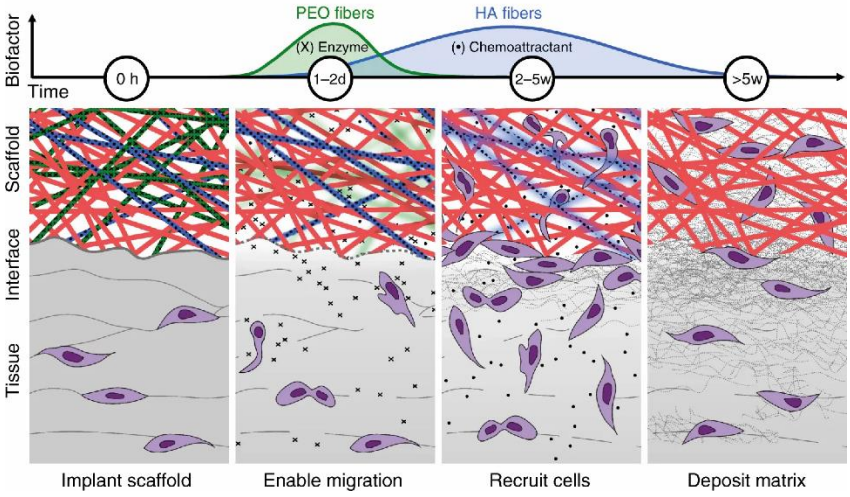
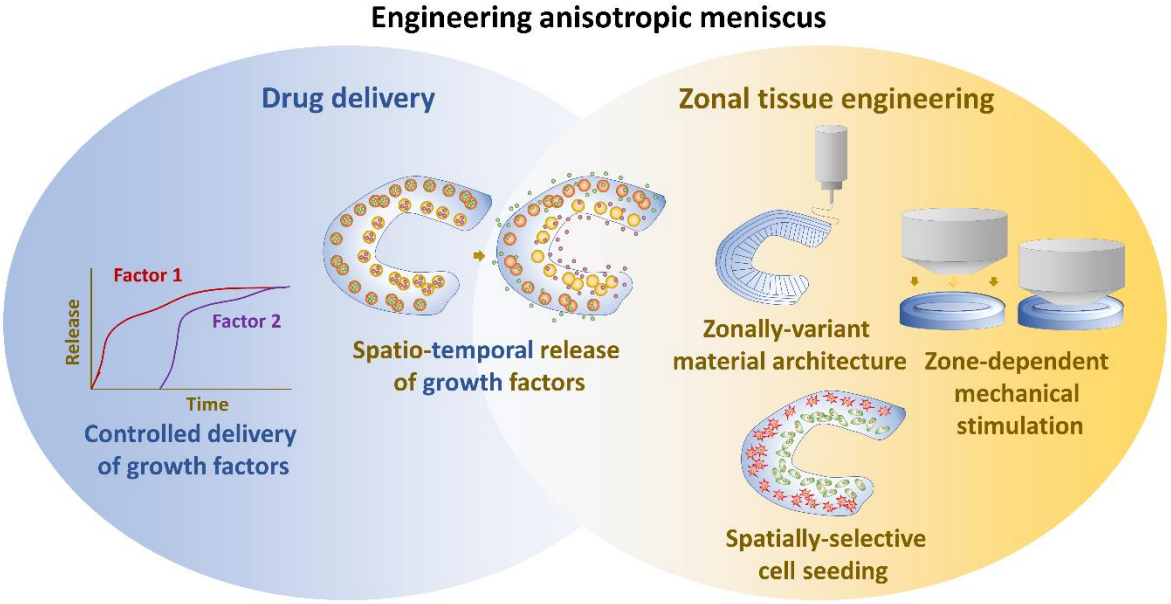


Figure 4



Graphical abstract



Tables

Table 1. ECM-derived scaffolds for the controlled delivery of GFs for fibrocartilage regeneration. (In all the studies, the meniscus is the targeted tissue, except for[31], where the TMJ osteochondral tissue is the target).

Biomaterial	**Growth factor(s)**	**Model**	**Outcome**	**Ref.**
Collagen scaffold	BMP-2	*In vivo* leporine orthotopic	Partial tissue healing (dose-dependent formation of hyaline-like matrix)	[31]
Photocrosslinkable methacrylated gelatin hydrogel	TGF-β3	*In vitro* human adipose-derived stem cells *In vitro* bovine meniscus organ culture	Chondrogenic differentiation Tissue healing (collagen I and II)	[36]
Photocrosslinkable methacrylated gelatin hydrogel	TGF-β3	*In vivo* ovine orthotopic	Tissue healing (proteoglycans); Mitigation of osteo-chondral degeneration	[37]
Glutaraldehyde-crosslinked gelatin hydrogel	PRP	*In vivo* leporine orthotopic	Tissue healing (proteoglycans)	[38]
Glutaraldehyde-crosslinked gelatin hydrogel	FGF-2	*In vivo* leporine orthotopic	Improved meniscal cell density and tissue healing (proteoglycans)	[39]
Fibrin hydrogel	CTGF	*In vivo* leporine orthotopic	Capillaries and ECM (Collagen I and II) formation	[43]
PRF membrane	PRF GFs (in combination with PRP injection)	Clinical trial orthotopic	Improved post-operatory score; No difference between PRF group and non-PRF group	[44]

ECM: extracellular matrix; GFs: growth factors; BMP-2: bone morphogenic protein 2; TMJ: temporomandibular joint; TGF-β3: transforming growth factor beta 3; PRP: platelet-rich plasma; FGF-2: fibroblast growth factor 2; CTGF: connective tissue growth factor; PRF platelet-rich fibrin.

Table 2. Scaffolds based on other natural polymers for the controlled delivery of active factors for meniscus regeneration.

Biomaterial	Growth factor source	Model	Outcome	Ref.
Chitosan scaffold	PRP	*In vivo* ovine orthotopic	Tissue healing; Scaffold integration	[47]
Silk fibroin sponge	PRG	*In vivo* leporine orthotopic	Cell infiltration and matrix synthesis	[49]

PRP: platelet-rich plasma; PRG: platelet-rich gel.

Table 3. PLGA-based scaffolds for the delivery of growth factors in fibrocartilage regeneration.

Targeted tissue	Growth factor(s)	Model	Outcome	Ref.
Meniscus	PRP	*In vivo* murine ectopic xenotransplant (human meniscus explants)	Attachment of seeded articular chondrocytes and partial meniscal healing	[55]
TMJ osteochondral tissue	TGF-β1 BMP-2	*In vivo* leporine orthotopic	Osteochondral tissue regeneration	[56]

PLGA: poly-(lactic-co-glycolic acid); PRP: platelet-rich plasma; TGF-β1: Transforming growth factor beta 1; BMP-2: Bone morphogenic protein 2; TMJ: temporomandibular joint.

Table 4. Scaffolds based on hybrid materials for the controlled delivery of growth factors for fibrocartilage regeneration. (In all the studies, meniscus is the targeted tissue, except for[68], where the IVD is the targeted tissue).

Biomaterial	**Growth factor(s)**	**Model**	**Outcome**	**Ref.**
Fiber-reinforced collagen/chondroitin sulfate matrix crosslinked via EDC/NHS chemistry	PRP	*In vitro* human meniscal cells	Increased cell number; Aggrecan, collagen I and elastin upregulation	[64]
Matrigel™/hyaluronic acid scaffold	VEGF	*In vivo* ovine orthotopic	No tissue healing	[65]
Collagen/ carboxymethylcellulose scaffold	BMP-7	*In vivo* ovine orthotopic	Partial tissue healing	[65]
Heparin-conjugated decellularized meniscus scaffold	PDGF-BB	*In vitro* bovine meniscus organ culture	Cell migration and proliferation; Tissue integration;	[66]
Thiol-modified hyaluronic acid/ polyethylene glycol diacrylate hydrogel	PDGF-BB	*In vivo* leporine orthotopic	Decreased disc degeneration; Inhibition of cell apoptosis and of deposition of collagen III;	[68]
Glycol-chitosan/ multialdehyde-polyethylene glycol hydrogel	TGF-β1	*In vitro* leporine bone mesenchymal stromal cells *In vivo* leporine orthotopic	Cell proliferation; Expression of fibrochondrogenic markers Fibrocartilaginous tissue growth (collagen I and II)	[70]

IVD: intervertebral disc; EDC/NHS: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide; PRP: platelet-rich plasma; VEGF: vascular endothelial growth factor; BMP-7: Bone morphogenic protein 7; PDGF-BB: platelet-derived growth factor BB; TGF-β1: transforming growth factor beta 1.

Table 5. Film-coated suturing wires for the controlled delivery of growth factors for meniscus regeneration.

Biomaterial	**Growth factor**	**Model**	**Outcome**	**Ref.**
PLA suture coating	VEGF	*In vivo* ovine orthotopic	Presence of endothelial cells; No angiogenesis; No meniscal healing	[15]
PLA suture coating	VEGF	*In vivo* ovine orthotopic	No angiogenesis; No meniscal healing	[74]
Gelatin suture coating	FGF-2	*In vitro* human meniscus organ culture	Proliferation of meniscal cells; Inhibition of meniscal death	[76]

PLA: poly-(lactic acid); VEGF: vascular endothelial growth factor; FGF-2: fibroblast growth factor 2.

Table 6. Studies aiming at mimicking zonally-variant meniscus architecture by controlling porosity.

Technology	**Strategy**	**Biomaterial**	**Model**	**Outcome**	**Ref.**
Multi-step freeze-drying	Multi-layered scaffold with varying composition and pore size	Genipin-crosslinked gelatin/chitosan hydrogel	*In vitro* human meniscal cells	Cell infiltration and proliferation; Composition-dependent mechanical properties, density and pore size	[91]
Unidirectional cryostructuring	Oriented micropores formation; Zone-dependent hydrogel composition and pore size	Collagen I and II/chondroitin sulfate hydrogel	*In vitro* human mesenchymal stromal cells and endothelial cells	Cell migration and good metabolic activity Zone-specific matrix deposition	[92]
Thermally-induced phase separation	Oriented channel-like microporosity vs random microporosity	PLA/ PCL/polyurethane scaffold	*In vivo* murine ectopic	Scaffold microarchitecture-dependent tissue ingrowth	[94]

PLA: poly-(lactic acid); PCL: polycaprolactone

Table 7. Studies aiming at mimicking zonally-variant architecture of meniscus by using 3D printing.

Strategy	**Biomaterial**	**Model**	**Outcome**	**Ref.**
Circumferential/radial fiber alignment	PCL scaffold	*In vitro* cell-free structural and mechanical testing	Tunable equilibrium modulus based on architectural parameters (fiber spacing and orientation, interlayer offset)	[82]
Perpendicular fiber organization in scaffolds with varying strand spacing	PCL scaffold	*In vivo* murine ectopic	Strand spacing-dependent morphology of recruited cells and collagen alignment in newly formed matrix	[83]
Circumferential/radial fiber arrangement (combined with biochemical and biomechanical stimulation)	PCL scaffold	*In vitro* leporine bone marrow-derived mesenchymal stem cells *In vivo* leporine orthotopic	Deposition of zone-dependent ECM components; Zone-specific matrix deposition and cell phenotypes	[89]
Perpendicular PCL strand orientation (combined with zone-dependent hydrogel composition and zonally-variant mechanical stimulation)	PCL/agarose/methacrylated gelatin scaffold	*In vitro* porcine fibrochondrocytes	Zone-specific collagen/GAG deposition*;* Protective effect of hydrogel on cells against mechanical stress	[93]

Perpendicular/circumferential PCL strand orientation (combined with zone-dependent hydrogel composition)	PCL/agarose/methacrylated gelatin scaffold	*In vitro* human fibrochondrocytes	Cell alignment; Zone-dependent deposition of ECM components	[90]
Circumferential/radial carbon nanotubes alignment	Carbon nanotubes /polymer resin nanocomposite	*In vitro* cell-free structural and mechanical testing	Anisotropic mechanical properties	[84]
Circumferential/radial fiber reinforcement	Alginate/acrylamide gel and UV-curable, epoxy-based reinforcement	*In vitro* cell-free structural and mechanical testing	Anisotropic mechanical properties	[85]
Perpendicular strand organization	Methacrylated gelatin scaffold	*In vitro* human meniscal cells *In vitro* human meniscus organ culture	Cell viability and cellular alignment; Fibrocartilage-like tissue formation and scaffold integration	[86]

ECM: extracellular matrix; PCL: polycaprolactone; GAG: glycosaminoglycans; UV: Ultraviolet.

Table 8. Studies aiming at mimicking zonally-variant fibrocartilage architecture by using PCL-based electrospinning. (In all the studies, meniscus is the targeted tissue, except for[107], where fibrocartilage in general is the targeted tissue).

Strategy	**Model**	**Outcome**	**Ref.**
Aligned fiber orientation; (combined with dual cell seeding)	*In vitro* bovine mesenchymal stem cells and meniscus fibrochondrocytes	Domain-dependent collagen matrix deposition, cellular nuclei morphology, mechanics and intracellular calcium signaling	[107]
Different fiber orientation in single-layer and multi-layer scaffolds	*In vitro* bovine mesenchymal stem cells	Fiber orientation-dependent scaffold stiffness and direction of newly formed collagen; Fiber orientation-dependent collagen amount and cell infiltration; Positive mechanical reinforcement by multilayered circumferential scaffolds	[88]
Circumferential fiber orientation	*In vitro* bovine mesenchymal stem cells	Cell alignment along fiber direction; Regionally-variant mechanical properties	[87]

PCL: polycaprolactone

Table 9. Studies aiming at mimicking zonally-variant fibrocartilage architecture by using different mechanical stimulation technologies. (In all the studies, meniscus is the targeted tissue, except for[114], where the TMJ disc is the targeted tissue).

Strategy	**Stimulation**	**Biomaterial**	**Model**	**Outcome**	**Ref.**
Zone-dependent mechanical stimulation and zone-dependent hydrogel composition	Dual (compressive and tensile) dynamic loading	3D printed PCL scaffold impregnated with agarose (IZ) and methacrylated gelatin (OZ)	*In vitro* porcine meniscal chondrocytes	Zone-specific collagen/GAG deposition*;* Protective effect of hydrogel on cells against mechanical stress	[93]
Zone-dependent mechanical stimulation	Dual (compressive and tensile) dynamic loading	Collagen hydrogel	*In vitro* bovine meniscal fibrochondrocytes	Native-like collagen organization, GAG distribution and cell morphology; Anisotropic tensile properties	[97]
Biomechanical and biochemical stimulation combined with biomimicking fiber organization	Dual (compressive and tensile) dynamic loading	3D printed PCL scaffold	*In vitro* leporine bone marrow derived-mesenchymal stem cells *In vivo* leporine orthotopic	Deposition of zone-dependent ECM components; Zone-specific matrix deposition and cell phenotypes	[89]
Mimicry of meniscal attachment at the tibia	Mechanical anchoring at the horns	Collagen hydrogel	*In vitro* bovine meniscal fibrochondrocytes	Circumferential/radial fiber organization; Anisotropic tensile properties	[115]
Mechanical stimulation and	Hydrodynamic flow using perfused	Non-woven mesh of esterified	*In vitro* bovine and human	Bi-zonal engineered tissue varying in GAG,	[95]

chemical stimulation (TGF-β3, ascorbic acid and insulin)	rotary cell culture system	hyaluronic acid	articular chondrocytes	collagen distribution and cell morphology	
Mechanical stimulation and chemical stimulation (TGF-β3, ascorbic acid and insulin)	Hydrodynamic flow using perfused rotary cell culture system and mixed flask	Non-woven mesh of esterified hyaluronic acid	*In vitro* bovine articular chondrocytes	Bi-zonal tissue architecture and zone-dependent mechanical properties	[96]
Mechanical stimulation and chemical stimulation (Chondroitinase ABC and TGF-β1)	Passive axial compression	TMJ shape-specific agarose well	*In vitro* bovine articular chondrocytes and meniscal cells	Anisotropic neo-tissue formation	[114]

TMJ: temporomandibular joint; 3D: three-dimensional; PCL: polycaprolactone; OZ: outer zone; IZ: inner zone; GAG: glycosaminoglycans; ECM: extracellular matrix; TGF-β: transforming growth factor beta.

Table 10. Studies aiming at mimicking zonally-variant meniscal architecture by spatially-selective seeding of different cell types.

Strategy	**Biomaterial**	**Model**	**Outcome**	**Ref.**
Different cell type seeding and use of aligned or randomly distributed electrospun fibers	PLA electrospun fiber scaffold + hydrogel made of collagen II, chondroitin sulfate and hyaluronic acid	*In vitro* human meniscal cells from vascular (OZ) and avascular zone (IZ)	No significant differences in gene expression between cell types; Fiber orientation-dependent cell morphology and alignment	[108]
Different cell type seeding and use of material layers with different porosity	Fibrous silk protein scaffold	*In vitro* human dermal fibroblasts (OZ) human articular chondrocytes (IZ)	Cell-type dependent GAG and collagen production; Porosity-dependent mechanical properties	[116]
Different cell type seeding	Scaffold-free approach	*In vitro* bovine articular chondrocytes and meniscal cells (OZ) bovine articular chondrocytes (IZ)	Cell-type dependent GAG and collagen production; Cell-type dependent mechanical properties of formed matrix	[117]

PLA: poly-(lactic acid); OZ: outer zone; IZ: inner zone; GAG: glycosaminoglycans.

Table 11. Scaffolds based on nanofibrous hybrid scaffolds for the controlled delivery of active factors for fibrocartilage reconstruction. (In all the studies, meniscus is the targeted tissue, except for[119], where fibrocartilage in general is the targeted tissue).

Biomaterial	**Enzyme/growth factor**	**Model**	**Outcome**	**Ref.**
Poly-(ethylene oxide)/PCL nanofibrous scaffold	Collagenase	*In vitro* bovine meniscus organ culture	Localized proteoglycans digestion; Increased tissue porosity and cell density; Dose-dependent integration	[118]
Poly-(ethylene oxide)/PCL/hyaluronic acid nanofibrous scaffold	Collagenase PDGF-AB	*In vitro* bovine meniscus organ culture *In vivo* murine ectopic xenotransplant (bovine meniscus explants)	Scaffold porosity-dependent cell infiltration Localized collagen degradation; Cell migration; Collagen deposition; Integration	[27]
BSA (core)/PLA-polyethylene glycol (shell) nanofibrous scaffold	PDGF-BB	*In vitro* human meniscal and synovial cells	Increased cell proliferation and upregulation of relevant genes	[120]
PCL/PLGA/BSA nanofibrous scaffold	TGF-β3	*In vitro* bovine synovium-derived stem cells	Increased cell proliferation; Dose-dependent matrix synthesis (collagen, proteoglycans)	[119]

PCL: polycaprolactone; PDGF-AB: platelet-derived growth factor AB; BSA: bovine serum albumin; PLA: poly-(lactic acid); PDGF-BB: platelet-derived growth factor BB; PLGA: poly-(lactic-co-glycolic acid); TGF-β3: transforming growth factor beta 3.