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REVIEW

Regeneration of the anterior cruciate ligament: Current strategies in tissue engineering

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Abstract

Recent advancements in the field of musculoskeletal tissue engineering have raised an increasing interest in the regeneration of the anterior cruciate ligament (ACL). It is the aim of this article to review the current research efforts and highlight promising tissue engineering strategies. The four main components of tissue engineering also apply in several ACL regeneration research efforts. Scaffolds from biological materials, biodegradable polymers and composite materials are used. The main cell sources are mesenchymal stem cells and ACL fibroblasts. In addition, growth factors and mechanical stimuli are applied. So far, the regenerated ACL constructs have been tested in a few animal studies and the results are encouraging. The different strategies, from *in vitro* ACL regeneration in bioreactor systems to bio-enhanced repair and regeneration, are under constant development. We expect considerable progress in the near future that will result in a realistic option for ACL surgery soon.

Key words: Anterior cruciate ligament; Tissue engineering; Orthopedic; Ligament regeneration; Stem cell

Core tip: This article reviews the current research strategies in anterior cruciate ligament tissue engineering and highlights the most promising strategies in this field.

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INTRODUCTION

Knee injuries frequently result in ruptured ligaments, typically through high-pivoting sporting activities such as skiing, football and basketball. In 2005, around 400000 physician office visits in the United States were related

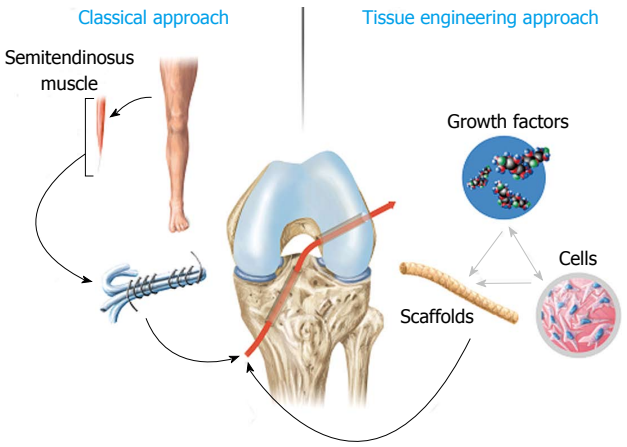

Figure 1 Comparison of the current clinical strategy in anterior cruciate ligament surgery to tissue engineering approaches. The current "golden standard" in the clinical routine is the use of autologous tissue grafts such as semitendinosus (depicted in the figure) or patellar tendon. In tissue engineering approaches, scaffolds alone or in a combined fashion with cells or growth factors are used to improve tissue regeneration.

to knee injuries[1]. The worldwide estimation of young sports players that require surgery following a knee injury lies between 17%-61%[2]. The anterior cruciate ligament (ACL), a main stabilizing structure of the knee, is one of the most commonly injured ligaments. In the United States alone, around 350000 reconstructive surgeries of the ACL are performed annually. According to the National Center for Health Statistics, the annual costs for the acute care of these injuries are around $6 billion[3].

Historically, the treatment of ACL injuries involved different strategies, from non-operative care to several surgical procedures[4]. Simple primary suturing in the 1970s was abandoned due to bad clinical results. Augmented ACL repair using natural as well as synthetic grafts leads to somewhat improved results. Synthetic grafts were popular in the 1980s but resulted in serious complications and bad clinical results. From the early 1990s onwards, ACL reconstruction with autograft or allograft material has become the method of choice for most surgeons (Figure 1). Despite the ongoing success of autografts, problems mostly associated with donor site morbidity remain, such as anterior knee pain, infrapatellar contracture, tendonitis, patellar fracture, muscle weakness and limited graft availability[5]. In terms of allograft material, the risk for transmissions of blood-borne diseases and the delayed biological incorporation were mentioned as the main disadvantages[6]. In addition, relatively high failure rates of ACL reconstruction, especially in young and active patients, have been reported for allografts[7]. An incidence of osteoarthritis as high as 50% within 7-14 years after injury and reconstruction of the ACL is still the main drawback of this surgical strategy. The development of osteoarthritis following ACL injury is not fully understood and may be caused not only by the limitation of the current grafts, but also by the initial joint trauma and the trauma caused by the surgeon. However, this has resulted in enormous ongoing research interest in that topic[8,9].

The regeneration of musculoskeletal tissues has become increasingly popular in the field of orthopedic research. Typically, structures that are injured or lost due to trauma and disease are the ideal candidates to be engineered. Tissue engineering as a multidisciplinary field includes strategies of engineering, material science and biology, with the aim of regenerating tissues that not only recreate the morphology, but also restore the normal function. In the late 1980s, Langer and Vacanti[10] first described the classic four basic components that are needed in tissue engineering: a structural scaffold, a cell source, biological modulators and mechanical modulators[10].

The ACL, with its limited healing capacity and the consequent need for reconstructive surgery, certainly is an appealing but also challenging structure for tissue engineering. In contrast to extra-articular ligaments, such as the medial collateral ligament, the intra-articular location of the ACL apparently prevents its primary healing. The disruption of the synovial sheath does not allow local hematoma formation crucial for the onset of the inflammatory response that would stimulate primary healing[11]. In addition, the complex three dimensional structure of the ACL, with different tensioning patterns throughout the knee path of motion, contributes to the difficulty of regenerating this ligament in terms of form and function.

It is the purpose of this article to review the current approaches in tissue-engineering of the ACL, to provide an overview of the current problems and limitations, and to present future directions of this evolving research technology.

SCAFFOLDS FOR ACL REGENERATION

Many different biomaterials have been introduced as a potential scaffold for ACL tissue engineering. Ideally, the scaffold has to be biocompatible and its mechanical properties should mimic the natural ACL as closely as possible. It also needs to be biodegradable to enable tissue ingrowth, which is crucial for the new ligament to form. Biological materials, biodegradable polymers and composite materials have all been or still are under evaluation for ligament regeneration[11].

Dunn *et al*[12] and Bellincampi *et al*[13] developed scaffolds made of collagen fibrils. They showed that ACL fibroblasts adhered to these scaffolds and remained viable, *in vitro* as well as *in vivo*. Unfortunately, after 6 wk the constructs were completely resorbed. Goulet *et al*[14] reported on the decreasing mechanical strength of collagen scaffolds seeded with ACL fibroblasts. Murray *et al*[15] demonstrated that a collagen-glycosaminoglycan composite scaffold supported cell growth and the expression of fibroblast markers. Several techniques have been explored to improve the mechanical properties of collagen-based scaffolds, including cross linking the collagen or a special braid-twist design[16-18]. However, despite considerable improvements of the mechanical properties, collagen-based scaffolds thus far have not been able to mimic the strength of the natural ACL.

Similar challenges regarding the mechanical strength have also been reported for other biological materials, such as alginate, chitosan and hyaluronic acid[19-25]. Many different composites of these materials have been explored and it has been shown that some of them may be an interesting option in terms of cell attachment and cell proliferation. However, the mechanical insufficiency of these biological materials remains a considerable problem for their routine practical use in ligament regeneration. To overcome the mechanical weakness, Panas-Perez *et al*[26] developed a collagen-silk composite and concluded that a scaffold with > 25% silk provides sufficient mechanical support very close to the properties of the native ACL.

The use of silk in ligament scaffolds is not restricted to combinations with other biomaterials. In various studies, its functionality in diverse tissue engineering approaches, especially in the musculoskeletal field, has been proven. The properties that make silk an attractive candidate as biomaterial are its remarkable strength and toughness compared to other natural as well as synthetic biomaterials[27-34]. The majority of studies dealing with silk as raw material for scaffold production use fibers from cocoons of the mulberry silkworm *Bombyx mori*. Due to biocompatibility issues, silkworm silk requires removal of the surface protein layer sericin, which can elicit adverse immune responses[35,36]. Once sericin is removed, the remaining silk fibroin fibers are non-immunogenic, biocompatible and capable of promoting cell adhesion, growth and, in the case of progenitor cells such as mesenchymal stromal cells (MSCs), differentiation. The classical way to remove this protein layer is to boil raw silk fibers in alkaline solutions such as sodium carbonate. Recently, Teuschl *et al*[36] successfully removed sericin from a compact and highly-ordered raw *Bombyx mori* silk fiber scaffold using borate buffer based solutions. The possibility of removing sericin after the textile engineering process eases the production of complex 3D structures in TE applications because the gliding properties of the silk fiber due to the gum-like sericin assist during textile engineering steps (*e.g.*, braiding and weaving). The pioneers in using silk fibers as raw material for ACL scaffolds are Altman and Kaplan, who demonstrated that the mechanical properties of their twisted fiber scaffolds match that of the native human ACL[37]. Moreover, Horan *et al*[38] demonstrated the processability of silk fibers with a huge number of different textile engineering techniques, enabling the generation of complex hierarchical structures with defined properties. Another characteristic that makes silk an attractive candidate for ACL tissue engineering is its slow rate of biodegradation (proteolytic degradation). Thus, ACL scaffolds made out of silk fibroin can provide the primary stability over an extended period of time, allowing ingrowing cells to rebuild neoligamentous tissue without exposing the knee joint to periods of instability. Moreover, the gradual transfer of stabilizing properties from the silk scaffold to the new forming tissue should allow a neotissue formation similar to the initial native tissue regarding collagen alignment, vascularization, *etc*.

In the literature, silk-based ligament grafts have been tested in animal models in only a few studies[26,39-41]. Historically, former ACL studies with synthetic materials have shown that the extrapolation of findings from animal data to humans needs large animal studies, like goat, sheep or pig models. To the best of our knowledge, only two studies have already tested silk-based ACL grafts in large animal studies with encouraging results[42,43]. In a pig model, Fan *et al*[43] demonstrated that their woven silk ligament scaffold in conjunction with seeded MSCs supported ligament regeneration after the 24 wk post implantation period. In conclusion, these very promising *in vivo* studies suggest that ACL scaffolds fabricated from silk fibroin have great potential for the translation into clinical applications. Moreover, clinical trials of silk-based ACL grafts proving functionality and safety in human knees have already been documented[44].

Apart from biological materials, synthetic biodegradable polymers have been introduced in ligament tissue engineering. Petrigliano *et al*[45] mentioned the advantages of synthetic polymers as proper selection and different manufacturing techniques allow for exact adaptation of the mechanical properties, cellular response and degradation rate[45]. Lin *et al*[46] used a scaffold composed of polyglycolic acid coated with polycaprolactone. Buma *et al*[47] worked with a braided polydioxanone scaffold in an *in vivo* animal study but reported an early loss of mechanical properties. Lu *et al*[48] compared different synthetic braided materials and concluded that poly L-lactic acid (PLLA) scaffolds had the best results in terms of mechanical properties as well as fibroblast proliferation. Laurencin *et al*[49] also developed a PLLA scaffold in a 3 dimensional braided fashion with distinct regions for the bony portions and the intra-articular portion of the construct. The same group consequently compared a different PLLA scaffold with different manufacturing techniques and demonstrated that a braid-twist scaffold had the most favorable viscoelastic properties[50,51]. In another study, a polyethylene glycol hydrogel was added to the PLLA scaffold which resulted in even better viscoelastic performance of the construct, but on the other hand, this also led to a decreased pore size of the scaffold which may negatively influence cell proliferation[52].

More recently, electrospinning has been used for the development of scaffolds for ligament tissue engineering[53]. This technique can be used to produce very thin fibers in the nanometer to micron range. This allows for a more exact adaptation of the mechanical properties. Some of the studies using this technique reported better cell proliferation and extracellular matrix production[53,54]. However, these techniques are under constant investigation and while early *in vitro* studies show interesting results, the overall biological and mechanical performance still has to be examined further to draw any conclusions for a later clinical use of these materials.

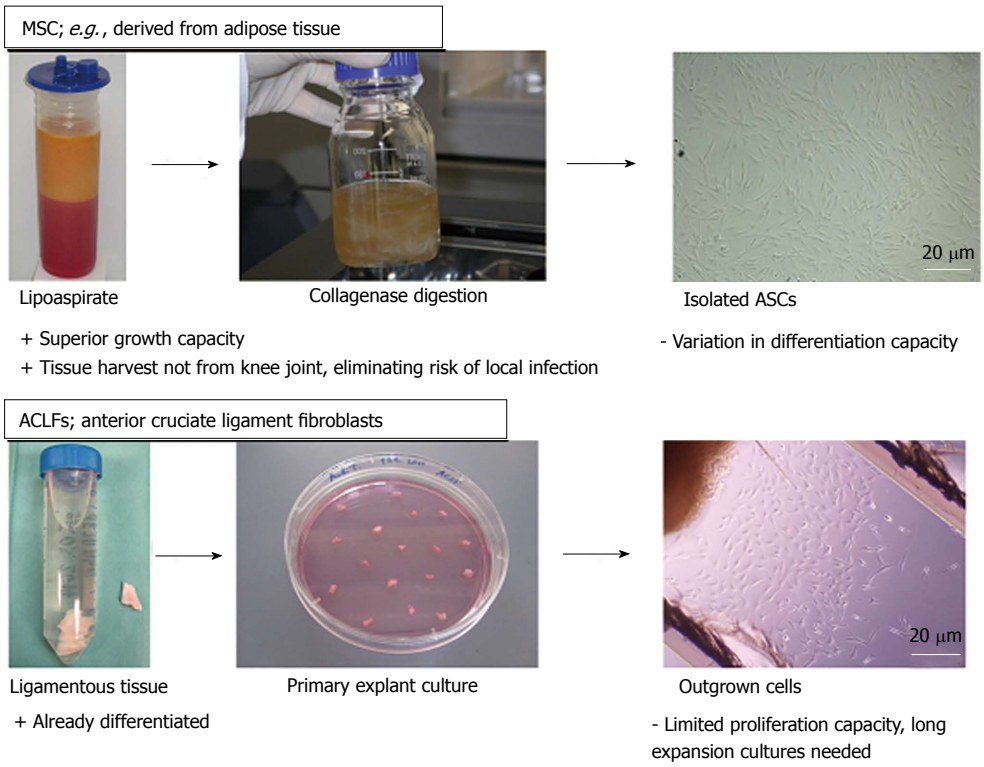

Figure 2 Overview of the main cell types used for anterior cruciate ligament tissue engineering approaches. Two different types of cells are mainly regarded as the primary choice for anterior cruciate ligament (ACL) regeneration: mesenchymal stem cells (MSC) and ACL fibroblasts. Since MSCs can be isolated from adipose tissue (in our studies in cooperation with the Red Cross Blood Transfusion Service of Upper Austria, Linz, Austria) or bone aspirates, their harvest is less delicate than cells isolated from ligamentous tissue. Further advantages of MSCs over ACL fibroblasts are their superior growth capacity and capability of differentiating into the appropriate cell types. Nevertheless, due to their origin, ACL fibroblasts would be the accurate cell type to build up neoligamentous tissue. ASCs: Adipose derived stem cells.

CELL SOURCES FOR ACL REGENERATION

Two different types of cells are mainly regarded as the primary choice for ACL regeneration: mesenchymal stem cells (MSC) and ACL fibroblasts[55].

MSCs are present in almost all tissue types of the body[56,57]. However, for cell therapeutic purposes, bone marrow and adipose tissue are regarded as the main feasible sources to isolate MSCs[58,59]. The potential of MSCs to differentiate into various mesenchymal lineages, including fibroblastic, osteogenic, chondrogenic and myogenic, was proven in numerous studies. Furthermore, MSCs have already been effectively applied to enhance repair in different musculoskeletal tissues, in particular in bone and ligaments (Figure 2)[40,60-62].

The use of ACL fibroblasts involves the risk of local infection in the knee during biopsy harvesting. From the view that the seeded cells should rebuild the ligament tissue by deposition of extracellular matrix, the appropriate cell type would be ACL fibroblasts since they are the native cell type in intact ligament tissue. Therefore, they are used as control cells for cell behavior such as protein expression, especially in *in vitro* studies. Interestingly, different studies have demonstrated that the ACL tissue contains populations of cells sharing MSC characteristics, such as clusters of differentiation markers or multipotency[63,64]. Although stem cells are present in the ACL tissue, their regenerative capacity is too restricted to be capable of healing ruptured ligaments. As ACL tissue can only be harvested reasonably in diagnostic arthroscopic procedures after ACL rupture, other ligament fibroblast sources have been discussed, such as the medial collateral ligament[65]. Nevertheless, the majority of studies involving cell therapy approaches in ACL tissue engineering uses mesenchymal stromal cells as a cell source since they can be obtained much more easily in higher numbers and, moreover, MSCs show higher proliferation and collagen productions rates compared to ligament fibroblasts[66,67].

From a cellular view, the knee joint comprises different sources of cells[68] (ligament tissue, synovium, *etc.*) that have been shown to participate during the ligament regeneration process, such as the above described ACL fibroblasts or MSCs that are natively recruited after ligament ruptures or tears. The activation and recruitment of regenerating cells can be augmented mechanically, for instance by the surgical procedure (*e.g.*, drilling of bone holes for the graft which gives access to the vasculature of bone tissue) or biochemically, by the use of growth factors or gene-based therapeutic approaches.

GENE-BASED THERAPEUTIC APPROACHES AND GROWTH FACTORS

Growth factors can either be directly applied *via* inserted cells (producing these biochemical signal molecules *in*

situ), *via* local delivery of growth factors or *via* gene-based therapeutic approaches where vehicles are encoding the chosen growth factor.

The most frequently used factors belong to proteins that directly affect the deposition of extracellular matrix proteins, such as the bone morphogenetic proteins (BMPs) or the degradation of ECM components assisting in remodeling impaired tissue. BMPs belong to the TGF-β superfamily. Their most prominent characteristic is to induce the differentiation of MSCs into the chondrogenic and osteogenic lineage. A special class of the BMPs, the growth and differentiation factors (GDF) 5/6 and 7, has been shown to be able to ectopically induce neotendon/ligament formation *in vivo*[69]. Furthermore, Aspenberg *et al*[70] (1999) demonstrated the enhanced regenerative effect of GDF 5 and 6 in an Achilles tendon rat model[70]. Interestingly, from a mechanistic point of view, the effects of GDFs depend on the mechanical loading of the injection site. Forslund *et al*[71] (2002) showed that the injection of GDF 6 in unloaded Achilles tendon defects led to the induction of bone formation[71], which in contrast was not observable in control groups of loaded tendons. This clearly indicates the interaction of the effect of growth factors and mechanical stimulation.

Other factors that have also been used to enhance the repair of tendon/ligament structures but are not directly associated with ECM turnover are insulin-like growth factor 1 (IGF1)[72,73], vascular endothelial growth factor (VEGF)[74], epidermal growth factor (EGF)[75] and platelet derived growth factor (PDGF)[76-79]. For instance, VEGF is well known to be a powerful stimulator of angiogenesis and the main function of IGF1 is mainly attributed to an anti-inflammatory effect[80] since functional analysis revealed a decreased recovery time but no biomechanical improvement in an Achilles tendon injury model.

An autologous and already clinically applied approach to augment tendon and ligament healing with growth factors is the use of platelet-rich plasma (PRP). PRP is obtained by plasma separation and constituents of platelets, blood proteins such as fibrin and a mixture of diverse growth factors (PDGF, VEGF, TGF-β, IGF, *etc*.) involved in general healing processes. Beside its autologous nature, another advantage generally attributed to PRP is its combination of growth factors in native proportions[81,82]. This feature of PRP is noteworthy as various studies have proven the synergistic effects of different growth factor combinations. Although beneficial effects of PRP have been demonstrated in cell culture studies as well as in *in vivo* models on tendon/ligament regeneration, the effectiveness of PRP in clinical use is still debated due to varying outcomes[81,83-87]. In a review by Yuan *et al*[87], these variances were mainly attributed to non-optimized treatment protocols.

Another strategy to trigger the healing capacity is to deliver therapeutic genes, either *in vivo* with vehicles or *ex vivo* in cells which are subsequently implanted. Wei *et al*[74] demonstrated that autologous graft remodeling in an ACL rabbit model can be enhanced by local administration of *TGFβ-1/VEGF165* gene-transduced bone MSCs, leading to superior mechanical properties compared to solely *TGFβ-1* gene transduced cells. In another very promising study by Hoffmann *et al*[88], MSCs were genetically modified to coexpress Smad8 and BMP2. These genetically modified MSCs enhanced the regeneration of the Achilles tendon in a mouse model. Taken together, the co-expression of growth factors is more efficient and potent than single gene therapeutic approaches.

MECHANICAL STIMULATION IN ACL REGENERATION

Mechanical stimuli and dynamic loading are necessary for ligaments to maintain their strength. In a number of studies, Woo *et al*[89] demonstrated that immobilization leads to weakened mechanical properties of ligaments[89-91]. From a mechanistic point of view, it is known that cells react to mechanical stimuli *via* integrin-mediated focal adhesions and cytoskeleton deformation[92-94]. Altman *et al*[95,96] demonstrated that mechanical stimuli are able to influence stem cell differentiation as well as the production of extracellular matrix (ECM). Mechanical strain resulted in the differentiation of MSCs into fibroblast-like cells, as seen by the upregulation of ligament markers tenascin-C, collagen types I and Ⅲ, and the formation of collagen fibers[95,97]. Petrigliano *et al*[98] showed that uniaxial cyclic strain of a three-dimensional polymer scaffold seeded with MSCs resulted in upregulated tenascin-C, collagen type I and Ⅲ. Berry *et al*[99] reported on the proliferative effect of uniaxial strain on young fibroblasts. Park *et al*[100] demonstrated that 8% cyclical strain in ligament fibroblasts leads to higher cell proliferation and collagen production compared to a 4% strain and unloaded controls. In their review, Leong *et al*[11] mentioned that despite the known fact that mechanical stimuli play an important role in ligament tissue engineering, the timing, direction and magnitude of the stimuli as well as the cell type can all be of significant influence on the cellular response. As an example, they discussed a study by Moreau *et al*[101] in which MSCs were stimulated immediately after seeding and showed an inhibited expression of collagen I and Ⅱ. In contrast, the opposite effect was observed when the mechanical loading was applied at the peak of MSC proliferation. Leong *et al*[11] mentioned that in case of ACL tissue engineering, additional investigation is required to elucidate the mechanotransduction pathways that are necessary for tissue formation and maintenance. They also stated that, to date, it is not known if any mechanical stimulation is required prior to implantation of tissue engineered ACL constructs.

FUTURE DIRECTIONS IN ACL REGENERATION

In a recent questionnaire study by Rathbone *et al*[102], 300 orthopedic surgeons were asked if they would consider a tissue engineered ACL if it were an available option.

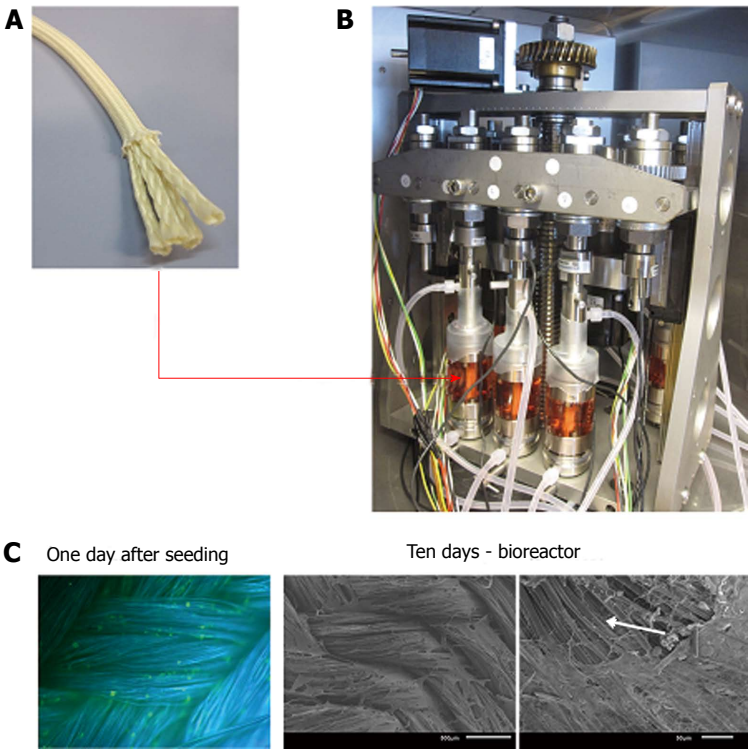

Figure 3 Adipose-derived stem cells cultured on silk-based ligament grafts (A) produce sheets of extracellular matrix proteins (C) under mechanical stimulation *via* a custom-made bioreactor system (B: design and construction in cooperation with the Technical University of Vienna, Institute of Materials Science and Technology). A: The silk-based anterior cruciate ligament (ACL) scaffold is produced of *Bombyx mori* silk fibers in a wire-rope design; B: The scaffold is seeded with ASCs for 24 h and then transferred into bioreactor and cultured under linear and rotational displacement for 10 d. The mechanically stimulated ACL scaffolds show sheets of extracellular matrix. The arrow in the bottom panel indicates an artefact of scanning electron microscopy preparation. In this area, the covering extracellular matrix sheet has been flushed away due to too intense flushing, allowing the view to the underlying silk fibers.

Eight-six percent answered positively if the construct demonstrates biological and mechanical success. For 63%, improved patient satisfaction was important and 76% of the participants mentioned that a tissue engineered ACL would be superior to any of the currently used autograft materials. It was also clearly stated that a fully load-bearing construct for implantation is needed and that several ACL tissue engineering strategies should address this need for mechanical integrity. This seems to be of crucial importance as the presently used ACL reconstruction techniques with autograft or allograft material provide an immediate load bearing environment. It seems obvious that, until the results of any regenerated ACL can compare with the current relatively successful autograft methods, patients are likely to prefer the autograft. As most surgeons do not require immobilization after reconstructive surgery, immobilization is likely to be unacceptable.

Another important aspect that will need consideration is the timing of the tissue engineering process and consequent implantation. In recent studies, our group focused on the mechanical stimulation of silk grafts with a custom-made bioreactor system[103] in order to increase the maturity of cell-loaded grafts prior to implantation (Figure 3). In accordance with a study by Altman *et al*[95], we triggered MSCs to produce layers of ECM on silk-based grafts. Our hypothesis is that the applied mechanical stimulation triggers the MSCs into ligamentous cells which in conjunction with the cells' own secreted ECM leads to more functionality of the cell/scaffold construct and therefore will superiorly fulfil its tasks once it is implanted.

Future studies using a combination of *in vitro* bioreactor engineering with consequent *in vivo* implantation are certainly needed to get a clearer picture of this complex topic. On the other hand, engineering mechanically appropriate scaffolds that are implantable at any time also seems to be a good option. Future research efforts may also demonstrate which cell type seeded on these scaffolds is the ideal candidate for direct *in vivo* implantation in this case. Furthermore, there is also some interest in exploring the regenerative potential of solely implanted scaffolds that would recruit *in vivo* cells, provided there is the appropriate mechanical and physiological environment. Just recently, Murray *et al*[104] proposed the strategy of repair and regeneration. Here, tissue engineering efforts are undertaken to overcome the obstacles to native ACL healing. This group proposed a bio-enhanced ACL repair technique that uses a collagen scaffold saturated with platelet-rich plasma. In a number of animal studies, they demonstrated improved mechanical and biological healing of the ACL[84,105-107]. In a recent randomized large animal trial, bio-enhanced ACL repair had equal results compared with ACL reconstruction. It was also shown that the knees treated with enhanced ACL repair had a lower rate of osteoarthritis in contrast to those treated with ACL reconstruction which developed osteoarthritis in 80% after one year[108]. Despite these interesting findings, it may be problematic to draw direct conclusions as osteoarthritis is not common a year after ACL injury in humans.

CONCLUSION

There is a growing research interest in the tissue engineering of the ACL and the clinical need seems obvious. Different strategies from *in vitro* engineering of ACL grafts to bio-enhanced repair and regeneration are followed. For the

surgical community, any type of engineered ACL may be a future option provided that it is easy to implant, does allow for at least the same aggressive rehabilitation protocol as currently used and will lead to better patient satisfaction and outcome.

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