TISSUE ENGINEERING APPROACHES FOR THE REPAIR AND REGENERATION OF THE ANTERIOR CRUCIATE LIGAMENT: TOWARDS 3D BIOPRINTED ACL-ON-CHIP

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Abstract

The anterior cruciate ligament (ACL) is the most frequently injured ligament in the knee. The current method to treat the injured ligament is reconstruction using autografts and allografts. Reconstruction requires the regeneration of ligament, bone and their interface to ensure proper recovery. Recently, researchers have focused on using tissue-engineered scaffolds made of synthetic materials and biomaterials — such as collagen, decellularised tissues, silk and synthetic polymers produced following different manufacturing methods — for ACL reconstruction. Different materials can be easily processed using various fabrication methods for mimicking the mechanical properties of the ACL. The advances in technologies play an important role in the production of constructions that can mimic native ACL. The present review addresses integrative scaffold design, different challenges in the potential materials and manufacturing methods as well as future strategies for ACL repair. Furthermore, the review provides a road map to 3D printing combined with organ-on-chip technology to demonstrate the potential for cost-effective and user-friendly fabrication methods for ACL engineering. Finally, it underlines the potential of 3D bioprinting and organ-on-chip technologies for micro-engineering of ligaments and their associated environment.

Keywords: Anterior cruciate ligament, 3D (bio)-printing, relative gene expression, collagen content, platelet-rich-plasma, platelet-rich fibrin, organ-on-chip.

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List of Abbreviations

3D	three dimensional
AM	additive manufacturing
ACL	anterior cruciate ligament
BEAR	bridge-enhanced ACL repair
bFGF	basic fibroblast growth factor
BPTB	bone-patella-tendon-bone
BLB	bone-ligament-bone
BMSC	bone-marrow-derived mesenchymal stem cell
dECM	decellularised extracellular matrix
ECM	extracellular matrix
FCS	foetal calf serum
FDA	Food and Drug Administration
FGF	fibroblast growth factor
FRESH	freeform reversible embedding of suspended hydrogels
GAG	glycosaminoglycan
GPS	gravitational platelet separation
HA	hydroxyapatite
HG DMEM	high glucose Dulbecco's modified Eagle's medium
HYP	hydroxyproline
IgE	immune globuline class E
IGF	insulin-like growth factor
IL-1	interleukin-1
ITS	insulin-transferrin-selenium
LC	ligamentocyte
L-PRF	leukocyte-rich platelet-rich fibrin

L-PRP	leukocyte-rich platelet-rich plasma
LG-DMEM	low glucose Dulbecco's modified Eagle's medium
MCL	medial collateral ligament
MEW	melt electrowriting
MKX	mohawk homeobox transcription factor
MMP	matrix metalloproteinase
MPC	mesenchymal progenitor cell
MSC	mesenchymal stromal cell
NEAA	non-essential amino acids solution
OA	osteoarthritis
P/S	penicillin-streptomycin
PBS	phosphate-buffered solution
PCL	posterior cross ligament
PDGF	platelet-derived growth factor
PEG	polyethylene glycol
PEGDA	polyethylene glycol diacrylate
PGA	polyglycolic acid
P(DTD DD)	poly(desaminotyrosyl-tyrosine dodecyl dodecanedioate)
PDS	polydioxanone
PLGA	polylactic-co-glycolic acid
PLLA	poly L-lactic acid
PL	patellar ligament
PLT	platelets
PPF	poly(propylene fumarate)
PPP	platelet-poor plasma
PRF	platelet-rich-fibrin
PRP	platelet-rich-plasma
RBC	red blood cells
RFU	relative fluorescence units
rhBMP	recombinant human bone morphogenetic protein
qPCR	reverse transcription-polymerase chain reaction
SB	single-bundle
SCXA	scleraxis A
SCXB	scleraxis B
SEM	scanning electron microscope
TC	tenocyte
TGF-β1	transforming growth factor beta 1
TNC	tenascin C
TNMD	tenomodulin
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor
WBC	white blood cells

Introduction

Rupture of the ACL is one of the most common knee-ligament injuries due to the increase in physical activity (Nwachukwu *et al.*, 2019). Over 200,000 patients per year are diagnosed with ACL ruptures worldwide (Buller *et al.*, 2014). Furthermore, ACL insufficiency further leads to instability and subsequent degenerative joint diseases (Simon *et al.*, 2015). Therefore, surgical treatments are crucial for restoring functional stability (Ateschrang *et al.*, 2018).

ACL rupture is treated surgically by reconstructing the ACL using a graft. The goal is to restore the stability of the knee and joint and potentially reduce the risk of subsequent articular damage (Kiapour and Murray, 2014). Historically, primary ACL repair was attempted by suturing the damaged ends of the ligament together (Duchman *et al.*, 2017; Palmer, 2007). This approach was abandoned and replaced by graft reconstruction procedures due to the significant failure rate of the sutures during the cyclic stress of natural knee movements. Nowadays, clinically used grafts are divided into three categories: autografts, allografts and synthetic grafts (Lin *et al.*, 2020; Shaerf *et al.*, 2014). Autografts include BPTB and hamstring tendons. Allografts are usually taken from the posterior tendon, Achilles tendon, *Tibialis anterior* tendon, BPTB and *Peroneus longus* tendons (Iosifidis and Alexandros, 2012). Puji *et al.* (2017) demonstrated that BPTB autografts have better outcomes following surgery, as assessed by the presence of knee and kneeling pain. Although autografts and allografts have excellent initial mechanical strength and encourage cell proliferation and new tissue growth, these techniques present some disadvantages: autografts require a second surgery for tissue harvesting while allografts can cause immunogenic responses (Duchman *et al.*, 2017; Panos *et al.*, 2020). In a recent study on the decellularisation of allografts, release of IgE and IL-1 at the wound site was considerably lower at the early stage, indicating a decreased immunological response. However, tissue regeneration is likely to require proper inflammatory responses, which may be limited by high levels of proinflammatory cytokine (Li *et al.*, 2020).

The history of ACL repair grafts

In the early 1980s, non-degradable synthetic grafts were employed to provide stability to the knee joint due to the concerns over the use autografts and allografts for ACL reconstruction. The FDA has approved different synthetic grafts for ACL reconstruction. However, these synthetic grafts are not recommended for primary ACL repair (West and Harner, 2005). Also, the use of permanent synthetic grafts can cause complications, including long-term rupture, foreign-body response and poor tissue integration (Chung *et al.*, 2017). Table 1 provides an overview of synthetic ligaments used for ACL repair and lists their advantages and disadvantages. For instance, Gore-Tex® is a single-strand polytetrafluorethylene fibre that provides higher tensile strength than native ACL (Ventura *et al.*, 2017). In addition, the use of other biomaterials such as collagen, which is the most common natural ligament ECM component, has been proposed (Bi *et al.*, 2015; Del Pizzo *et al.*, 1977; Legnani *et al.*, 2010; Ouyang *et al.*, 2002; Perrone *et al.*, 2017; Roth *et al.*, 1985; Ventura *et al.*, 2017).

Table 1. Advantages and disadvantages of synthetic ligaments for ACL repair.

Synthetic ligaments	Advantages	Disadvantages	References
Gore-Tex®	Higher tensile strength than in the native ACL	The construct leads to progressive long-term loosening	Ventura *et al*., 2017
Dacron	Higher tensile strength than in the native ACL	Leads to short-term stable low abrasion resistance	Legnani *et al*., 2010
Leeds-Keio artificial ligament	The stiffness of this ligament is similar to the native ACL	Inclusion of fibrous tissue was identified An alignment of longitudinal collagen fibres was found	Del Pizzo *et al*., 1977; Roth *et al*., 1985
Collagen	It promots more cellular ingrowth	"Pure" mechanical properties	Perrone *et al*., 2017
Silk	It provides sufficient mechanical properties for ACL reconstruction	The construct restricts neoligament regeneration	Bi *et al*., 2015
Various polymeric scaffolds	Higher mechanical and structural properties of native ACL	The constructs generally loose mechanical strength over time	Ouyang *et al*., 2002

Tissue-engineering approaches for ACL repair

Tissue-engineering strategies typically aim to combine ACL reconstruction with bioactive molecules, such as cytokines and growth factors, that might improve the ACL healing process by promoting cellular differentiation and proliferation (Butler *et al*., 2008; Tolikas *et al*., 2017). For this purpose, clinicians have been using PRP to treat ruptured ACLs (Lopez-Vidriero *et al*., 2010; Mazzucco *et al*., 2009; Murray *et al*., 2006; Schnabel *et al*., 2007). PRP is the plasma fraction of autologous blood containing a high concentration of platelets and growth factors. According to Figueroa *et al.* (2015), PRP treatment of grafts could be a synergic factor in the faster maturation of grafts when compared to untreated grafts. The beneficial effects of PRP are demonstrated on ACL-derived LC *in vitro* (Cheng *et al*., 2012; Fufa *et al*., 2008; Krismer *et al*., 2017; Mastrangelo *et al*., 2011; Murray *et al*., 2009; Yoshida *et al*., 2014). However, it is critical to determine which technique to use when obtaining the PRP, as there are significant variances in the number of platelet obtained and whether or not white blood cells, such as leucocytes, are included (Krismer *et al*., 2017; Yoshida *et al*., 2014). For the classification of different PRPs, the reader is referred to Mishra *et al*. (2012). However, in the clinics, to the best of the authors' knowledge, only one study found a significant beneficial healing effect with the addition of PRP (Vogrin *et al*., 2010). The authors showed more anteroposterior stability in ACL reconstructions when PRP was used. Other studies could not demonstrate such beneficial effects on ACL healing (Bissell *et al*., 2014). Recently, mainly four growth factors have been used in ACL treatment, *i.e.* bFGF, TGFβ, VEGF and PDGF (Table 2) (Amiel *et al*., 1995; Joshi *et al*., 2009; Kobayashi *et al*., 1997; Kondo *et al*., 2005; Madry *et al*., 2013; Mastrangelo *et al*., 2010; Nin *et al*., 2009; Spindler *et al*., 2006; Takayama and Kuroda, 2017; Takayama *et al*., 2015; Vavken and Murray, 2011; Wei *et al*., 2011). Table 2 summarises the outcomes and provides an overview of the different growth factors and their effects as observed in several *in vivo* and *in vitro* studies. Murray *et al*. (2016) and Karamchedu *et al.* (2021) were interested in the healing metabolism of the ACL. They compared the healing process of the MCL and ACL in a canine model (Murray *et al*., 2004; 2006; 2007). In canine knees, Murray *et al*. (2006) generated central defects in the MCL and/or patellar ligament as well as an intra-ACL. Then, the histological response to injury was assessed at 3, 7, 21 and 42 d. When compared to ACL defects, MCL and patellar ligament defects had significantly more filling of the wound site and significantly higher levels of fibrinogen, fibronectin, PDGF-A, TGF-1, FGF-2 and vWF at the wound site at all time points. As a result, the study validated the hypothesis that there is a lack of provisional scaffold in the ACL's intra-articular wound site and the loss is linked to a decrease in essential ECM proteins and cytokines. Murray *et al.* (2007) hypothesised that MCL and ACL have two different healing mechanisms. In the healing process of MCL, the ruptured ends are connected by a fibrin-platelet clot, which provides an environment for tissue ingrowth and remodelling. In addition, the ruptured ACL does not form a fibrin clot due to the upregulation of urokinase plasminogen activator in synoviocytes (Bakirci *et al*., 2020; Cesari *et al*., 2010; Flevaris and Vaughan, 2016). Urokinase plasminogen activator converts plasminogen into the active form of plasmin. Plasmin degrades fibrin and the clot loses the connection between the two ruptured ends. The loss of a clot could be the main reason for inhibition

Table 2. An overview of research on growth factors for ACL treatment.

FGF	*In vitro* - stimulates cell proliferation and collagen production on TCs	Amiel *et al*., 1995 Madry *et al*., 2013
	In vivo - enhances neovascularisation and formation of granulation tissue	Kobayashi *et al*., 1997
TGF	*In vitro* - increased both collagen and non-collagenous protein synthesis in TCs	Kondo *et al*., 2005
	In vivo - improves the structural properties of the construct - promotes angiogenesis of the reconstructed ligament	Mastrangelo *et al*., 2010 Wei *et al*., 2011
VEGF	*In vitro* - reduces angiogenesis - promotes graft maturation and biomechanical strength	Takayama *et al*., 2015
	In vivo - increases the matrix synthesis during its remodelling and healing processes	Vavken and Murray, 2011
PDGF	*In vivo* - releases growth factors with a similar spatial and temporal sequence to healing extra-articular tissue - promotes the proliferation potential of osteoblasts on the tendon-bone interface or TCs	Joshi *et al*., 2009 Spindler *et al*., 2006 Takayama and Kuroda, 2017
	Clinical - no discernible clinical or biomechanical effect was found	Nin *et al*., 2009

of tissue regeneration in ACL ruptures (Kiapour and Murray, 2014). Kiapour and Murray (2014) proposed that the presence of plasmin in the synovial fluid could be a primary reason for the poor wound-healing in the ACL. However, recently, Bakirci *et al.* (2020) have not found any support for this relatively plausible hypothesis when performing real-time imaging and experimental wound healing assays using primary ACL cells from patients undergoing full-knee prosthesis surgery. Nevertheless, tissue-engineered scaffolds combined with cells and growth factors should still be considered as a novel approach for replacing the fibrin clot in ACL injuries. Proffen *et al.* (2015) reported the use of a collagen scaffold soaked with PRP in combination with a novel bio-enhanced primary repair technique using a suture stent, called BEAR technique.

Preclinical studies

Preclinical studies have shown that biomechanical properties of ACL replaced following the BEAR technique are equivalent to other ACL reconstruction techniques after 3, 6 and 12 months of healing in an animal model. Karamchedu *et al*. (2021) also studied the effects over 1 year of different surgical treatments on post-traumatic OA using a porcine ACL transection model. Results showed that surgical methods that protect the knee from maximum body-weight load during movement minimise cartilage damage within 1 year. Furthermore, the first human trial of the BEAR technique showed that results depend on both patients' features and surgical choices (Murray *et al*., 2019). In a randomised trial, the performance of this scaffold-based technique was compared to that of traditional reconstruction, revealing similar results in terms of subjective scores and arthrometric measurements: based on these findings, this scaffold-based approach for ACL healing may have the potential to reduce morbidity associated with tendon harvesting, maintain knee proprioception and shorten recovery times (Murray *et al*., 2019; 2020).

PRP was also used for the activation of MSCs (Veronesi *et al*., 2018). The presence of a population of perivascular tissue-specific stem cells in the septum between the two bundles of the ACL is the basis for their use: if these cells are activated properly, they may have fibroblastic potential, which could accelerate ligament healing. Furthermore, both PRP and MSCs have immunomodulatory properties that help the ACL recover by reducing intra-articular inflammation (Roubelakis *et al*., 2014). Despite substantial preclinical research on the use of biological treatments to promote ACL healing in *in vitro* and *in vivo* models, the clinical literature is fragmented, with few clear recommendations for the future.

Seijas *et al.* (2014) published a retrospective case series in which they focused on a small group of 19 football players who were treated with an arthroscopic intra-ligament injection of 4 mL of leukocyte-poor PRP, followed by a 6 mL intra-

articular injection at the end of surgery. The results were outstanding, with 16 out of 19 patients returning to former sport activity having stable knees, with Tegner Score patients returning to sport the fastest (Seijas *et al*., 2014). Centeno *et al.* (2015) published the results of a prospective trial in which patients were treated with a fluoroscopically guided intra-ligament injection of PRP, platelet lysate and BMSCs. Overall, the results were positive, with 7 out of 10 patients showing signs of ACL recovery on an MRI scan 3 months following therapy. This evidence suggested that this strategy would be clinically effective but this would have to be demonstrated in a larger study.

Recently, advancements in engineering led to the development of complex biological systems able to reproduce the functionality of an ACL. Ligament and tendon engineering has been using different technologies such as 3D bioprinting, electrospinning and cell sheet (Li *et al*., 2019; Sensini *et al*., 2021), which allow for placing different cell types and growth factors in the right locations to mimic the anatomical structure of the natural ACL. On the other hand, organ-on-chip devices are miniature systems in which cells are cultured in microfluidic channels. Microfluidics keeps the cells alive by flowing the culture medium for several weeks, creating conditions similar to those *in vivo* (pH, flow, pressure and nutrients). Researchers can also target one of these conditions or test the effect of various drugs on cell behaviour. They generate functional data for preclinical testing of potential drugs at the earliest stages. These technologies are also exciting as they open new possibilities for the field of orthopaedics, overcoming the so-called "valley-of-death" (Fig. 1) (Butler 2008; Tolikas *et al*., 2017). In contrast, the present review focuses, for the first time, to the best of the authors' knowledge, on ACL engineering from fundamental to 3D bioprinting and organ-on-chip technologies (Hao *et al*., 2016; Marieswaran *et al*., 2018). In the first part, the healing mechanisms in ACL and the future of integrating different approaches to ligament tissue engineering are discussed; then challenges and potential future directions for the field are reviewed. Moreover, the future path of 3D printing combined with organ-on-chip technologies is envisioned to develop the next generation of ligament tissue engineering.

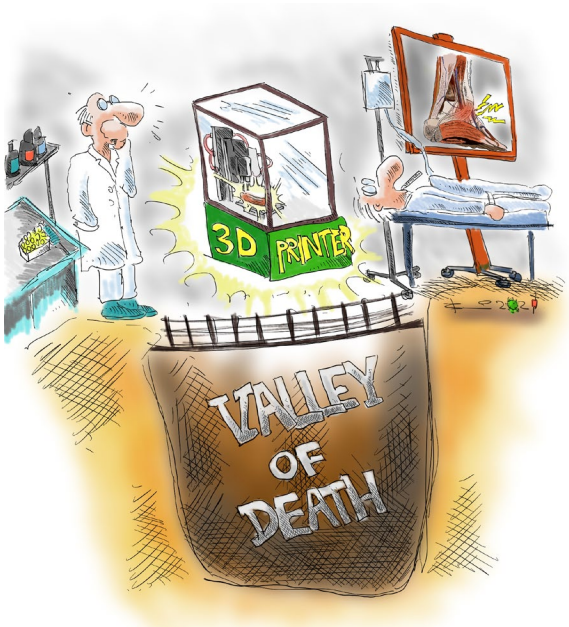

Fig. 1. Cartoon illustrating how 3D printing could bridge the so-called "valley-of-death" providing innovation from basic science to the patient's bedside (Butler, 2008).

The healing mechanism of ACL ruptures

Injuries to the ACL are common and the result of high levels of sportive activities. Early reports on primary ACL repair demonstrated unsatisfactory outcomes, leading to unanimous abandonment of suture repair and widespread adoption of ACL reconstruction approaches (Murray *et al*., 2013). Currently, ACL reconstruction is considered to be the gold standard for the treatment of ACL injuries, especially in the active group of patients with symptomatic instability. Recent advances in tissue engineering and regenerative medicine have generated renewed interest in preserving the remnant ACL to maintain its native biomechanical properties (Murray *et al*., 2013; Taylor *et al*., 2015). Novel approaches rely on an understanding of the pathophysiological processes that occur within the wounded ligament and its surroundings (Hao *et al*., 2016; Taylor *et al*., 2015). Most studies that have investigated these processes have been performed in animals. Few research studies were conducted on human ACL tissue. Murray *et al.* (2000) studied 23 human knee ACL remnants following rupture and identified 4 distinct histological stages of healing, including inflammation, an epiligamentous repair phase, proliferation and remodelling. The absence of a fibrin clot, the formation of a synovial cell layer with retraction capability on the surface of the ruptured ends and also the lack of any tissue bridging the rupture site were all highlighted as differences in response to injury from other dense connective tissues. At 3 to 10 weeks after surgery, some of the ligaments were extracted. Furthermore, Crain *et al.* (2005) examined 48 patients who had undergone ACL reconstructions. These researchers defined and divided the appearance of ACL remnants into 4 morphological kinds. The ACL remnant had adhered to the posterior cruciate ligament in the first type, the roof of the notch in the second, the lateral wall of the notch in the third and there was no scarring in the fourth. Crain's classification of the ACL remnant has been utilised as a standard since it was published and is proof of the ACL biological healing potential. Kirizuki *et al.* (2018) examined the ACL residual tissues taken from patients who had undergone initial ACL restoration within 3 months following damage and used the Crain's classification to determine the potential for proliferation and differentiation of the tissues. They showed that

during the subacute stage, in the non-reattachment group, ACL remnant tissue may be more likely to heal than in the reattached group. Nguyen *et al.* (2014) discovered that the human proximal third ACL had a natural healing ability similar to MCL in 5 patients. Recently, the morphological and histological changes of a ruptured ACL showed a short reparative phase within 3 months of injury, followed by a long remodelling phase of the ruptured ACL that ended with the attachment of the remnant to the posterior cruciate ligament (Haviv *et al*., 2018).

As discussed above, the ACL healing mechanism is a multi-factorial process that takes place in a dynamic environment (Murray and Fleming, 2013). Another fundamental challenge is the complex kinematics of the joint (Murray *et al*., 2013), which is cyclic loading during natural knee motion. Cyclic loading of a rigid fixation system leads to its failure (Buschmann and Bürgisser, 2017). For example, sutures are a rigid form of fixation, which often fails under cyclic loading (Bakirci *et al*., 2020; Vavken *et al*., 2013). In conclusion, knee motion and the biology of the ACL's response to complete rupture are rather complicated processes (Murray *et al*., 2013; Perrone *et al*., 2017). There is an epi-ligamentous reparative phase and no bridging scar formation, which (Murray *et al*., 2000) distinguishes it from tissues that successfully repair. Additionally, the formation of a synovial layer over the epi-ligamentous tissue, which consists of cells with a contractile actin isoform, may partially account for the retraction of the remains that is unfavorable to a reparative bridging tissue (Murray *et al*., 2000). ACLs, differently from other intra-articular tissues that do not repair (such as articular cartilage), respond to rupture with a proliferative fibroblastic and angiogenic response.

Bioreactors and ACL culture

Bioreactors have been successfully proven to be useful devices for testing pre-clinical scenarios of how partial tendon explants respond to mechanical loading. Mainly uni-axial loading devices were designed that allow a strain-controlled culture of tendon explants (Butler *et al*., 2008; Cook *et al*., 2016; Dyment *et al*., 2020; Gantenbein *et al*., 2019; Janvier *et al*., 2020; Riehl *et al*., 2012; Stoffel *et al*., 2017; Wang *et al*., 2013). Experiments using *ex vivo* bioreactor systems are instructive about specific mechanobiological loading regimes that seem to be beneficial for cultured tissues, such as loading frequencies around 1 Hz, which correspond to relaxed walking (Benhardt and Cosgriff-Hernandez, 2009; Hohlrieder *et al*., 2013; Krismer *et al.* (2016) Strain-controlled organ culture of intact human anterior cruciate ligaments – an *ex-vivo* model to investigate degenerative and regenerative approaches. Proceedings of the ORS Annual Meeting, Orlando, FL, USA, conference abstract; Riley, 2008; Snedeker and Foolen, 2017; Woo *et al*., 2008). The design and engineering of how tendons are clamped to the loading device are essential (Snedeker and Foolen, 2017; Steiner *et al*., 2012; Wunderli *et al*., 2020). Specific bioreactors were also designed for ACL culture (Gantenbein *et al*., 2019; Hohlrieder *et al*., 2013). Most devices are based on a linear stage to control for displacement and force to infer way-stress diagrams (Wunderli *et al*., 2017). Most studies used sinusoidal loading regimes and only in rare cases random loading profiles were investigated but keeping net energy uptake of the tendon tissue constant over time (Steiner *et al*., 2012) (Fig. 2). This assumes that human movement in most cases is of a sinusoidal nature (Buschmann and Bürgisser, 2017). Furthermore, in terms of frequency and duration of loading, a consensus is still missing. However, it is assumed that about 1 Hz loading is associated with walking in humans (Bramson *et al*., 2021). Fewer studies focused on the importance of culture media and whether perfusion of the culture chamber is essential or whether static cultures are sufficient. Concerning the effects of increasing glucose concentrations towards a more hyperglycaemic/diabetic condition, there are a few reports (Gautieri *et al*., 2017; Snedeker 2016). These studies demonstrated a stiffening effect and, in particular, effects on the collagen fibre orientation by an increase in glucose concentration (Snedeker and Gautieri, 2014). In terms of hydrogel cultures used for mechanical stretching, also relatively few studies investigated these effects (Gautieri *et al*., 2017; Issa *et al*., 2011; Snedeker, 2016). It seems to be a common conclusion that some level of basic mechanical loading is required to maintain the homeostasis of tendon explants (Schubert *et al*., 2021; Wang *et al*., 2021).

The effect of mechanical loading upon ligament homeostasis

The cells inhabiting the niche of the ligament are referred to as LCs for ligaments and as TCs for tendons (Murray *et al*., 2013). Morphologically, these are referred to as fibroblast-like cells, mainly producing collagen type I/III and, to some extent, proteoglycans. TCs and LCs are found in dense connective tissues, which are well-organised and arranged in rows between parallel thick fibres. They provide the primary resistance to tensile force (Van Eijk *et al*., 2004). Also, tenomodulin is a type II transmembrane glycoprotein specific to the development and maturation of tendons and ligaments. Interestingly, TCs and LCs have a lower proliferation and migration rate (Amiel *et al*., 1995) and lower responsiveness to growth factors (Spindler *et al*., 1996) and adhesion strength (Yang *et al*., 1999) compared to other cell types. As for different central ECM genes of the tendon, tenascin C is highly relevant, as is MKX (Liu *et al*., 2015; Otabe *et al*., 2015). MKX overexpression by an adenovirus induces MSC differentiation and stimulates collagen type I/III and SCX expression (Otabe *et al*., 2015). Recently, MKX has been identified as a critical player in the regulation of mechano-sensing properties of tendon (Kayama *et al*., 2016). Moreover, essential genes for tissue homeostasis are scleraxis and tenascin C

(Berthet *et al.*, 2013; Hasegawa *et al.*, 2013; Nichols *et al.*, 2018) and cyclic mechanical loading onto LCs does have a beneficial effect on the cell phenotype by modulating MKX (Nam *et al.*, 2015; Otabe *et al.*, 2015; Xu *et al.*, 2015).

The mechanical behaviour of TCs and LCs was extensively studied by several research groups (Buschmann and Bürgisser, 2017). The fundamental question of general interest is whether mechanical loading is beneficial and would help accelerate the wound healing potential in the case of tendinopathy. Understanding the latter is a big challenge and there are many unsolved questions. Many studies stated that the trigger from anabolism to catabolism is the initiator where the ACL and/or tendons lose their healing capacity. Thus, most of the literature stated that ligaments seem to have a very poor wound healing mechanism (Ackermann and Hart, 2016; Snedeker and Foolen, 2017).

It is essential to recognise the different biologically relevant microenvironments (Yates *et al.*, 2012). Snedeker and Foolen (2017) pointed out the significant difference between the "extrinsic tendon compartment" that represents "synovium-like" tissues that connect to the immune, vascular and nervous systems and the "intrinsic tendon compartment" that involves the single fascicles. The extrinsic compartments are usually ignored for the organ culture experiments, so an active immune system or nerves and their cells are excluded to reduce the number of cells.

The cell population in tendon and ligament also contains some progenitor cells, which exhibit universal stem-cell characteristics, such as clonogenicity, a high proliferative capacity and multi-differentiation potential (Costa-Almeida *et al.*, 2015; Hirzinger *et al.*, 2014). It is important to note that cell-specific differences exist between LCs and TCs. Bi *et al.* (2007) reported that fibromodulin and biglycan, two major components of the ECM, provide a niche environment for stem cells. Tendon and ligament-specific progenitor cells were identified using cluster of differentiation marker expression and flow cytometry. How these cells can be used to improve tendinopathy is yet unclear and needs to be elucidated.

Challenges in ligament tissue engineering

ACL reconstruction is a very common surgery (Murray, 2021; Pujji *et al.*, 2017). The focus of surgery is to restore the functional stability of the ACL, which provides for flexion strength and tibial rotation (Duchman *et al.*, 2017; Perrone *et al.*, 2017). Recently, engineered ligaments have shown promise in overcoming the drawbacks of autografts, allografts and synthetic grafts (Nau and Teuschl, 2015; Perrone *et al.*, 2017). However, the unique mechanical properties and poor healing capacity of ACL are the main limitations for tissue engineering solutions for ACL ruptures (Murray *et al.*, 2013). The stiffness of ACL is around 242 N/mm in samples from 22 to 35 year old donors (Dargel *et al.*, 2007). The ultimate load and stiffness of the ACL decrease with age (Woo *et al.*, 1991). It is difficult to find the proper material for reconstruction. This depends not only on the bulk mechanical properties but also on the anisometric structure and viscoelastic properties, which allow for differential load support during knee movement. Until now, none of the materials used as grafts has been able to reproduce these complex mechanical properties. Current research has focused on using biological and synthetic polymers, such as collagen, silk and polymeric scaffolds, to overcome the failure of autografts and allografts. The ideal grafts should

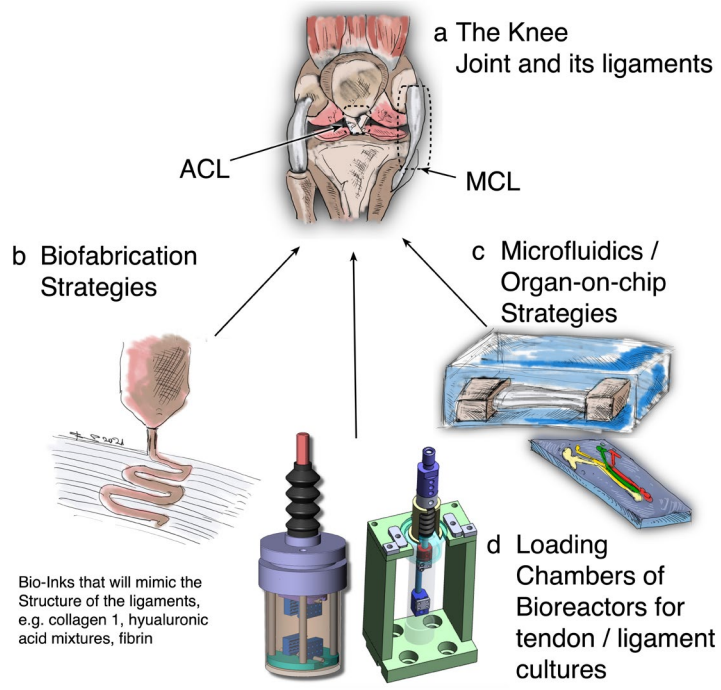

Fig. 2. Scheme of current problems and proposed strategies to regenerate the knee and its ligaments. (**a**) Overview of the knee joint. (**b**) Extrusion-based 3D-printing strategies to create a tendon/ligament-like microenvironment using bio-inks. (**c**) Microfluidics lab-on-chip approaches for high-throughput screening. (**d**) Loading chambers of bioreactors for tendon / ligament organ explant cultures.

provide immediate joint stability and should also gradually degrade and reduce in strength as the ligament regenerates and remodels. The selection of the suitable material and the manufacturing methods are critical factors for the regeneration of ligaments. In recent years, collagen, silk, composite materials and biodegradable synthetic polymers have all been studied as potential materials for ACL reconstruction.

Scaffolds in ACL engineering

Ideal scaffolds for ACL reconstruction should be biocompatible 3D constructs with mechanical properties capable of withstanding the forces normally experienced by the original tissue. A wide range of biology-based materials, such as alginate (Majima *et al*., 2005), chitosan (Sarukawa *et al*., 2011), collagen (Gantenbein *et al*., 2015), silk (Snedeker and Foolen, 2017; Teh *et al*., 2011) or hyaluronic acid (Huang *et al*., 2007), has been used in tissue engineering and regenerative medicine. Also, composite materials have been investigated for ACL reconstruction in order to improve the mechanical properties and biocompatibility. This part of the review provides an overview of the biomaterials that have been used for ACL regenerations.

Natural biomaterials and scaffolds

Many collagen-based constructs have been used in ACL reconstruction because of their chemical and structural similarity to native tissue, since the primary and natural component of the native ACL matrix is collagen type I (Goulet *et al*., 2011). In the late 1990s, Bellincampi *et al.* (1998) used the viability of rabbit ACL fibroblasts and their adhesion property to extruded collagen fibrils to form scaffolds for *in vivo* and *in vitro* studies. The results showed that the potential benefit of these constructs was limited because of the decreasing cell numbers over time. More recently, Robayo *et al.* (2011) reported that the mechanical strength of collagen scaffolds decreases over time. Several approaches have been investigated to increase the mechanical properties of collagen scaffolds. Crosslinking of collagen using UV or chemical reagents significantly improves the scaffolds' mechanical properties (Caruso and Dunn, 2005). The advantage of using crosslinkers is to retard the scaffold's degradation (Caruso and Dunn, 2005). However, the mechanical strength of the collagen scaffold is still weaker than native tissues. Due to the difficulty in mimicking the native crosslinking, the predominantly used crosslinking agents are chemical, such as glutaraldehyde, formaldehyde, acyl azide, carbodiimides and hexamethylene diisocyanate (Zeugolis *et al*., 2009). The main drawback of these chemicals is their potential toxicity. Fleming *et al.* (2008) reported no significant improvement in suture repair with the use of a collagen scaffold alone. However, the authors further demonstrated that ACL repair significantly improves by combining a collagen scaffold with autologous platelets, when compared to using them separately (Fleming *et al*., 2009). The mechanism behind this is unclear; however, it may be due to a synergistic effect between collagen, PRP and other ECM molecules. Previous work demonstrated the cell compatibility of commercially available porcine- or bovine-derived collagen types I and III patches with human ACL cells and bone-marrow-derived MSCs (Gantenbein *et al*., 2015). The results showed that the combination of commercial collagen patches with a dynamic intraligamentary stabilisation system would be a novel technique to repair ACL ruptures.

Another natural material used in ligament tissue-engineering is silk as it has remarkable strength and toughness compared to other natural and synthetic biomaterials (Kasoju and Bora, 2012; Petrigliano *et al*., 2006). In addition, it degrades slowly *in vivo*, allowing adequate time for host-tissue infiltration and eventual stabilisation (Leong *et al*., 2014). For biocompatibility, *Bombyx mori* silk requires the removal of the surface protein layer sericin, which is known to cause immune responses (Fan *et al*., 2009). After removal of the sericin, the silk fibres support cell attachment, migration, cell proliferation and differentiation (Meinel and Kaplan, 2012). The use of silk has also been promising for many *in vivo* studies due to good biocompatibility, slow degradability and remarkable mechanical properties (Chen *et al*., 2010; Cornish and Musson, 2013; Fan *et al*., 2008; Li and Snedeker, 2013; Li *et al*., 2014a; Li *et al*., 2014b; Panas-Perez *et al*., 2013; Petrigliano *et al*., 2006; Seo *et al*., 2009). The unique mechanical properties of silk combined with different fabrication methods, such as braiding or knitting, make silk an attractive candidate for ACL reconstruction. Altman *et al.* (2002) were the first to employ a braided silk fibroin scaffold seeded with MSCs for ACL reconstruction in 2002. Then, Chen *et al*. (2006; 2010) demonstrated that modifying silk fibroin with polypeptide chains could increase cellular attachment and proliferation. Furthermore, Murphy *et al.* (2008) changed the functional groups on the tyrosine residues in silk derivatives to promote cell proliferation. However, the limited internal space in twisted or braided fibre scaffolds restricted the neo-ligament tissue regeneration. Therefore, the use of different materials is attempted to investigate possible means of ACL reconstructions. Collagen and hyaluronan were used to improve silk substrates. Collagen- and hyaluronan-treated substrates increase the rates of cell migration when compared to silk scaffolds alone. Specific composite scaffolds seemed to favour angiogenesis, which is crucial for the initial repair phase of damaged ligaments and solving the blood supply problem in damaged ligaments (Panas-Perez *et al*., 2013; Seo *et al*., 2009). Recently, Dong *et al*. (2020) demonstrated that use of laponite/regenerated silk fibroin hybrid fibres using wet-spinning techniques worsened the mechanical properties of regenerated silk fibroin fibres. Furthermore, an *in vivo* study in a rat ACL reconstruction model showed

that the presence of laponite could significantly improve the graft osseointegration process (Dong *et al*., 2020). In comparison to other synthetic or natural biomaterials, silk clearly has a dominant role in biomedical applications (Fan *et al*., 2009; Kasoju and Bora, 2012; Meinel and Kaplan, 2012). For silk-based materials with more intricate designs, current breakthroughs in nanofabrication technology, as well as multilayer alterations, seem promising (Wu *et al*., 2021). Furthermore, efforts should be made to design manufacturing methods that are eco-friendly, easy to scale up, simple and time-saving as well as have low batch-to-batch variations, in order to transition from academic research results to clinical settings. Furthermore, the ability of highly concentrated regenerated silk aqueous solution feedstock to be stored for a long time should be considered. Another inherent problem of natural polymers is that they are brittle.

In this part of the review different natural biomaterials have been introduced as potential scaffolds for ACL tissue engineering. Ideally, the scaffold must be biocompatible and its mechanical properties should be as close as possible to natural ACL. It must also be biodegradable to allow tissue ingrowth, which is critical for the formation of the ligament.

Synthetic biomaterials and scaffolds

Several polymeric scaffolds such as PGA, pDTD DD, PDS, PLGA and PLLA are used for ligament tissue-engineering due to the possibility of modifying their physicochemical and mechanical properties (Silva *et al*., 2020). Different manufacturing methods such as knitting, braiding, bioprinting and electrospinning allow these polymers to have improved mechanical properties. Cells are proven to spontaneously orientate along the direction of the fibres, leading to abundant ECM secretion rich in collagen type I and III. Ouyang *et al*. (2002) studied the adhesion and proliferation of ACL cells and MSCs on different polymer substrates. The primary outcome of the study was that MSCs proliferate faster on PLGA and D-PLA and have a higher degree of cell attachment and proliferation than ACL cells on all other polymer substrates studied. Tovar *et al*. (2010) compared p(DTD DD) and PLLA fibre for the potential use of ACL reconstruction scaffolds. Results showed that p(DTD DD) possesses greater strength, less stiffness as well as more favourable degradation rate and cellular compatibility than PLLA. Although polymeric scaffolds have shown excellent mechanical strength, they did not facilitate enough cell adhesion, proliferation and subsequent function because of the lack of signalling molecules and hydrophobicity. Lu *et al*. (2005) fabricated a 3D braided scaffold of PGA, PLAGA and PLLA to investigate the effects of fibre formulation on mechanical properties and biodegradability. PLGA and PLLA filaments and fibronectin were used to improve cell adhesion. SEM results showed that cells seeded on PLLA-Fn and PLAGA-Fn scaffolds produced the most ECM. Rapid degradation of PGA *in vitro* resulted in matrix disruption and cell death over time. The rate of degradation of the scaffold must match the speed of tissue growth. In addition, the elastic and viscoelastic mechanical properties of the native ligament should be considered. Furthermore, improving the strength and biological integrity of the ligament-to-bone interface should be critical in developing a novel scaffold and *in vitro* model.

In most of the studies considered, synthetic materials promote continuous tissue remodelling using different functional and biological agents. Additionally, the most important properties of these materials are biocompatibility, chemical stability, degree of polymerisation, absence of soluble additives, low water absorption and mechanical properties, which are higher or similar to that of native ACL.

The engineered bone-ligament interface

The integration of an engineered ligament into bone tunnels is still a significant problem for ACL reconstruction (Saccomanno *et al*., 2016). Current fixation methods do not provide the biomimetic properties of an interface tissue. A heterogeneous scaffold design, with a gradient in chemical composition and cellular content, is necessary to replace the tissue to minimise the stress concentration and mediate load transfer between soft and hard tissues. Therefore, the research focus has been changed to produce a multi-tissue unit for ACL reconstruction. Several materials have been investigated as an alternative to native interface tissue, such as the composite of polylactic acids and polydopamine tyrosyl-tyrosine ethyl ester carbonate (Bourke *et al*., 2013). Paxton *et al*. (2009) investigated the potential of PEGDA hydrogel incorporated with HA and the cell adhesion peptide RGD as a material for the BLB interface. Results demonstrated that HA increases the mechanical strength of the hydrogel and generates a better interface formation, with a more significant proportion of calcium phosphate in the interface tissue. Wang *et al.* (2021) developed an *in vivo* model for ligament-bone regeneration using decellularised rabbit tendons and genetically modified osteoblasts and chondrocytes. The outcomes showed that decellularised tendon grafts improve cellular interaction and matrix formation in the bone-ligament interface. In addition, Li *et al.* (2016) fabricated a triphasic silk scaffold customised with three regions mimicking the ligament, fibrocartilage and bone layer in the ligament-bone insertion. They modified each region with a different coating: silk fibroin coating for the ligament; silk fibroin, chondroitin sulphate and hyaluronic acid sodium salt for the fibrocartilage region; silk fibroin solution and HA for the bone region (van Hengel *et al*., 2020). Three different cell types, such as BMSCs, chondrocytes and osteoblasts, were seeded onto the ligament, cartilage and bone regions of the three-phase silk scaffold,

Table 3. Additive manufacturing methods for ACL engineering.

Additive manufacturing methods	Possible materials to use for ACL reconstruction	Advantages (+) and disadvantages (-)	References
Stereolithography	PEG, PEGDA, HA, decellularised ECM, CaP	+: high resolution and cell viability -: crosslinking demands translucent and photosensitive bioinks, which limits the selection of adjuncts and cell density	Le Guéhennec *et al.*, 2020 Luo *et al.*, 2020 Pitaru *et al.*, 2020 Zorlutuna *et al.*, 2011
Volumetric printing	Photo crosslinking hydrogels, gelatine methacryloyl	+: fast processing, high resolution, no need for support -: complex systems	Bernal *et al.*, 2019 Grigoryan *et al.*, 2019 Loterie *et al.*, 2020 Regehly *et al.*, 2020
Fused deposition modelling	Thermoplastic polymer and composites (PCL, PLGA, thermoplastic polyurethane	+: no post-processing needed, cheap -: chance of degrading materials due to the high processing temperature	Choi *et al.*, 2016 Ge *et al.*, 2009 Merceron *et al.*, 2015
MEW	Thermoplastic polymers and their composites, hydrogels	+: high resolution to mimic collagen fibres -: relatively slow printing speed	Castilho *et al.*, 2021 Hochleitner *et al.*, 2018
Extrusion-based 3D bioprinting	Hydrogels, cells, bioglass	+: incorporate bioactive molecules -: slow processing	Bakirci *et al.*, 2017 Kajave *et al.*, 2020 Toprakhisar *et al.*, 2018

respectively. The cells seeded onto the silk scaffold showed high proliferation ability and enhanced differentiation capacity into the corresponding cell line. On the other hand, surgeons use a bone-to-bone technique for ACL reconstruction using autograft; it is a gold standard and bone-to-bone repairs heal better than other methods. Recently, metal screws are used primarily to fix tendon grafts, which cause pain stress-shielding phenomena and local irritation. Also, metal screws might be associated with tissue destruction and osteoporosis in the surrounding bone tissues. Therefore, this procedure requires secondary surgery to remove the screws. Nevertheless, bioresorbable and biodegradable screws have been considered as an effective fixation system with several advantages over metal screws, such as no corrosion and no need for second surgery.

Towards 3D bioprinted ACL-on-chip

3D bioprinting is the automated manufacture of tissues and organs to address medical problems. It combines cells, hydrogels and materials into a single construct that can replace damaged or wounded tissue, using AM. The final product is frequently complex, containing a variety of structural and cellular components (Mironov *et al.*, 2009).

Over the last two decades, advances in 3D printing have provided excellent opportunities for personalised treatment and production of *in vitro* models due to several advantages, among which the technique low cost and ease of use. Table 3 provides an overview of recent advances in AM in the field of tendon research. AM can be used for ACL reconstruction (Bakirci *et al.*, 2017; Bernal *et al.*, 2019; Castilho *et al.*, 2021; Choi *et al.*, 2016; Ge *et al.*, 2009; Grigoryan *et al.*, 2019; Hochleitner *et al.*, 2018; Kajave *et al.*, 2020; Le Guéhennec *et al.*, 2020; Loterie *et al.*, 2020; Luo *et al.*, 2020; Merceron *et al.*, 2015; Pitaru *et al.*, 2020; Regehly *et al.*, 2020; Toprakhisar *et al.*, 2018; Zorlutuna *et al.*, 2011). Studies focused on the potential of using AM technologies to successfully, rapidly and economically print customised implants at medical clinics (van Hengel *et al.*, 2020; Youssef *et al.*, 2017) (Fig. 2). In addition, AM allows for biomaterials and cells to be reassembled in their natural order, provides structurally and mechanically heterogeneous scaffold designs, improves cell attachment and growth and promotes cell interaction and matrix heterogeneity (Afghah *et al.*, 2019; Groen *et al.*, 2017).

Recent advances in AM also provide new fabrication opportunities for *in vitro* models (Miri *et al.*, 2019). Some studies have shown the use of AM for ACL reconstruction. Liu *et al.* (2016) used a 3D bioprinter to produce screw-like scaffolds, combined with MSCs, to fix tendon grafts and promote tendon graft healing within the bone tunnel in a rabbit ACL reconstruction model. Results showed that PLA/HA scaffolds loaded with MSCs facilitate the healing process of tendon grafts in the bone tunnel in terms of the high level of bone ingrowth and bone graft interface formation. The study is promising for using easily operated, low-cost 3D-printed structures. Recently, Parry *et al.* (2017) developed a 3D-printed PPF scaffold with delayed delivery of rhBMP encapsulated on PLGA microspheres. The scaffolds improve bone filling after bioabsorbable implant fixation. The study has provided promising results

concerning the use of 3D-bioprinted bioabsorbable screws in ACL reconstruction treatments. The future milestones for the development of ideal bioabsorbable screws include optimising their composition material, production properties and degradation characteristics to improve fixation and bone integration while overcoming the tissue's foreign body response. Similar to Parry *et al.* (2017) work, Park *et al.* (2018) investigated the effectiveness of using 3D-bioprinted scaffold sleeves of MSCs to increase osteointegration. Results showed improved osteointegration between tendon and tunnel bone in a rabbit model ACL reconstruction as assessed by haematoxylin and eosin staining, immunohistochemical staining of type II collagen and micro-computed tomography (Park *et al.*, 2018). Recently, a BLB construct has been described that recapitulates the bone-ligament interface (Fig. 3) (Lui *et al.*, 2019; Mussig *et al.*, 2010). The authors succeeded in controlling deposition of different cell types, which could produce a bone-ligament interface (Ede *et al.*, 2018). Gwiazda *et al.* (2020) used MEW for the development of a bone-ligament construct to mimic cell alignment in native tissues. They investigated MSCs orientation on aligned, crimped and random MEW fibres for 4 weeks. The scaffolds were rolled in 3 braided bundles combined with the bone component, osteogenically induced hMSCs on MEW scaffolds. The mechanical properties of non-cellularised and cellularised bone-ligament constructs were tested under both quasi-static and cyclic conditions. Results showed that *in vitro* maturation leads to significantly softer tissue in the constructs and that the mechanical properties improved the resilience due to ECM production. As previously mentioned, the ACL is composed of a highly organised collagen matrix and progress in the development of collagen printing could also help print ACLs that are similar to the natural tissue (Lee *et al.*, 2019; 2021).

Advanced *in vitro* models, such as organ-on-chip systems, combine cell culture settings with cutting-edge methodologies to create more consistent 3D microenvironments that simulate the physiological, chemical and mechanical characteristics of native tissues. These systems pioneer the way by stimulating and controlling critical tissue properties in a single device, including concentration gradients, shear stress, cell patterning, tissue borders and tissue-organ interactions (Kiener *et al.*, 2021; Miri *et al.*, 2019; Yang *et al.*, 2022). Organ-on-chip technologies (Bhatia and Ingber, 2014; Guenat *et al.*, 2020; Tavakol *et al.*, 2021) aim to reproduce the cellular microenvironment to mimic the physiological and pathological conditions, such as tension and flow-induced shear, that can help to develop a more feasible model for ACL treatment. Cells can be exposed to a variety of biomechanical and biochemical cues in organ-on-chip devices, which can better mimic *in vivo* cell response. Biomechanical cues can be passive or active on organ-on-chip devices and they are often external to the cell. Substrate stiffness, geometric confinement and topographic signals are examples of passive biomechanical stimuli, whereas tensile stretch and compression, fluid shear stress, interstitial fluid flow and hydrostatic pressure are all active stimuli (Mainardi *et al.*, 2021). Several microfluidic platforms aimed at exposing tissues

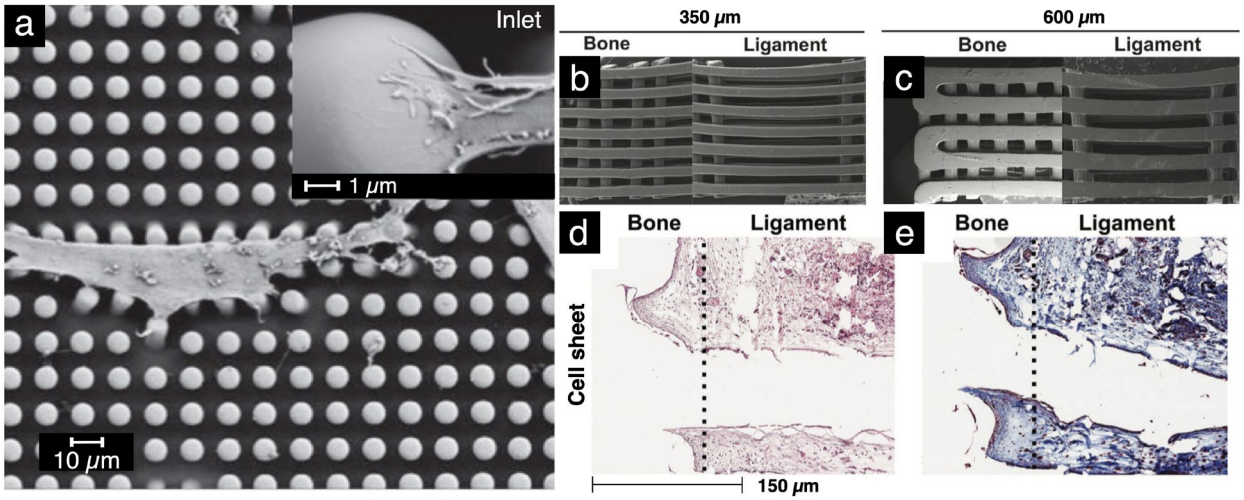

Fig. 3. Recent advancement of bio-engineered microenvironments of the interface between bone and ligament. (**a**) SEM image of adhesion of a gingival fibroblast on micropillar heads with pillar distances of 5 mm according to Mussig *et al.* (2010). The inlet shows a higher magnification of the direct interactions between the adhesion sites of the gingival fibroblast and the immobilised fibronectin on the pillar tops. Gingival fibroblasts were seeded onto a micropillar surface at a density of 7×10^3 cells/mL and cultivated for 24 h. (**b**,**c**) SEM images of multiphasic BLB scaffold at two different magnifications with a porous interface using 3D-printing and cell-sheet technology for the reconstruction of the dorsal scapholunate interosseous ligament made of medical-grade polycaprolactone scaffolds (Lui *et al.*, 2019). (**b**) Bottom view of 350 μm bone compartment. (**c**) Bottom view of 600 μm bone compartment. (**d**,**e**) Haematoxylin and eosin and Masson's Trichrome staining of BLB interphase in samples implanted for 8 weeks including cell sheets (Lui *et al.*, 2019). All figure parts were reproduced with permission from the publishers.

to mechanical forces have been reported in the last decade (Guenat and Berthiaume, 2018). Fascinatingly, they managed to integrate compression and elongation or mimic the cyclic strain of breathing motion (Zamprogno *et al*., 2021) status reply.

In the last decade, organ-on-chip platforms have rapidly evolved, combining previously specified technologies (iPSCs, biomaterials, bioreactors and AM) to replicate both healthy and pathological situations in numerous organs and tissues (Dyment *et al*., 2020). Various organs have been recreated on a chip, including lung, liver, heart, gut, muscle, arteries and bone. These have been created for purposes such as toxicological evaluation, vascularisation and drug testing on tissue-specific functions. These models have been designed to examine the basic mechanisms of organ function and disease.

Combined with AM and other biofabrication techniques, organ-on-chip technologies will provide a dynamic environment to perfuse, vascularise and cyclic load the bioprinted tissue. Therefore, organ-on-chip technology can better recapitulate the complex interactions between ligaments, tendons and bones in health and disease. In addition, as a small number of cells is needed in these systems, patients' cells will be cultured in parallel, allowing for the optimisation of culture parameters and screening of therapeutic compounds. Organ-on-chip systems combine cell culture settings with cutting-edge methodologies to create more consistent 3D microenvironments that simulate the physiological, chemical and mechanical characteristics seen in native tissues. These systems pioneer the way by stimulating and controlling critical tissue properties in a single device, including concentration gradients, shear stress, cell patterning, tissue borders and tissue-organ interactions. Lyu *et al.* (2020) produced an organ-on-chip that provides a concentration gradient of osteogenic induction medium. They established a method that optimises the ratio between the different stem cells to obtain the best outcomes for the composition transition under the medium concentration gradient as maintained by the microfluidic chip *in vitro*. Then, the scaffolds were implanted into a rat model of tendon tear injury to evaluate the effectiveness of the repair. Results showed that their method is a powerful approach to mimic the native tissue and has the potential to be translated for patient treatment.

3D printing and organ-on-a-chip technologies have the potential to reform and advance biomedical research by leading to the production of highly accurate and functional *in vitro* tissues, organs and disease models (Gold *et al*., 2019). It is critical to mimic the microenvironment as well as the 3D spatial distribution of cells and ECM to ensure native-like functionality at both the single-cell and tissue/organ levels. Researchers are still far from fully solving these problems and both 3D printing and organ-on-a-chip technologies have their limitations. Nevertheless, due to their different advantages, both technologies have real applications in different areas of biomedical research and they are complementary rather than alternatives to each other. It is well known that 3D printing methods that combine deposition modelling and stereolithography are mainly used for the fabrication of microfluidic devices (Au *et al*., 2016). 3D printing technologies greatly simplify the processing of traditional lithography methods by minimising the need for experimental procedures with operators, while dramatically reducing processing costs and time due to automated operating software and a user-friendly interface. These are the main reasons for the progress in the use of 3D printed organ-on-chip devices. There are three ways to combine 3D printing technology with organ-on-chip devices: i) fabrication of microfluidic devices through 3D printing technology; ii) printing of ACL cells onto prefabricated organ-on-chip devices; iii) direct printing of the entire organ-on-chip constructs, including mechanical microfluidic devices and biofunctional units.

Ingber (2022) mimicked different organs outside the body even though he established multiple organ-on-chip devices to investigate the crosstalk between organs. This study can provide a good foundation for future integration of the ACL-on-chip inside the joint-on-chip and checking the crosstalk between different ligaments and the dynamic environment of the ACL healing. The future direction requires a novel organisational model so that the fundamental discovery can be used in medical technology and commercial translation at the academic-industrial interface (Tolikas *et al*., 2017).

Conclusion

The number of people who suffer from ACL injury is constantly increasing. New options for ligament repair are necessary to overcome the limitations of current treatments. Interest in ACL reconstruction has persisted over the years due to the drawbacks of surgical techniques. Despite successful recovery reports of acute ACL injuries (Ateschrang *et al*., 2018), the treatment of chronic ACL insufficiency with tissue deficiency has not yet been achieved. The recent advances in manufacturing methods and materials science have facilitated the further development of ligament tissue engineering. Treatment approaches to such lesions would necessitate biocompatible scaffolds that incorporate sufficient mechanical properties with controllable degradation. The specific characteristic of the ACL, such as distinctive expression of ECM components, different cell morphology and function as well as higher metabolic rate, should be considered for future scaffold designs. The progress of high resolution 3D bioprinting facilitates further complexity, such as creating complex multi-material structure designs and controlling spatial distributions of cells and bioactive molecules. Also, organ-on-chip technology is essential for producing more physiologically

relevant environments. Such environments could provide a better understanding of the complex interactions between ligaments, tendons and bones in both health and disease conditions. The technology could revolutionise drug discovery and personalised treatment for ACL healing.

In summary, understanding the ACL's biological structure and healing metabolism is an essential step toward clinical treatments. In addition, advances in engineering, materials science and organ-on-a-chip technologies enable designing novel ACL reconstruction methods, fixation screws and development of drugs.

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Authors' contributions

E. Bakirci: study conception, primary literature research and drafted the manuscript. O. T. Guenat: wrote the organ-on-chips part, edited the manuscript and provided funding. S.S. Ahmad: drafted and approved the manuscript. B. Gantenbein: produced the figures, drafted as well as edited the manuscript and acquired funding. All authors read and approved the final manuscript.

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Discussion with Reviewers

Eithne Comerford: How near is this development to ultimate clinical translation?

Authors: The BEAR implant is the only option currently available in the clinic for ACL reconstruction

using allografts, autografts or suture-only. Unlike standard reconstruction, this implant does not require the use of harvested tendons for ACL repair. The BEAR implant is an absorbable implant. It bridges the space between the patient's torn ACL ends, is made of bovine collagen and is sutured into place. During the surgical procedure, the patient's blood is infused into the implant to form a protected clot that promotes the body's healing process. It is absorbed and replaced by the body's own tissues within approximately 8 weeks after BEAR implantation. Another concrete example is the use of bovine collagen membranes, which are already being used in clinics. However, advanced manufacturing methods such as 3D printing can improve the design of the implant, such as alignment of collagen fibres and different scaffolds, and shorten the healing process.

Eithne Comerford: How do the authors think this structure will react to the osteoarthritic environment of most knee joints in which it will be placed?
Authors: Injuries to the ACL frequently lead to early-onset osteoarthritis. Furthermore, current ACL reconstruction practice seems unable to stop these degenerative changes despite the recent advances. However, to the best of our knowledge, anatomic graft placement is crucial to reproducing normal knee kinematics and might slow the progression of joint degeneration following ACL reconstruction.

Rahul Gawri: Gold standard autografts are tendons implanted at the ACL site for reconstruction. What are the authors' thoughts on using stem cells and their differentiation towards TCs over LCs? Does differentiation towards TCs offer a better integration at the bone interface or will LCs offer similar integration results?
Authors: Stem cell therapy is a very exciting and yet challenging approach to regenerate joints in general (Nöth *et al.*, 2005, additional reference; Wang *et al.*, 2021). The problems of this research field lie in the superficial understanding of how these additional transplanted cells will get the right survival factors and micro-environmental parameters to thrive again to a fully differentiated LC or TC. In the cell therapy field open questions reside in the selection of patients that would suit this personalised therapy. Furthermore, outcome criteria, such as shortened time of disability, pain scores and other parameters to judge a good to very-good outcome for the patient, are unclear. The joint connective tissues generally presents a lower cell density compared to other tissues. Furthermore, the tissue homeostasis is relatively slow, in the range of years, to renew the ECM. Also, if these tissues are encapsulated, for instance in the knee joint, then, these are called "immune-privileged" regions, which are usually not so much exposed to immune cells. Of course, then, mechanical damage and pathology lead to inflammation and possible onset of arthritis. Thus, "boosting" the healing potential of these regions might sound tempting at first sight but as long as the fate and proper differentiation of such additional transplanted MSCs or even endogenous progenitor cells into the "native" LCs or TCs cannot be confirmed, the therapy might be causing more problems than provide benefits. MSCs can reduce the inflammatory response indirectly by the release of vesicles and a specific secretome. Maybe secretomes of such regenerative cells will be key for future therapy.

Rahul Gawri: ACL ruptures are not immediately repaired due to swelling in the joint as well as loss of range of motion post-injury and repair is performed after some time. The intra-articular inflammation post-injury also causes inflammation in the synovial membrane and adipose tissue, which might contribute to weakened/failed graft integration at the bone site due to the release of inflammatory adipokines. Would the authors like to comment on incorporating adipocytes under inflammatory challenge in the organ-on-chip model for ACL repair evaluation to gain insights into the repair potential and osteointegration of the candidate material under near physiological conditions?
Authors: So far, there are not many studies on that topic. For instance, Matsumoto *et al.* (2021, additional reference) showed that adipose-derived stem cells "sheets" surrounding transplant grafts applied during ACL reconstruction enhance tendon-bone healing and biomechanical strength. In addition, organ-on-chip devices might be used in the near future to study the different inflammatory molecules and their effects on ACL due to their capacity to mimic the microenvironment of tissues. In this respect, biofabricated ACL constructs could be produced *in vitro* with innovative methods with specific inflammatory molecules concentration gradients and speed through the microfluidic device. For instance, ACL could be printed using the FRESH method or by MEW biofabrication methods (Böhm *et al.*, 2022; Dufour *et al.*, 2022; Shiwarski *et al.*, 2022, additional references). FRESH is known as the gold standard for collagen printing, which is the most common ECM component in the ACL. MEW is one of the highest resolution biofabrication methods already used in *in vitro* studies for ACL reconstruction.

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