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MAKING CONNECTIONS: USING ANATOMY TO GUIDE TISSUE ENGINEERING APPROACHES AT THE ENTHESIS

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Abstract

The enthesis demonstrates a distinct highly ordered zonal microanatomy at the osteotendinous/osteoligamentous tissue connection that allows for the smooth transmission of mechanical forces between tissues. Interfacial tissue engineering (ITE), a subset of the interdisciplinary field of tissue engineering, is directed at replicating this complex transitional anatomy of the enthesis *in vitro*. Yet, the limited understanding of tissue boundaries, gradients and structural relationships at specific anatomical locations hampers the development of novel therapeutic strategies for bespoke enthesis regeneration, thus reducing their direct clinical applicability. This review provides an overview of ITE approaches for repair of the osteotendinous/osteoligamentous junction and highlights the importance of complementary inclusion of direct anatomical research. The cross-disciplinary collaboration across an array of experts, including anatomists, involved in the design, development and utilisation of bioengineered tissues will enhance the properties of such tissues and improve their clinical relevance. More specifically, a detailed anatomical analysis of the region of interest should drive the *in vitro* design and enable researchers to develop anatomically and clinically relevant tissue-engineered replacement tissues for human implantation. Finally, the present review discusses the challenges and future directions of the ITE field and highlights the importance of anatomically driven tissue engineering as an emerging tool in clinical translational research.

Keywords: Anatomy, tissue engineering, interfacial tissue engineering, enthesis, tendon-bone insertion, osteotendinous junction, bioengineered scaffolds, tendons, ligaments.

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List of Abbreviations

BMP	bone morphogenetic protein
BMSC	bone mesenchymal stem cell
COL2A1	collagen type II alpha 1 chain
COMP	cartilage oligomeric matrix protein
CRISPR	clustered regularly interspaced short palindromic repeat
ECM	extracellular matrix
ESWT	extracorporeal shock-wave therapy
FDA	Food and Drug Administration
FDP	flexor digitorum profundus
FGF	fibroblast growth factor
FGFR2	fibroblast growth factor receptor 2
GDF-5	growth and differentiation factor-5
Gli1	GLI-Kruppel family 1
HA	hyaluronic acid
iPSC	induced pluripotent stem cell
ITE	interfacial tissue engineering
LIPUS	low-intensity pulsed ultrasound
MCL	medical collateral ligament
nHA	nano hydroxyapatite
PCL	polycaprolactone
PGA	poly(glycolic acid)
PLGA	poly(lactic-co-glycolic acid)
Ptc 1	patched 1
PTHrP	parathyroid-hormone-related protein
Runx2	runt-related transcription factor 2
Scx	Scleraxis
Sox9	SRY-box transcription factor 9
TGF	transforming growth factor
TNMD	tenomodulin

Introduction

Anatomy

Anatomists study the form, structure and function of the human body. As the oldest scientific discipline of medicine, anatomy has existed for centuries. Yet, a recurring theme is present throughout the study of the human anatomy: the doctrine "form follows function". It is evident that anatomical forms and structures are perpetually connected to physiological functions (Mandrycky *et al*., 2017). The human body is a sophisticated, coordinated, multi-component system made of a complex arrangement of cell and tissue types. When an injury or disease disrupts the normal anatomical structure of a tissue or organ, it indubitably affects its associated function too.

A plethora of medical advances has revolutionised the clinical and biomedical landscape in the past decades, enabling structural and functional alterations to be treated by surgical or pharmaceutical intervention. However, treatment is not available in all clinical cases, since the tissue or organ is already too damaged in its structure and related functional capacity. Organ transplantation from a donor might be considered as an alternative; however, issues related to tissue rejection and donor availability severely hamper this opportunity. These problems, combined with the pressures of an ageing population, have led to the rapid growth of the interdisciplinary field of tissue engineering.

Tissue engineering

Tissue engineering, as one of the pillars of regenerative medicine, is the term used to describe the scientific field dedicated to investigating the manufacture of tissues or organs *in vitro*. Langer and Vacanti (1993) described tissue engineering as "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ". In fact, tissue engineering can be described as a new age of anatomically related research focussing on the synthesis of new tissues rather than their analysis (Mironov and Markwald, 2001). Significant clinical advances in organ-specific regeneration have taken place within this field since its infancy, with *in vitro*-manufactured organs, such as the bladder and skin, being implanted into human patients with success (Atala *et al.*, 2006). In the orthopaedic field, tissue engineering has witnessed some progress in enabling the commercial availability of some FDA-approved biological augmentation products, such as Carticel®, which consists of autologous chondrocytes introduced to repair focal articular cartilage defects (Bentley *et al*., 2003; Knutsen *et al*., 2004; Mao and Mooney, 2015). Despite this, the tissue engineering field still has to make substantial progress towards the definitive goal of providing specific anatomically correct patient implants for a wide range of tissue and organ types, particularly in areas of tissue connections.

The development of replacement organs or tissues and their architecture is a complex task, as the elegant structure formed during embryonic development is often difficult to replicate and remains a major challenge. Different tissue engineering strategies can be employed for tackling this issue. One such strategy is the top-down or traditional approach, involving the seeding of cells on a porous biodegradable scaffold, which in turn accommodates cell proliferation as well as ECM production and eventually degrades to form tissue constructs (Connon, 2015). This can usually be achieved with the aid of perfusion (Gaspar *et al*., 2012; Niklason, 1999; Zhao *et al*., 2018), chemical stimulation, such as by growth factors (Caballero Aguilar *et al*., 2019; Gooch *et al*., 2002; Kowalczewski and Saul, 2018; Wang *et al*., 2017) and hormones (Aramwit *et al*., 2017; Doostmohammadi *et al*., 2019; Samsudin, 2003), or mechanical stimulation (Boublik *et al*., 2005; Salinas *et al*., 2018). In contrast, another strategy called bottom-up or modular approach focusses on the structural microarchitecture to develop modular tissues with a repetition of functional units, which can be regarded as initial building blocks to assemble larger tissues (Connon, 2015 ; Nichol and Khademhosseini, 2009). By mimicking the native microarchitecture of these functional units, bottom-up approaches have the potential to manufacture more biomimetic engineered tissues (Nichol and Khademhosseini, 2009).

Anatomical connections

The interconnected field of tissue engineering calls for the expertise of many different specialities, from developmental biologists to mechanical engineers. Establishing a multidisciplinary team with the related and complementary expertise required is key to replicate the anatomical complexity found *in vivo* (Fig. 1). Indeed, the tissue engineer must first establish the tissue/organ they are going to engineer, understand its normal anatomical structure and function before designing a replacement tissue/organ. Pivotal to future development across the field of tissue engineering is the anatomist, who has the knowledge as to what comprises normal anatomy and to establish whether the engineered tissue complies with the tissue's native anatomy, function and related properties. In this way, the anatomist can aid in scaling up laboratory tissue-engineered models to integrated, functional, macroscopical implants, which is often overlooked when considering original model designs. Having a haptic appreciation of the regional anatomy is vital in tissue and organ replication and equally significant for human implantation. Should a tissue/organ be considered suitable for implantation, knowledge of the topographic anatomies should similarly be considered. Hence, anatomists should be regarded as a vital part of a tissue engineering team as not only can they evaluate the suitability of a potential engineered tissue based on structure, function and related properties, but also have the knowledge of the regional anatomy as well as the

system-wide anatomical connections (Mironov and Markwald, 2001).

In tissue engineering, not enough attention is given to the fact that no tissue or organ is isolated from the others within the normal human anatomy. This creates a significant clinical challenge. Not only the engineered tissue or organ needs to replicate structure and function of its intended replacement but it must also be able to connect and work with the surrounding anatomical topography. Therefore, tissue-connection points at the host implantation sites are also of critical interest when designing replacement tissues for an intended implantation. This is of particular importance within the musculoskeletal system, where the purpose of each component tissue type is to produce, transmit or absorb force. The specific challenges of tissue engineering for the musculoskeletal system are addressed below.

Anatomy, development and functions of musculoskeletal tissue connections

Musculoskeletal tissue connections are vital for human movement and locomotion. Muscles are attached to bones through connective tissue straps called tendons, which anchor the muscles firmly to the bone. For movement to occur, muscle contraction is initiated and the resulting force must then traverse the tendon to the moveable bone. In contrast, ligaments act to restrict movement between two bones, a necessary feature for stability in the joints and a vital component to ensure normal locomotion. In addition, articular cartilage on the surface of bones plays an essential role in maintaining normal locomotion by minimising friction between adjacent joint surfaces and acting as a shock absorber, an example of which is the articular cartilage found in the knee joint (Musumeci *et al*., 2014). Deficiencies in each component part of the musculoskeletal system (muscle, tendon, ligament, bone and cartilage) can cause severe pain and debilitation for patients. In fact, musculoskeletal injuries and diseases are commonly associated with acute and long-term pain, work limitations and functional disability (Brooks, 2006), with problems only increasing due to an ageing population. Therefore, methods to engineer replacement musculoskeletal tissues are in great demand. The limited functional treatment options and comparatively well characterised structure and function of the musculoskeletal system make these tissues particularly appropriate for tissue engineering endeavours (Rothrauff and Tuan, 2014).

The main sites where musculoskeletal tissue types meet are the muscle-tendon (the myotendinous junction), the bone-tendon/ligament (the enthesis) and the bone-cartilage (the osteochondral junction) connections. The present review will concentrate on the bone-tendon/ligament connection, the enthesis, describing its unique anatomical and functional roles before examining the progress in replicating this tissue-junction site in the laboratory.

The enthesis: bone-tendon/ligament connections

The anatomy of the enthesis

The anatomical connection between tendon/ligament and bone is called the enthesis (Benjamin

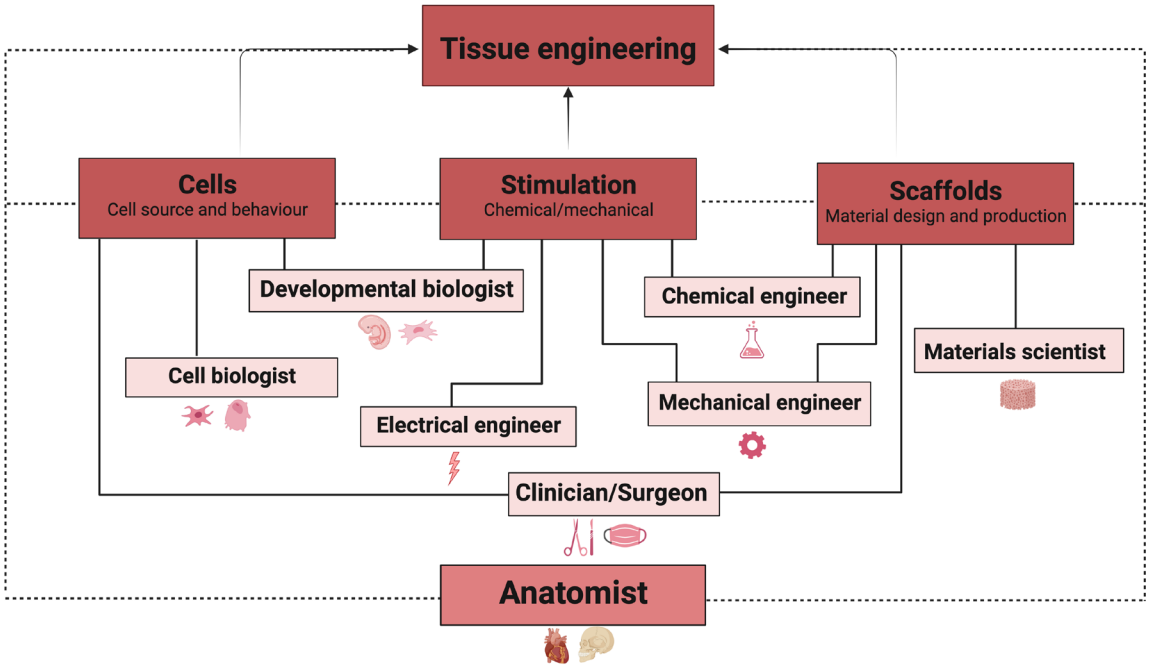

Fig. 1. Making connections in tissue engineering. Cross-disciplinary collaborations between expert researchers significantly strengthens the design and development of musculoskeletal tissue transitions and enhances the translatability of the engineered tissue. Created using Biorender.com.

and Ralphs, 2001; Benjamin *et al.*, 2002) (Fig. 2). As introduced above, the connection between tissues of the musculoskeletal system is fundamental to their overall function. At the enthesis, soft, flexible tendon/ligament tissue anchors to hard, rigid bone tissue. This mismatch in mechanical properties between the soft and hard tissues presents a significant engineering challenge, as the force of muscle contraction must travel through the tendon and bone for bone movement to occur. In engineering terms, a sudden change in mechanical properties would lead to a situation known as impedance mismatch and failure is likely to occur at the connection between hard and soft tissue. However, nature has engineered an ingenious solution to this mechanical problem by carefully constructing fibrocartilaginous entheses into several zones, each with an incremental improvement in mechanical properties. In fact, the fibrocartilaginous enthesis comprises four separate regions: 1) tendon/ligament (dense fibrous connective tissue), 2) uncalcified fibrocartilage, 3) calcified fibrocartilage, 4) bone (Benjamin *et al.*, 2002; Benjamin *et al.*, 2006; Benjamin and Ralphs, 1998; Benjamin and Ralphs, 2001) (Fig. 2). The fibrocartilage region adapts the mechanical properties at the interfacial region and this, combined with notable changes in collagen fibre alignment, collagen type and a gradual increase in mineral content, modifies the rigidity of the interface region from soft (tendon/ligament) to hard (bone) (Benjamin *et al.*, 2002; Benjamin and Ralphs, 1998; Doschak and Zernicke, 2005; Spalazzi *et al.*, 2013; Thompoulos *et al.*, 2010). In addition, the abovementioned heterogeneity favours the heterotypic cellular interactions required for retaining homeostasis and function (Zhao *et al.*, 2017). The traditional morphological description of the enthesis stated that the outer limit of mineralisation was denoted by the tidemark, classifying a sharp transition between calcified and uncalcified zones of the enthesis (Benjamin *et al.*, 2002; Benjamin and Ralphs, 1998; Benjamin and Ralphs, 2001). Raman spectroscopy analysis has revealed that minerals are, in fact, gradually distributed across the enthesis (Genin *et al.*, 2009; Thomopoulos *et al.*, 2010; Wopenka *et al.*, 2008), further substantiating the fact that a smooth transition between the two tissue types exists. Furthermore, the calcified fibrocartilage zone interdigitates with the bone in a highly irregular manner (Milz *et al.*, 2002) and, thus, the surface area of contact between soft tissue and bone greatly increases (Benjamin *et al.*, 2002; Benjamin and McGonagle, 2009; Doschak and Zernicke, 2005). This jigsaw-like, multi-directional interlocking at the true anatomical tendon-bone junction resists failure in any one direction (Shaw and Benjamin, 2007) and, with the gradual changes in biological and mechanical properties across the interface reducing strain and decreasing impedance mismatch, these morphological adaptations reduce the chance of failure between the soft and hard tissue (Benjamin *et al.*, 2002; 2006; Doschak and Zernicke, 2005; Paxton *et al.*, 2012a).

Development of the enthesis

The development of the enthesis occurs as the tissue precursors of both tendon and bone undergo the

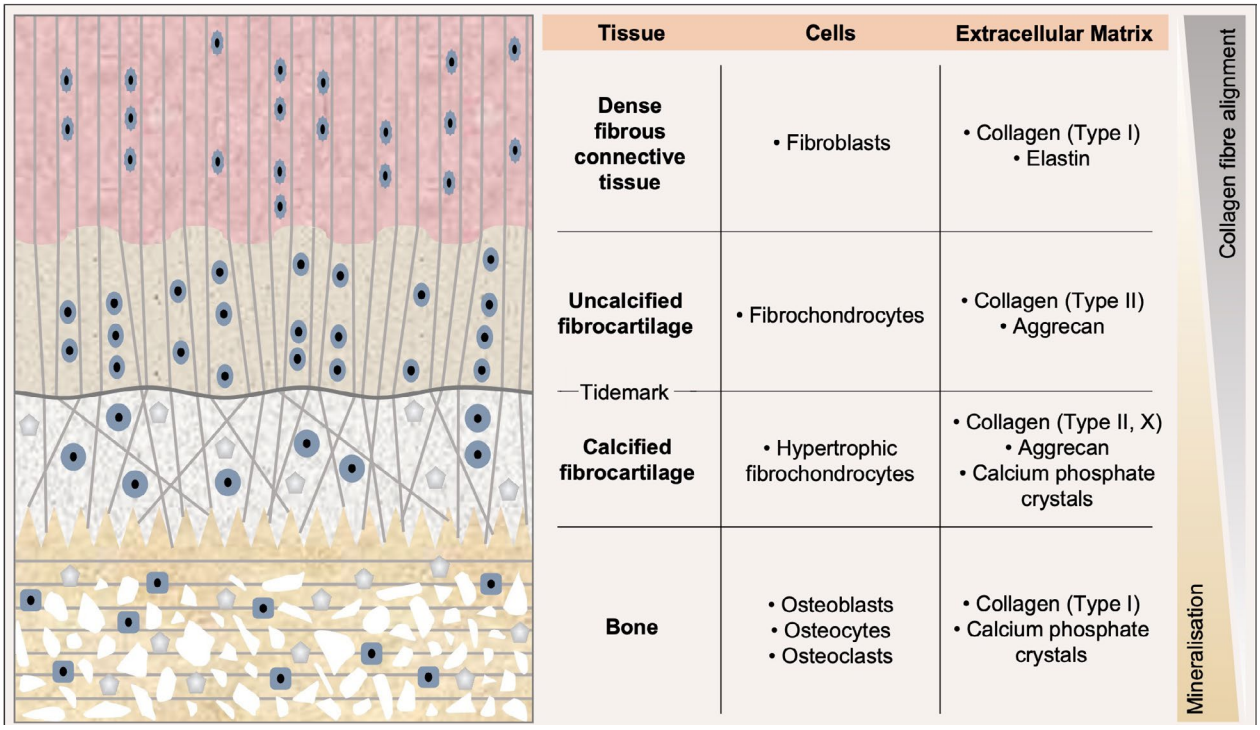

Fig. 2. The fibrocartilaginous enthesis. Visual summary of the key cellular, as well as ECM constituents and structure, of the fibrocartilaginous enthesis. Mineralisation is increased and collagen fibre alignment is decreased moving from dense fibrous connective tissue to bone, accompanied by a decreased longitudinal arrangement of cells.

process of fusion, in a series of carefully orchestrated events relying heavily on a combination of complex cell signalling events and mechanical stimulation. The specific events occurring during formation of the interface has been elegantly reviewed by numerous research groups (Galatz *et al.*, 2007; Lu and Thomopoulos, 2013; Mackie *et al.*, 2008; Roberts *et al.*, 2019; Schwartz *et al.*, 2012; Thomopoulos *et al.*, 2010; Zelzer *et al.*, 2014), with the most recent update published by Bobzin *et al.* (2021). These reviews are highly recommended for specific details concerning the morphogenesis of the bone-tendon/ligament insertion. However, for the purposes of the present review, a brief description of the subject is provided below.

The formation of a fibrocartilaginous enthesis starts with the process of endochondral ossification, the process by which many long bones form from a cartilaginous template (Mackie *et al.*, 2008; Schwartz *et al.*, 2012). The tendon and bone precursor models arise as separate components in the embryo,~ 15.5 d post conception in the mouse (Galatz *et al.*, 2007), equivalent to ~ 8 weeks of human development. Connection of the two precursor models occurs when the tendon cell condensations attach to the hyaline cartilage that, at this point, is forming the cartilaginous bone template (Mackie *et al.*, 2008). Development of the graded, transitional region does not occur until the early postnatal period and maturation of the enthesis continues throughout postnatal development. The beginning of ossification or mineral deposition occurs postnatally, with mineral gradients at the developing bone-tendon junction evident from postnatal day 7 in the mouse (Schwartz *et al.*, 2012). These mineralisation fronts are thought to co-exist with the mineralisation events occurring during endochondral ossification (Schwartz *et al.*, 2012) and, as described in the adult enthesis, persist throughout development until maturity. As the enthesis is developing, the mineral gradient that is already evident early in postnatal development becomes separated from the tendon by a layer of cartilage from the epiphyseal growth plate (Zelzer *et al.*, 2014). This cartilage is not yet calcified but undergoes mineralisation during the first few weeks of postnatal growth (Schwartz *et al.*, 2012; Zelzer *et al.*, 2014), eventually forming a mature graded mineral fibrocartilaginous enthesis by 21 d in the mouse (Galatz *et al.*, 2007).

The molecular mechanisms involved in orchestrating these events are complex and several factors have been implicated in the creation of a functional bone-tendon connection. Tendon and cartilage cells express Scx and Sox9, respectively, and a gradient-like expression of these, or related, factors was originally thought to be responsible for the formation of the enthesis (Thomopoulos *et al.*, 2010). However, studies have established that a progenitor pool of enthesis cells that co-express Scx and Sox9 exists at the junction (Blitz *et al.*, 2013; Sugimoto *et al.*, 2013; Zelzer *et al.*, 2014). GDF-5 is also expressed by the cells in this enthesial progenitor pool and, under control of the Hedgehog signalling pathway, these cells transform into mineralising fibrochondrocytes, responsible for producing calcified tissue and the mature graded enthesis (Dyment *et al.*, 2015; Felsenthal *et al.*, 2018). More specifically, $Gli1^+$ cells are the precursors of fibrocartilage and calcified fibrocartilage (Felsenthal *et al.*, 2018). Further work by Killian and Thomopoulos (2016) has also confirmed Scx as a vital player in the formation of a functional enthesis and many other factors such as TGF-β, PTHrP and Ptc 1 have also been implicated (Zelzer *et al.*, 2014). Moreover, it has been revealed that Scx^+ cells actively contribute and control the development of tendon-bone insertion units in the mandible of mice at postnatal day 2 and 7, respectively (Roberts *et al.*, 2019). The same group demonstrated that FGFR2 signalling guides development of tendon-bone interfaces in the mandible, where loss of FGFR2 signalling diminishes Scx expression in Scx^+/$Sox9^+$ progenitors and encourages differentiation of $Sox9^+$ chondrocytes over Scx^+ tenocytes, leading to ectopic endochondral bone formation exterior to the boundary of the mental eminence of mice (Roberts *et al.*, 2019). This finding is in line with other published studies showing that proteins of the FGF family can activate Scx expression and tenocyte cell fate in chick somites and limbs (Brent *et al.*, 2003; Brent and Tabin, 2004; Havis *et al.*, 2016).

In conjunction with the complex signalling events that are involved in generating a functional enthesis *in vivo*, mechanical factors also play a crucial part in this process. When normal mechanical movement is restricted or lost at the developing enthesis, formation of the mature fibrocartilaginous enthesis is severely disrupted. Botulinum-toxin-induced muscle paralysis neatly demonstrates this reduction in functional development, as toxin-injected muscles have been shown to attach to bone through an underdeveloped enthesis, with inferior mineral composition and related mechanical properties across the enthesis compared to saline controls (Schwartz *et al.*, 2013; Thomopoulos *et al.*, 2007). These experiments demonstrate the sensitivity of musculoskeletal tissues to their mechanical environment, not only in their own development and maturation phases, but in the important processes of making connections with others. Furthermore, it highlights the importance of considering the native environment when tissue engineering an equivalent substitute.

Injury and repair of the enthesis

The most problematic characteristic of the bone-tendon/ligament connection point is that, despite its carefully evolved anatomical form, damage due to mechanical overuse or inflammatory conditions disrupts the structure and, therefore, function of the enthesis. Some of the most common anatomical locations of frequent injury include intra-articular entheses – such as the rotator cuff tendons, the glenoid labrum, the anterior cruciate ligament – and

extra-articular entheses – such as the Achilles tendon, the patellar tendon as well as the medial and lateral collateral ligaments of the knee (Derwin *et al*, 2018). Resulting pathologies of injured entheses include rotator cuff disease, tennis elbow, jumper's knee and Achilles tendinosis (Derwin *et al*, 2018). Rotator cuff disease remains one of the most challenging enthesis-related pathologies, accompanied by an increasing incidence with age due to higher susceptibility to degeneration (tendinosis) of the avascular rotator cuff complex (Apostolakos *et al*, 2014; Dang and Davies, 2018; Derwin *et al*, 2018).

Tendon/ligament ruptures commonly occur during sports, usually affecting young, active members of the population who require fast and effective methods for repair and rehabilitation. Complete tendon rupture at the bone-tendon interface (avulsion) requires surgical reattachment of the soft tissue directly onto the hard bone. However, healing processes form a disorganised fibrovascular scar at the enthesis instead of the ordered fibrocartilaginous transition (Galatz *et al*, 2006; Liu *et al*, 1997; Rodeo *et al*, 1993; Silva *et al*, 2006). The surgically treated interface is biomechanically inferior (Galatz *et al*, 2006; Rodeo *et al*, 1993; Thomopoulos *et al*., 2003) and fails to recreate the gradient in collagen fibre orientation and minerals (Derwin *et al*, 2018; Gulotta *et al*., 2009; Rodeo *et al*, 1993; Silva *et al*, 2006). Then, when the patient attempts to return to normal movement, the repair site is particularly prone to partial or full detachment, risking further pain, loss of function and debilitation.

Regeneration of the four-zoned enthesial structure has been attempted in many animal models, using exogenous application of growth factors, novel materials and stimulation techniques (for reviews see Akyol *et al*., 2014; Boys *et al*., 2017; Font Tellado *et al*., 2015; Patel *et al*., 2018; Paxton *et al*., 2012a; Valencia Mora *et al*., 2015). Local application of growth factors, such as BMPs, FGFs and TGFs, has shown varied results in *in vivo* studies aimed at recreating the zoned, fibrocartilaginous enthesis following tendon reattachment surgery. While some studies have reported an increase in fibrocartilage deposition (Chen *et al*., 2011; Ide *et al*., 2009; Kim *et al*., 2011a) or mechanical strength (Chen *et al*., 2011; Hashimoto *et al*., 2007; Seeherman *et al*., 2008) of the repaired enthesis with growth factor addition, Gulotta *et al.* (2011) reported no significant difference between control and experimental groups, suggesting that further investigation into the utility of growth factors on enthesial repair is necessary. Several studies have also investigated the application of additional cell types at the tendon-bone repair site (for review see Rothrauff and Tuan, 2014), for example, chondrocytes (Nourissat *et al*., 2010; Wong *et al*., 2004), mesenchymal stem cells (Gulotta *et al*., 2009; Gulotta *et al*., 2010; Nourissat *et al*., 2010; Soon *et al*., 2007; Tornero-Esteban *et al*., 2015) or adipose-derived stem cells (Valencia Mora *et al*., 2014). Again, the results of these studies are variable, with no one technique restoring the specific anatomical attachment of bone-tendon as is observed before injury *in vivo*.

Novel stimulation techniques aimed at improving the anatomical connection between tendon and bone in *in vivo* models have also been used, such as ultrasound application or ESWT. Significant evidence for the positive effect of LIPUS on the healing potential of the bone-tendon interface has been provided by Lu and colleagues, who stated that LIPUS improves the quality of the bone-tendon interface based on several factors related to bone regeneration, such as increased mineral deposition as well as increased hardness and osteogenesis and/or chondrogenesis at the interfacial region (Lu *et al*., 2016; Lu *et al*., 2008; Lu *et al*., 2015; Lu *et al*., 2009; Qin *et al*., 2006a; Qin *et al*., 2006b). Despite a series of studies by Wang and colleagues (Wang *et al*., 2003; 2005; 2008) reporting the effectiveness of ESWT on bone-tendon interface healing, this technique has ceased to be under investigation, as its usefulness in restoring the anatomical connection of the enthesis still remains unclear.

Current interfacial tissue engineering approaches for repair of the enthesis

The *in vitro* engineering of the bone-tendon or bone-ligament connection has received much attention over recent years. While many groups focussed their research on the tendon or bone independently, some groups began to realise the importance of the functional transition between hard and soft tissues and the need for a suitable implant to counter the effects of poor hard-soft interface healing *in vivo* following injury. The advent of many synthetic options for replacement of tendons and ligaments being produced *in vitro* – acknowledging the fact that the overall success of an implant will largely be dictated by the strength and function of its connection with the host tissue – has led to the foundation of the subset of ITE for hard-soft tissue repair. The major challenge in ITE lies beyond homogenous tissue replication but in replicating a highly heterogenous, complex structure of small size [characteristically 0.1-1 mm (Lu and Thomopoulos, 2013)] that provides native mechanical functionality. In-depth reviews of tissue engineering options for the bone-tendon and bone-ligament interfaces have been published (Calejo *et al*., 2019; Boys *et al*., 2017; Patel *et al*., 2018; Armitage and Oyen, 2015; Font Tellado *et al*., 2015; Rothrauff and Tuan, 2014; Lu and Thomopoulos, 2013; Lu *et al*., 2010; Smith *et al*., 2012; Yang and Temenoff, 2009), with a summary of the major developments and techniques in this field described below.

Co-culture systems

Simple single-cell-type culture models are used widely in bone tissue engineering and tendon/ligament engineering to engineer distinct tissue models (Abbah *et al*., 2014; Borciani *et al*., 2020;

Calejo *et al.*, 2018; Frimat *et al.*, 2011; Hsieh *et al.*, 2016; Hsieh *et al.*, 2018; Kook *et al.*, 2017; Li *et al.*, 2019; Moysidou *et al.*, 2021; Paxton *et al.*, 2009; Paxton *et* al., 2010b); however, one of the fundamental approaches in ITE is the introduction of co-culture models, which can enable the possibility of cell-cell interactions in tissue culture in a spatially and temporally regulated manner. In general, co-culture of different cell types allows heterogenous cell-cell contact and communication to provide closer *in vivo* models of multicellular tissues (Goers *et al.*, 2014; Kook *et al.*, 2017). The technique exploits paracrine signalling as a method for tissue engineering and, since heterotypic cellular interaction is implicated in enthesis development, it is a prominent ITE technique.

In the simplest terms, a co-culture can be established in standard 2D tissue culture, with both cell types initially cultured either side of a divider, which is then removed to allow the two cell types to grow towards each other to form an interfacial region (Wang *et al.*, 2007). Wang *et al.* (2007) attempted to replicate the bone-ligament interface co-culturing fibroblasts and osteoblasts with this model system, and although expression of some enthesis specific markers was evident (collagen type II, aggrecan and COMP) a mature fibrocartilaginous transition was not produced. This useful model of the bone-tendon interface has been adopted elsewhere to study the role of BMP-7 (Schwarting *et al.*, 2015a) and cyclooxygenase (Schwarting *et al.*, 2015b) *in vitro*.

Although there is some utility in studying the interaction of cells in 2D culture, it is now well established that cell behaviour in 2D culture differs from that in 3D culture (Antoni *et al.*, 2015; Edmondson *et al.*, 2014) and, in fact, 3D cell culture is known to represent the physiological environment of the cell population with more accuracy than 2D culture (Antoni *et al.*, 2015). Hydrogels have shown significant potential in supporting 3D co-cultures across the tissue engineering field. Hydrogel scaffolds are made of crosslinked polymeric network structures that swell in water but do not dissolve (Kook *et al.*, 2017; Nikolova and Chavali, 2019). Their hydrophilicity, biocompatibility and other physical properties have resulted in their widespread use as biomaterials. To this end, an *in vitro* model system was developed using hydrogels as scaffolds to enable a more elaborate investigation of co-cultures in 3D (Alsaykhan and Paxton, 2020). While the authors examined several different natural hydrogels (agarose, gellan, fibrin and collagen) and orientation options (vertical *versus* horizontal orientation) of the 3D co-cultures, cell-encapsulated fibrin hydrogels arranged in a horizontal plane were proven to best aid cell proliferation and migration to form a basic model of the tendon-bone interface *in vitro* (Alsaykhan and Paxton, 2020). While the use of hydrogels comprises one successful approach to investigate 3D cultures, several techniques used to generate 3D scaffolds will be explored in the present review.

Bioengineered scaffolds

Scaffolds provide the 3D framework for cell and tissue growth (Castillo-Henríquez *et al.*, 2021; Doberenz *et al.*, 2020) and scaffolds manufactured in other forms can also be used to guide tissue formation both *in vitro* and *in vivo*. Some such common fabrication methods suitable for generating bioengineered scaffolds include electrospinning, freeze-drying, solvent casting and 3D bioprinting. Electrospinning is a promising approach used to manufacture fibrous and porous scaffolds by means of an electrical field (Beachley and Wen, 2009), which resemble the hierarchical organised structure and architecture found in the native ECM (Carvalho *et al.*, 2019; Kook *et al.*, 2017). Another technique which has gained significant attention in the last decade is 3D bioprinting, a computer-aided layered manufacturing technique, involving the combination of the primary ingredients known as bio-ink, acting as a biological scaffold, and various cell types (Chowdhury *et al.*, 2020; Genova *et al.*, 2020; Guvendiren *et al.*, 2016; Kim *et al.*, 2020).

Scaffolds can be produced from naturally occurring biomaterials, including collagen, gelatine, alginate, HA, fibrin and acellular ECM from allogenic and xenogenic sources, as well as synthetic biomaterials, such as PGA (Mochizuki and Ochi, 2015) and PLGA (Zhao *et al.*, 2014; Chen *et al.*, 2019), or inorganic ceramics, such as calcium phosphates (Chan and Leong, 2008; Rahmati *et al.*, 2018). Scaffold properties should always be considered when designing and manufacturing bioengineered scaffolds for different applications and these are largely dictated by the anatomical structure and function of the tissue type to be fabricated. These include, but are not limited to, high material surface-area to volume ratio, porosity, pore size, biocompatibility and degradation (Amado *et al.*, 2017; Nikolova and Chavali, 2019), which can be controlled by the fabrication method. These properties allow for the development of a biological network suitable for cell migration, cell attachment, nutrient transportation and ECM production (Amado *et al.*, 2017; Rahmati *et al.*, 2018).

The following sections will explore options for scaffold design for interfacial repair by focussing on multiphasic scaffolds, gradient scaffolds, whole 3D tissue constructs and scaffolds manufactured through decellularisation, sharing the functional and structural similarity to the native tissue or organ. Moreover the importance of retaining, or developing, the natural anatomical transition for predicted functional success will be highlighted.

Biphasic/multiphasic scaffolds

Based on the zonal composition and structure of the complex tendon/ligament-bone interface, as demonstrated in Fig. 2, biphasic/multiphasic scaffolds are more applicable to ITE compared to single-phase scaffolds. Each constituent layer/phase can be manufactured to exhibit various properties that replicate the architecturally and compositionally

distinct zones of the enthesis and permit the creation of phase-specific cell phenotypes and/or mechanical environments (Lei *et al*., 2021). Much focus has been placed on the development of multiphasic scaffolds that recapitulate the different regions of the enthesis. A series of studies by Spalazzi *et al.* (2006a; 2008; 2006b) examined the construction of three-layered scaffolds to mimic the soft tissue region, fibrocartilaginous region and bone region of the native interface, achieved using PLGA knitted sheets, PLGA microspheres and PLGA/bioactive glass microspheres, respectively. Once formed, the scaffolds were seeded with fibroblasts in the tendon portion and osteoblasts in the bone portion and cells were anticipated to infiltrate into the interfacial region of the scaffold (Spalazzi *et al*., 2006b). In early studies using this approach, cell infiltration occurred into the central portion but no fibrocartilaginous-like tissue was formed in this region (Spalazzi *et al*., 2006b). However, the addition of a third cell type, chondrocytes, into the triphasic scaffold resulted in fibrocartilage-like tissue production and the utility of this scaffold in maintaining region-specific heterogeneity has been assessed in an *in vivo* model (Spalazzi *et al*., 2008). The long-term aims of this work were to provide a 3D scaffold for implantation at the graft healing site to assist with graft infiltration in soft-to-hard tissue repair.

The ECM is essential for providing cell support and maintaining the cellular microenvironment *in vivo* (Kook *et al*., 2017). More novel approaches include the use of several materials and techniques to produce nanoporous multiphasic scaffolds *in vitro*, resembling the architecture of the native ECM, including electrospun fibres and hydrogels. In a recent review by Lei *et al*. (2021), the authors examined the effective use of biomimetic scaffolds for tendon/ligament-to-bone interface regeneration. Promising results in developing nanoporous multiphasic scaffolds were described in a study by Sun *et al*. (2016). The authors produced co-electrospun dual nano-scaffolds made of PLGA and PCL that were enhanced using different materials to increase biocompatibility: collagen was merged with PLGA fibres, while nHA was integrated into PCL fibres. *In vivo* testing in a rabbit rotator cuff tear model revealed that the PLGA/collagen scaffolds encouraged better collagen ingrowth compared to the simple PLGA scaffold, while the use of the PCL/nHA scaffold exhibited increased new bone formation compared to the PCL-only scaffold. Furthermore, the PLGA/collagen-PCL/nHA dual nano-scaffold was proven to be biomechanically superior to the PLGA-PCL scaffold (Sun *et al*., 2016).

In a recent study, Gottardi *et al*. (2021) described the successful manufacture of biphasic 3D-printed PLGA scaffolds with differential pore alignment (tendon end, aligned; bone end, random) that mimics the native tendon-to-bone insertion. Adult human mesenchymal stem cells were seeded on the scaffolds and, then, cultured for 21 d in dual fluidic bioreactors, allowing tenogenic and chondrogenic differentiation media to be delivered in a region-specific manner (Gottardi *et al*., 2021). Enthesis-associated genes such as *SOX9* were found to be expressed in the cartilaginous compartment of the scaffold, while at day 21, the cartilage matrix marker *COL2A1* and the tendon-specific marker *TNMD* were observed to be upregulated in both cartilaginous and tendinous compartments of the scaffold (Gottardi *et al*., 2021). The authors were successful in engineering a biphasic scaffold recapitulating some essential aspects of the zonal transition of the enthesis. While these are promising findings, the constructed dual fluidic system will need to be further optimised.

A further study has detailed the generation of similar 3D triphasic scaffolds (Mosher *et al*., 2015). A distinct advantage of this technique is that each phase consists of the same bulk material and, therefore, can form seamless transitions with each neighbouring phase through thermal fusion, a feature that decreases the potential risk of delamination (Mosher *et al*., 2015). Seamless phase transitions in both mechanical properties and matrix components are key factors that will be vital for recreating the anatomical complexity of the bone-tendon and bone-ligament interfaces. In their review, Lei *et al*. (2021) reported on a study by Li *et al*. (2016) describing the design of a triphasic silk scaffold to simulate the ligament-to-bone insertion site. To replicate the transitional tissue zones between ligament and bone, the three regions of the silk scaffold were coated with different materials, namely silk fibroin (ligament); silk fibroin, chondroitin sulphate and HA (fibrocartilage); and silk fibroin and HA (bone). Integration of the triphasic scaffold into a rabbit ACL reconstruction model successfully displayed multizonal tissue formation through matrix deposition with improved bone ingrowth and enhanced tissue mechanical properties (Li *et al*., 2016).

Gradient scaffolds

In the process of developing 3D scaffolds for bone-tendon repair, harnessing the ability of the scaffold to control differentiation of the cellular component of the construct has been an interesting method applied to form a gradient of cell phenotypes within a scaffold using just one initial cell type. Gradient scaffolds are most commonly fabricated using a bottom-up approach by allowing minerals to grow in a soft scaffold or by mixing them into a soft material (Boys *et al*., 2019; Lipner *et al*., 2014). Phillips *et al.* (2008) seeded fibroblasts onto a polymeric scaffold containing a gradient of the bone-specific transcription factor Runx2. As Runx2 plays a critical role in bone mineralisation, this study demonstrated that the osteoblastic differentiation of the cells within the scaffold could be controlled through spatial distribution of Runx2 (Phillips *et al*., 2008). Furthermore, this gradient of osteoblastic

differentiation was mirrored by improved mechanical properties of the scaffold (Phillips *et al.*, 2008), similarly to the coupled association of mineral distribution and mechanical properties at the native enthesis (Moffat *et al.*, 2008; Schwartz *et al.*, 2012). In addition to these achievements, following 14 d of implantation in an ectopic site, the scaffold displayed regional organisation of bone- and tendon-like phenotypes, demonstrating potential for a bone-tendon interface scaffold (Phillips *et al.*, 2008). A similar approach was observed in nanofibre scaffolds, whereby spatial control of osteoblastic differentiation of osteoblast precursors (MC3T3-E1) (Li *et al.*, 2009) and adipose-derived stem cells (Liu *et al.*, 2014) has been controlled through the distribution of mineral content along the length of the scaffold. This approach appears to be a promising method for generating gradient interfaces with potential for recreating the anatomical complexity of the bone-tendon interface. In a more recent study by Boys *et al.* (2019), the authors described the successful design and production of a scaffold for cell seeding comprising an apatitic mineral gradient that incorporated the inherent hierarchical structure of trabecular bone. In contrast to the typical bottom-up approach, these scaffolds were fabricated using a top-down approach, by stripping the mineral content from decellularised bone. The resulting scaffold was reported to exhibit structural and compositional gradients and an intact collagen structure (Boys *et al.*, 2019). Overall, the gradient design is a useful means for creating tissue engineered interfaces for orthopaedic applications.

3D whole-tissue constructs

Conventional tissue engineering approaches comprised of scaffolds, cells and the introduction of stimulatory factors (*e.g.* growth factors and/or mechanical stimulation) have demonstrated limited success in creating complex 3D shapes as well as facilitating *in vivo* tissue and organ regeneration and, thus, have been deemed unsuitable for clinical applications from both a logistical and financial point of view (Agarwal *et al.*, 2020). Furthermore, the 3D architecture of the ECM poses a high level of difficulty when attempting to replicate its intricate structure using traditional 2D culture systems (Griffith and Swartz, 2006; Kook *et al.*, 2017). The methodologies described above focus on the development of scaffolds aiming to study and recapitulate the enthesis. Another approach in ITE involves the development of functional, whole 3D tissue constructs mimicking native tissue for replacement of damaged tissues or whole organs. Such tissue constructs usually resemble the anatomy of the native tissue and are of clinically relevant size, shape and structural integrity (Agarwal *et al.*, 2020; Kang *et al.*, 2016).

Considerable progress on the engineering of 3D tissues that incorporate tissue connection sites has been reported in a series of studies by the Larkin group. A bone-to-bone ligament replacement was engineered using a scaffold-free method, incorporating BMSCs with bone and ligament tissues formed in isolation. Following formation of the individual tissue types, the bone and ligament tissues were co-cultured to create the composite construct (Ma *et al.*, 2009). The engineered bone-ligament connection remained intact both *in vitro* and following implantation into rats (Ma *et al.*, 2009) and sheep (Ma *et al.*, 2012), as MCL and ACL replacements, respectively. Importantly, the neo-enthesis did not fail under physiological tensile loading (Ma *et al.*, 2009; 2012). An interesting phenomenon is that the engineered tissues also increased in size and showed improved mechanical properties during implantation (Ma *et al.*, 2009; 2012), suggesting further development and remodelling of the engineered tissue *in vivo*. In addition, the neo-enthesis showed an anatomical structure similar to that found in native tissues, with a fibrocartilaginous region present (Ma *et al.*, 2012). More recent developments using this model system include an investigation into using allogenic or autogenic BMSCs to form the bone-ligament-bone constructs, with little variation between cell sources in terms of tissue formation, mechanical properties or performance (Mahalingam *et al.*, 2015a). An additional study has examined the potential use of frozen (and subsequently stored) bone-ligament-bone constructs *versus* the fresh constructs described above and have noted no significant difference in either mechanical or histological results (Mahalingam *et al.*, 2015b), indicating that frozen constructs may be applicable as a source of tissue replacement in the future. Both these recent studies demonstrated the versatility of this system and the success of the large-animal model trials demonstrated significant potential for clinical replacement option for both ligaments and bone-ligament interface *in vivo*.

Several other groups have investigated the production of whole tissue constructs that include a tissue-transition region. An example is the body of work produced by Paxton *et al.* (2010b), who devised an *in vitro* model of a ligament with bony regions at either end. The soft tissue region was formed through contraction of a fibrin hydrogel, mediated by seeded tendon fibroblasts (Paxton *et al.*, 2009). Initial work focussed on developing an existing tendon *in vitro* model (Calve *et al.*, 2004) into a bone-to-bone ligament model by including calcified polymer hydrogels as bone anchors in the *in vitro* system (Paxton *et al.*, 2009). Incorporation of minerals into the polymer anchors proved essential for the formation of an artificial bone-ligament interface (Paxton *et al.*, 2009) and, therefore, the bone anchors were replaced by calcium phosphate (brushite) bone cement plugs in following studies, to establish the current bone-to-bone-ligament model system (Paxton *et al.*, 2010a; 2010b). Further investigation into the formation of the bone-to-bone ligaments has included increasing their collagen content with both the addition of promotors of the collagen synthesis pathway (Paxton *et al.*, 2012c)

and defined periods of uniaxial stretch and rest regimes in a custom-built bioreactor system (Paxton *et al*., 2012b). Increasing the collagen content and, therefore, mechanical properties of the bone-to-bone ligaments is vital for consideration of these bone-ligament structures as surgical implants. In addition, it is important to recognise that preformation of a bone-ligament/tendon interface *in vitro*, as opposed to single separate tissues, may improve the biological response of the implanted structures, as bone-to-bone healing occurs quicker and with more clinical success (Doblare *et al*., 2004) than bone-ligament/tendon healing (Weeks *et al*., 2014). Therefore, establishing the tissue connection site *in vitro* could be of clinical benefit.

Decellularised scaffolds

Due to the complexity of the microanatomy at the enthesis, it is often challenging to create bioengineered constructs with similar heterogenous tissue transition zones mimicking the native tissue structure. The ECM has a critical role in tissue maintenance and renewal, providing a location for cell adhesion and also important cues that can direct cell behaviour. It is not surprising that decellularised tissue is gaining popularity in tissue engineering applications, as the natural ECM can provide an ideal microenvironment, or niche, for stem cells (Chen and Lv, 2018). During the past decades, decellularised allogeneic and xenogeneic ECMs for tendon-to-bone repair were a strong focus due to their excellent structural profile, biodegradability, high biocompatibility and lack of immunogenicity in host tissue (Chen and Lv, 2018; Lee *et al*., 2016). Indeed, using the existing matrix is an excellent way to explore true anatomical tissue engineering possibilities and deserves attention for investigations in this area. Most commonly, acellular (decellularised) ECM derived from mammalian ECM, specifically porcine small intestinal mucosa and dermis, has been successfully employed in the repair of rotator cuff tears (Hakimi *et al*., 2013; Zhang *et al*., 2012; Zhao *et al*., 2017). Several studies have investigated the preparation and use of acellular tendon and fibrocartilage in tissue engineering (Chen *et al*., 2019; Liu *et al*., 2018; Ning *et al*., 2012; Ning *et al*., 2015). Ning *et al*. (2012; 2015), for example, decellularised Achilles tendon slices by processing the tissue through repetitive freeze-thaw cycles, frozen sectioning and nuclease treatment to produce tissue-engineered scaffolds with an intact native microstructure. These scaffolds demonstrated advanced biocompatibility and satisfactory mechanical properties while the use of different growth factors endorsed cell growth and tenogenic differentiation of tendon-derived stem cells and BMSCs. In the Liu *et al*. (2018) study, decellularised Achilles tendon slices were used to repair large rotator cuff tears in a rabbit model. Histological assessment at 8 weeks post-implantation showed an increase in cell ingrowth and osteogenesis, optimised tissue integration and enhanced mechanical strength. Equally, it has been reported that decellularised tendon-bone composite grafts can be implanted to substitute the injured enthesis (Farnebo *et al*., 2014a; Farnebo *et al*. 2014b; Su *et al*., 2019), thus evading the challenges of re-creating the structurally complex enthesis. Farnebo *et al*. (2014a; 2014b) employed decellularised tendon-bone grafts originating from both the Achilles tendon and calcaneus for enthesis reconstruction. Such grafts exhibited improved mechanical strength at earlier healing time points, with decreased immunogenicity and a structured ECM, as compared to untreated grafts (Farnebo *et al*., 2014). Recently, Su *et al*. (2019) developed triphasic decellularised bone-fibrocartilage-tendon composite scaffolds using differential decellularisation methods. These scaffolds allowed BMSCs to undergo regional differentiation *in vitro* and maximised ossification in a rabbit model with femur-tibia defects *in vivo*. Ultimately, the potential for anatomical tissue engineering from decellularised scaffolds holds great promise for providing tissue-engineered implants that already possess the key anatomical tissue architecture that has proven difficult to engineer following the bottom-up approach.

Conclusions and future perspectives

The importance of anatomical analysis

It is imperative to conduct a formal anatomical study when intending to develop *in vitro* therapeutic strategies using the abovementioned tissue engineering approaches. Such a study may include the investigation of the macrostructure and microstructure, histopathology and molecular composition of tissue specialisations of the enthesis or, indeed, any other tissue or organ replacement. Different imaging techniques, including ultrasound, computer tomography and magnetic resonance imaging scans have been employed over the years to investigate primarily animal tissue interfaces. However, only a handful of studies since the 2000s have focused on characterising human entheses by performing anatomical investigations. Examining the native anatomy of the human entheses is a vital step for enhancing the translatability of tissue engineering research. Table 1**a**,**b** provides a comprehensive list of studies examining human bone-tendon/bone-ligament interfaces. The authors have used a mix of anatomical analysis approaches, including morphometric measurements and histological investigation techniques, to describe the morphology of the tendon/ligament insertion and determine enthesis classification, respectively.

The authors' research group is interested in investigating these complex anatomies found at tissue interfaces, particularly at the osteotendinous/osteoligamentous junction, and is specialising in engineering replacement tissues for human implantation. Hence, it is of paramount importance

Table 1a. List of studies examining human entheses to date (continued as Table 1b).

Functional unit	Anatomical location of osteotendinous/ osteoligamentous junction	References
Head and neck	Atlas/transverse ligament complex	Milz *et al.* (2001a)
	Masticatory muscles tendons insertion site	Hems and Tillmann (2000)
Upper limb: shoulder girdle	Pectoralis major insertion site	Huang *et al.* (2020)
	Subscapularis tendon insertion site and capsuloligamentous complex	Kordasiewicz *et al.* (2016)
	Coracoacromial ligament insertion site	Milz *et al.* (2008)
Upper limb: arm	Biceps brachii, triceps and brachialis tendons insertion site	Benjamin *et al.* (1992)
Upper limb: elbow	Lateral and medial humeral epicondyles tendon-collateral ligament complex	Milz *et al.* (2004)
Upper limb: hand	Flexor digitorum profundus tendon insertion site	Chepla *et al.* (2015) Mortimer *et al.* (2021) Wilkinson (1953) Xu *et al.* (2021)
	Flexor pollicis longus insertion site	Wilkinson (1953)
	Opponens pollicis architecture and insertion site	Williams-Hatala *et al.* (2016)
	Extensor muscles insertion site	Tan *et al.* (2007)
	ECM enthesis associated with pisiform bone	Adamczyk *et al.* (2008)
Thorax	Scapula/suprascapular ligament complex	Moriggl *et al.* (2001)
Abdomen	Abdominal muscles (rectus abdominis, pyramidalis and anterior and posterior aponeuroses) insertion site	De Maeseneer *et al.* (2019)
Lower limb: hip	Ligamentum capitis femoris insertion site	Shinohara *et al.* (2014)
	Pubovisceral muscle insertion site	Kim *et al.* (2011b) Kim *et al.* (2015)
	Acetabulum/transverse ligament complex	Milz *et al.* (2001b)
	Adductor longus, brevis and gracilis tendon insertion site	Davis *et al.* (2012)
	Adductor magnus, brevis and longus tendon insertion site	De Maeseneer *et al.* (2019)
Lower limb: posterior thigh	Hamstring tendon insertion site	Grassi *et al.* (2013)

to use precise anatomical model systems to direct the design and development of tissue-engineered human entheses. The approach, detailed in Fig. 3, is grounded on completing an in-depth anatomical analysis by dissecting the human tissue interface and, subsequently, performing a detailed histological and morphological characterisation. This concept is further outlined in a study by Mortimer *et al.* (2021). The authors examined the FDP enthesis in macroscopical and microscopical detail by employing anatomical dissection and histomorphological analysis techniques. The investigation of the surrounding topography through dissection can provide an irrefutable strategy in aiding the scale up of laboratory tissue-engineering models. This contrasts with the use of traditional imaging modalities to produce anatomically relevant models through 3D printing as a basis for desired model scale (Appel *et al.*, 2013; Ballyns *et al.*, 2008; Nam *et al.*, 2015.) More specifically, the knowledge gained from this vital evaluation can drive tissue-engineered design and development using the traditional ITE approaches described above to reconstruct the highly ordered anatomy of the human enthesis *in vitro*. Ultimately, the aim is to produce anatomically and clinically relevant tissue-engineered constructs aimed at human implantation at the site of interest. However, these 3D constructs can aid in the discovery of novel therapeutic treatments for enhancing musculoskeletal repair following injury, while the

Table 1b. List of studies examining human entheses to date (continuation of Table 1a).

Functional unit	Anatomical location of osteotendinous/ osteoligamentous junction	References
Lower limb: knee	Quadriceps tendon and patellar ligament insertion site	Evans *et al.* (1990) Evans *et al.* (1991) Toumi *et al.* (2012) Toumi *et al.* (2014)
	Anterior cruciate ligament femoral and tibial enthesis	Beaulieu *et al.* (2015) Beaulieu *et al.* (2017) Dai *et al.* (2015) Iwahashi *et al.* (2010) Mochizuki *et al.* (2014) Sasaki *et al.* (2012)
	Posterior cruciate ligament synovio-entheseal complex	Binks *et al.* (2014)
	Meniscal attachment complex	Abraham *et al.* (2014) Benjamin *et al.* (1991) Donahue (2013) Villegas and Donahue (2010) Wang *et al.* (2009)
	Tibialis posterior insertion site	Bloome *et al.* (2003) Moriggl *et al.* (2003) Petersen *et al.* (2003) Willeger *et al.* (2020)
	Tibialis anterior insertion site	Willegger *et al.* (2017)
	Extrinsic calf muscle tendon insertion site	Frowen and Benjamin (1995)
	Calcaneal tendon insertion site	Ballal *et al.* (2014) Chao *et al.* (1997) Doral *et al.* (2010) Edama *et al.* (2016) Lohrer *et al.* (2008) McGonagle *et al.* (2008) Rufai, Ralphs and Benjamin (1995) Toumi *et al.* (2016)
	Ankle ligament complex	Wenny *et al.* (2015)
	Lesser metatarsophalangeal joint plantar plate	Gregg *et al.* (2007)
Multiple functional units	Entheses at multiple sites (28+)	Benjamin *et al.* (1986) Benjamin *et al.* (2004a) Benjamin *et al.* (2004b) Benjamin *et al.* (2007) Benjamin and McGonagle (2007)

obtained anatomical considerations can equally improve the understanding of the pathogenesis of enthesopathies, enthesitis and rheumatoid arthritis.

Overcoming the challenges of interfacial tissue engineering

The ultimate goal for repair and regeneration at the osteotendinous/osteoligamentous junction is to restore the normal anatomy of the tendon/ligament-to-bone enthesis. As described above, the enthesis has an intricately complex architecture and, although the exact process of how tissue boundaries and gradients in cell phenotype and ECM are established and maintained are not yet fully understood, it is vital to understand boundary development between regions to design appropriate replacements (Zhao *et al.*, 2017). While morphological studies on single component tissues can help to direct studies on more complex tissue transitions, it is argued that the cell type at the enthesis have a distinct phenotype when compared with those from tendon/ligament, cartilage and bone cells alone (Lu and Thomopoulos, 2013). ITE approaches rely heavily on the integrated study of cellular and molecular biophysical mechanisms in response to both endogenous and exogenous factors at the enthesis. Until these are fully elucidated, ITE strategies will remain experimental.

Despite the multitude of ITE strategies focusing on modelling and recreating the enthesis, there is currently no optimal design. However, the development of materials processing and establishing gradient properties has witnessed significant progress.

The ideal current theoretical design will incorporate a combinatory approach of cell, scaffold, growth factor and mechanical stimuli techniques. For example, an optimal cell environment could be accomplished by combining nanoscale and high-resolution 3D bioprinting techniques for precision control of gradient scaffold structure and the development of smart biomaterials with adjustable chemical, physical and biological properties will aid in the regulation of spatial or temporal biochemical delivery (Font Tellado *et al*., 2015). In addition, spatially guided local cell differentiation for phenotype gradients could be achieved using advanced techniques such as gene therapy, including CRISPR gene editing, and iPSCs (Boys *et al*, 2017). Furthermore, enthesis-specific biochemical and/or mechanical cues could be delivered using custom-built bioreactors and tailored optimisation of engineering parameters could reduce experimental time and enhance construct functionality.

Generally, there are no standard protocols in ITE and there can be significant overlap in methodology. However, the aim of the ITE approach is to translate research from bench to bedside. In prospect of reaching the implantation stage, certain criteria must be fulfilled. Once satisfactory evidence for the effectiveness of a designed 3D model *in vitro* has been collated, the developed method must be applied *in vivo* using small-animal models (*e.g.* mice, rats, rabbits) and at later stages large-animal models (*e.g.* sheep, goats, horses). Several studies have shown that the use of large-animal models will draw more attention to key questions regarding optimal engraftment and associated immune reactions as well as allow the testing of implants under similar biomechanical conditions (Ribitsch *et al*., 2020). If good results are yielded from the use of small- and large-animal models, the next stage on the translational scale will involve the collaboration with healthcare institutions or companies to design and produce the tailored 3D tissue construct aimed for patient implantation. Yet, a positive result in small-animal models will not necessarily signify that this will be translated to similar effects in large-animal models or humans (Schindeler *et al*., 2017). All in all, animal preclinical models are useful and cost-effective means in testing whether a hypothesised approach is suitable to progress towards a more advanced and expensive level and ultimately reach the clinical trial end stage.

Concluding remarks

The carefully structured anatomical morphology present at the enthesis functions across the boundary

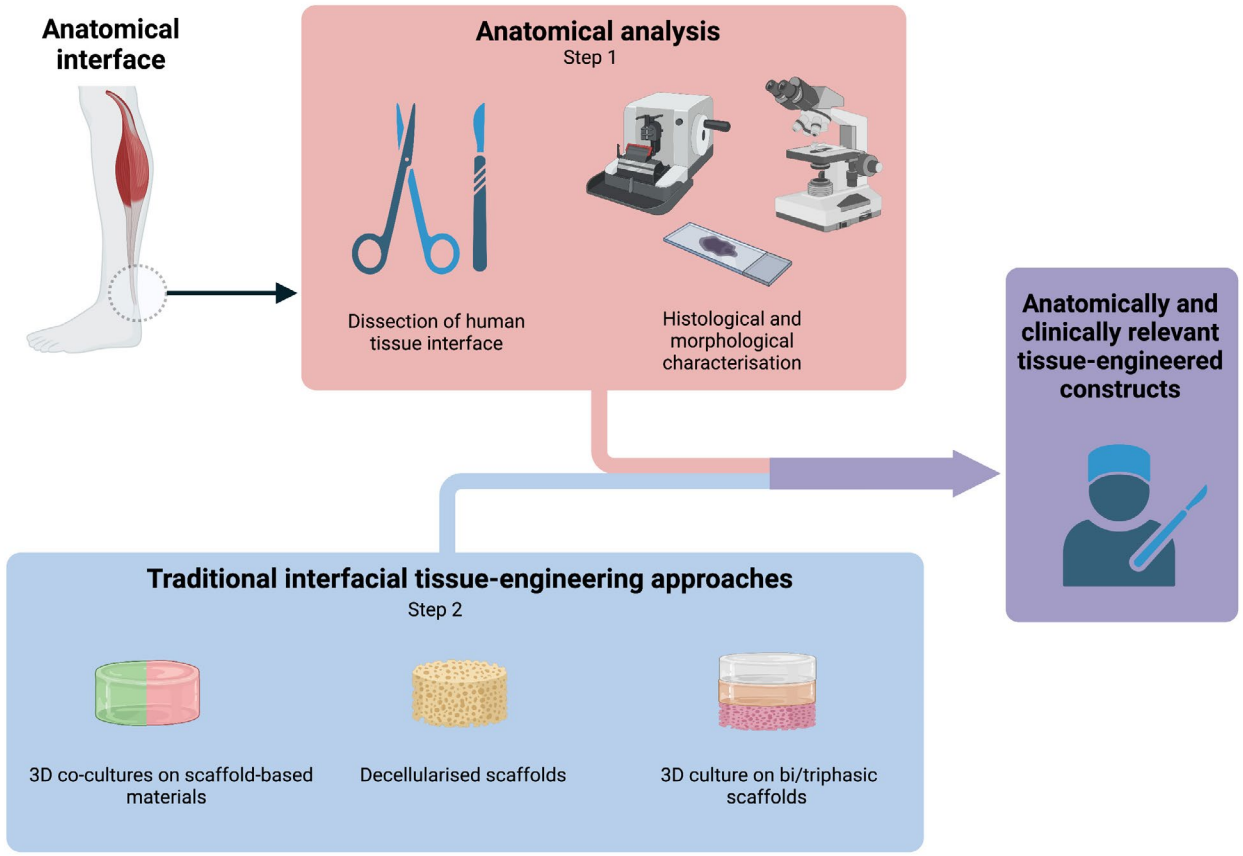

Fig. 3. Anatomically driven tissue engineering research. The suggested approach is based on completing an in-depth anatomical analysis by dissecting the human tissue interface and subsequently performing a detailed histological and morphological characterisation to help better guide this area of anatomically and clinically relevant tissue-engineered research. Created using Biorender.com.

between tissues with differing mechanical properties. As tissue engineering seeks to provide suitable alternatives for body tissues for replacement following injury or disease, recreating the specific anatomical structure and function of tissues is paramount to their success as tissue replacements. Cutting-edge ITE techniques complemented by an initial anatomical analysis will not only replicate the surrounding, or connecting, anatomies of the region but also allow the engineered tissue to function as the native tissue should.

Many research groups are now acknowledging the need for engineering suitable anatomically relevant tissue transition points for successful orthopaedic applications and highlight the need for cross-disciplinary research collaborations involving anatomists, biologists, engineers and chemists to encompass all the areas of expertise required. With this in mind, the idea of making connections applies on two levels: i) for the physical engineering of tissue transition sites *in vitro*; ii) to highlight the strength of cross-disciplinary collaborations for achieving the ultimate success in tissue engineering of musculoskeletal tissue transitions.

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Discussion with Reviewers

Reviewer 1: Can the authors speculate where they think most progress will be made in ITE in the next decade? Are there standardised pre-clinical models/ methods for ITE?

Authors: We believe that the combination approach of anatomical analysis, as a key prerequisite for design and development of tissue-engineered structures, with cutting-edge techniques in tissue-engineered scaffold fabrication, is where the most significant progress will be made. Specific as well as regional anatomical considerations are crucial for translating technologies to the clinic. As an additional consideration, the further investigation and utilisation of decellularisation techniques to maintain anatomically accurate scaffolds is likely to expedite scaffold design in a way that is both anatomically and clinically relevant.

Reviewer 2: What is the specific knowledge of anatomy that can indeed bring forward the tissue engineering field?

Authors: The knowledge achieved by conducting anatomical analyses will aid in the correct representation of the native macro and micro anatomy of the tissue in question, providing information on both morphological and biomechanical properties, therefore enhancing the applicability and function of tissue-engineered therapeutics.

Reviewer 2: Why do you think anatomical knowledge can aid in the engineering and scaling up of complex structures?

Authors: Anatomists are known for studying the form, structure and function of the human body, and following the doctrine "form follows function". Anatomists are equally interested in chiefly investigating the mechanics before attempting to provide solutions to repair disrupted anatomical

structures. In addition, a thorough anatomical analysis can increase the translation of interfacial tissue engineering models. It is crucial to regard the tissue/organ as a whole but also in relation to surrounding tissues/organs. As such, anatomically relevant tissue-engineered constructs can be developed to function as surgical grafts/implants.

Editor's note: The Guest Editor responsible for this paper was Manuela Gomes.