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Current Progress in Enthesis Repair: Strategies for Interfacial Tissue Engineering

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Abstract

Complex interfaces have evolved at the junction between tissues of the musculoskeletal system to overcome the problems of impedance mismatch. In this review, we have revisited the anatomy, development, injury and repair of the interface between bone and tendon/ligament, also known as the osteotendinous/osteoligamentous junction or enthesis. Specifically, the existing options for repair have also been discussed along with the current progress of interfacial tissue engineers aiming to manufacture multiphase tissue constructs for repair of these complex interfaces *in vitro*.

Introduction

Injury at the interface between tissues is the result of the mismatch between the mechanical properties of the materials. In engineering, this is termed impedance mismatch, where the resistance in the source and load are different resulting in power loss. In the musculoskeletal system, the main source of impedance is not electrical but mechanical: the difference in stiffness between muscle, tendon and bone. If there were not a gradual mechanical transition between the tissues, the result would be a concentration of strain, where one tissue stretches to a greater extent than the adjacent tissue. The end result of strain concentration is mechanical failure, as repeated loading results in damage and eventually injury. To overcome this, complex interfaces have evolved at the junctions between the tissues of the musculoskeletal system [1-7]. These interfaces have a multiphasic tissue heterogeneity that allow smooth transmission of force between the two tissues, minimizing the formation of stress and strain concentrations [3]. Such boundaries exist between muscle and tendon (myotendinous), bone-tendon (osteotendinous), bone-ligament (osteoligamentous) and bone-cartilage (osteochondral), each with their own biochemical and morphological adaptations to smooth the transmission of force between tissues.

To this end, a new field of tissue engineering research has emerged over the past 5 years called interfacial tissue engineering (ITE). ITE is aimed at attempting to recreate these tissue interfaces *in vitro* to allow the realistic prospect of implantation of musculoskeletal tissues after disease or trauma.

This review will focus on two of the aforementioned interfaces, the junction between bone and the soft tissue sinews, bone and ligament. However, due to the similarity in composition and mechanical properties of tendon and ligament, the soft tissue junction described will be referred to as the enthesis and will relate to both bone-tendon and bone-ligament interfaces, unless otherwise stated. The structural anatomy of the enthesis in health and disease and the important developmental events occurring during enthesis formation will be revisited before discussion of previous attempts of *in vivo* repair of this complex interface. These achievements will then be examined in relation to important studies in the new field of ITE, where these complex structures are engineered *in vitro*.

The Osteotendinous/Osteoligamentous Junction – The Enthesis

Anatomy of the enthesis

The osteotendinous/osteoligamentous junction or enthesis is designed to allow smooth transmission of force between tendon or ligament and bone [4,8-10]. An enthesis can be either fibrous or fibrocartilaginous, formed by intramembranous or endochondral ossification, respectively [10]. Indirect fibrous insertions are characterized by soft tissue attachment to the periosteum, and the presence of Sharpey's fibers that extend directly from the tendon/ligament to the bone [8,9] (Figure 1A). Fibrocartilaginous enthesis are more complex. Tendons develop from fibrous outgrowths of the cartilaginous primordial bone prior to its ossification [10], therefore the transition from tendon to bone in a fibrocartilaginous entheses is an indirect attachment with a zonal arrangement, comprising four separate regions: 1) tendon; 2) fibrocartilage; 3) mineralized fibrocartilage; and 4) mineralized bone [4,10] (Figure 1B). Traditionally, the outer limit of calcification was demarcated by a tidemark, signifying a sharp transition between the calcified and non-calcified regions of the enthesis [8,11], however recent work using Raman Spectroscopy has demonstrated that mineral is gradually distributed across the insertion site [12,13]. The zone of calcified fibrocartilage interdigitates with the bone thereby greatly increasing the surface area for attachment of soft tissue to bone [4,5,10]. This results in a reduction in strain concentrations and a decrease in the impedance mismatch [4,9,10]. The fibrocartilaginous structure is so well suited to the loading that it experiences that tendon and ligament failures tend to occur in the subchondral bone and not at the interface [11]. This strongly implies that the bone is weaker in tension than the enthesis and that the zonal arrangement of the enthesis is particularly effective at dissipating stress uniformly and performing well in situations of extreme tensile loading. In addition to a gradual increase in mineral content from tendon to bone, collagen fiber alignment changes as the sinew approaches the bone [13,14]. A combination of the increase in mineral content and the reduced collagen alignment mean that the mechanical properties of the enthesis differ along its length and, just like the tendon as a whole

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[15], the enthesis is more compliant at the tendon end than at the bone end [10,14,16]. These functional differences are strongly related to structural adaptations across the enthesis and serve to decrease injury to the transition [16]. It is generally accepted that the enthesis can be classed as an "enthesis organ" and that the surrounding tissues of the enthesis also work together to achieve the common goal of stress reduction at the interface [4]. These include the fibrocartilage layer, a bursa, fat pad and the bone (Figure 2) [5,17,18]. The best developed enthesis organ is found at the Achilles' tendon insertion site [18], although others are found throughout the body [5,17].

Development of the enthesis

ITE aims to engineer a structure similar in arrangement and mechanical properties to native tissue *in vitro*. Therefore, it is important to examine the factors that are thought to be key to establishing the complex interface *in vivo*. The development of the enthesis has been extensively reviewed by Thomopoulos et al. [13] however, for the purposes of this review, a brief description of the morphological events occurring during interface formation is provided below. The formation of a fibrocartilaginous enthesis is thought to occur in three phases by endochondral ossification. First, the tendon/ligament attaches to the hyaline cartilage comprising the bone precursor [5,19]. Next, ossification occurs, the hyaline cartilage becomes eroded and regions of fibrocartilage become visible in the tendon/ligament [5,19]. The appearance of fibrocartilage is as a consequence of fibroblast metaplasia, probably as a result of the mechanical stimulus at the

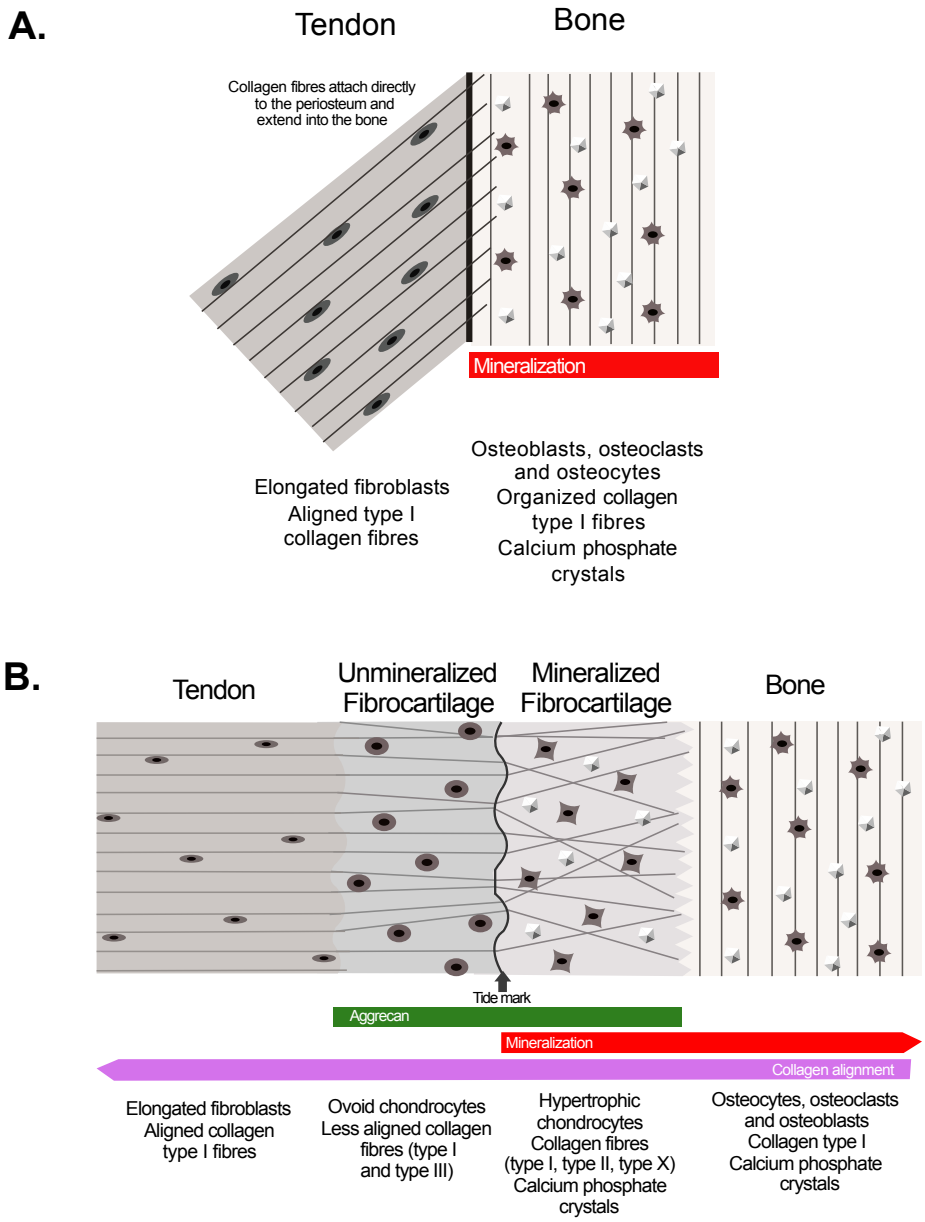

Figure 1: Types of enthesis. A) Indirect fibrous bone-tendon interface. Tendon joins directly to the bone by collagen fibres inserting into the periostuem layer of the bone (Sharpey's fibres). B) Direct fibrocartilaginous bone-tendon interface. Tendon transitions through fibrocartilage regions before reaching the bone. Collagen fibres become more disorganised and mineral content increases as the soft tissue reaches the bone.

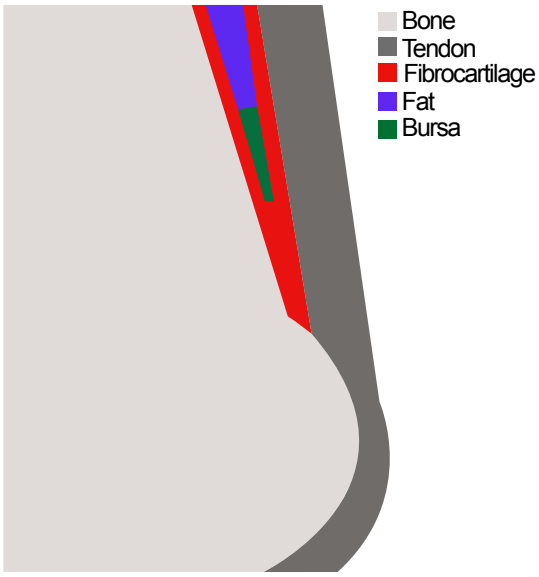

Figure 2: The enthesis organ. Diagramatic representation of the enthesis organ at the human Achilles tendon. All components of the organ work together to decrease strain at the bone-tendon insertion site.

insertion site as the tendon/ligament moves relative to the bone [5,19]. Finally, the hyaline cartilage of the rudimentary bone is resorbed. However, fibrocartilage continues to form as a result of ongoing fibroblast metaplasia [5,19] resulting in the formation of the graded enthesis described above. *In situ* hybridization studies have attempted to examine enthesis development to elucidate the mechanisms involved in establishing this complex insertion [20]. Galatz et al. [20] described how, while the separate tissue masses of tendon and bone were evident from 15.5 days post conception (dpc) in mice, a transition zone between the two tissues was not present until 7 days post-natally and only formed a mature fibrocartilaginous insertion site 21-days post-natally [13,20]. Interestingly, a subsequent study by the same group has shown that the maturation of a fibrocartilaginous interface is strongly influenced by mechanical factors [21]. Paralyzing the adjoining muscle of the shoulder with botulinum toxin, resulted in a less mineralized insertion point, fibrocartilage development was severely impaired and collagen alignment was disrupted at the interface when compared to the saline-injected control group [13,21]. These studies highlight the sensitivity of musculoskeletal tissues to their mechanical environment and the need for appropriate stimulation during formation and maturation. Furthermore mechanical and chemical stimuli are also important in providing the necessary cues for enthesis development [13]. Several proteins have been identified as important mediators of interface formation and maturation, such as parathyroid hormone related protein (PTHrP), Indian hedgehog homolog (Ihh), Patched 1 (Ptc 1) and Scleraxis (Scx), all of which have been localized to tendon-bone insertion sites (for review see [21,22]) and SOX-9, an important transcription factor involved in chondrogenesis [13,22]. For example, Scx expression has been shown to regulate BMP-4 expression in tendon cells at the insertion site to bony ridges in the developing enthesis [23]. It is likely that gradient-like expression of these factors in combination with mechanical forces is important in forming the gradient structure present in the mature fibrocartilaginous bone-tendon/ligament insertion [13].

Injury at the enthesis

For an in-depth description of enthesis injury, readers are referred to a review by Benjamin et al. [4], however a brief summation has been provided here. The most common injuries occurring at the enthesis are sporting injuries, such as the overuse injuries, tennis/golfer's elbow and jumper's knee [4,9]. Tennis elbow occurs at the junction of the flexor tendons to the humerus and is characterized by the formation of a disorganized collagen matrix at the interface [4] whereas Jumper's knee affects the interface of the patellar tendon with the lower portion of the patella [4]. In addition to overuse injuries at the enthesis, other disorders at the enthesis can also occur. Enthesesopathies are considered insertional disorders and inflammatory disorders are termed enthesitis [4,24]. Entheseopathies can occur in conditions such as diffuse idiopathic skeletal hyperostosis (DISH), a degenerative condition whereby excess bone in deposited at the insertion site [4]. Enthesitis is commonly present in the enthesis region of patients with inflammatory disorders such as seronegative spondyleoarthropathies [17,24].

Further to the conditions that affect the enthesis, there is considerable interest in avulsion fractures occurring at the insertion of tendon or ligament to bone. As mentioned previously, injuries at tendon or ligament insertion sites tend to occur in the subchondral bone region rather than the join between the soft and hard tissue [11]. Avulsion fractures can cause overuse injuries at sites of apophysitis (inflammation of bony outgrowths) in children [4], however, in adults, are more commonly caused by extreme force [4].

Although, tendon avulsion has not been widely investigated, it is extremely relevant to this review, since a major problem in the surgical repair and regeneration of tendon and ligament attachment sites is the failure to establish a suitable interface region on implantation of a donor tendon/ligament. Although injury at the enthesis is not common, many other musculoskeletal injuries can occur that result in the partial or complete rupture of the tendinous/ligamentous tissue, for example, the anterior cruicate ligament, Achilles' tendon or rotator cuff tendon (see later)

Adaptation at the enthesis

As tendons, ligaments and bone are all dynamic tissues, and therefore capable of being remodeled, it is also likely that the enthesis in susceptible to changes in load patterning [10]. Zumwalt [25] examined the effect of endurance exercise of the size of bony attachment sites of six different muscles [25] and, contrary to previous ideas; the size of the attachment site did not increase following exercise even when the muscle underwent hypertrophy [25]. Similarly, a more recent study has examined the effect of training and sudden detraining on the enthesis and found that the insertion site size did not differ between untrained, trained and detrained rats [26]. However, the proteoglycan and collagen content of tendons in the detrained group were significantly less that the untrained and trained groups, indicating a severe loss in tissue quality and mechanical properties following a sudden change in activity [26]. This may have important consequences for immobilization of tendons following injury. Furthermore, the effect of microgravity on the enthesis has been reported to lead to significant bone loss at the insertion point and a weakness in the join between tendon and bone [27]. This further indicates that as the contributing tissues to the enthesis are known to be responsive to exercise or immobilization, the enthesis then becomes susceptible to injury.

Repair of the Injured Enthesis

As mentioned previously, the architecture of the tissues of the enthesis is so well adapted that failure between the tendon/ligament

and bone is extremely rare. However, to understand the events taking place during healing of tendon/ligament to bone, in the case of surgical repair of soft tissues, it is important to examine healing in both tendons and bone individually. Although there is currently a lack of understanding about the specific events occurring during bone-tendon healing, it is thought that combinations of the events described below will occur concurrently at the site of repair.

Since tendons and ligaments are subject to frequent loading and are relatively avascular, degenerative changes are common. In contrast to bone, which has a high capacity for self-repair, tendons and ligaments heal slowly, taking up to one year to restore function to that of native, uninjured tendon/ligament [28,29]. The low vascularity and the low cellular composition of tendons and ligaments are probable reasons for the slow and unsatisfactory healing of these tissues [28-31]. Tendon/ligament healing occurs in 3 overlapping phases: 1) Haemostasis/inflammatory; 2) proliferative/fibroplasia; and 3) remodeling/maturation [28,31]. The inflammatory phase commences immediately after injury and involves the formation of a haematoma which is responsible for initiating a cascade of vasodilators, platelets and pro-inflammatory chemicals from mast cells. The clot is then broken down as fibroblasts invade the wound site. Angiogenesis is initiated, promoting the formation of capillary networks within the wounded area. During the inflammatory stage, there is an increase in DNA, fibronectin, glycosaminoglycan and water content and a concomitant increase in type III collagen which acts as a temporary scaffold to stabilize the wound site [28,29,31]. After a few days, the proliferative phase is initiated. At this stage, the wound site is filled with granulation tissue, mainly in the form of disorganized type III collagen, but this is gradually converted into type I collagen throughout the following weeks [28,29,31]. After six to ten weeks of healing, the remodeling phase is reached. During this final phase, fibroblasts reduce their matrix synthesis and collagen fibers re-orientate along the long axis of the tendon. The normal ratio of type I to type III collagen is restored, in addition to a return to normal levels of collagen cross linking, glycosaminoglycans, water and DNA content [28,31]. A year of healing is usually required to approach normal function, however normal tendon function and biomechanics are rarely completely restored after injury [29].

In contrast to tendon, which undergoes the repair process following injury, bone undergoes a regenerative process, restoring functional capacity after fracture [32]. Bone fracture healing can be separated into four phases, 1) Inflammatory stage, 2) Fibrocartilage formation, 3) Primary bone formation and 4) Secondary bone formation [33]. In the inflammatory stage, a haematoma is formed at the fracture site and inflammatory cells infiltrate the site. These inflammatory cells then secrete a host of growth factors and cytokines that initiate the further stages of the regeneration process, such as TGFβ, PDGF, VEGF, FGF-2, M-CSF, IL-1, IL-6, BMP, TNFα. Granulation tissue is formed at the fracture site which becomes vascularised. In the second stage, a fibrocartilaginous template is formed from chondrocytes and fibroblasts and acts as a temporary mechanical support at the fracture site. Then, in the next phase of primary bone formation, osteogenesis begins and mineralized bone matrix is laid down. Concurrently, the fibro cartilaginous soft callus is gradually removed as the woven bone is deposited. Finally, in the phase of secondary bone formation, the existing woven bone is remodeled into cortical or trabecular bone [33]. The whole process of fracture repair can occur within 11-12 weeks [34], much shorter than the year-long modeling time of tendons [35], and usually results in a regenerated bone matrix similar in both composition and mechanical properties to uninjured bone [34]. Perhaps the differing healing rates of tendon and bone can be part of the cause for the lack of tissue integration experienced at the site of repaired tendons/ligaments to bone.

Although the healing processes in bone and tendon have been described, it must be acknowledged that healing at the bone-tendon/ligament interface may not be the same as in each tissue type individually. During bone-tendon/ligament healing, the formation of a direct enthesis, where a zonal, fibro cartilaginous region is present between the soft tissue and the bone, does not occur. Instead, healing at the interface usually results in the formation of an indirect enthesis with a lower mechanical strength than before injury [36,37]. The lack of fibrocartilaginous tissue formation at the junction between two tissues with different mechanical properties results in stress concentrations and often, failure occurs at the interface site. The formation of a fibrocartilaginous tissue insertion is now the main aim in both *in vivo* and *in vitro* repair options for the enthesis and will be discussed in detail later.

Current limitations in the clinical repair of common tendon/ligament ruptures

As mentioned previously, the most common soft tissue injuries requiring surgical repair and regeneration of bone-soft tissue attachment sites are the anterior cruciate ligament (ACL), the Achilles' tendon and the rotator cuff group.

The anterior cruciate ligament (ACL) is a key component of the knee joint, connecting the femur and the tibia [38,39]. The function of the ACL is to stabilize the knee by resisting anterior tibial translation and restraining internal rotation [38,39]. ACL injury is common, with over 100,000 occurring annually in the US alone [40], usually in the young, active population [41,42]. The cost of ACL repair surgery was recently estimated at approximately $17,000-$25,000 [43-45], and unfortunately, the low success rate of current repair strategies and failure due to tendon pullout, or mechanical breakdown of the implanted synthetic graft structure [46] usually results in additional surgery and long rehabilitation periods. Current methods of ACL repair can include autografts, allografts or synthetic grafts. The most common form of ACL reconstruction is the use of an autografting technique, using bone-patellar tendon-bone (BPTB) grafts. The central third of the patient's own patellar tendon is excised with portions of the femoral and tibial bone blocks remaining [47-50]. This complete bone to bone graft is then inserted into a bone tunnel and fixed in place, usually with interference screws. [47,50-53]. A similar technique is also employed using a hamstring tendon graft [53-55], either as a single-strand semitendinosus tendon graft or a quadruple-strand semitendinosus/gracilis tendon graft [50]. Both the BPTB grafts and the hamstring grafts are routinely used as biological tissue replacements. Due to the concerns over donor site morbidity, substantial attention has been given to the use of allograft tissues, procured from cadaveric specimens [50,56-58]. Tissues available for ACL reconstruction are bone-patellar tendon-bone, Achilles tendon, fascia lata, tibialis anterior and posterior tendons and hamstring grafts [50,56,57]. Allograft reconstructions reduce problems associated with autograft tissue harvest, and decrease surgical time, but introduce other problems such as tissue rejection, disease transmission, delayed biological incorporation and insufficient supply of available donors [56,58].

The use of synthetic polymers to create artificial ligaments for ACL reconstruction was once thought to have several advantages over

either autograft or allograft techniques, such as the ability to preserve the patient's own tendinous tissues and avoiding potential disease transmission [59]. Synthetic polymers that have been evaluated as ACL grafts include polytetrafluoroethylene (Gore-Tex), carbon fibres (Integraft), polyethylene tetraphatalate (Dacron; Stryker-Meadow Ligament and Leeds-Keio Ligament) and braided polypropylene (Kennedy Ligament Augmentation Device) [58]. Unfortunately, the synthetic grafts mentioned above share many undesirable traits, such as mechanical breakdown over time resulting in pain, synovitis, sterile effusions and osteoarthritis at the implant site [46]. Although studies have shown a good repair of initial function, the inability to restore the mechanical behavior of the native ligament leads to eventual deformation and graft failure [46,59,60]. With the poor performance of synthetic grafts, biological grafts are still the gold-standard method of repairing injured ACLs.

The Achilles' tendon connects the soleus and gastrocnemius muscles to the calcaneous to allow plantar flexion at the ankle joint [61]. Achilles tendon rupture occurs in approximately 18 in 100,000 people [61-63], typically in males aged between 30-40 [63] and repair can be performed surgically (open or percutaneal), or non-surgically (immobilization with braces or casts) [62]. Should the tendinous tissue become detached from the bone, and repair with either the injured tissue or an autograft, allograft or synthetic replacement necessary, the attachment of the tissue/material to the bone must be augmented. Options for autografting in Achilles' tendon repair include the semitendinous or gracilis tendons or the gastrocnemius aponeurosis flap [64]. Allografts are also used in Achilles' tendon repair, as are several commercially available synthetic products [65].

The rotator cuff refers to a group of four muscles (supraspinatus, infraspinatus, teres minor and subscapularis) and their accompanying tendons [66,67]. Tears of the rotator cuff are a very common cause of shoulder pain, but unlike ACL and Achilles' tendon rupture, rotator cuff tears are most common in the older population [42,66] and it is generally accepted that instead of trauma being the main cause, most rotator cuff ruptures are the result of degeneration of the bone-tendon interface [68]. Degenerative changes occur within the tissues, such as chondroid metaplasia in the bone and calcification, vascular proliferation, fatty infiltration and inflammation occurring in the tendon [68]. As with the other tendinous and ligamentous tears described above, tears to the rotator cuff can be managed surgically, or conservatively, however, tendon to bone healing is poor, and the zonal arrangement of the enthesis region is not formed *in vivo* after repair [66,69].

Options for repair of the enthesis *in vivo*

There has been much interest in the repair of the enthesis due to the poor capacity for regeneration and lack of normal enthesis structure following healing. Unlike the direct enthesis, where a zonal, fibrocartilaginous region is present between the soft tissue and the bone, healing at the interface usually results in the formation of an indirect enthesis with a lower mechanical strength than before injury [36,37]. In the case of tendon-to-bone repair, this becomes problematic as the soft tissue fixation site to bone is now the weakest point in the system, and tendon pull-out and graft failure can often occur [70], leading to further surgery and a longer rehabilitation time [70]. Several attempts have been made to improve repair of the enthesis *in vivo* with varied results (Table 1).

Growth factors: The use of growth factors has proved very popular, based on the wide array of growth factors and cytokines that are secreted during tendon and bone healing. The local application of specific growth factors thought to induce endochondral ossification has been used to induce the formation of a neo-enthesis. Bone morphogenetic proteins (BMP) can induce ectopic endochondral ossification in *in vivo* models [71], therefore it was hypothesized that local application of BMP-2 would provide the required stimulus for enthesis formation in a rabbit model [72]. As predicted, the ultimate failure loads of BMP-2 generated entheses were higher than the control group, suggesting that BMP-2 administration could assist in re-establishing the enthesis *in vivo*. BMP-2 incorporated into fibrin gels or collagen and hyaluron sponges have also accelerated healing at the enthesis [73,74], however, BMP-13-expressing cells had no positive effect on the healing enthesis [75]. Furthermore, using BMP-2 incorporated polyethylene glycol hydrogels with periosteum progenitor cells resulted in the formation of increased fibrocartilage and bone layers, elevated markers for cartilaginous tissue expression and increased mechanical properties when compared to control groups [76]. While many groups have reported the positive effects of BMP-2, when used in combination with platelet-rich plasma and fibrin, BMP-2 was shown to have no significant effect on the maximum loads of bone-tendon interfaces until the 8th week postoperatively [77]. Other growth factors have also been employed at the site of repair to aid in the formation of a fibrocartilaginous transition. The use of granulocyte colony-stimulating factor (G-CSF) incorporated within a gelatin hydrogel has been shown to increase the ultimate tensile strength of the soft tissue insertion at four weeks [78], with this increase in strength being attributed to enhanced osteogenesis and angiogenesis, as upregulation of osteogenic factors and increased capillary density were both seen in the G-CSF treated groups [78]. TGFβ has also been investigated at the healing bone-tendon interface with differing results [73,79]. While TGF-β1 and TGF-β3 were shown to have no significant effect on bone-tendon healing in rat and rabbit models [73,79], a recent study by the same research group has reported the positive enhancement of bone-tendon healing following sustained application of TGF-β3, with an increase in the strength of the interface compared to the control group [80]. This increase in interface strength was still lower than the uninjured bone-tendon interface [80]. Finally, both FGF-2 and PDGF-BB have been shown to increase the production of a fibrocartilaginous region at the interface [81,82], although only FGF-2 application resulted in improved mechanics [81].

Materials: Novel materials are also being used to augment healing at the bone-tendon junction in animal models. Bone cements, most commonly used to fill large bone defects, are now being used to fill the gap between the tendon and bone during surgical repair, providing an osteoconductive material to induce bone ingrowth at the site of repair [83,84]. An initial study reported the use of α-BSM calcium phosphate cement to aid fixation at the tendon-bone repair site [85]. Although fixation strength was not improved, the authors strongly advocated the use of a bone cement to provide a carrier for growth factors which, as described above, could expedite interface healing [85]. This technique was employed in a later study by the same group, where TGFβ3 was added to a calcium phosphate cement [86]. In general, increases in ultimate failure strength when compared to control groups have been reported with both a calcium phosphate bone cements [84,86], a magnesium-based bone cement (Osteocrete) [83] and a brushite bone cement [87]. Additionally, the use of bone cements resulted in positive histological effects at the junction with new bone formation [83,84], improved regeneration of the fibrocartilaginous insertions, and reduced scar tissue formation [83]. Furthermore, with the use of brushite bone cement, at 6 weeks postoperatively, the strength and

Mode of repair	Site of repair	Animal Model	Observations	Studies
Growth factors				
Bone Morphogenetic protein-13 (BMP-13) expressing MSCs	Rotator cuff	Rat	No difference in strength, stress or stiffness when compared to control group.	[75]
BMP-2 injection at repair site	Flexor tendon	Rabbit	Ultimate failure load increased in BMP-2 group.	[72]
rhBMP-2 in hyaluron or collagen sponge	Rotator cuff	Sheep	Increased mechanical properties of the interface when treated with rhBMP-2 sponge (either hyaluron or collagen sponge) when compared to sponge alone.	[74]
BMP-2 loaded polyethylene glycol diacrylate (PEGDA) hydrogel with periosteum progenitor cells	Rotator cuff	Rabbit	Increased fibrocartilage and bone layers and increased pull-out strength at 4 and 8 weeks postoperatively compared to control group	[76]
Platelet rich plasma and BMP-2 in fibrin glue	Achilles tendon	Rabbit	No significant increase in maximum loads at 2 or 4 weeks, but becomes significant at 8weeks when compared to control groups. Woven bone and fibrocartilage produced.	[77]
Granulocyte colony stimulating (G-CSF) factor in gelatin	Flexor tendon at ACL site	Dog	Increased mechanical strength at the interface in the treatment group	[78]
Platelet derived growth factor-BB (PDGF-BB) coated suture anchors	Rotator cuff	Sheep	Increased histologic score at insertion site but no difference in treatment to control Group	[82]
Fibroblast growth factor -2 (FGF-2) in acellular dermal matrix graft	Rotator cuff	Rat	Increased fibrocartilage and increased load to failure in the FGF-2 group	[81]
TGFβ3 released from fibrin glue delivery system	Rotator cuff	Rat	Increased levels of disorganised scar tissue. Increased interface strength compared to surgical repair but not matching levels of uninjured bone-tendon insertion.	[80]
$TGF\beta_1$, $TGF\beta_2$, $TGF\beta_3$	Rotator cuff	Rat	$TGF\beta_1$ increased type III collagen but no significant increase in mechanics compared to paired control. $TGF\beta_3$ showed no differences between treatment and control groups.	[79]
TGF β and BMP-2 in fibrin glue carrier	Achilles tendon	Rabbit	TGF_β had no effect, but BMP-2 accelerates healing at interface	[73]
Ultrasound/shock wave therapy				
Low intensity pulsed ultrasound (LIPUS)	Patellar tendon	Rabbit	Increased hardness of new bone, fibrocartilage and tendon	[101]
Extracorporeal shock wave therapy	Patellar tendon	Rabbit	Regeneration of fibrocartilaginous zone and increased osteogenesis in treated group	[132]
Low intensity pulsed ultrasound (LIPUS)	Patellar tendon	Rabbit	Increased healing at the interface in treatment group	[100]
Inhibition				
α2-macroglobulin	Semitendinosus tendon at ACL site	Rabbit	Enhanced bone healing at the interface in the treatment group.	[96]
α2-macroglobulin	Rotator cuff	Rat	No difference in mechanics between treatment and control groups but increased collagen in treatment group.	[97]
Doxycycline (MMP-13 inhibitor)	Rotator cuff	Rat	Increased load to failure in treatment group	[98]
Tumour necrosis factor-α (TNF-α) blockade	Rotator cuff	Rat	Increased strength, stiffness and fibrocartilage levels compared to control group	[99]
Bone cements				
α-BSM Calcium phosphate cement	ACL	Pig	Significantly lower pull out strengths than control group	[85]
Calcium phosphate matrix with and without $TGF\beta_3$	Rotator cuff	Rat	Increased Collagen I/Collagen III ratio and increased strength at interface in treatment group	[86]
Magnesium-based bone cement	Hamstring graft at ACL site	Rabbit	Increased cartilage and failure strength in treatment group.	[83]
Brushite bone cement	Brushite bone cement	Rabbit	Increased strength and stiffness at interface in treatment group.	[87]
Cells				
Chondrocyte pellet	Patellar tendon	Rabbit	Fibrocartilage zone structure in treatment group.	[95]
Bone marrow stromal cells	Rotator cuff	Rat	No difference in cartilage production, collagen organization or failure strength in treatment group.	[36]
MTI-MMP transduced MSCs	Rotator cuff	Rat	No differences noted at 2 weeks, but Increased fibrocartilage, load, stress and stiffnes than control group at 4 weeks	[94]
MSCs in fibrin gel	Achilles tendon	Rat	Increased failure load compared to control at 45 weeks. Organised enthesis structure produced	[93]

Chondrocytes in fibrin gel	Achilles tendon	Rat	Increased failure load compared to controls but lack of organization at the enthesis	[93]
Scleraxis-transduced MSCs in fibrin glue	Rotator cuff	Rat	Increased failure load, stiffness, stress and fibrocartilage at 4 weeks compared to control group	[37]
Autologous MSCs in fibrin glue	Achilles tendon at ACL site	Rabbit	Zone of fibrocartilage and increased load to failure in treatment group	[92]
Other				
Polyglycolic acid (PGA) sheet	Rotator cuff	Rabbit	Increased fibrocartilage, load to failure and strength at interface when compared to polycaprolactone (PCL) control, but not tendon control.	[88]
Polyurethane patch (Artelon® Tissue Reinforcement graft)	Achilles tendon	Cadaveric human	Increased load to failure in treatment group	[91]
Demineralised bone matrix	Patellar tendon	Sheep	Increased fibrocartilage and mineralized fibrocartilage in treatment group	[89]
Paralysis with Botulinum toxin	Rotator cuff	Rat	Decreased muscle weight and volume over 8 weeks, but recovered by 24 weeks in treatment group. Increased collagen fiber alignment in treatment group.	[102]
Delayed mechanical conditioning	ACL	Rat	Increased maximum loads in delayed groups when compared to immediate loading or immobilized groups	[103]
Controlled mechanical loading	ACL	Rat	Low levels of controlled loading in immediate post- operative period does not significantly impair healing	[104]
Autologous cartilage plug	Patellar tendon	Goat	Increased fibrocartilage production at the interface.	[90]

Table 1:

stiffness of the tendon had increased by 117% and 102% respectively compared to the contralateral untreated knees [87]. In addition, at 12 week postoperatively, failure of the bone-tendon complex occurred in the inter-articular mid-substance region of the graft in the brushite treated groups compared to in the tibial or femoral tunnels in the untreated controls [87].

Polyglycolic Acid (PGA) sheets have also been used to facilitate healing at the bone-tendon interface by providing a rapidly degrading scaffold that stimulates cell growth and supports the newly forming tissue [88]. Although this method does promote the formation of a fibrocartilaginous interface, the mechanical strength of the interface is lower than in a normal tendon model, thus indicating that while the transition between the tendon and PGA is improved, the PGA-bone interface remains weak resulting in decreased mechanical function [88]. Demineralized bone matrix (DBM) is another material proposed to enhance healing at the bone-tendon interface by providing a robust, osteoinductive scaffold at the site of repair [89]. After 6, 9 and 12 weeks, DBM treated animals showed significantly more fibrocartilaginous tissue than the control group in an ovine patellar model [89]. While the insertion site of the DBM group contained more fibrocartilage and mineralized fibrocartilage than the control group, the amount of bone formed in both groups was similar, indicating that DBM may produce a more physiologically similar enthesis [89]. However, the mechanical strength of the interface has yet to be determined. Similar to the idea of implanting a scaffold at the insertion site, Wong et al [90] have reported that an autologous cartilage plug can be inserted between the soft and hard tissue at the repair site, and that this will increase fibrocartilaginous tissue formation. A commercial graft, originally developed as a joint spacer, has also been reported to increase load to failure in a human Achilles' tendon cadaveric model [91]. The Artelon® Tissue Reinforcement graft consists of a polyurethane patch that is sutured to ruptured tissue at the repair site [91].

Cells: The addition of cells to the insertion site at the healing bone-tendon interface has had limited success in regenerating a fibrocartilaginous transition. Addition of bone marrow stromal cells (BMSCs) resulted in no difference in cartilage production, collagen orientation or failure strength in the treatment group of a rat rotator cuff repair model [36]. Conversely, coating the ends of a tendon allograft with BMSCs in a fibrin gel leads to the formation of a zone of fibrocartilage and increased mechanical properties at the interface in a rabbit ACL replacement model [92] and BMSC addition in fibrin glue alone can also increase the mechanical properties of the interface and result in an organized enthesis at 45 days after surgery in a rat Achilles tendon model [93]. In addition, BMSCs that had been transduced with scleraxis were applied to the insertion site in a fibrin glue and resulted in a positive effect on bone-tendon healing, with increased failure load, stiffness, strength and fibrocartilage production after 4 weeks when compared with the control group [37]. Similarly, MSCs transduced with a matrix metalloproteinase (MMP) gene exhibited increased fibrocartilage, load, stress, and stiffness at the insertion site after 4 weeks [94]. Finally, a chondrocyte pellet facilitated the formation of a fibrocartilaginous zone in a rabbit model [95], however, while addition of chondrocytes within a fibrin glue matrix resulted in increased mechanical properties when compared to controls, there was a lack of organised tissue structure at the interface [93].

Inhibitors: While promotion of certain factors may prove beneficial to interface formation, it is also clear that inhibition of other factors may also lead to a favorable healing response. To this end, the MMP inhibitor α2-macroglobulin has been applied at the bone-tendon site in rabbit and rat models. Enhanced bone healing [96] and increased collagen levels [97] have been reported, but no difference in the mechanics of the treatment and control group was noted [97]. However, another MMP inhibitor, doxycycline, has led to an increased load to failure in the treatment group when compared to the control [98]. Similarly, inhibition of Tumor necrosis factor α (TNFα) leads to increased mechanical properties and fibrocartilage formation in the treatment group of a rat model [99].

Physical treatment options: Some treatments have also proved to be beneficial in accelerating healing at the osteotendinous junction. Low intensity pulsed ultrasound (LIPUS) can promote healing at the interface in both rat and rabbit patellar tendon models [100,101], whereas extracorporeal shock wave therapy can also increase osteogenesis and fibrocartilaginous tissue formation in the treatment groups [102]. Interestingly, another treatment, paralysis caused by botulinum toxin, has had mixed results when used to enhance healing at the osteotendinous junction. Although paralysis seemed to increase collagen alignment in the treatment group, it also resulted in the reduction in muscle weight and volume over 8 weeks. This was recovered after 24 weeks, but it is thought that the longevity of muscle paralysis was detrimental to interface formation and that a shorter length of paralysis would be more beneficial to recapitulation of the normal enthesis structure [102]. The effect of mechanical loading following surgery has also been under investigation recently. While one study has determined that delayed mechanical loading results in increased maximum loads at the bone-tendon interface when compared to immediate early loading or immobilization [103], a follow-up study by the same research group has stated that low-levels of controlled loading in the immediate post-operative period are not detrimental to bone-tendon junction healing [104]. It is clear that future work in this area is extremely important for improving the quality of bone-tendon healing in a clinical setting. Understanding the effects of post-operative conditioning is pertinent to establishing the most successful rehabilitation regimes to provide the best possible outcome for the patient following surgery.

Although modifying the local environment with growth factors and osteoinductive materials has shown some promising results, the problems faced by surgeons to allow formation of multi-tissue gradients at the enthesis remain challenging. As stated, the formation of a fibrocartilaginous zone is a vital process absent from enthesis healing. It has also been mentioned that cartilaginous tissue lacks a bloody supply and therefore exists in a physiologically hypoxic environment. Interestingly, a recent review has highlighted the importance that the hypoxic environment may have on regeneration of the enthesis by stimulating chondrogenesis [105]. Hypoxia has been shown to be vital for the processes of chondrogenesis, osteogenesis and angiogenesis [105,106] and the fact that the native bone-tendon interface region is lacking in vascularisation heavily suggests that the enthesis is in a physiologically hypoxic environment [105]. Furthermore, as initiation of chondrogenesis is stimulated by hypoxia-inducible factor 1-α (HIF1-α), recent evidence has suggested that establishing a hypoxic environment during bone-tendon interface healing may provide an appropriate environment for generation of a fibrocartilaginous enthesis [105]. To this end, Zhao et al. [105] have suggested two important prospects for the achieving the formation of hypoxia at the interface site. The first of these is a model involving the creation of a haematoma in the bone tunnel during bone to tendon fixation [105]. The second is the use of HIF-targeted drug therapy, aiming to prolong the action of HIF-1α in the bloodstream [105]. These options seem particularly promising and could potentially give an exciting step forward in the understanding and repair of bone-tendon junction sites *in vivo*.

Interfacial tissue engineering (ITE) options for repair – *in vitro* studies

While several methods have been described as augmenting the strength of the ligament-bone graft fixation site, the fact that the majority of failures still occur at the transition of graft to native tissue, highlights the need for an appropriate transition between tissues if implants are to be successful. In the case of manufacturing artificial tissues for implantation, this would involve the engineering of the complex tissue interface *in vitro*.

As described earlier, the relatively new field of ITE is aimed at attempting to recreate complex tissue interfaces *in vitro*. Achievement of this goal would propel the prospect of implantation of musculoskeletal tissues after disease or trauma towards being a clinical reality. Several groups have realized this importance of engineering composite tissue constructs for the repair of musculoskeletal tissues and are producing innovative solutions to the gradient design and multi-tissue requirements for tissue-engineered musculoskeletal constructs. While some groups have chosen to focus on the production of a scaffold to be inserted as an adjunct to native tissue repair, in the form of a multi-phase multi-cellular scaffold or plug [40,107,108], others have attempted to engineer graded interfaces with one cell type [109] or have engineered a whole multiphasic tissue from end to end with the goal of implantation to the injured site [110-114]. Each of these techniques will be discussed and evaluated as possible methods of musculoskeletal tissue repair.

Developing multiphasic scaffolds for replication of the insertion site: The Lu group has developed a triphasic scaffold to mimic the fibrocartilaginous transition seen at the insertion site of tendons/ligaments to bone [40,107,108]. A 3-layered scaffold was produced using polylactide-co-glycolide (PLGA) knitted sheets, PLGA micro spheres and PLGA/bioactive glass micro spheres to mimic the soft tissue, fibrocartilaginous, and bone regions respectively. Following fabrication of the scaffold, fibroblasts were seeded onto the PLGA sheets, osteoblasts were seeded onto the bioactive glass, and although cell migration into the central core was observed [40], there was no formation of a fibrocartilage-like region in the central core. The specifics of fibroblast/osteoblast interactions were next investigated in a 2D study, where fibroblasts and osteoblasts were co-cultured on either side of a hydrogel divider, before the divider was removed and both cells migrated into the central portion of the dish [115]. This novel approach permitted evaluation of the interaction of fibroblasts and osteoblasts as would be found *in vivo* at the bone-ligament insertion. Interactions between the different cell types reduced the proliferation rate, altered the alkaline phosphate (ALP) activity of osteoblasts and had a positive effect on the expression of matrix proteins present at the enthesis, such as collagen type II, aggrecan and cartilage oligometric matrix protein (COMP) [115]. However, despite fibrocartilage-like markers being present, a fibrocartilaginous interface region was not formed in culture. [110]. In subsequent studies, chondrocytes were included both in 2D and 3D [107,110]. In both situations, a fibrocartilage-like region was formed during triculture [107], thus developing a transition that replicates native tissue structure at the insertion. It is thought that by using these techniques, the triphasic scaffold will promote biological fixation of soft tissue grafts to bone and re-establish the gradual transition of soft tissue to bone in such a way as to transfer load smoothly. However, the tensile properties of this triphasic scaffold have not been reported, nor has the attachment potential of the scaffold with either the hard or soft tissues. Establishing the tensile properties will help to determine whether introducing a triphasic scaffold provides a mechanically sound attachment between ligament or bone or whether the three phases of the scaffold are mechanically distinct, and therefore increasing the number of interface regions and the propensity for implant failure.

Another research group has successfully manufactured a continuous bone-soft tissue mimetic interface, using only one cell type (fibroblasts) seeded onto polymeric scaffolds containing a gradient of the transcription factor runt-related transcription factor 2 (Runx2) [109]. Runx2 is a bone specific transcription factor that plays a critical role in bone development and osteoblast differentiation. By controlling the spatial distribution of Runx2 within the scaffolds, osteoblastic differentiation and mineralized matrix deposition occurred in a graded fashion throughout the scaffold [115]. The mechanical properties of the scaffolds were examined by uniaxial tensile testing and increased mineral deposition resulted in increased construct stiffness, demonstrating the ability to produce a construct with graded mechanical properties with only one cell type, as opposed to the multicellular, multilayer constructs described elsewhere [40,107,108]. Furthermore, the 3D gradient resulted in a zonal organisation of fibroblastic and osteoblastic cell phenotypes *in vivo* after 14 days of implantation into an ectopic site [109], achieving an important recapitulation of the native transition. However, the fibrocartilaginous region was not present in these constructs therefore these constructs do not fully replicate the native bone-ligament insertion [109]. Nevertheless, future development of this technique may allow for fibrocartilaginous tissue induction to duplicate the native tissue transition for enthesis repair.

Developing whole multi-tissue constructs: The authors group has previously reported the production of a full artificial ligament construct for potential repair of the anterior cruciate ligament [111-113]. Initial work focused on establishing an attachment between artificial "anchors" to guide the formation of a cell-embedded fibrin gel into a bone-ligament-bone construct [111]. By forming an attachment between the anchor and the soft tissue, the fibrin gel is held under tension, known to be important in the formation of fibripositors and production of aligned collagen fibers within this system [112]. Polyethylene glycol diacrylate (PEGDA) was chosen as the anchor material in this initial study, however, there was little interaction between the PEGDA anchor and the fibrin gel, most probably due to the fact that PEG is non-adhesive to proteins without functionalization [111,113]. However, when incorporated with hydroxyapatite, the anchors attached to the fibrin-based ligament construct, with the length of attachment dictated by the hydroxyapatite content of the anchors [111]. The finding that calcium phosphate was vital in the formation of a bone-ligament interface and the primary determinant of interface longevity, led to the development of our current engineered bone-to-bone ligament system, where the anchors are constructed from a resorbable bone cement, brushite [114]. Brushite ($CaHPO_4 \bullet 2H_2O$) cements are formed from the combination of an appropriate calcium phosphate salt, most often β-tricalcium phosphate (β-TCP; $Ca_3(PO_4)_2$) with an acidic source of orthophosphate ions ($H3PO_4$) and the resultant matrix is formed of brushite with some unreacted β-TCP [116,117]. Figure 3 depicts the process by which our tissue engineered ligament-like constructs form. The fibroblast seeded fibrin gel contracts between the two fixed brushite cement anchor points and is therefore held under tension. Over time, the fibroblasts contract and remodel the fibrin gel and secrete their own collagenous extracellular matrix (Figure 3A-D). An investigation into the effect of shape of the brushite cement anchors on interface longevity revealed that simple shapes without complex areas likely to result in stress and strain concentrations at the bone-ligament insertion site were preferred for the manufacture of bone-to-bone ligament constructs [118]. Furthermore, we have reported that by increasing the collagen content of the soft tissue portion of the construct using promoters of collagen synthesis (ascorbic acid, proline and TGFβ) leads to an increase in strength of the artificial hard/soft tissue interface [118]. The engineered hard/soft tissue interface will remain intact until at least 18 weeks *in vitro* (Figure 3E). Engineering an in vitro musculoskeletal hard/soft tissue interface has also improved prospects for the production of functional tissues for potential implantation. While many groups have focused on the engineering of tendon/ligament tissue in isolation [119-128] and very few have focused on generating the whole tissue with appropriate interfaces for implantation, generation of functionally adequate tissues through use of bioreactors is also a requirement. Engineered tissues that lack the interface between the ligament and the bone and use various materials to hold the tissue during development [119,120,128] have synthetic interfaces that are prone to mechanical failure [112]. Any ligament construct that lacks a bony interface would have to be fixed to either the soft-tissue remnants of the injured ligament or by direct attachment of the engineered tissue to bone, which is more prone to failure due to tendon/ligament slippage and lack of bone-tendon/ligament

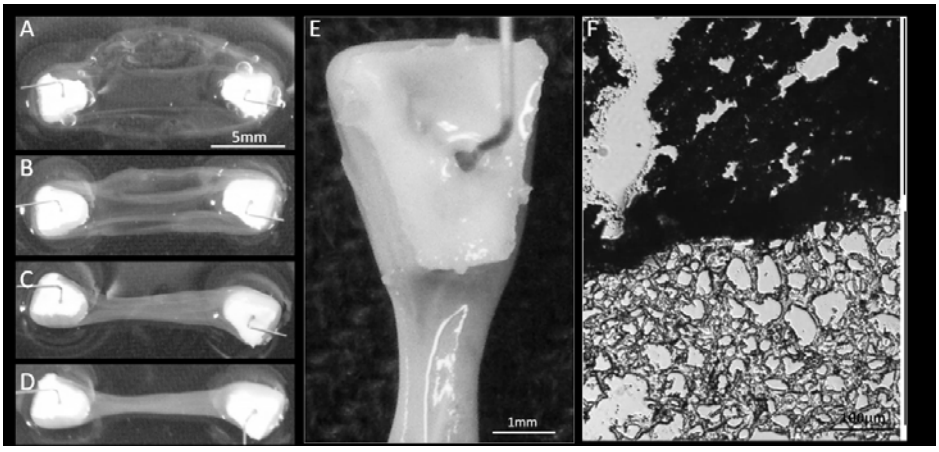

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Figure 3: Bone-ligament bone constructs. Two brushite anchors are immobilised on the surface of a Petri dish and the fibrin gel is remodelled over time until a multi-component tissue is formed. A) Day 3, B) day 7, C) day 21, D) day 35 of formation. E) Gross morphology of the hard/soft tissue interface at 18 weeks of formation. The interface between the brushite anchor and the collagenous soft tissue is still intact. F) Histological section of the hard (top)/soft (bottom) tissue interface after 6 weeks in culture. N.B. Section was left unstained to maintain the integrity of the cement portion.

interface formation. This is also true for attachment to bioreactors, as a synthetic interface is also required to attach the artificial tissue to a grip. However, by forming an *in vitro* hard/soft tissue interface, gripping of the constructs by the hard tissue portion in a bioreactor [129] or tensile tester [115,118] becomes straightforward and by using this method, the functional effects of stretch on our engineered bone-to-bone ligament constructs has also been recently reported [129]. We are currently investigating the process of interface formation with a range of different anchor materials, histological techniques (Figure 3F) and novel imaging modalities with a goal of establishing graded mineral deposition and fibrocartilaginous region at the hard/soft tissue interface as observed at the natural enthesis.

The furthest progress reported to date on engineering three-dimensional multiphasic tissues from bone-to-bone has been reported by Ma et al. [130,131]. A scaffoldless method has been used to engineer bone and ligament separately from bone marrow stromal cells and once the two tissues have been formed, they are co-cultured to form bone-ligament-bone constructs with viable entheses that remain intact *in vitro* and *in vivo* [130] and do not fail under tensile loading in the physiological range [131]. In addition, following implantation, the constructs significantly grow in size and increase their mechanical properties. These bone-ligament-bone constructs have been implanted in both rat [130] and sheep models [131] as replacement medial collateral ligament (MCL) and ACL respectively. Although these studies report a very promising method of construct formation with the advantage of engineering an intact enthesis that performs well under tensile loading, the complexity of the manufacturing process and the length of time required to produce such a construct could limit the success of this method of soft tissue repair. Nevertheless, this technology describes an important step-forward in engineering transitions between tissues of varying properties, as they have successfully managed to replicate the zonal transition with a region of fibrocartilage with aligned chondrocytes at the enthesis region [131].

Future prospects in ITE : For the development of suitable strategies for the advancement of ITE, it is important to analyze the most promising methods of *in vivo* enthesis repair and implement them in *in vitro*. As highlighted in this review, *in vivo* studies have already provided a wealth of information on the effect of growth factors on enthesis formation that could be utilized in ITE. Our research group has already applied the positive effect of bone cements at the enthesis repair site [83,87] to manufacture bone-to-bone ligament analogues that have significant potential for ACL repair [115,118,129]. To build on this, it appears the inclusion of growth factors could provide a promising means of establishing a fibrocartilaginous transition *in vitro*. Based on the *in vivo* studies, BMP-2 incorporation could potentially result in fibrocartilage production [72-74,76,77] and warrants investigation within our tissue-engineered ligament system. Similarly, based on previous studies, the effects of FGF-2 and PDGF may also be interesting to investigate [81,82]. Modification of the local environment by other means is also a key direction for interfacial tissue engineers to explore with respect to the formation of multi-tissue constructs. Of particular interest is the investigation of ultrasound and shock wave therapy [100,101,132], as it is envisaged that these physical cues could possibly be incorporated into bioreactors for functional tissue engineering. Furthermore, development of regional variation in the culture environment has strong potential for the development of graded, multi-tissue constructs *in vitro*, as the importance of oxygen tension for the development of different tissues types has been highlighted [105,106]. This could be an exciting development for the future engineering of multi-phase tissue constructs. It is also important to use the information gathered about the stages of enthesis development to provide suitable physical environments for formation of a similar structure *in vitro*. As highlighted, the important study by Galatz et al. [20] demonstrates the need for mechanical stimulation during the postnatal period of development and the significance of an appropriate mechanical environment during development [20]. Similarly, paralysis of the muscle unit affects enthesis structure during the postnatal period [21] again indicating that to replicate the native enthesis structure *in vitro* requires creation of suitable mechanical environments. Also of note is the knowledge that fibrocartilage can form in tendons and ligaments in areas subject to compression [11,133], a theory that has been already been explored in tendon engineering *in vitro* [134]. Therefore, it strongly appears that to achieve success in ITE, researchers need to establish ways to create suitable mechanical environments, perhaps in isolated regions, for the tissue constructs to achieve multiphasic tissue distribution. Again, incorporating such stimulation protocols into bioreactors either alone or in combination with modifying the local environments described above may assist in the goal of achieving fibrocartilaginous transitions and multi-tissue construct *in vitro* for implantation.

Summary and Conclusions

The complex interfaces that are present at the boundary of musculoskeletal tissues have an important role in force transmission and stress dissipation between tissues of varying properties. The structure and morphological adaptations present at the enthesis act to smooth the force caused by muscle contraction through the tendon and to the bone to produce movement. These adaptations are not recapitulated during repair at the bone-tendon junction and are the primary reason attributed to the low success rates of graft implantation. With an increase in musculoskeletal injury in the developed world, it is important that clinicians and interfacial tissue engineers study the anatomy, development and repair of the enthesis to optimize the prospects for clinical advancement in enthesis repair strategies.

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