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Advances in tissue engineering approaches for repairing and rehabilitating the myotendinous junction

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Abstract

The myotendinous junction (MTJ) acts as a bridge between muscle and tendon; yet its high stiffness relative to muscle fibers renders the tissue susceptible to injuries due to eccentric loading disparities. The limited regenerative capacity of MTJ tissue and potential for postsurgical scarring and reinjury necessitates complementary therapeutics that can enhance cellular interactions, restore mechanical properties, and support tissue rehabilitation.

This review explores various approaches to engineer the MTJ utilizing biomaterial scaffolds and cellularized materials that mimic structure and function. While biomimetic materials show promise, challenges remain due to the interface's complexity and differing patient- and location-specific structure–function characteristics, necessitating further research to address these gaps. This review also highlights the importance of studying MTJ injuries in women's health and craniofacial reconstruction. Furthermore, engineered MTJ models provide versatile platforms for investigating trauma and degeneration, thus offering potential for advancing research across multiple fields, shedding light on interactions at tissue interfaces, and shaping the future of MTJ rehabilitation.

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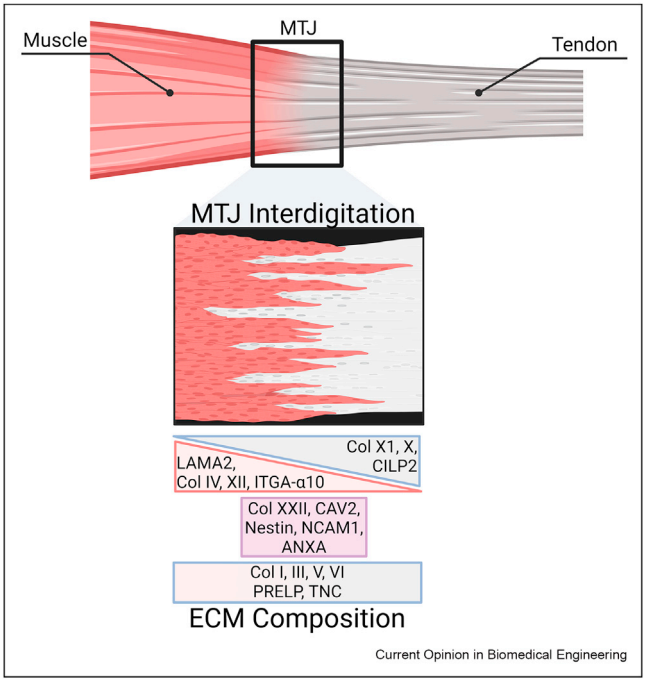
Introduction

Despite the high prevalence of musculoskeletal disorders, affecting nearly half of the adult population in the United States and accruing healthcare costs that surpass those associated with major diseases such as diabetes and cardiovascular diseases, research into reparative and regenerative treatments for musculoskeletal disorders remains notably underfunded [5,6].

A recent systematic review focusing on the muscle–tendon–bone complex revealed that failures at the myotendinous junction (MTJ) were linked to 28% of the musculoskeletal injuries studied [8]. The MTJ, also referred to as the musculotendinous junction, is the tissue bridge between a muscle and tendon that transfers tensile and contractile forces between muscle filaments and collagen fibers in tendon in a healthy homeostatic state (Figure 1). However, the high stiffness of tendon tissue relative to the compliant muscle fibers makes this region especially susceptible to injuries because the differences in loading can result in tearing of the interfacing tissues [9,10]. Of the reported injuries, the mean age of the population was 33.7 ± 0.5 years as these patients were significantly younger than those suffering from tendon or enthesis injuries [8]. While only 8% of these injuries were associated with evident trauma, a significant 92% were instigated by noncontact events and occurred in well-trained athletes. This highlights that MTJ injuries can occur in younger populations during non-traumatic events, emphasizing the need for research funding in this field [5,8].

MTJ injuries can be broadly characterized by two categories: minor strains and complete rupture, which occur when overloading causes major tissue separation [9]. Treatment options for minor MTJ injuries, such as the rest, ice, compression, elevation (RICE) method, nonsteroidal anti-inflammatory drugs (e.g. Advil® or ibuprofen), muscle relaxants (Flexeril® also known as cyclobenzaprine), anticonvulsant medications (e.g. gabapentin), and physical therapy, provide symptomatic relief but do not significantly promote restoration. Furthermore, limb immobilization, which is a treatment option for generic musculoskeletal injuries, results in altered tendon–muscle integration leading to reduced MTJ surface area, intramuscular fibrosis, muscle cell atrophy, and diminished tensile properties [11,12]. Conversely, subjecting the MTJ to

Figure 1



Schematic of the myotendinous junction (MTJ) highlighting the interdigitation at the interface of the muscle and tendon tissues necessary for mechanical transmission. Distinctive extracellular matrix (ECM) components of native tissue regions [2,3] are presented for each tissue type of muscle (red triangle), tendon (gray triangle), the overlapping region of the MTJ (purple rectangle), and components found throughout all regions (long red-to-gray gradient rectangle). Created with BioRender.com.

mechanical load demonstrates remarkable adaptability, thus increasing its junctional interface to accommodate different load intensities [13]. Complete MTJ ruptures often necessitate surgical intervention to repair the torn interfacial tissue, but there is a risk of rerupture and scar tissue formation at the site of injury due to the poor intrinsic reparative characteristic of the hypocellular and hypovascular tendinous end [14]. While specific mechanisms of MTJ injury and reinjury remain to be elucidated, tendon reinjury is well documented. Thus, MTJ reinjury in this review is referenced within the context of tendon. Despite advancements in surgical interventions, approximately 80% of reinjuries occur at the initial injury site, which highlights an unmet need in treating MTJ injuries [15]. Therefore, there has been a shift in the paradigm toward complementing surgery with a tissue-engineered solution to rehabilitate the MTJ.

In this review, we describe current engineering technologies to recapitulate the MTJ mechanics and microarchitecture with the goal of repairing and rehabilitating the interfacial tissue. The technologies discussed are biomimetic materials, such as acellular scaffolds and cell-based constructs. We also aim to elucidate potential directions for further MTJ research, as well as current gaps in the field and ways to include factors such as genetic disease, biological sex, and anatomical location into future efforts toward regenerative rehabilitation.

Criteria for designing engineered MTJ platforms

Composition and architectural analysis as it pertains to engineering the MTJ

The ultrastructure and microenvironment of musculoskeletal tissues are uniquely adept at transferring mechanical forces; therefore, understanding these interactions at the MTJ is crucial to engineering functional MTJ therapies. The MTJ is made up of complex hierarchical structures and organized microstructural components that contribute to its function. The MTJ is broadly characterized by three regions: muscle, tendon, and the overlapping transitional region [3,9,14], as depicted in Figure 1.

Ultrastructure and extracellular matrix composition of skeletal muscle

Skeletal muscle is a highly vascularized, innervated connective tissue composed of hierarchical myotubes that facilitate internal force generation. These myotubes are supported by extracellular matrix (ECM) proteins, including collagens, laminins, fibronectin, glycoproteins, proteoglycans, and elastins [16,17]. The endomysium, the connective tissue surrounding each muscle fiber within a bundle, is rich in collagen type IV and laminins, which provide an anchoring point at the MTJ region [18,19]. More broadly, collagen type I and collagen type III provide structural support, whereas laminins and elastins interface with cell surface proteins to aid in force transduction between the bulk tissue and the individual cells. At the cellular level, force transduction at the ECM and cellular interface is modulated through laminins, dystrophin-glycoprotein complex, integrins, and a signal transduction cascade. Proteins such as dystrophin and desmin play a role in contractile functions, whereas proteins such as vinculin and talin are involved in F-actin cytoskeletal function [20,21]. Muscle tissue contraction is specifically driven through the interactions of cell surface proteins that transmit signals to the sarcolemma through the dystrophin-associated glycoprotein complex [21,22]. Alterations in the viscoelasticity of the ECM through injury, disease, or scar tissue formation can disrupt signals and influence muscle contractility, as well as cytoskeletal rearrangement [23]. As mentioned, skeletal muscle is specialized in force generation;

however, active movement and locomotion are facilitated by force transmission to the tendon.

Ultrastructure and ECM composition of tendon

Contrary to skeletal muscle, tendons are hypocellular, sparsely vascularized tissues that are purely reactive to forces transmitted from muscles to bone. In the tendon region, tendon fibroblasts are surrounded by a primarily collagen type I matrix. This matrix also contains small amounts of collagen types III, V, XI, XII, and XIV [24,25]. The collagen forms fiber-like structures aligned along the tendon's length, providing excellent uniaxial mechanical strength. Various noncollagenous proteins are also present, including proteoglycans, glycoproteins, and glycoconjugates. Tendinous proteoglycans, decorin, biglycan, fibromodulin, and lumican play a crucial role in assembling collagen fibrils and maintaining tendon's mechanical integrity [9]. Other proteoglycans, such as aggrecan, increase water content in the tendon to provide compression resistance. Glycoproteins found in the tendon include lubricin, which aids in fiber sliding, tensacin-C (Tnc), and tenomodulin (Tnmd), which is a marker for tendon maturation [24]. Upon injury, tendons exhibit disrupted microarchitecture with disorganized collagen type III fibers, reduced fibril diameter, and increased cellularity. The ECM of injured tendons also have altered composition, elevated proteoglycan content, and disrupted collagen cross-linking, contributing to compromised mechanical properties and impaired tissue healing. The uniqueness of tendon and muscle, respectively, culminate in a unique structural and microenvironmental gradient in the MTJ.

Ultrastructure and ECM composition of MTJs

The ultrastructure of the MTJ involves special structures such as the Z-line from the muscle fiber and sarcoplasmic invaginations at the interface, which increase the contact area between muscle and tendon [18,26,27]. The myofilaments from the terminal sarcomeres also connect to the muscle's outer membrane. While tendons mainly have collagen fibers aligned lengthwise, the MTJ fibers closer to muscle are in multidirectional orientations. When observed in three dimensions, the muscle shows furrow-like indentations, whereas the tendon has ridge-like protrusions [28].

The interfacing region between muscle and tendon consists of finger-like muscle projections that extend into the tendon ECM (Figure 1), making up the bulk of the MTJ region [18,26]. Aligned actin-binding proteins and filaments increase the junctional surface area, efficiently transferring eccentric loads from myofibrils to the tendon's larger area. These interdigitations enhance force transfer, prevent slippage during muscle contraction, and ensure that force transmission remains parallel to the muscle basement membrane to reduce shear stress and prevent interface failure. The complex interdigitations and folds enable a mechanically responsive interface, with altering gene and protein expression levels occurring in response to increased loads [28].

The tissue composition of muscle, tendon, and MTJ have many common ECM components, such as collagen I, but unique differential expression of the respective tissues is shown in Figure 1 [2,3,26]. Collagen type XXII is exclusively found at the MTJ and displays interdigitated folding into the muscle fiber membrane that assists with force transmission and structural stability [18,29–31]. In fact, a recent study on zebrafish MTJs by Malbouyres et al. revealed Col XXIIa1 knockout samples displayed reductions in locomotion and posture due to diminished force transmission capabilities [31]. Proteomic analysis of the MTJ also identified Caveolin 2 (CAV2), neurepithelial stem cell protein (Nestin), Annexin A1 (ANXA), and neural cell adhesion molecule (NCAM1) as being uniquely expressed in the MTJ region [2,3,32]. Collagen types I, III, and VI are present throughout all regions of the MTJ. Collagen type VI extends the endomysium to the MTJ and enhances attachment [28]. Collagen type I contributes to MTJ stability and adaptability to increased loads, while collagen type III is involved in ECM remodeling after injury [13,18]. Collagen type IV and laminin are the main components of the muscle basement membrane that connect the MTJ region to the muscle endomysium [18,19,21]. In addition, collagen type IV can assist in connection between the MTJ region and the tendon [25]. Novel MTJ-enriched proteins of cartilage intermediate layer protein and integrin alpha 10 (ITGA10) were found to be located near the muscle end to assist with ECM binding at the MTJ interface [3].

Similar to other musculoskeletal tissues, cells in the MTJ interface region respond to local stresses in the ECM via integrin binding, transmitting external signals through collagens and laminins to membrane bound and intracellular proteins such as tensin, vinculin, paxillin, and talin [13,19,33–35]. Furthermore, it is demonstrated that MTJ ECM development is dependent on muscle contraction, potentially during embryonic maturation [36]. It has been shown that the MTJ microenvironment varies among tissue location, as well as anatomical differences between species and individual patients [37–40]. For example, there is a lack of desmin or nestin I in the extraocular MTJ, whereas these are proteins are highly expressed in high-load-bearing MTJs [39]. MTJs in the mdx mouse, the mouse model used to study muscular dystrophy, showed greater collagen type III deposition [40]. It has also been shown that the structure, mechanics, and composition of MTJs in the pelvic floor vary with biological sex and change drastically during

pregnancy, thus highlighting the subjectivity of anatomical location [37,38]. Collectively, the ECM and cellular interactions within the MTJ contribute to the structural integrity, force transmission, and coordinated functioning of the muscle–tendon interface, while recognizing that age, biological sex, and anatomical location may introduce variations in these characteristics. These elements are crucial in preventing injury at the MTJ and rationalize a need for tissue-engineered constructs to recapitulate such components.

Soluble factors and intertissue communication

Cells within skeletal muscle secrete a variety of growth factors and cytokines, such as insulin-like growth factor-1 (IGF-1) and transforming growth factor-beta (TGF-β), throughout development, during tissue homeostasis, in response to exercise and in response to injury, with known variations as a function of biological sex and anatomical location [41–47]. Cells within tendon tissue are also known to express similar growth factors (e.g. IGF-1) and cytokines during tissue development, tissue homeostasis, and response to injury [24,48–51]. As with all tissues, the concentration and bioavailability of these signals varies with time. Similarly, both skeletal muscle and tendon tissues respond to soluble cues from blood vessels (e.g. vascular endothelial growth factor) or from immune cells responding to injury (e.g. platelet-derived growth factor) [18,52–57].

Specifically, within the MTJ, these growth factors and cytokines can participate in the activation of both muscle and tendon fibroblasts, among other cells, influencing MTJ adaptability under mechanical load. In rat models undergoing a fast-runner exercise regime, there is a demonstrated 2-fold increased expression of peroxisome-proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1α) and enhanced expression of TGF-β in the muscle portion of the MTJ, alongside an observed upregulation of the IGF-1 receptor in the tendinous portion [13]. The enhanced expression of these soluble signals at the MTJ also correlated with morphological modifications that enabled increased muscle–tendon interactions at the interface [13]. Thus, a solid understanding of these signaling cascades can inform the use of soluble factors in the development of *in vitro* MTJ model systems.

It is also important to consider the bioavailability and active signaling that occurs within healthy tissue vs. injured tissues. The bioavailability of these factors is tightly regulated by binding to ECM and proteoglycans [58,59]. Growth factors and other soluble signals such as insulin play pivotal roles in MTJ development, homeostasis, and healing and are critical to regenerative rehabilitation via tissue engineering strategies. Thus, incorporation of these soluble signals into engineered solutions may improve clinical outcomes.

Mechanical and structural characteristics of MTJ platforms

The MTJ exhibits specific mechanical and structural properties that are critical for function and efficient force transfer. Comparable to other mechanoresponsive tissues, the MTJ has an aligned ECM that absorbs and disperses external loading forces transferred in the tissue [18,19]. The matrix alignment contributes to the transition of viscoelastic behavior from the highly compliant contractile muscle to the stiffer tendon. Thus, the role of the MTJ is to appropriately transmit external loads between muscle and tendon, though the expected loads may vary across anatomical locations and fiber types, biological sex, comorbidities, and species (e.g. mouse vs. human) [39,60,61].

Muscles are embedded in the tendinous ECM with varying ratios of tendon-to muscle-lengths, which is contingent on the location within the body and patient features. Furthermore, the efficiency of the load transfer depends on the tendon–fiber-to-muscle-fiber ratio [27]. Therefore, the relative length of tendon and muscle fibers in the MTJ should be considered when engineering MTJ scaffolds. These regionalized tissue-length ratios culminate in varied mechanical properties that align more closely with the predominant tissue concentration. To ensure the seamless integration of a multicomposite scaffold that can support the mechanical interface of the two regions, it is crucial to consider the mechanical properties of native human tendon and skeletal muscle. For example, the Young's moduli of native human tendon and skeletal muscle in the lower limbs are reported to be in the range of 660–1200 MPa and 60–290 MPa, respectively [14,62]. While elastic moduli values for muscle and tendon drastically transition from a passive state to a tensile, flexed state, results consistently demonstrate that tendon is at least one order of magnitude different in its measured properties [63]. It should thus be noted that musculoskeletal tissue mechanics are not always easy to quantify, as demonstrated in a tendon ultrasonography study by Finni et al. [64]. Though noninjured Achilles tendons displayed a 5.8% strain at maximum volumetric contraction compared to injured counterparts strained to 3.1%, the method by which tendon length was normalized caused significant changes in the results [64]. Analyzing tendon strain from the aponeurosis proximal to the MTJ rather than the MTJ itself results in significantly higher strains and displacements, which are measurements of the aponeurosis rather than the tendon itself [64]. The role of the aponeurosis and MTJ in tendinous mechanical observation further confounds the necessary mechanical properties required of a therapy due to the variability and subjectivity of collection methodology. Therefore, it is critical that an engineered biomaterial must be able to sufficiently mimic and withstand the mechanical loading within the MTJ by adapting to various loads and promoting

functional reparative processes. However, mechanical measurements of the MTJ vary as a function of the location, role, and species (e.g. mouse or rat compared to human). This underscores the importance of integrating multicomposite scaffolds that can accurately replicate the mechanical properties of the native tissue and effectively support the unique mechanics of the different MTJs through structural mimicry at the interface.

Cellular components of healthy and pathological MTJs

The complex cellular milieu in the MTJ is a heterogeneous population of resident and infiltrating cells from the muscle and tendon tissues [18,65,66]. The MTJ's intricate cellular composition ensures effective integration and force transfer between the two structurally different muscle and tendon tissues, but populations and structure can shift due to injury or exercise [26,67,68]. In healthy muscle, the primary functional unit is the multinucleated myofiber, which is created by myoblast fusion during development or repair and facilitated by skeletal muscle satellite cells [18,27,69]. The complex muscle tissue is also comprised of stromal cells, endothelial cells, and immune cells such as macrophages [47,57], and nerve cells to facilitate contraction and movement [70–72]. Healthy tendons are characterized by tenocytes, tendon fibroblasts, and tendon/stem progenitor cells, which are all vital for maintenance and repair. These cells, residing among collagen fibrils, are crucial for the tendon-healing process [73,74]. Healing involves both intrinsic tenocytes and extrinsic cells, including immune cells for inflammation management and endothelial cells for angiogenesis and delivering nutrients to the repair site [58,75,76]. Cellular diversity is evident in the transition from the muscle to the tendon components of the MTJ, where each play a vital role in shaping its structural integrity and functionality. Tenoblasts and tenocytes are actively involved in the synthesis and maintenance of the tendon's ECM, emphasizing the inherent characteristics of this hypovascular tissue [77]. Myoblasts and myotubes are present at the MTJ and form the transitional area where muscle fibers merge into the tendon tissue. Satellite cells, which are crucial for muscle repair, are also found in the MTJ, aiding in tissue regeneration upon injury [78]. Furthermore, endothelial cells emerge as vital agents in the repair process for both muscle and tendon components [79]. Surprisingly, adipocytes, or fat cells, are present in healthy MTJs and are suspected to facilitate MTJ remodeling via cytokine signaling [9,80]. Yan et al. identified a novel subpopulation of muscle–tendon progenitor cells (MTPs), characterized by their dual expression of muscle and tendon marker genes. These MTPs, capable of bridging the cellular and functional divide between muscle and tendon tissues, demonstrated significant regenerative properties. When implanted into an MTJ injury model, the MTPs exhibit the ability to facilitate tissue repair and regeneration, underscoring their potential for bidirectional differentiation [66].

When injuries occur at the MTJ, these diverse cell types work together to initiate repair processes, restore tissue function, and aid in the recovery of both the muscle and the tendon components of the junction [73]. Furthermore, studies such as those by Rieu et al., along with others [81,82], provide valuable insights into cellular preferences and interactions within the MTJ. Recognizing that accurate ECM influences are pivotal in shaping cellular phenotype and maintaining proper cellular function are imperative for engineering MTJ materials that will support homeostasis and minimize inflammatory responses. By understanding the role of exercise and injury on cell adaptations within the MTJ, engineered muscle–tendon units can be constructed and enhanced, yielding promising advancements in the development of *in vitro* MTJ model development and validation.

Rehabilitative strategies for the MTJ via biomimetic engineering

When developing strategies to repair, augment, or protect the MTJ during injury or invasive surgery, it is essential to consider the native tissue microarchitecture, such as porosity and alignment. The MTJ region is an interface between two drastically different tissues, in terms of structural organization. Muscle tissue is aligned and compliant, with substantial cellular complexity to enable muscle contraction. In contrast, tendons enhance axial mechanical properties. When subjected to mechanical stress, the MTJ experiences a transition from contractile, force-generating muscle tissue to stiff load-bearing tendon [17].

Biomimetic scaffolds capture the intricacies of tissue microarchitecture and mechanics, offering precise control over these properties at the microscale and nanoscale.Ideally, a biomimetic MTJ scaffold should mirror the muscle–tendon mechanical gradient and the transfer of forces across the interface. Techniques to create nanofibrous- and microfibrous structures, such as electrospinning [74], ice templating [83–86], and three-dimensional (3D) printing, can be used to achieve these structural and functional features. In addition to mimicking the MTJ structure, the cellular interaction between tenocytes and myotubes is significant in the repair and maintenance of the MTJ interface [87]. Therefore, a biomimetic scaffold should support cell attachment, maintenance, and differentiation upon implantation, and promote organization and integration of the resident cells. Decellularized extracellular matrices [7,32] provide an ideal biomimetic environment for cell attachment adhesion by providing the

relevant adhesion proteins such as laminin and collagen. The following section describes current biomimetic approaches to recapitulate the native MTJ environment within *in vitro* and *in vivo* model systems.

Utilizing electrospun scaffolds for mechanical mimicry in the MTJ

Electrospinning is a scaffold fabrication method for tissue engineering, which has been widely leveraged to target the repair of tissue junctions, such as the enthesis interface connecting tendon to bone [88]. However, only one study has investigated the use of electrospinning for the MTJ [74], despite substantial investigations of materials for the aponeurosis [88]. Electrospinning offers precise control over nanofibrous fiber diameter and orientation, porosity, degradation rate, and mechanical strength, which makes this technique attractive in fabricating the MTJ structure. Electrospinning can also be tuned to spatially deposit nanofibers of varying compositions to mimic the triphasic MTJ structure and mechanical gradient. Ladd et al. developed triphasic nanofibrous scaffolds for MTJ repair using a polycaprolactone/collagen (PCL/col) and poly (l-lactide)/collagen (PLLA/col) mixture [74]. The scaffold featured distinct regions with varying porosity and alignment mirroring native MTJ microarchitecture. The PLLA region exhibited an elastic modulus of 27.62 MPa while rupturing at a strain percentage of 35%, whereas the PCL region had an elastic modulus of 4.5 MPa and a 130% strain at failure [74]. The interfacing region displayed intermediate properties (elastic modulus: 20 MPa). While the scaffold failed to precisely match MTJ mechanics, the novel design supported cell attachment and differentiation as NIH3T3 fibroblasts and C2C12 myoblasts attached and differentiated into myotubules [74]. This work also established comparable trends in the mechanical profiles of the interface and highlights the potential of the use of integrated scaffolds for engineering MTJ composites.

Application of decellularized scaffolds in MTJ regeneration

Composite scaffolds for interfacial tissue engineering face challenges replicating interfacial muscle−tendon microarchitectures. Abnormalities in MTJ development or regeneration can lead to functional disability and unforeseen outcomes [29,31]; therefore, it is imperative that homeostatic tissue ultrastructure and mechanics are as closely replicated as possible. Intact decellularized extracellular matrix (d-ECM) may address these issues by preserving the native ECM structure and components, comparable to native MTJ ultrastrcture and composition, as described in Figure 1. While mammalian ECM materials have been used extensively in tissue repair [75], research on decellularized scaffolds for MTJ regeneration remains limited [58,76]. Developing an optimal decellularization protocol that maintains native architecture and elasticity while fully removing cellular components could lead to better recapituclation of native MTJ structures within *in vitro* engineered MTJs. Moreover, understanding how these decellularization protocols preserve the native ECM composition is critical as proteomic profiling of MTJ regions revealed that Col XXII, CAV2, and ANXA are differentially expressed in the MTJ region compared to the skeletal muscle and tendon regions [2,3,31,32].

Zhao et al. examined a decellularized porcine Achilles tendon MTJ (D-MTJ), preserving tissue structure and integrity [32]. The D-MTJ retained mechanical strength and promoted new myofiber formation 30 days post implantation in a rabbit-muscle-defect model. It facilitated muscle regeneration, as evidenced by the expression of key regulatory genes such as myoblast determination protein (MyoD) and myosin heavy chain (MyHC), both of which are crucial for regeneration and functionality [32]. Complementary to proteomics assessments of native skeletal muscle, the D-MTJ structure was found to have upregulation of laminins, annexins, and myosins relevant to force transduction [2,3,32]. However, assessment of the tendon or MTJ-specific proteins was not performed on the D-MTJ structure, and future success of this material should consider all regional compositions of the MTJ [32]. Compared to native-MTJ controls, the D-MTJ resulted in a thinner fibrotic capsule and less inflammation [32]. This study was the first to assess the biological response of a decellularized MTJ scaffold [32]. However, further research is needed to assess long-term effects as well as explore degradability and force generation in large animal studies. Nevertheless, Zhao et al.'s results are consistent with other results using decellularized ECM-based materials, where decellularied ECM is able to effectively restore 3D structure, ultrastructure, vascular networks, and ECM components in whole organs such as skin, lungs, and kidneys while minimizing inflammation and promoting resident cell infiltration [89−91].

Decellularized tissues can also be used as compositional or adhesive cues, even when the original tissue architecture is lost during processing (e.g. milling, digestion) for applications in electrospinning, spray coating, 3D bioprinting, among other applications [77,92−95]. For MTJ reconstruction, Turner et al. used xenogeneic small intestinal submucosa (SIS) ECM in a canine model following gastrocnemius MTJ resection [78]. The authors decellularized xenogeneic canine SIS using chemical and mechanical methods and created a particulate material that was vacuum pressed into a layered structure [78]. The results showed the xenogeneic d-ECM scaffold recruited progenitor cells and promoted vascularization and innervation of the MTJ six months post implantation [78]. These finding suggest the

biological influence of the decellularized ECM in improving regeneration and cell infiltration.

Electrospinning and d-ECM are both useful approaches for recapitulating the muscle–tendon interface due to the ability of each to mimic the structure and environment of native tissues. As the development of new cellularized *in vitro* MTJ models emerge, we expect that investigators will utilize these material formats in their construct development.

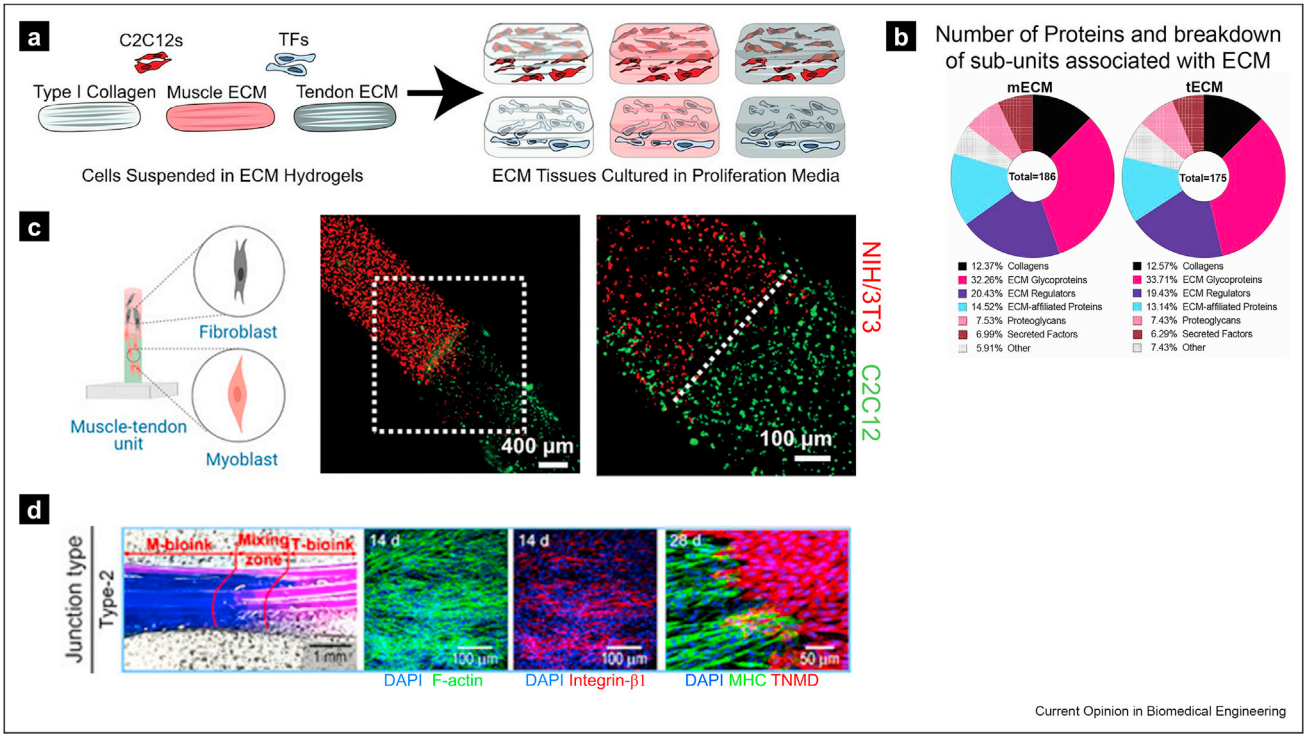
Cellular and structural biomimicry in MTJ engineering

Cell-based scaffolds aim to recreate native tissue environments by promoting cellular adhesion and optimizing matrix deposition and degradation rates. When combined with a scaffold designed to replicate specific tissue structures, cell-laden constructs may enhance repair, provide insights into fundamental interactions, and serve as platforms for investigating disease. In the context of the MTJ, where muscle and tendon tissues intricately interface, cell types such as myoblasts and tenocytes play pivotal roles. Tendon cells contribute to a collagen I matrix, whereas muscle cells form myofibers that exert lateral forces on the tendon matrix, aligning tenocytes and collagen fibrils along the force direction. When cell-based scaffolds can organize these essential cell populations, researchers can begin to understand how these interactions are interrupted during injury and disease, thus, improving our understanding of cell material interactions, cell phenotypes, and neotissue formation within the MTJ. Creating cellular, structurally mimetic constructs holds the potential to unveil complex tissue interactions at interfacial tissues, exemplified by current cell-seeded, biomimetic MTJ constructs, with recent work highlighted in Figure 2.

Employing hydrogels for 3D cell-culture constructs

Polymeric hydrogels can be formulated as injectable, 3D printable, and controllable materials that provide a suitable environment for cellular encapsulation, promoting the incorporation of relevant cell types involved

Figure 2



Schematics adapted from ** papers highlighting recapitulation of MTJ microarchitecture (**a**) Cellularized aligned hydrogel formation in decellularized muscle extracellular matrix (mECM) and tendon extracellular matrix (tECM) [1] utilizes the native microenvironment to support relevant tissue growth. (**b**) Proteomics analysis of mECM and tECM [1] match the proteomics of native tissue supporting the author's use of decellularized hydrogels as a successful construct for MTJ rehabilitation. (**c**) Cryo-bioprinted cell-laden hydrogel demonstrating distinct aligned muscle, tendon, and MTJ regions [4]. Fluorescence imaging shows regional distribution and overlap of relevant muscle and tendon cells throughout the aligned cryo-bioprinted structure [4] confirming the precise control of microstructure and cellular orientation. (**d**) Images of a three-dimensional printed muscle-bioink and tendon-bioink structure with contact in the mixing zone during printing [7]. Interdigitation of the muscle- and tendon-bioink at day 28 [7] mimics the microarchitecture of native MTJ. Cell growth over 14 and 28 days shows the potential long-term applications of this construct not usually found in traditional bioprinting. Abbreviation: MTJ = myotendinous junction.

in MTJ regeneration. 3D printable hydrogels enable the creation of patient-specific structures, enhance tissue integration, and support regeneration. Hydrogel matrices can be engineered to deliver controlled release of bioactive molecules, including growth factors and cytokines, which support tissue remodeling; however, hydrogels as a whole exhibit poor load-bearing capabilities and would not be directly used as a therapy for MTJ rupture.

Despite not being widely used for MTJ regeneration due to limited mechanical properties, they are often used to study fundamental cell—cell and cell—matrix interactions [87,96,97]. Early investigations, such as those by Swasdison and Mayne in 1991, used collagen type I hydrogels to study MTJ development mechanisms, observing spontaneously contractile muscle fibers surrounded by continuous basal lamina, yet the direct insertion of collagen fibers into muscle fibers remained unconfirmed [98]. Recent efforts by Gaffney et al. utilized porcine-derived muscle and tendon ECM-based hydrogels to explore interface-specific interactions [1]. The utility of the ECM-derived hydrogels allows for relevant microenvironments to be retained and the mechanical property modulation of specific tissue regions. The different hydrogel and cell types evaluated (Figure 2a) allowed for proteomic evaluation of the tissue constructs. Proteomic analysis (Figure 2b) identified similar protein expression as previously mentioned for native tissue (Figure 1) [1,2], but also a few proteins with lower levels of expression possibly caused by the decellularization process [1]. Over a five-day culture period, tendon-ECM (tECM) hydrogels led to the highest paxillin expression in C2C12 cells, with myoblasts and tendon fibroblasts also showing elevated paxillin expression compared to controls [1]. Collagen type XXII was present in tECM but not in muscle-ECM, and tendon fibroblasts displayed significantly higher collagen XXII staining in tECM hydrogels than in controls [1]. As previously mentioned, collagen XXII is an MTJ-specific protein implicated in force generation and transmission [31]. Though the mechanisms distinguishing collagen XXII expression in tECM hydrogels compared to muscle-ECM hydrogels remain unclear, the increased expression of this MTJ-specific protein highlights the therapeutic potential of these scaffolds to mimic the microenvironment of native MTJ. These hydrogel-based studies shed light on MTJ-related protein expression and interactions, despite the limitations of hydrogels in MTJ regeneration.

Cell-based hydrogel scaffolds are created to counteract the limited regenerative capabilities and concerns regarding scar formation, impaired mechanics, and infiltration of fatty tissue during MTJ reconstruction [99]. Notably, Luo et al. demonstrated the creation of a cell-laden hydrogel scaffold with anisotropic, interconnected microchannels using a cryo-bioprinting technique [4]. These microchannels have the potential to enhance nutrient diffusion and replicate the MTJ interface. Additionally, the method offers precise printing control and cryogenic preservation, ensuring architectural accuracy and scaffold stability [4]. Luo et al. successfully created a construct that mimics the MTJ structure, as shown by their incorporation of myoblasts and fibroblasts and use of alignment [4]. The authors applied precise structural control and demonstrated a multiregion construct for the muscle, tendon, and overlapping MTJ regions (Figure 2c, [4]) similar to the regional distributions of proteins described in Figure 1. Despite the success of these *in vitro* experiments, it is crucial to acknowledge the existence of challenges and possible limitations. These include the absence of examination regarding cell differentiation impact, which could potentially affect overall cell function, limited scalability, and demands for technical expertise. Further research through *in vivo* studies is essential to advance cryo-printing technology.

Bioprinting approaches in MTJ tissue engineering

Bioprinting offers a promising avenue for MTJ reconstruction, enabling the study of interfacing tissue components such as inflammation and force dispersion. Merceron et al. created a 3D bioprinted construct comprising muscle, tendon, and an interface region using a 3D computer-aided design and four extrusion materials containing myoblasts and fibroblasts [100]. The scaffold consisted of three regions: a muscle region with an elastic modulus of 0.39 ± 0.05 MPa, a tendon region with an elastic modulus of 46.67 ± 2.67 MPa, and an interface region with an elastic modulus of 1.03 ± 0.14 MPa. Cell viability studies on day 7 revealed 94% viability for myoblasts and 83.8% for fibroblasts, with the development of aligned myotubes on the muscle side and successful ECM generation by fibroblasts on the tendon side [100]. Although promising for a reparative, biomimetic construct, further research is needed to investigate collagen deposition and focal adhesions, which anchor the intracellular cytoskeleton to the ECM, in the MTJ. Focal adhesions at the MTJ, which are commonly associated with integrin signaling pathways, are responsible for cell—matrix attachment and respond to force transmission throughout the ECM [18,101]. Optimizing 3D printing parameters to closely resemble native tissue may improve cell—cell and cell—matrix interactions and improve adhesion in the intermediate tissue gradient. These scaffolds, while not equivalent to native MTJ, can replicate desired interactions at interfacing tissues, including injury response, inflammation, and regionalized force loading.

Similarly, Laternser et al. developed an automated bioprinting technique for muscle and tendon tissue models, using a photopolymerized bioink with biocompatibility, precise structure, and scalability [100]. They

used a GelMA-PEGDMA-based ink containing skeletal muscle cells and tenocytes, resulting in bioprinted tissues that displayed proliferation, differentiation, and muscle contractions in response to mechanical stimulation [100]. Histological analysis confirmed muscle and tendon differentiation, with gene expression markers including collagen type I, III, and Tnmd for tendon, and myogenin, MyHC-2, and alpha-actinin 2 (ACTN2) for muscle [100], resembling MTJ-like interactions reported by others [1,18]. Originally designed for drug screening, this bioprinting technique to create cell-laden constructs shows promise in MTJ research, allowing precise spatial deposition of desired micro-architecture and analysis under mechanical load [100].

Bioprinting the MTJ is challenging due to the necessary replication of the physiological environment. Kim et al. addressed the challenge of bioprinting the MTJ by utilizing d-ECMs and human adipose-derived stem cells (hASCs) to create a 3D model comprising muscle, tendon, and interfacing units [7]. Following tissue decellularization, muscle bioink and tendon bioink, derived from decellularized muscle and tendon, respectively, were mixed with hASCs and extruded to form materials [7]. Immunofluorescence staining and reverse-transcription polymerase chain reaction (RT-PCR) analysis showed upregulation of myogenic and tenogenic genes, suggesting successful differentiation. MTJ-associated genes were also observed to be upregulated in the constructs with the most overlap of the muscle and tendon bioinks (Figure 2d) [7]. These results indicate the scaffold's capability to replicate MTJ regions induces hASC differentiation and aids in understanding MTJ development and reconstruction approaches.

Development of advanced 3D constructs for MTJ recapitulation

Three-dimensional tissue-engineered constructs that structurally and biologically mimic the MTJ can serve as both a therapeutic to restore the MTJ tissue and as an *in vitro* model to study the complex interactions at the interface. *In vitro* MTJ models are essential for investigating tissue homeostasis, muscle—tendon crosstalk, and MTJ-related gene expression. To this end, Gaffney, Fisher, and colleagues uncovered the role of mechanotransduction at the MTJ on cellular phenotype [102]. Muscle tissues were engineered in a novel bioreactor to evaluate paxillin and type XXII collagen expression. C2C12 myoblasts were cultured in collagen type I hydrogels or tECM around movable anchors for 10 days, followed by cyclic stimulation over 2 and 4 weeks alongside static cultures. The protein expression of paxillin remained unchanged between hydrogel materials in static cultures but increased by 62% in tECM under mechanical stimulation relative to the collagen hydrogen. Furthermore, type XXII collagen protein expression was present in all tissues after 2 weeks, with greater abundance observed in the mechanically stimulated tissues, which is supported by studies conducted on human MTJ, where type XXII collagen expression upregulated in the MTJ compared to the individual muscle and tendon tissues [26]. Interestingly, the static tECM-cultured group resulted in an increased expression compared to the collagen hydrogel, demonstrating the influence of the substrate-material properties on protein expression [102]. Findings from this study elucidate the cellular crosstalk and protein modulation in the MTJ in physiological loading conditions using a tissue-engineering MTJ model, which is an avenue for potential future work in studying MTJ development.

Schon et al. also developed an MTJ-biomimetic construct to improve MTJ surgical outcomes. The construct was a novel biologically active composite surgical mesh containing a polyglycolic acid scaffold and multipotent stem cells (MSCs) within an alginate hydrogel to facilitate cellular delivery and address the challenge of poor suture retention in muscle during tension-free repair [103]. Utilizing a mesh-embedded hydrogel allows for controlled cellular delivery and enhanced alignment, offering a promising approach for effective MTJ regeneration via soluble and structural cues. The tissue-engineering MTJ was evaluated in a murine Achilles-gastrocnemius/soleus junction injury mode. By day 14, the MSC-loaded surgical mesh displayed tissue alignment and increase collagen type 1 expression, similar to tendinous tissue [103]. Overall, this study demonstrated the potential of the MSC-loaded surgical mesh as an integrated approach for enhancing tendon repair and regeneration in the Achilles-gastrocnemius/soleus junction of murine models.

Engineered constructs have also been evaluated in preclinical models. VanDusen et al. created scaffold-free skeletal muscle units (SMUs) using isolated muscle cells from a rat soleus muscle and tissue-engineered bone anchors from residual bone marrow cells [104]. The resulting SMUs were implanted into murine models for 28 days to assess the therapeutic efficacy following a 30% resection of the tibialis anterior muscle. Although the desired application was for volumetric muscle loss, the group demonstrated a fully integrable therapy capable of muscle regeneration, angiogenesis, and innervation. Laminin was found in high concentrations in the SMU ECM and upregulated paxillin expression, suggesting MTJ development [104]. Another hallmark exhibited by the SMUs was the distinct alignment of muscle fibers [104], which is imperative in regenerating musculoskeletal tissues, and lack of immune response. The therapeutics were unable to replicate the full mechanical profile of native MTJ and muscle; however, VanDusen et al. was able to demonstrate the efficacy of their SMUs with respect to

innervation, blood supply, and overall cellular interactions [104].

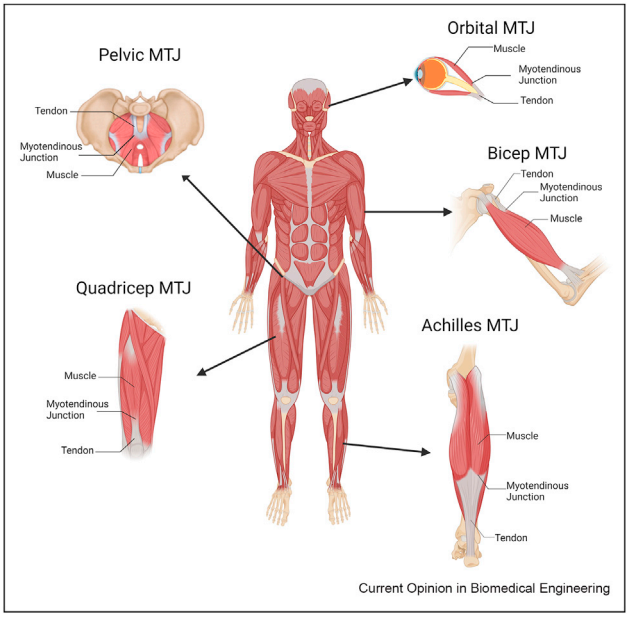
In addition to studying MTJ development or evaluating implantable materials, 3D tissue-engineered constructs are pivotal for restoring MTJ structure and function. Reconnecting muscle and tendon are vital for regenerating the ruptured MTJ, including bridging nerve connections and maintaining sufficient vasculature. Hashimoto et al. sought to focus on these challenges via use of skeletal-muscle-derived multipotent stem cell (Sk-MSC) sheet pellets for MTJ regeneration in rodents with complete tibialis anterior ruptures [105]. Primarily focused on muscle recovery, Sk-MSC sheet pellets containing essential myogenic, neurotrophic, and vascular growth factors ruptures, labeled as "bio-bonds," were overlaid on the ruptured MTJ, and evaluation for short- and long-term biological outcomes [105]. The engrafted cells formed an interconnected network of tendon and muscle fibers. Mechanical bridging of the tendon and muscle was observed through engrafted fibroblast migration ruptures [105]. To further improve the impact of this strategy, efforts could be made to comprehensively address both muscle and tendon components to offer more effective treatments for musculotendinous injuries, given the promising potential of Sk-MSC sheet pellets for MTJ regeneration. While the study by Hashimoto et al. offers significant information about cell-based 3D tissue-engineering MTJ construct, future work should focus on the investigation of the mechanics at the interface, which are crucial for maintaining force transmission at the MTJ. Furthermore, the initial work predominantly concentrates on muscle mass and tetanic tension [106], and future investigations should include functional assessments, such as evaluation of range of motion. Future work in the development of cellularized constructs should also evaluate ECM production and composition, working to understand how ECM composition or ECM secretion changes over time, given the shifts observed following exercise and injury in preclinical and clinical evaluations [36,102] and recent MTJ proteome profiling [2,3].

Future directions in MTJ tissue engineering

Repairing and rehabilitating the MTJ remains a challenge due to the complexity of the tissue structure and function. Understanding the cellular function and multi-tissue interactions, which are influenced by the compositional gradient along the MTJ, is critical in advancing the field of MTJ tissue engineering. This review addresses the knowledge gap by providing detailed information about the ECM composition, microstructure, mechanical properties, and cellular components that contribute to the maintenance, repair, and regeneration of the muscle, tendon, and MTJ. The variations in MTJ structure and function, which are dependent on anatomical location, sex, age, and disease, are also highlighted in this review to inform researchers of the importance in considering these anatomical differences when developing MTJ-specific tissue-engineering approaches (Figure 3). Moreover, the development of *in vitro* MTJ models to study cellular-tissue interactions at the interface and the development of MTJ-like materials to repair and restore the MTJ's structure and function are discussed in this review.

The current MTJ tissue engineering approaches for clinical treatment are centered around mimicking the MTJ microarchitecture and mechanics to provide structural cues for the innate endogenous healing cells and developing cell-based scaffolds to promote early cell–cell interactions upon implantation. Microscale and nanoscale fabrication techniques, such as electrospinning and bioprinting, replicate the intricate transitional MTJ microarchitecture and provide a biomimetic structure to promote cellular attachment, integration, and organization of resident muscle and tendon-healing cells, while decellularized materials serve as biologically derived templates with native ECM proteins to further promote restoration of MTJ structure and mechanics. However, a limitation of these acellular strategies is the absence of the initial cell–cell interactions and subsequent release of growth factors (e.g. IGF-1); both of

Figure 3



Schematic of the myotendinous junction (MTJ) at various locations throughout the body. While recent work has focused on larger muscles in the legs, future considerations should consider other MTJ locations, where expression of key proteins and overall load bearing are different from the MTJs found in the limbs. Created with BioRender.com and licensed Adobe® Stock Photos.

which are critical for MTJ development and homeostasis, healing cascade initiation, and tissue integration. Combining these materials with cellularized components, such as cell-laden hydrogels, would promote early repair via exogenous and endogenous cell communication and provide structural and molecular cues for tissue restoration and improved function.

As this field continues to move forward, evaluating the long-term biological response and functional outcomes of tissue-engineering MTJ constructs is limited by the lack of validated preclinical MTJ injury models. Studies by Perucca Orfei et al. [107] that laid the groundwork for recent efforts by Yamamoto et al. [108] to use collagenase to develop an *in vivo* MTJ injury model in mice are the first steps in this direction. Yamamoto et al. made significant contributions to our understanding of MTJ injuries and the repair process through histological and morphological evaluation in mice. However, in the model development and validation, Yamamoto et al. did not evaluate or address functional outcomes. Understanding how these morphological changes translate into functional recovery is crucial for developing and validating future rehabilitation strategies within a collagenase-based injury model. It is necessary to understand if a regenerative therapy can move toward restoration of the intricate muscle to tendon load transfer and movement. Additionally, variability of the injury and therapeutic response due to species-specific healing mechanisms, degree and location injury, and selection of evaluation timepoints warrants the need for the validation and standardization of a preclinical MTJ injury model to evaluate and compare tissue-engineered MTJ therapies. More specifically, longitudinal studies with emphasis on the postinjury cascade to probe the dynamic cellular interactions and structural composition throughout healing will provide insights for interventions to target specific stages of the reparative and rehabilitation process.

Future work should investigate engineering MTJs for applications beyond the traditional areas of interest within the upper and lower extremities [109]. While these areas are prone to sports-related injury, many patients suffer from complications at the muscle−tendon interface due to non−sports related activities, such as genetic disease or complications related to childbirth (Figure 3). For example, engineered MTJ *in vitro* models can contribute significantly to understanding and treating women's health conditions, particularly related to childbirth and pelvic floor injuries [110−114]. Building engineered tissue models of pelvic floor MTJs or MTJs in cervical tissues could be leveraged to understand the impact of mechanical loading as a simulate for exercise or physical therapy, on the repair of the tissue following childbirth, other traumatic injuries, or fluctuations in hormone levels from menopause [68], endometriosis, or polycystic ovary syndrome. Alternatively, development of healthy and dystrophic MTJ *in vitro* models [40] could aid in understanding the cascade of symptoms and muscle weakness in patients with muscular dystrophy. Furthermore, MTJs in the jaw region, connecting facial muscles to the temporomandibular joint, and ocular MTJs attaching extraocular muscles to the sclera [39,113−117], are other essential areas where overuse and strain often lead to injury and reduced quality of life for patients. Engineered MTJ models can replicate overuse or injury of these tissues and aid in understanding the relationship between the mechanical load and pathophysiology. Furthermore, an *in vitro* MTJ model can be utilized to evaluate and validate treatments, thus advancing the craniofacial and ocular tissue engineering fields. In conclusion, the future of MTJ tissue engineering, whether to repair and rehabilitate or model the complex interactions at the interface, is promising with broad biomedical implications.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Brittany Taylor reports equipment, drugs, or supplies and travel were provided by Burroughs Wellcome Fund. Mariah Turner reports financial support was provided by Burroughs Wellcome Fund.

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* of special interest
** of outstanding interest

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This study investigates muscle and tendon injuries using three-dimensional tissue models containing tissue-specific extracellular matrices (ECM) to understand their interactions during injury and recovery. By using ECM materials from Achilles tendon and gastrocnemius muscle, unique tissue-specific hydrogels were created, affecting cell behavior, and significantly increasing the expression of the muscle-tendon junction marker, paxillin, in cells cultured within them. This research emphasizes the need to analyze cell-matrix interactions in

muscle-tendon junctions and offers insights for future multi-tissue engineering methods for studying the muscle-tendon unit.

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The authors examine how the MTJ forms and organizes and identifies muscle and tendon specific extracellular matrix (ECM) components with the goal of designing effective regenerative therapies to restore functionality to damaged muscle–tendon units. This work uncovers the spatial distribution of ECM proteins in the muscle, tendon, and MTJ through comparative analysis of the ECM proteome.

3. * Karlsen A, Gonzalez-Franquesa A, Jakobsen JR, Krogsgaard MR, Koch M, Kjaer M, Schiaffino S, Mackey AL, Deshmukh AS: **The proteomic profile of the human myotendinous junction**. *iScience* 2022, **25**:103836. 103836.
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