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Extracellular Matrix at the Muscle – Tendon Interface: Functional Roles, Techniques to Explore and Implications for Regenerative Medicine

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Abstract

The muscle-tendon interface is an anatomically specialized region that is involved in the efficient transmission of force from muscle to tendon. Due to constant exposure to loading, the interface is susceptible to injury. Current treatment methods do not meet the socioeconomic demands of reduced recovery time without compromising the risk of reinjury, requiring the need for developing alternative strategies. The extracellular matrix (ECM) present in muscle, tendon and at the interface of these tissues consists of unique molecules that play significant roles in homeostasis and repair. Better, understanding the function of the ECM during development, injury and aging has the potential to unearth critical missing information that is essential for accelerating the repair at the muscle-tendon interface. Recently, advanced techniques have emerged to explore the ECM for identifying specific roles in musculoskeletal biology. Simultaneously, there is a tremendous increase in the scope for regenerative medicine strategies to address the current clinical deficiencies. Advancements in ECM research can be coupled with the latest regenerative medicine techniques to develop next generation therapies that harness ECM for treating defects at the muscle-tendon interface. The current work provides a comprehensive review on the role of muscle and tendon ECM to provide insights about the role of ECM in the muscle-tendon interface, and discusses the latest research techniques to explore the ECM to gathered information for developing regenerative medicine strategies.

Keywords

extracellular matrix; muscle-tendon interface; clinical significance; mass spectrometry; protein labeling; ECM visualization; regenerative medicine

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Author Contributions

All authors contributed to the conception and design of the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

Introduction

The musculoskeletal system, which is comprised of bone, connective tissue and skeletal muscle, provides the body with structure and facilitates movement. The central nervous system triggers signals via motor neurons to muscle, which results in a transmission of force from contracting muscle fibers through tendon to bone, enabling motion [1]. The force is transmitted from muscle to tendon across an anatomically specialized interface called the myotendinous junction (MTJ) [2]. Due to constant exposure of force during physical activity, the MTJ is susceptible to injury. Eccentric stretching of the muscle beyond the normal range of motion can result in injury, and the severity depends on the extent of force experienced at the muscle-tendon interface. Traditional treatment methods for acute injuries include PRICE (protection, rest, ice, compression and elevation), NSAID (non-steroidal anti-inflammatory drugs) administration and physical therapy, which assist in the repair and recovery process. However, the persistent socioeconomic burden these injuries present necessitates a need for alternate treatment options that reduce recovery time without comprising the risk of injury recurrence. Moreover, severe injuries resulting in a complete tear at the MTJ lack standardized clinical treatment methods.

Understanding the assembly of MTJ during development, repair and aging has the potential to provide essential information for the development of new strategies that address injuries at the muscle-tendon interface. Structurally, the MTJ consists of the subsarcolemma of the muscle cell membrane and transmembrane, intercellular and extracellular proteins that connect myofibers to the collagen-rich tendon matrix [3]. The overall force transmission at the MTJ is dependent on the relationship between the structural units present in muscle and tendon [3]. Challenges in the clinical translation of engineering technologies targeted towards the muscle-tendon interface arise due to the difficulty in integrating two disparate tissues to form a seamless MTJ [4]. The extracellular matrix (ECM), representing the three-dimensional (3D) network of molecules within the tissue, forms a continuum that integrates muscle and tendon. Developmental studies identified that some ECM components play a role in cell migration, tissue formation, alignment and guidance at the MTJ [5]; however, there is limited knowledge regarding the role and overall composition of the ECM at the MTJ either during morphogenesis or in adults during repair and aging. Understanding the role and composition of the ECM present in muscle and tendon can provide information regarding the ECM at the muscle-tendon interface. Skeletal muscle ECM provides chemical, physical and biological cues that modulate tissue homeostasis [6], tissue remodeling [7], and cell-ECM interactions that regulate myoblast migration[8], alignment[9], proliferation[10] and differentiation[11]. Similarly, tendon ECM plays a role in adaptation to mechanical loading [12], modulation of the tendon stem cell niche [13], and cell-ECM interactions leading to tenocyte migration[14], proliferation[14] and differentiation[13]. Therefore, better understanding the roles of the ECM that integrates muscle and tendon at the MTJ can unearth important information that is beneficial for developing novel regenerative medicine strategies. This review focuses on describing the existing knowledge on the structure of the MTJ, clinical relevance to injuries at the MTJ, and the role and distribution of ECM in the muscle and tendon during development, homeostasis, repair and aging to provide insights about the ECM at the muscle-tendon interface. Finally, the review highlights the latest techniques employed for exploring ECM

composition and dynamics that have the potential to deliver guidance for developing novel regenerative therapies.

The Myotendinous Junction

The MTJ is a highly specialized and architecturally complex structure, where the sarcolemma of the muscle meets the ECM of the tendon (Figure 1). At the junction, muscle fibers break into subdivisions of several sarcomeres by the inside-out folding of the sarcolemma. Myofilaments of these subdivisions insert into the plasma membrane at the subsarcolemmal layer [15]. Structurally, the MTJ comprises of actin microfilaments and actin binding proteins that extend from the last z-band to the sarcolemma, the transmembrane proteins that connect the cytoskeletal components to the basement membrane, and the proteins that link the basement membrane with the tendon ECM [16]. The finger-like processes at the MTJ are critical in force transmission and are hypothesized to reduce stress at the interface by increasing the surface area by 10–20 fold [15]. In addition, the angle of incident stress is lowered in such a way that shear stress is experienced, which leads to force transmission parallel to the basement membrane, improving the strength at the MTJ and preventing failure [17,18].

MTJ Assembly During Development—MTJ formation begins during embryogenesis and continues to develop and mature through the neonatal period (Figure 1). During embryogenesis, tendon and muscle progenitors coexist together at the site of the future MTJ [19]. Then, tendon progenitors and ECM components are thought to align along the direction of the contractile force experienced during muscle development [19]. Cross-talk between muscle and tendon progenitors, via cell-cell interactions, endocrine signaling and paracrine signaling, activate key signaling pathways that are critical for the proper maturation and functioning of the MTJ. For example, the neuroregulatory ligand vein is secreted by muscle progenitors in *Drosophila*, and activates the EGF signaling pathway in tendon progenitors, which in turn determines the fate of tendon cell differentiation [20]. Similarly, FGF4 signaling from muscle progenitors induces expression of scleraxis and tenascin-C in tendon progenitors, which modulates tendon maturation [21]. In addition, retinoic acid signaling between these two populations was previously established to play an important role in muscle cell apoptosis and myotendinous assembly [22]. Several other proteins (*e.g.* Moleskin, slowdown, thrombospondin-4) and microRNAs (*e.g.* miR-9a) are also thought to be involved in the cross talk between muscle and tendon progenitors to regulate proper assembly of the MTJ during development [23–26]. Further details on the role of cell communication during the assembly and maturation of the MTJ is reviewed elsewhere [3, 5].

Interactions between muscle and tendon progenitors are not limited to cellular and molecular crosstalk during MTJ assembly. Several studies indicate that the assembly of the MTJ is also mediated by factors autonomous from the muscle and tendon progenitors. For instance, when the transcription factor Pax3 is knocked out, the embryos develop muscle-less limbs, but with appropriate tendon formation [27]. Similarly, when the transcription factor Scleraxis is knocked out, disrupting tendon differentiation, condensation of skeletal muscles still occurs in the correct locations [28]. These studies indicate that there could be factors such as

other cell populations and/or the ECM that direct the tendon and muscle progenitors during the assembly of the MTJ.

Adaptation of MTJ to Mechanical Loading—The MTJ adapts to changes in homeostasis such as in response to exercise and aging. Previous studies demonstrated that eccentric strength training, aerobics and running can shift the position of the MTJ and change the angle of both the finger-like processes and muscle fascicles, promoting increased force transmission and muscle hypertrophy [29]. In addition, a recent study showed that a fast running training regime upregulated the expression of TGF-β and type III betaglycan receptor genes in tendon proximal to the interface, which are correlated with an increase in type I collagen deposition, suggesting the ECM in the MTJ is adapting to the increase in loading [30]. On the contrary, studies on the aging MTJ revealed shortening and atypical sarcolemma invagination and alterations in collagen fiber arrangement and deposition, leading to conditions similar to muscle atrophy [31], indicating a compromise in force transmission and stabilization due to muscle inactivity [31]. Moreover, prolonged bed rest led to muscle atrophy and significant decrease in muscle-tendon stiffness [32]. These studies suggest that exercise can induce changes in the ultrastructure of the MTJ and is hypothesized to be a countermeasure for muscle atrophy. Understanding the communication between muscle and tendon when there is an injury at the MTJ could help identify factors that influence biomechanics post repair. Additionally, comprehending the changes in muscle and tendon biomechanics post MTJ injury could help in prognosis. Several studies indicate that muscle and tendon homeostasis is altered when there is an injury in either of the tissues. In a recent study, significant changes in tendon ECM was demonstrated in response to injury in the gastrocnemius muscle indicating that muscle and tendon affect each other due to change in tissue mechanics [33]. Conversely, a clinical study provided evidence for changes in the gastrocnemius muscle post Achilles tendon injury [34].

Together, these studies indicate that muscle and tendon communicate with each other bidirectionally with functional consequences. Therefore, exploring the changes in the ECM at the interface between muscle and tendon during development and post injury at the MTJ could aid in advancing clinical prognosis.

Clinical Challenges for Repairing Injuries at the Muscle-Tendon Interface

MTJ injuries can arise from diverse events, including daily activities, physical work, sports and trauma. These typically occur due to excessive eccentric force that results in muscle strains [35]. The strain localizes to the tapering fibers of the interface leading to microscopic and macroscopic damage at the MTJ [36] (Figure 1). Notably, eccentric forces greater than 20% of the average maximum isometric force are sufficient to induce a rupture at the muscle-tendon interface, which is why this type of injury predominantly occurs during physical activities [37]. This presents a significant challenge for sports medicine, where one-third of all sports-related injuries are due to muscle strains [38]. In modern day economics, sport is a profession and is recognized as an industry [39]; therefore, MTJ injuries preventing an athlete from training and competing impose a considerable economic and psychological influence on the athlete and the team [40]. In addition, aging presents a tremendous impact as studies demonstrate that the recovery time for muscle injuries are significantly increased

with advanced age [41], and the economic burden is skewed towards the aging population [42]. A recent report indicated that by 2050, 25% of the population will be 65 years or older [43], suggesting that demographic shifts will challenge the health care infrastructure. Taken together, muscle strain injuries, and the effect on the MTJ, will continue to be a significant contributor to the long term functional deficit, pain and economic burden on the world scale [44, 45].

Unfortunately, there is no definitive time frame for treating muscle strain injuries as scientific reports are contradictory. In general, the PRICE protocol is adopted immediately after injury to reduce the bleeding at the injury site [46]. As far as medication is concerned, NSAIDs and glucocorticoids are prescribed to aid in the initial phase of the recovery process. However, recent studies demonstrated that anti-inflammatory drugs hinder muscle regeneration and may pose detrimental effects [47]. After the initial treatment phase, isometric, isotonic and isokinetic training exercises are advised to regain the range of motion and functionally condition the injured muscle [48]. Surgical interventions are very rare for muscle strain injuries [49]. Currently, there are no standard clinical surgical intervention methods for a complete tear at the MTJ, and suturing techniques are typically employed to treat these occurrences in the clinic [50].

Regenerative Medicine Strategies for the Muscle-Tendon Interface

Regenerative medicine is an interdisciplinary strategy that focuses on repair, replacement and regeneration of cells and tissues to reverse the effects of genetic defects, injury and aging and restore functionality [51]. Complete rupture of the muscle-tendon interface is an uncommon occurrence; yet there are instances of clinically reported cases for a complete rupture [49, 50]. Unfortunately, surgical suturing methods are currently the only available clinical options. Notably, the efficacy of these studies is still questionable as there are no long-term data available to provide conclusive evidence that they work. Scaffold-based regenerative medicine strategies offer an alternative to address the current limitations in circumstances of a complete rupture at the muscle-tendon interface. Engineered scaffolds (acellular and synthetic) have the potential to be tailored based on the tissue type and are successfully used clinically for bone [52] and muscle [53]. However, there are no clinically available scaffolds for treating injuries at the muscle-tendon interface. This can be attributed to the complexity of the physical, chemical and biological properties at the interface. Understanding of the mechanical properties at the muscle-tendon interface is inadequate and is limited to mathematical models predicting the strain [36]. Moreover, it is challenging to design and develop scaffolds that have properties tailored to satisfy three distinct regions; muscle, tendon and the MTJ. To this end, electrospinning was employed to design a scaffold with three distinct materials within a continuous system and the results demonstrated that the designed scaffold had properties mimicking the native muscle-tendon interface [54]. Similarly, a decellularized muscle-tendon interface ECM scaffold was evaluated for exhibiting biphasic structural and mechanical properties to mimic the natural MTJ microenvironment [55]. Both the studies provide critical information about the rational design of scaffold-based regenerative medicine strategies to restore the muscle-tendon interface. However, the current lack of a successful intervention indicates that the appropriate cues are not being incorporated. Taken together, there are limited regenerative strategies addressing the muscle-

tendon interface and there is an immediate need to improve the current available methods for treating MTJ injuries.

From the current literature, it is evident that injuries at the muscle-tendon interface impose physical, mental and financial burdens on society, apart from posing a challenge for the medical community. There is space for improvement in injury prevention and repair/recovery strategies, which open up multiple research avenues. Limitations in treatment options are due to the difference in the tissue properties between muscle and tendon at the interface. The ECM presents a unique niche of molecules that form a continuous material that bridges the MTJ and plays a critical role during development, homeostasis, repair and aging. Therefore, understanding the role and composition of the ECM at the MTJ has the potential to provide critical information that can be used to develop biomimetic constructs that accelerate repair at the muscle-tendon interface.

Role of ECM at the Muscle-Tendon Interface

The ECM is a complex, molecular network that is present in all tissues and organs. There are three major molecular classes of ECM: glycosaminoglycans (GAGs) such as hyaluronic acid (HA) and chondroitin sulfate; proteoglycans (PGs) such as perlecan and decorin, and fibrillar/network forming proteins such as collagens, elastin, fibronectin and laminins [56, 57]. These components act as scaffolds on which cells reside, and provide physical, chemical and biological cues for modulating cell behavior, migration, proliferation, differentiation, growth and homeostasis [58]. Importantly, the ECM plays a pivotal role in regulating development, repair, aging and disease progression [59].

In the adult muscle-tendon unit, type XXII collagen is thought to be restricted to the MTJ [60], and play a role in strengthening the MTJ by interacting with the muscle basal lamina via integrin α7β1 [61]. Knockdown of *col22a1* in the zebrafish caused a reduction in the MTJ folds and resulted in contraction-induced muscle fiber detachment, leading to muscle weakness and a muscular dystrophy-like phenotype [61]. A study that used laser-capture microdissection to isolate the murine MTJ from cryosections found several other ECM proteins such as collagens, biglycan, fibromodulin, prolargin and laminin were located in the MTJ [62]. While their specific roles in the MTJ have yet to be determined, understanding the roles of these ECM molecules in muscle and tendon can potentially inform about their roles in the muscle-tendon interface (Figure 1). Several fibril-forming collagens are present in skeletal muscle, and are oriented both along the axis of muscle contraction and surrounding the muscle fibers [63]. Collagen fibrils provide mechanical stability to skeletal muscle and regulate cell adhesion and differentiation [64]. The majority of the tendon ECM is comprised of fibril-forming collagens (~60–85% of dry weight) [65], which are organized into different hierarchical levels aligned along the axis of mechanical loading to provide structural integrity [66]. Assembly of collagen fibrils is modulated by small leucine rich proteoglycans (SLRPs), such as biglycan, fibromodulin and decorin, which also regulate cellular functions [67]. Biglycan modulates myofiber anchorage to the surrounding ECM by binding with dystrophin-glycoprotein complex. Moreover, biglycan regulates utrophin expression to compensate for the disruption of dystrophin protein complex in Duchene muscular dystrophy [68]. In tendon, biglycan is essential for maintaining collagen fibril structure,

realignment and mechanical properties in the adult [69]. Fibromodulin modulates myogenesis of skeletal muscle by inhibiting the activity of myostatin, a negative regulator for muscle growth [70]. In tendon, fibromodulin binds to collagen fibrils and upregulates fibrillogenesis by mediating the stabilization and growth of fibrils. During collagen fibril maturation, fibromodulin displaces another SLRP, lumican, and facilitates fusion of individual collagen fibrils; thus, increasing the fibril diameter [71]. Prolargin functions as a basement membrane anchor by interacting with perlecan present in the basement membrane and type I collagen in the interstitial connective tissue [72]. In tendon, prolargin was reported to be involved in collagen fibrillogenesis [73]; however, the role has yet to be established. Laminin-211 is predominantly observed in the basement membrane of skeletal muscle and its interaction with α-dystroglycan is essential for maintaining the structural integrity and force transmission [74]. Along with other ECM proteins such as nidogens, perlecan, agrin and type IV collagen, laminin-211 forms the niche for muscle specific stem cells called satellite cells in the basal lamina, a layer of basement membrane adjacent to the cell surface [75]. Similar to satellite cells, tendon progenitor cells reside within a unique ECM niche, comprised of biglycan and fibromodulin, that regulates their function [13]. A basement membrane consisting of laminin, nidogen-1, perlecan and type IV collagen is also found surrounding the flexor tendon and is essential for preventing tendon adhesions between tendon and synovial sheath [76]. Therefore, enrichment of laminin at the muscle-tendon interface suggests that an ECM niche that supports satellite cells and tendon-specific stem cell could be present. This would provide a local source of cells that can immediately contribute to repair at the interface. Indeed, prior reports demonstrated that satellite cells were localized at the remodeling muscle-tendon interface in individuals undergoing heavy resistance training [77].

Besides the ECM identified at the MTJ, there are many other matrix molecules that play important roles in muscle and tendon. Several GAGs such as HA and heparan sulfate are present in the interstitial matrix of skeletal muscle. HA helps facilitate tissue hydration and regulates the viscoelasticity of the tissue [78]. In addition, HA levels were found to be significantly increased during development and hypertrophy, suggesting potential roles in modulating myogenesis [79, 80]. Heparan sulfate binding PGs are also involved in regulating several key processes during myogenesis. For instance, glypican-1 binds with fibroblast growth factor 2 (FGF-2), a negative regulator for myoblast proliferation and differentiation [81], and sequesters FGF-2 from its receptors. Glypican-1 is also involved in hepatocyte growth factor mediated signaling, which is essential for myoblast migration [8]. Furthermore, skeletal muscle ECM consists of various other PGs such as decorin, and lumican that interact with the collagens to maintain the ECM structural organization [63]. In addition, these PGs play other important roles in skeletal muscle homeostasis. For example, decorin binds transforming growth factor beta (TGFβ), an inhibitor for myogenesis [82], sequestering its bioavailability [83]. Decorin also plays a role in maintaining collagen fibril structure realignment in the direction of load and mechanical properties in the mature tendon [69]. Furthermore, decorin is differentially upregulated during exercise training; thus, modulating collagen assembly and leading to increased tendon strength [84]. Additionally, glycoproteins (tenascin-C, lubricin and tenomodulin) and elastic fibers (elastin, fibrillin-1 and −2) are also present within the tendon ECM [66] and play key roles. Lubricin plays an important role in tendon gliding, a process that dictates motion, and knockout of lubricin significantly

increases tendon gliding resistance [85]. Tenomodulin localizes to collagen type I fibrils and promotes collagen assembly during adaptation [86]. Elastic fibers are widely distributed within the tendon ECM and provide elastic recoil and resilience [87]. Further, they are part of the pericellular matrix surrounding the tenocytes and promotes their cell attachment by forming an intermediate link by binding to integrin α5β3 and type VI collagen [88].

Understanding the roles of the ECM at the muscle-tendon interface during various biological processes is essential in providing information for developing regenerative medicine strategies based on the type of injury repair. The following sub-sections detail the roles played by the ECM during development, repair, and aging.

ECM in the Developing MTJ—The ECM is secreted by cells from the early stages of embryogenesis. It is thought that ECM deposition and assembly in the embryonic spaces provide structural integrity and binding cues, as well as modulate cell migration [89]. Studies in developing zebrafish showed that fibronectin and laminin α2 are involved in the proper attachment of muscle fibers to the intersegmental boundaries (ISB), an anatomical location where the myoblasts and tenocytes condense to form the MTJ [90]. A recent study demonstrated that laminin-111 and fibronectin are reciprocally expressed by somites during the development of MTJ in the zebrafish [91]. Fibronectin is involved in the early assembly of somites during development. After somite formation, basement membrane consisting of laminin-111, type IV collagen, nidogen-1, nidogen-2 and perlecan is deposited [92]. Laminin-111 polymerization initiates signaling cascades that promote localization of matrix metalloprotease 11 (MMP11), which in turn downregulates fibronectin; thereby, leading to a laminin-rich basement membrane at the MTJ during development [91]. Similarly, thrombospondin-4 plays a role in the assembly of ECM at the MTJ and promotes muscle attachment to the ISB during zebrafish development [25]. As the MTJ matures, connective tissue cells secrete PGs and collagen that assemble into collagen fibrils, which are essential for providing structural stability to the MTJ and the tendon [5]. ECM proteins such as tenascin-C [93] and decorin [94] are involved in the maturation of the collagen fibrils, which dictate the mechanical properties of the muscle-tendon interface.

Further understanding on the role of ECM during the MTJ formation and maturation has the potential to provide crucial information to develop regenerative strategies that aid in the recovery from injuries that occur in the muscle tendon interface. For example, investigations into the basic biology of the ECM during development led to the generation of novel regenerative strategies for cardiac [95, 96] and neural [97–99] engineering. Fibronectin was shown to promoted cardiac precursor cell migration during development [100]. Using this information, scaffolds functionalized with fibronectin were constructed and shown to improve migration of cardiomyocytes to improve cardiac function *in vivo* [95]. Similarly, tenascin-C and laminin promote neural crest cell migration during development [101, 102]. Hydrogels that incorporated tenascin-C- and laminin-111 were designed and shown to facilitate migration of neuroblasts [103] and improve survival of neural stem cells [99], respectively, within the mouse brain.

ECM Changes in the Injured MTJ—Injury at the MTJ is characterized by damage to the ECM surrounding the muscle fibers at the interface with tendon, and the muscle fibers

partially separate from the surrounding matrix [104]. However, in extreme cases, muscle fibers are completely detached from the surrounding matrix and the tendon aponeurosis, creating a complete rupture of the muscle-tendon interface. Throughout the repair process, the ECM in the muscle and tendon undergoes characteristic changes to regulate different stages. After injury, the basal lamina enclosing the muscle fibers gets damaged and disintegrates. Then, proteases, including MMPs, digest type IV collagen and laminin-211 in the basal lamina, releasing fragments that play a role in modulating inflammatory cell migration [75]. Studies indicate that MMP9 induced by the inflammatory response leads to activation of satellite cells [105], due to the breakdown of their ECM niche. Several reports demonstrate that satellite cells secrete MMP2 and MMP9 to breakdown type IV collagen and laminin-211 present in the basal lamina [106]. Furthermore, migrating satellite cells express MMP13, which is involved in the degradation of collagens and fibronectin, to facilitate their migration [107]. Then, the satellite cells undergo asymmetric cell division, which is regulated by many factors including fibronectin, into myogenic precursor cells that trigger the muscle repair process, and daughter satellite cells that maintain the satellite cell pool [108]. Simultaneously, new ECM is deposited, predominantly by fibroblasts, to provide instructive cues for the newly regenerating muscle fibers. However, the deposited ECM must be remodeled during the repair to prevent fibrotic tissue formation, which restricts overall regeneration [109]. Fibrotic tissue presents a mechanical barrier and reduces the extensibility of the tissue, which renders the tissue susceptible for reinjury [110]. Vascular endothelial growth factor (VEGF) was previously demonstrated to reduce fibrosis in acute skeletal muscle injuries [111]. However, the factors responsible for VEGF-induced angiogenesis and reduced scar tissue formation in damaged skeletal muscle still remain elusive [112]. In the later stages of repair, heparan sulfate PGs play a role in regulating the differentiation of the myogenic precursor cells and fiber formation [113, 114]. Interestingly, tenascin-C, which is not typically expressed in uninjured adult skeletal muscle tissue was shown to be highly expressed in response to compensatory overloading in the plantaris muscle [79]. Furthermore, tenascin-C rich ECM deposition coincided with new myofiber formation during newt forelimb regeneration post amputation, indicating a potential role for tenascin-C in *de novo* muscle regeneration by facilitating myoblast migration and proliferation [115].

Similar to skeletal muscle, the ECM plays important roles during repair in tendon. Post injury, there is a significant increase in type III collagen deposition by the fibroblasts that migrated to the injury site [116]. Additionally, several SLRPs (*e.g.* decorin, biglycan) that play a role in collagen fiber organization are secreted during the proliferation stage of repair [117]. The final stage of repair is characterized by ECM remodeling and is subdivided into two stages. In the first phase, there is a decrease in ECM secretion and there is a gradual replacement of type III collagen by type I collagen, which aligns along the tendon axis of mechanical loading. ECM remodeling in tendon is orchestrated by MMPs (particularly MMP3), which mediate degradation of collagens deposited in the early stages of repair [118]. During the second phase of ECM remodeling, the collagen fibrils begin to cross-link; thereby, providing maturity to the recovering tendon [116].

Similar to development, cues from remodeling post-injury were used to develop scaffolds that elicited favorable tissue remodeling. For example, a recent study demonstrated that an acellular biological scaffold derived from porcine urinary bladder epithelial basement

membrane (MatriStem UMB™, Acell Inc.) facilitated constructive tissue remodeling by promoting migration of satellite cells at the site of injury when treated for volumetric muscle loss in a preclinical trial [119]. Similarly, an acellular dermal matrix scaffold containing skin epithelial basement membrane generated a pro-regenerative microenvironment by favoring M2 macrophage cells that are responsible for secreting MMPs to remodel the ECM during a full thickness cutaneous wound healing [120]. Therefore, future studies focusing on the ECM remodeling post injury at the muscle-tendon interface have the potential to contribute in developing regenerative medicine strategies.

Imaging techniques such as MRI reveal the location of the injury and help to distinguish if the injury is within the free tendon or at the ECM present in the muscle-tendon junction [104]. Depending on the location of the injury (tendon, MTJ or muscle), the architecture of the underlying ECM varies, which in turn determines the nature of the tissue repair and occurrence of reinjury. In addition to aiding diagnosis, these imaging techniques are also frequently employed for the prognosis of muscle strain injuries; however, they do not provide adequate and reliable information on the extent of the ECM damage at the muscle-tendon interface. Recent studies indicate that MRI can be used to understand the ECM damage and changes in tendon [121], articular cartilage [122], metastatic cancer [123], and ruptured aneurysms [124]. Furthermore, development of a GAG-based contrast agent led to high quality *in vivo* MRI of the cartilage in knee joints for diagnosing osteoarthritis [125]. Therefore, clinical imaging techniques directed towards the specialized ECM present in the muscle-tendon interface have the potential to significantly improve the diagnosis and prognosis of muscle strain injuries.

Additionally, the treatment procedures employed to address muscle strains influence ECM changes at the site of injury. Cryotherapy (topical application of ice) upregulated type I collagen and type III collagen gene expression levels in an injured rat tibialis anterior muscle but, it did not alter collagen deposition or ECM remodeling [126]. Interestingly, NSAID administration increased fibrosis in murine muscle repair studies [127, 128]; whereas, it improved recovery from acute muscle injuries in human clinical studies [129]. Furthermore, a proteomics study indicated that NSAID administration following acute skeletal muscle injury decreased laminin β2 protein levels [130], which plays an important role in regulating the presynaptic active zone formation in the neuromuscular junction [131]. Eccentric contraction exercises prescribed as a part of physical therapy significantly increased tenascin-C, COL1A1, COL2A1, and COL4A1 gene expression [132], which correlates with the observed increase of these ECM proteins during repair. Understanding the muscle-tendon interface ECM remodeling during repair has the potential to aid in the development of clinical treatment procedures that promote favorable ECM remodeling. Recently, a novel treatment based on low-level laser therapy was explored, and preliminary studies in rat models indicate that the therapy induced beneficial ECM remodeling by modulating type I collagen and type III collagen [133]; however, there are no clinical trials to date that validate the efficacy of this method.

ECM Remodeling in the Aging MTJ—As tissues age, the composition and physical properties of the ECM changes. There is an elevated MMP-assisted degradation and decreased synthesis of basement membrane proteins [134]. Simultaneously, there is an elevated

expression of reactive oxygen species, interleukins, cytokines and other inflammatory markers that lead to chronic inflammatory responses [135, 136]. The elevated MMP, plasminogen activator inhibitor and reactive oxygen species levels disrupt the integrity of elastin networks and modify the collagen matrix [59]. Taken together with the reduced production of GAGs [137], the overall integrity of the ECM is compromised as the tissues age [138, 139]. Several reports indicate that with aging there is an increase in the stiffness of skeletal muscle ECM due to modification of collagen networks and advanced glycation end-products [140, 141]. Furthermore, changes in the mechanical properties of the ECM with aging impairs satellite cell activation and renewal, as well as chemotactic and inflammatory responses, which are essential during skeletal muscle regeneration [142]. Acute resistance training in elderly men decreased gene expression levels of MMPs such as MMP3, MMP9 and MMP15 compared to young men; thereby demonstrating age-related ECM remodeling [143]. Age-related ECM gene expression was also observed in rat tendon, where differential expression of *Col1a1*, *Col3a1* and *Col5a1*, elastin and lubricin were observed [144]. Additionally, elevated gene expression of *Mmp2* and *Mmp9* were also reported in rat tendons; whereas, *Timp1* and *Timp2* were downregulated with aging [145]. Similarly, a recent study demonstrated significant decrease of GAG content in aged rat tendon compared to young tendon [146]. Interestingly, tendon cell proliferation was not affected during repair in aged mice; however, significant loss in fibrillar collagen deposition was observed [147]. While these studies provide some insight into how the ECM changes with time, additional studies on how aging affects the role, composition and 3D architecture of the tendon ECM are necessary for developing effective strategies to counteract the consequences of aging.

These studies indicate that the ECM plays a critical role in the development, homeostasis, repair and aging of muscle and tendon tissue. However, there remains a knowledge gap in understanding the specific roles played by the ECM at the muscle-tendon interface. Therefore, identifying how the composition, organization and function of the ECM at the muscle-tendon interface change during various biological process has the potential to provide better understanding of the repair process. The following sections detail the latest methods employed for understanding the composition, structure and role of ECM.

Techniques to Explore ECM

Traditionally, the *in vivo* roles of ECM molecules during development and repair were deciphered using knockout models[148], site-specific mutations[149], and rescue studies[150]. Over the years, several ECM loss of function mouse models were generated to elucidate the role of specific ECM molecules [89]. Moreover, techniques such as secondary harmonic generation microscopy and scanning electron microscopy were used to characterize the morphology of collagens and other ECM proteins [151]. However, these studies focused on the role and function of the ECM of interest based on the cellular response and are typically limited to the characterization of a few ECM components at a time. Nevertheless, these experimental models still hold a lot of promise to unearth new insights into the function of specific ECM molecules[148] when combined with recent advances in technology, including novel proteomics methods based on mass spectrometry, protein labeling and decellularization. The following sections will describe how these methods have the potential

to open new research avenues to explore and better understand the roles played by the ECM in development, homeostasis, repair and aging.

Mass Spectrometry—Mass spectrometry (MS) is a powerful and sensitive technique that measures the mass to charge ratio (m/z ratio) of ions. The instrument ionizes the sample molecules and segregates the corresponding ions to characterize the signal intensity based on the m/z ratio. For proteomics analyses, mass spectrometry is combined with chromatography techniques to improve the overall resolution. Typically, samples are enzymatically digested into peptides and loaded onto a liquid chromatography (LC) column, which separates the sample based on molecular specificity. Then the separated samples go through two rounds of MS, which sequentially measures the m/z ratio of the peptides then after peptide fragmentation. Over the years, different analysis methods/strategies such as metabolic labelling (SILAC) [152]and isobarically labelled peptides (iTRAQ) [153] were developed to quantify peptides characterized through LC-MS/MS. Subsequently, several software programs such as MaxQuant and MASCOT daemon were developed to identify peptides via *in silico* databases [154] and provide label free quantification. Detailed reviews on the principles behind LC-MS/MS proteomics are discussed elsewhere [155].

Mass spectrometry-based proteomics recently emerged as a leading candidate to characterize and analyze ECM composition in normal and diseased conditions [156]. Furthermore, ECM proteomics evolved into a strategy to unearth novel targets, therapeutic markers for diseases and as a tool to identify molecular mechanisms [157]. ECM molecules are usually large in size, covalently bound, cross-linked and glycosylated, thereby making them relatively insoluble and difficult to analyze using traditional biochemical methods [158]. However, researchers took advantage of the insoluble nature of the ECM molecules and developed fractionation techniques in combination with mass spectrometry to identify the ECM protein composition of various tissues [158, 159]. Tissue fractionation works on the principle that intracellular and soluble factors can be removed using buffers of increasing stringency; thus, leaving behind the ECM [158]. These studies resulted in the development of a bioinformatics platform called the Matrisome Database, which consists of an ensemble of genes that encode for the ECM and the associated proteins in different tissue types and disease conditions [156, 160].

As mentioned in a previous section, laser-capture microdissection was combined with mass spectrometry was employed to specifically isolate the MTJ in mouse limbs, and identify unique proteins localized at the muscle-tendon interface [62]. However, the tissues were not processed to enrich or characterize the ECM proteins localized to the MTJ. To date, there are no mass spectrometry-based proteomics studies conducted to identify tissue specific ECM in the MTJ under normal and injury/repair conditions. Critical information on the ECM at the muscle-tendon interface can be gathered by analyzing the ECM present in muscle and tendon through mass spectrometry-based proteomics. Analysis of equine forelimb tendon demonstrated that several key ECM proteins such as COL7A1, COL22A1, heparin proteoglycan-2, tenascin-X, dermatopontin were localized to the interfascicular matrix (softer outer region), whereas COL17A1, COL18A1, fibrillin-2 and thrombospondin-1 were identified in the tendon core. Moreover, ECM molecules such as biglycan, COMP, decorin, lumican, COL6A1, and COL6A3 were present in injured equine superficial digital flexor

tendon [161]. Studies on human tendon indicated that COMP was a major glycoprotein present in the insoluble ECM [162] and was differentially expressed during injury [163]. In addition, several ECM molecules were differentially regulated with age [164]. Mass spectrometry-based differential quantification of skeletal muscle ECM in response to injury has not been addressed to date; although, a recent study characterized skeletal muscle ECM proteins in adult mice [165].

Protein Labeling—Over the years, protein labeling methods provided researchers a way to monitor biological processes, and to quantify and detect protein modifications. Traditional protein labeling techniques involve the use of isotopes, mass tags and fluorescent labels [166]. Unfortunately, these methods cannot adequately detect or enrich for proteins that are in low abundance [167]. In addition, *in vivo* protein labeling methods require prolonged feeding time to enable proteome labeling [168]. To address these limitations, collaborative efforts in the fields of chemistry and biology led to the development of a protein labeling platform that uses non-canonical amino acids (ncAAs). For example, the methionine analogs homopropargylglycine (Hpg) and azidohomoalanine (Aha), contain a functional groups that can be modified via click chemistry to enrich for newly synthesized proteins that are otherwise difficult to identify and quantify as they are diluted by the unlabeled proteins from the same tissue [169]. The ncAAs are bioorthogonal, which means they are integrated into the biological system without reacting with the naturally occurring functional groups. In addition, the amino acid analogs are incorporated into the newly synthesized proteins using the native cellular machinery. This bioorthognal non-canonical amino acid tagging, or BONCAT [170], is used to enrich for and characterize the nascent proteome in various *in vitro* and *in vivo* systems using LC-MS/MS. Furthermore, the functional groups on ncAAs enables the labeling of newly synthesized proteins with fluorophores via click chemistry for visualization (fluorescent noncanonical amino acid tagging, FUNCAT) [171].

Previous studies demonstrated that the enriched biorthogonal ncAA labeled proteins can be analyzed via mass spectrometry-based analysis to map the nascent proteome of a range of model organisms [172]. The overall enrichment process involves the extraction of proteins, intracellular/extracellular or both, followed by a click reaction with biotinylated alkyne to tag the ncAA incorporated nascent proteins. The tagged proteins are then pulled down using streptavidin beads, which are further washed and cleaved to release the enriched ncAA labeled proteins that can be processed for mass spectrometry analysis [173]. This method of ncAA label assisted mass spectrometry-based proteomic analysis was utilized to understand protein turnover depending on the rate of nascent protein degradation during development [173] and cellular function [174]. Given the limited knowledge on the ECM at the muscle-tendon interface, ncAA protein labeling can be utilized to understand the intracellular and extracellular protein turn over in the MTJ during homeostasis, repair and aging. Prior research indicates that these methods have the potential to advance knowledge of the ECM composition and roles. For instance, a recent study demonstrated that nascent ECM secreted by mesenchymal stem cells and chondrocytes undergoing chondrogenesis can be visualized *in vitro* by fluorescently labeling Hpg and Aha present in the ECM [175]. Moreover, Aha labeling was used to understand how mesenchymal stromal cells interacted in a 3D microenvironment with the nascent ECM proteins that are involved in cell adhesion; thereby

providing information on cues that can used for designing 3D scaffold systems to enhance cell-material interactions that facilitates cell adhesion [176]. Furthermore, Aha and Hpg were successfully used to label newly synthesized protein in the developing mouse embryo with minimal impact on the developing proteome [172]. Additionally, a Aha labeling method was recently described to identify and quantify nascent ECM protein turnover in the different tissue fractions during development [173].

Visualization of the ECM—Traditional 2D imaging techniques do not resolve the architectural and surface topographical properties of the ECM, which are critical in understanding the ECM function. High-resolution 3D visualization provides structural details about the native ECM [177]; however, it is hindered by the opaque nature of the biological tissues. Hence, it is important to develop decellularization techniques that preserve the 3D organization of the ECM proteins while simultaneously removing the intracellular contents of a tissue. In general, decellularization involves the process of removing native cells, lipids and nuclear content from the tissue, while retaining the native 3D organization. The overall process utilizes various chemical, physical and enzymatic agents to effectively decellularize the tissue, as explained in detail elsewhere [178]. Perfusion-based decellularization techniques, a process where detergents are perfused through the blood vessels, is commonly used to decellularize organs with well-defined vasculature such as heart, liver, lungs and kidney [179]. Unfortunately, the muscle-tendon interface has limited vasculature; therefore, novel decellularization methods must be designed to achieve whole tissue decellularization efficiently without compromising the native ECM properties. To address this, a method was recently developed to decellularize soft tissues, like murine embryos, using a hydrogel that retained the native 3D conformation of the ECM [180]. Currently, confocal and multiphoton microscopy methods are employed to visualize the 3D spatial distribution of the ECM networks in the decellularized tissues [179, 180]. Furthermore, efforts are made to optimize antibody staining methods for the decellularized tissues to enable visualization of tissue specific ECM proteins [179]. Efficient decellularization techniques in combination with high-resolution 3D visualization can provide new information about the spatial distribution of tissue-specific ECM.

Implications for Regenerative Medicine—The techniques described above have the potential to provide important information about the composition, turnover and organization of ECM molecules across tendon and skeletal muscle that can be utilized while developing biomimetic constructs to restore the muscle – tendon interface. For instance, comparative analyses of the homeostatic and damaged/aging MTJ using mass spectrometry has the potential to inform about the overall *composition* of the ECM at the muscle-tendon interface, as well as identify key molecules that are upregulated and downregulated that compromise functionality and identify targets for intervention (Figure 2). Similarly, protein labeling methods have the capacity to present in depth details on the *dynamics* of newly synthesized ECM molecules that are critical for remodeling the interface during development, repair and aging. While these methods provide information about the identity of specific ECM it is critical for scaffold design to know the spatial distribution of components in 3D. Antibody staining combined with high resolution 3D imaging has the ability to deliver knowledge regarding the *organization* of an ECM molecule of interest. Finally, the mechanical

properties of the designed scaffold should ideally match the surrounding microenvironment at the muscle-tendon interface. Efficient decellularization methods provide an opportunity to characterize the mechanical properties of the ECM networks as previously reported [181]. However, new instrumentation to test the mesoscale mechanics of tissues needs to be developed. Taken together, better understanding of the structural, biophysical and biochemical properties of the ECM can provide essential information to guide the construction of scaffolds that can repair damaged muscle-tendon interfaces (Figure 2).

Limitations and Future Directions

Major challenges lie in the establishment of suitable research techniques that provide essential information about the ECM during repair and development. Advances in technology and novel techniques such as mass spectrometry-based proteomics analysis, ECM protein labeling and decellularization will help in exploring the role, composition and spatial distribution of the ECM proteins. However, obstacles remain in tissue processing, ECM protein extraction, visualization of ECM, and scaling up of decellularization for higher mammals. Therefore, continued research in the development and optimization of these areas will help advance the identification of the specific roles played by ECM present in the muscle-tendon interface during development, repair and aging.

Another hurdle lies in the efficient translation of engineering information obtained from ECM-based research to develop regenerative engineering strategies. Substantial advances have been made in bioengineering methods to characterize, design, develop and fabricate scaffolds for tissue engineering. However, biomimetic scaffolds developed for the MTJ repair do not recapitulate the architectural complexity and composition of the native ECM. Even though considerable strides were made in translating preclinical treatment methods, there remains a void in translating regenerative medicine strategies due to complications in the formulation, fabrication and scale-up of ECM based scaffolds. Therefore, future studies focused on preclinical and clinical translation of ECM based regenerative medicine treatment could significantly progress clinical treatment methods available for the muscle-tendon interface.

In conclusion, the ECM plays various roles in the development, repair, homeostasis and aging of the muscle-tendon interface. Therefore, understanding ECM function during repair and development has the potential to unearth important information that provides critical insight for developing regenerative medicine strategies aimed towards restoring the muscle-tendon interface.

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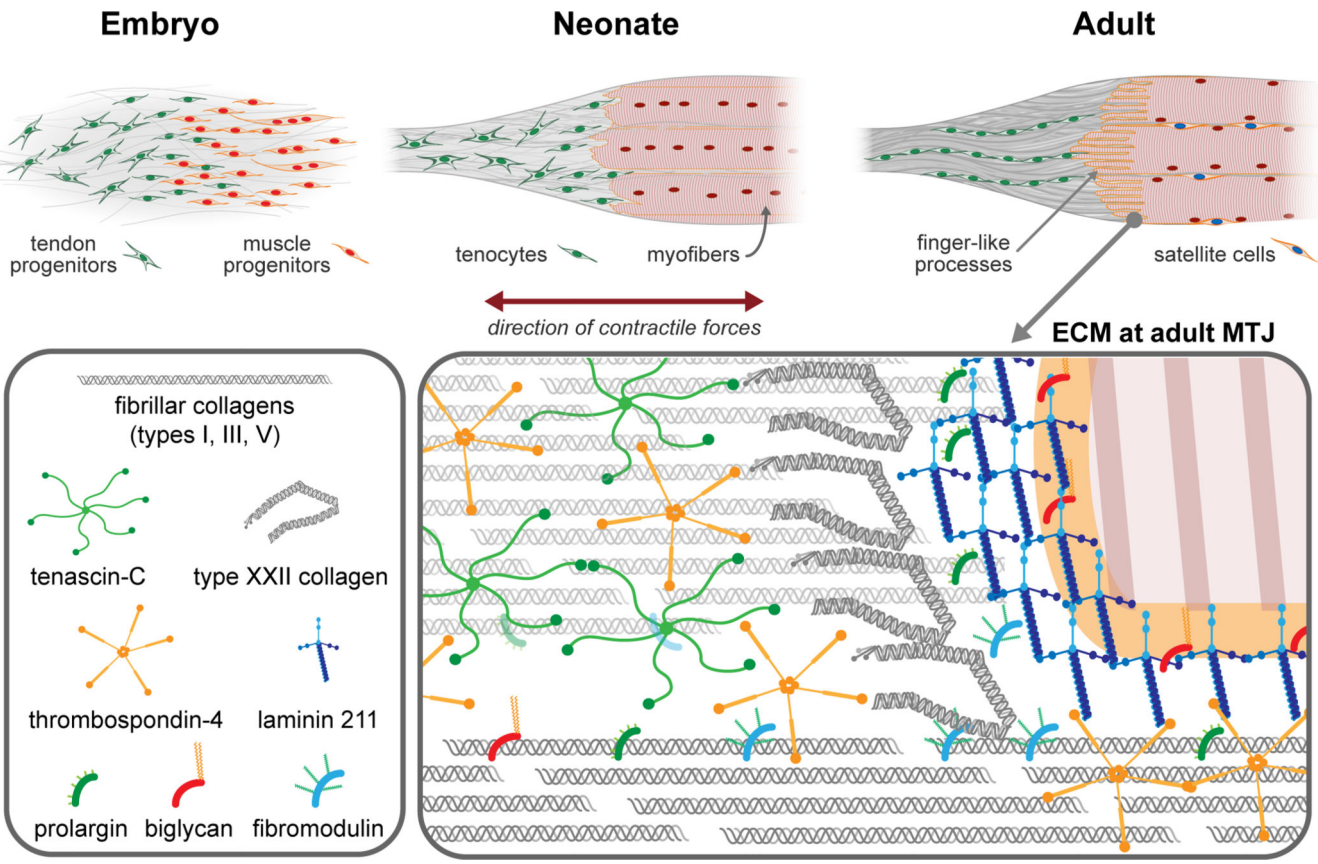

Figure 1:

Development of the MTJ (top). During embryogenesis, tendon and muscle progenitors condense at the site of future MTJ. In the neonate, the muscle - tendon interface appears smooth and there are very few finger-like processes present. In the adult, muscle fibers are mature and perfectly aligned at the MTJ. Finger-like extensions of the myofibers are well established and integrated with the tendon structure. The tendon ECM and cells are aligned along the direction of the load. Distribution of ECM discussed in this review within MTJ (bottom).

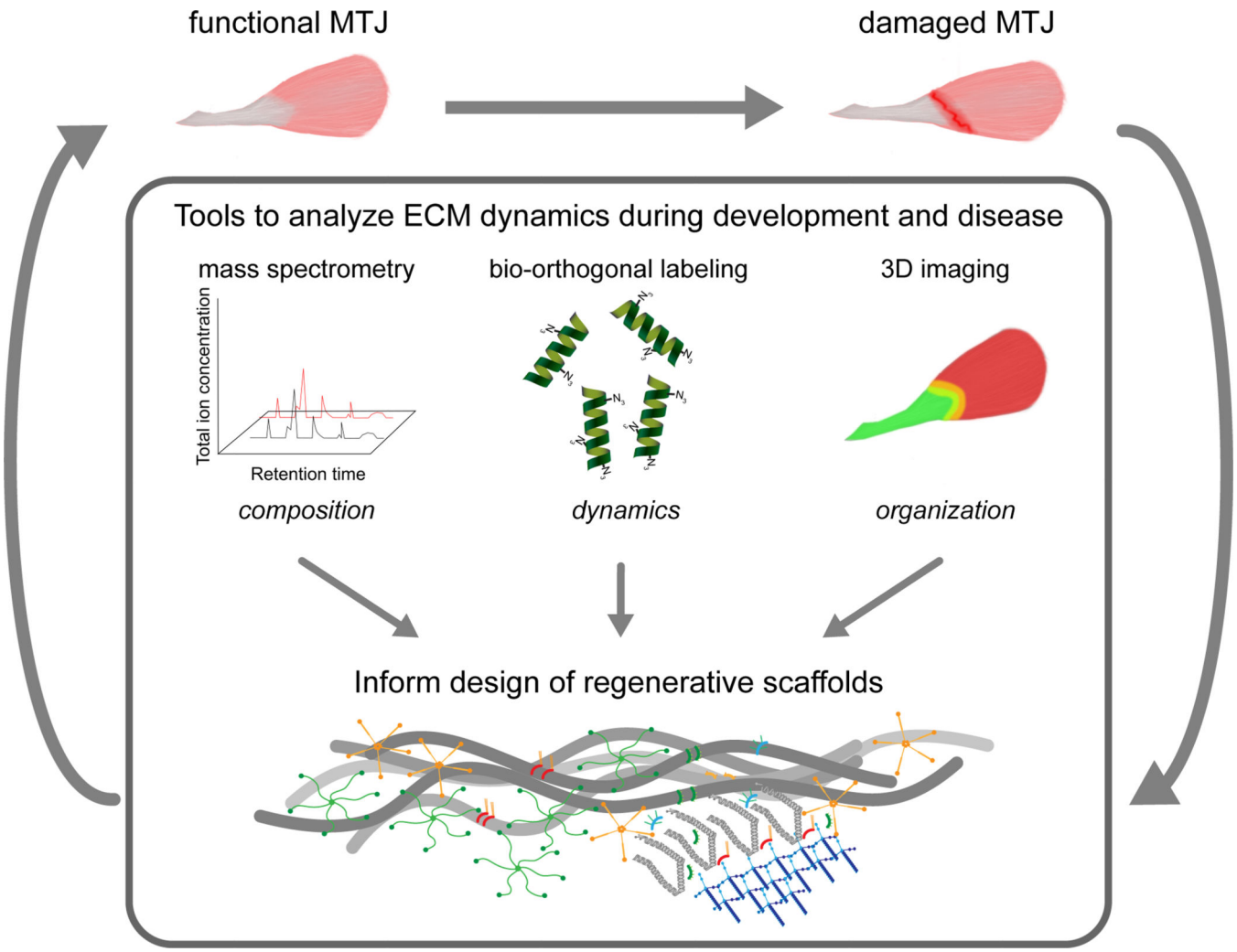

Figure 2:

The implication of ECM-based research in developing regenerative medicine for muscle-tendon interface. Knowledge regarding the composition and role of the ECM at the muscle-tendon interface will be advanced using techniques such as mass spectrometry, protein labeling and 3D imaging. This information will provide critical design information for developing ECM instructive scaffolds, which can be used alone or in combination with cells and growth factors. It is hypothesized that regenerative medicine strategies that involve the use of ECM instructive scaffolds will facilitate scar-free repair at the muscle-tendon interface.