The development of the myotendinous junction. A review

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Summary

The myotendinous junction (MTJ) is a complex specialized region located at the muscle-tendon interface that represents the primary site of force transmission. Despite their different embryologic origins, muscle and tendon morphogenesis occurs in close spatial and temporal association. After muscle attachment, muscle and tendon constitute a dynamic and functional integrated unit that transduces muscle contraction force to the skeletal system. We review here the current understanding of MTJ formation describing changes during morphogenesis and focusing on the crosstalk between muscle and tendon cells that leads to the development of a functional MTJ. Molecules involved in the formation of the linkage, both at the tendon side and at the muscle side of the junction are described. Much of this knowledge comes from studies using different animal models such as mice, zebrafish and Drosophila where powerful methods for *in vivo* imaging and genetic manipulations can be used to enlighten this developmental process.

Key words: myotendinous junction, DGC, basement membrane, integrin, collagen, development.

The myotendinous junction, an integrated mechanical unit

The transmission of mechanical force between the inside and outside of the muscle cells is based on structural relationships between the cytoskeleton proteins and components of the extracellular matrix (ECM). Skeletal muscles have two structures showing this function, the myotendinous junction (MTJ) and costamers. Both have similar junctional anchoring structures.

The MTJ is a specialized anatomical region that connects skeletal muscle to tendon (Fig. 1). Muscles and MTJ constitute an integrated mechanical unit. Structurally, the MTJ consists of subsarcolemal, transmembrane and extracellular protein complexes: actin microfilaments that extend from the last Z-line, actin-binding proteins that bundle actin filaments together, intracellular proteins that link the actin filament bundles to the sarcolemma, transmembrane protein complexes that connect cytoskeletal elements to basement membrane components, and proteins that link the basement membrane to the collagen fibril-rich matrix outside it. Muscle cell membrane (sarcolemma) is folded into finger-like extensions and invaginations to increase the interface area and to allow the membrane to resist to muscle contraction forces that are in the range of 1.8 to $3.5x10^4$ N/m^2 [1]. The function of the finger-like processes is to transmit muscle contraction force to the tendon. MTJ resistance to muscle contraction forces has been documented in numerous studies showing that muscle failure *in situ* is never associated with a separation at the interface between muscle and tendon, but rather occurs in the body of muscle cells, just proximal to the structurally defined MTJ[2].The ultrastructure of the finger-like processes is also well documented[3-7]. Finger-like processes are interdigitations of sarcolemma, where actin filaments that extend from the last Z line are connected to the subsarcolemmal proteins and indirectly interact with extracellular components[8]. The size of finger-like processes

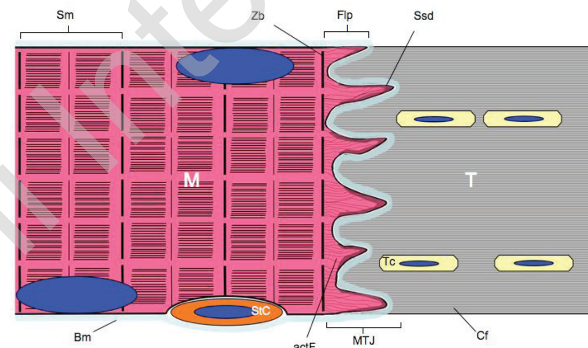

Figure 1. Schematic representation (parasagittal section) of adult muscle/tendon interface. The collagen fibers, produced by tenocytes, are anchored perpendicularly to the sarcolemma of the finger-like processes. The sub-sarcolemmal densities present at the tips of finger-like processes correspond to the muscle side of MTJ. These densities result from the massive recruitment of protein linkage-complexes that connect actin filaments from the last Z-band to the tendinous extracellular matrix. actF: Actin filament from last Z-band; Bm: Basement membrane; C: Collagen fibers; Flp: Finger-like process; M: Muscle; Sm: Sarcomer; Ssd: Sub-sarolemmal density; StC: Satellite cell; T: Tendon; Tc: Tenocyte; Zb: Z-band.

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differs among animal species and spatial changes may occur with exercise[8,9] and aging[10,11]. Interdigitations shorten with aging and the contact area between sarcolemma and extracellular components decreases resulting in muscular atrophy[10,12]. Their size differs also between the two muscle fiber types. Interdigitations are wider in slow muscle fibers than in fast muscle fibers[13].Two separate transmembrane-linkage systems have been described in the MTJ (Fig. 2). Both constitute a structural link between cytoplasmic actin and tendinous extracellular matrix proteins via laminin 211. The first linkage system contains the dystrophin-associated glycoprotein complex (DGC) also called dystrophin-associated protein complex (DAPC), and the second the α7β1 integrin. Integrins are the major membrane receptors of extracellular matrix proteins.

Several molecules are involved in the formation of the linkage, both at the tendon side and at the muscle side of the junction. Collagen I and tenascin-care the major molecules enriched at the tendon side[14]. Collagen VI is present in the endomysium extending to the MTJ[15]. On the muscle side, the sarcolemma is in close contact with the muscle basement membrane in which laminins and collagen IV are the major constituents[16]. Laminin 211 is common to both transmembrane linkage systems. It is strongly present in MTJ and represents the unique isoform that is found in adult. The laminin isoforms 221 and 411 are only expressed during embryonic development[17]. Some reports indicate that laminin α2 chain is exported at the sarcolemma where it regulates the deposition of both β and γ laminin chains to assemble extracellularly into laminin 211. The trimeric laminin 211 interacts then with dystroglycan and heparan sulphate proteoglycans and thus forms a structural link between sarcoplasmic actin filaments and the basement membrane[18-20]. The importance of laminin 211 in skeletal muscle function was highlighted by the merosin-deficient congenital muscular dystrophy type 1A (MDC1A), in which absence of the α2 chain of laminin 211 leads to skeletal muscle dysfunction highlights[21]. Mutations in LAMA2 gene, encoding the laminin α2 chain, lead to muscular dystrophy in humans, mice and zebrafish[22-24]. Laminin β1 and γ1 chains are expressed ubiquitously in basement membrane where they interact with collagen IV. They play an important role in basement membrane formation[25,26]. More recently, a new collagen, collagen XXII was identified in vertebrates and reported as

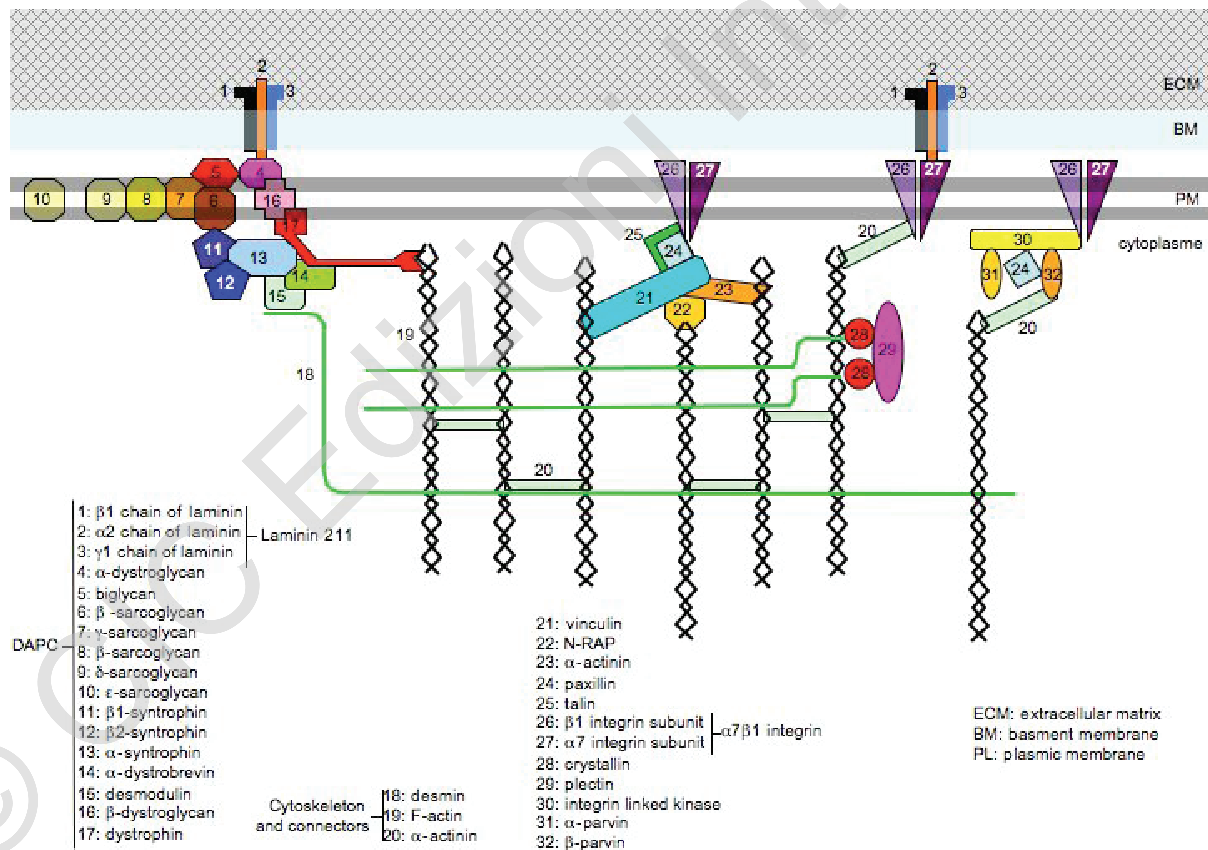

Figure 2. Schematic representation of the MTJ at the molecular level. Two types of linkage complexes are described so far: the dystrophin-associated protein complex (DAPC) also called dystrophin-associated glycoprotein complex (DGC) (proteins 4 to 17) and the protein complex containing the transmembrane α7β1 integrin (proteins 21-37 and 30-32). Both complexes connect the sarcomeric actin to the tendinous extracellular matrix (ECM) via the basement membrane laminin α2, most likely assembled into the laminin 211 trimer. These complexes are enriched at the MTJ and correspond to the sub-sarcolemmal densities observable in the finger-like processes with transmission electron microscopy. Both systems are interconnected *via* the intracellular proteins α-actinin or desmin.

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a marker of the MTJ[27]. Its function at the MTJ remains to be elucidated.
The DGC complex includes dystrophin, α- and β-dystroglycan, α, β, δ, γ and ε sarcoglycan, dystrobrevin, and α-syntrophin. Mutations in any genes of components of the DGC complex destabilize the linkage structure and result in different forms of myopathies[28-31]. In addition to its structural role, the DGC complex can serve as a scaffold allowing interactions with molecules implicated in the nitric oxide (NO) intracellular signalling pathway[32]. Dystrophin is connected to nitric oxide synthase (NOS) *via* dystrobrevin and α-syntrophin[33]. Syntrophin directly binds both dystrobrevin and dystrophin, and its C-terminal domain interacts with NOS[33]. Loss of NOS from the sarcolemma is observed in muscles of Duchenne muscular dystrophy (DMD) patients and mdx mice (which lackes dystrophin)[34]. NOS is also displaced from sarcolemma in mice that lack functional dystrobrevin or syntrophin[35,36]. The localisation of the dystrobrevin-syntrophin-NOS complex to the sarcolemma is regulated by biglycan, an ECM component associated with α-dystroglycan[37].These results show that muscle cells are able to regulate cell activity-dependant blood flow via the DGC complex. Many critical members of the DGC have been described in zebrafish[38], including dystrophin, β-dystroglycan, and β-, δ-, ε-, γ-, and ζ-sarcogly can indicating that DGC is highly conserved throughout evolution.
The second linkage system contains the α7β1 integrin. Integrins are the main ECM protein receptors that create structural links through the sarcolemmal membrane by anchoring actin filaments to the intracellular side and ECM molecules on the extracellular side. They play also an important role in cell signalling and they regulate cell activity. The α7β1 integrin complex is present throughout the sarcolemma, but is significantly enriched at the MTJ[39,40]. This integrin specifically binds laminin 211, which is the major laminin isoform found in muscle fiber basement membrane. Mice harboring a targeted disruption in the α7 integrin gene *(ITGA7)* develop a form of muscular dystrophy in which the MTJ is specifically affected[40,41]. Patients with α7 integrin deficiency exhibit congenital myopathy with variable clinical phenotypes[42]. The utrophin glycoprotein complex is another major laminin receptor in skeletal muscle[43]. Utrophin shows significant sequence homology with dystrophin and interacts with the same proteins but, contrary to dystrophin, it can bind to actin filaments at different sites[44]. In normal adult skeletal muscles, utrophin is restricted to the neuromuscular and myotendinous junctions[45]. During development, utrophin is expressed at extra junctional sites. Mice that lack utrophin exhibit a mild form of myastenia with reduced sarcolemmal foldings at the postsynaptic membrane of the neuromuscular junction[46,47]. To understand the functional overlap between α7β1 integrin and utrophin in skeletal muscles, Welser et al.[48] have generated double knock-out mice that lack both α7 integrin and utrophin. The additional loss of utrophin results in more severe MTJ structural defects than in mice in which only one laminin receptor is loss, suggesting that utrophin and α7β1 integrin compensate mutually. These data reveal important roles for α7β1 integrin and utrophin in maintaining the structural and functional integrity of the MTJ. The α7β1 integrin is known to be associated intracellularly with the tyrosine protein kinase FAK (Focal Adhesion Kinase)[49] as well as with other kinases, including the integrin-linked kinase ILK[50], the mitogen-activated protein kinase MAPK, and signalling molecules[51,52]. Several studies show that a number of other intracellular proteins associated with integrins such as tensin, paxillin, talin and vinculin also localize at the MTJ[53-61].

Development of the MTJ

Embryonic origin of the MTJ
The axial skeleton and skeletal muscles derived from distinct somitic compartments, the sclerotome and myotome respectively. Myogenic cells forming striated skeletal muscles of the body originate from the dorsal part the dermomyotome[62,63]. The medial and lateral parts of the dermomyotome give rise to axial and limb myogenic cells, respectively[64]. In vertebrates, muscle cells differentiate from the somitic mesoderm under the control of the paired homeodomain transcription factor Pax3 and the MyoD family of basic helix-loop-helix transcription factors[65-68]. The dermomyotome is patterned by the combined action of the signalling molecule Wnt produced by the dorsal neural tube and the ectoderm, and Shh produced by the notochord and the floorplate. Myf5, the first myogenic regulatory protein expressed in the skeletal muscle lineage acts in concert with Pax3 to activate in muscle precursors a network of myogenic regulatory factors, including MyoD, myogenin, and MRF4[68-69].
Tendon cells, called tenocytes, as well as axial cartilage and bone forming the vertebra and the ribs[70], originate from sclerotome, but tendon and cartilage progenitors are located in different places as shown by the expression analysis of specific early molecular markers[71-72]. Tenocytes associated with the axial skeleton are thought to originate from the dorsal region of the sclerotome, whereas limb tendons originate from the lateral plate[71].

Muscle and tendon markers
A large number of muscle (for review see[73]) and tendon (reviewed in[74]) markers are expressed during embryonic development and are evolutionary conserved among vertebrates. In vertebrate dermomyotome, myogenic precursors express Pax3 and Pax7 that are both required for muscle development. Depending on types of muscle fibers, distinct genetic pathways govern myogenesis. All signalling pathways implicate the major markers of muscle fiber determination and differentiation, Myf5, Mrf4, MyoD, and myogenin. It is not the scope of this review to relate the high complexity of muscle fiber differentiation (for review, see[73]).
A number of extracellular matrix genes are expressed in developing tendon. Among them, several collagens have been identified in zebrafish, chick, mouse, and human developing tendon, such as the fibrillar collagen type I[75-78], type III[75-76] and type V[75,79,80], and the non fibrillar collagens type VI[81,82], type XII[83-86], and type XIV[84,87]. In addition to collagens, a number of other proteins have been identified in tendon, such as tenomodulin and tenascin that are generally considered as good markers of early developing tendon cells[81]. Finally, quantitatively minor matrix components are also found

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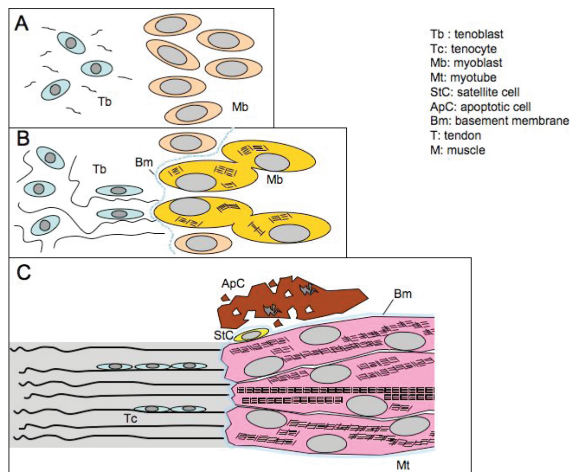

Figure 3. Schematic representation of MTJ formation during development. In developing somites, the myotome compartment that contains myoblasts is juxtaposed to the syndetome containing tenoblasts. In limb buds, myoblasts and tenoblasts are somite-derived cells. (A) Tenoblasts secrete a sparse fibrillar ECM adjacent to myoblasts. (B) Myoblasts fuse in contractile myotubes and a nascent basement membrane appears at the interface between myotubes and tendon. The first random contractions organize collagen fibers in parallel array. In the same time, the ECM provides a solid support, which constraints the thick and thin filaments of sarcomere to organize into parallel arrangement. (C) Sarcolemma resistance to the increasing contractile forces augments correlatively with the progressive formation of sarcomeres and stretching results in the progressive parallel alignment of collagen fibers. This process suggests a mechanical crosstalk between muscle and tendon. The size of collagen fibers increase and the finger-like processes begin to form. The deposition and assembly of a continuous basement membrane is finished. The myotubes that are not anchored to ECM are eliminated by apoptosis. Satellite cells are observed associated with myotubes and tenocytes are aligned along the collagen fibers. MTJ development ceases after birth. As shows the Figure 1, formation of finger-like processes and the recruitment of linkage complexes at the tips of sarcolemmal protrusions (corresponding to the sub-sarcolemmal densities observed with transmission electron microscopy), align sarcomeres parallel to collagen fiber axis and permit the transmission of muscle forces to skeleton through tendons.

in tendons such as elastin, emilin, fibrillin. However, none of them are tendon specific. The discovery of the bHLH transcription factor scleraxis provides an excellent marker of tenocytes in chick and mouse embryos[71,88]. However, it should be noted that scleraxis is also expressed in ligament[71]. The fact that scleraxis expression occurs normally in the absence of myogenic cells also shows that tendon can initiate their development independently of muscles. *Scleraxis* is essential for the initiation of tendon differentiation, but another transcription factor called Mohwak is necessary for the tendon maturation[89].

The main structural changes during MTJ development

To our knowledge, the development of the MTJ was first reported in 1948 by Wortham[90]. The author showed that muscle and tendon of chicken hind limbs are morphologically distinguished at E7 although no distinct boundary between these tissues was observable until E10. At E11, the MTJ membrane displays cytochemical specialization compared to non junctional membrane in that it is the only site where myogenic cells show acetylcholinesterase activity[91]. Then, electron microscopy observations show that MTJ formation begins with the accumulation of extracellular material at muscle ends[1,92]. In zebrafish, we observed that the first deposition of myoseptal matrix starts before the end of the segmentation period[93]. In vertebrates, there are five major events in MTJ formation (Fig. 3)[1]: close association of muscle cells with tendon cell precursors. This step is also characterized by a close association of cells with collagen fibers[2], zebrafish basement membrane formation[3], sarcolemmal folding[4], appearance of subsarcolemmal densities, and[5] myofibril association with subsarcolemmal densities.

Despite their different embryonic origins, the morphogenesis of muscle and tendon occurs in close spatial and temporal association. The development of functional musculature depends on the correct attachment of muscles fibers to the adjacent fibrous connective tissue, such as tetrapod tendons and fish myosepta. Tendons connect muscles to bones where as myosepta link muscles to adjoining muscles. After attachment, muscles and their tendons constitute integrated mechanical units that control the position and movement of joints and store and release elastic energy[94].

The earliest morphological modification at the incipient MTJ is the formation of close interactions between myogenic cells and fibroblasts, as described by Tidball and Lin[1]. Fibroblasts and myogenic cells, which are initially separated by several micrometers develop progressively interactions by formation of close appositions between extensive long villous processes of the fibroblasts and muscle cell membranes. Concomitant to these association processes, an accumulation of ECM components is observed between muscle fibers and tendon cells. The deposition of ECM proteins close to the sarcolemma forms the nascent basement membrane.

As shown in chicken[1] and zebrafish[93], the first deposited collagen fibrils are sparse and do not display any particular orientation. Later in development, tendon collagen fibrils are organized in mono-oriented fibril bundles in chicken and mammals, where as in zebrafish, collagen fibrils appeared organized in a typical orthogonal arrangement. This plywood-like structure appears first near the muscle cell membrane, then throughout the whole myoseptal matrix. The mechanism of the spatial arrangement of collagen fibrils in myosepta is unknown. In mammalian tendons, tenocytes and collagen fibrils start to align themselves in a mono-oriented direction in response to the contractile force of the developing muscle during the late embryonic and neonatal periods[94]. In zebrafish orthogonal arrangement of collagen fibril bundles might be initiated as the muscle starts to contract, i.e. at about 20 hours post fertilization (hpf). In myosepta, the specific array of these tendon-like structures is supposed to functionally increase the myoseptal mechani-

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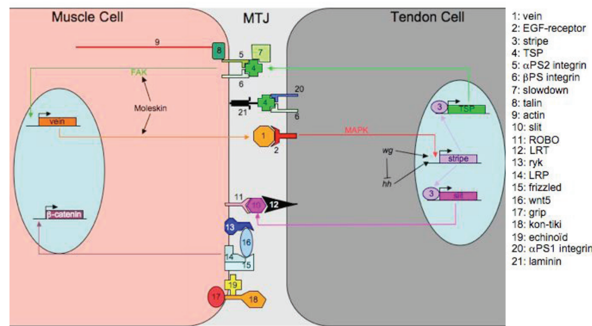

Figure 4. Cross-talk between muscle (pink) and tendon (dark grey) cells in *Drosophila*.
The MTJ (light grey) is formed through muscle-specific αPS2βPS integrin interaction with the tendon-secreted ECM component TSP and its regulator Slow. Laminin binds the αPS1βPS tendon-specific integrin *via* the molecular adaptator TSP. The αPS2βPS integrin intracellular signal is mediated by FAK and induced the expression of vein gene in muscle cell. *Vein* protein is deposited in intercellular space and links the tendinous EGF-receptor. *Vein* expression and deposition of the corresponding protein are regulated by moleskin. Interaction of Vein on EGF-R leads to the *stripe* gene expression *via* MAPK signaling. *Stripe*, which expression is also induced by *wingless (wg) hedgehog (hh)* pathway activation, is a central actor of tendon cell differentiation. This transcription factor induces the expression of *tsp* and *slit* genes. Slit binds ROBO at the muscle cell surface and LTR at the tendon cell surface. Wnt5 also provides a structural link between the muscular receptor ryk and the frizzled/LRP co-receptors.

cal resistance to muscle contractile forces and to be more appropriate to high-speed undulatory locomotion.
The anchoring of collagen fibers to the sarcolemma through direct or indirect interactions with transmembrane proteins that extend from the last Z-line leads to the formation of sarcolemma inter digitations.

The cross talk between muscle cells and tendon cells
The correct assembly of the MTJ is crucial for proper muscle and tendon function. The signals that mediate connection between muscles and tendons, and the mechanism governing mutual induction of the shared junction site shave not been elucidate yet. The differentiation of mesenchymal cells into tendon precursors, as evidenced by *scleraxis* expression, is independent of myoblasts, and *vice versa*. However, the maturation and segregation of tendon primordia into individual tendons is induced by muscle cell migration[95]. Conversely, muscle cells migrate in ectopic regions in absence of tendon precursors. These results indicate that signals from both tissues coordinate muscle and tendon development[95,96]. Some elements of the cross talk between myotubes and tendon precursors have been elucidated in Drosophila[96]. The sites of attachment of Drosophila muscles on epidermis are called tendon cells, by analogy with the tendon cells that link vertebrate muscle to bone. In Drosophila, tendon cells connect muscle cells to the chitinous exoskeleton. The earliest identified marker for tendon precursors is the zing-finger transcription factor stripe, which induces expression of most tendon cell-specific genes, including the markers of terminally differentiated tendon cells, β1-tubulin and delilah. Stripe for which two isoforms have been identified, SrA and SrB, is expressed in tendon cell precursors independently of muscle precursors. In order to mature properly, tendon precursors need to receive a signal from muscle cells. It has been demonstrated that the neuroreguling-like ligand vein, secreted by muscle precursors at the interface between muscles and tendons, activates EGF-signalling pathway in tendon cell precursors. Only cells that receive the vein signal will express β1-tubulin and delilah. Those that have not received the vein signal, dedifferentiate and loose the expression of markers [97,98]. A very interesting model was recently proposed by Liu and Geibrecht[99], to explain the mechanisms by which Drosophila muscle cells signal to the tendon cells to form and maintain a stable MTJ (Fig. 4).These authors show that moleskin, known as a nuclear import protein, is essential for muscle attachment. Mature muscles associate with their corresponding tendons *via* an ECM-rich matrix that interacts with muscle integrins. Moleskin is synthesized by muscle cells and modulates vein secretion, accumulation and/or localization at the site of the muscle-tendon attachment site. The binding of vein to the tendon cell receptor EGFR, results in MAPK activation. Activated MAPK is translocated to the nucleus and with SrA, an isoform of the epidermal growth factor-like transcription factor, activates downstream genes to induce terminal differentiation of tendon cells.
FGF4 and FGF6 (in mice) and FGF8 (in chicken) are implicated in the cross talk between muscle and tendon cells[100,101]. Fgf4 expression in the tips of muscle cells induces the expression of *scleraxis* and *tenascin-C* in tendon cells[102]. On the contrary, Fgf8 is expressed in tendon cells and diffuses in the developing tendinous matrix to repress muscle cell growth by down-regulation of the myogenic factor MyoD at muscle cell boundaries[100]. Contacts between myotubes and tendon cells result in the establishment of a stable MTJ.
The research of new factors regulated differentiation, orientation and attachment of individual muscle fibers allowed to identify several proteins such as the Roundabout (ROBO) protein (Fig. 4). Robo, identified in Drosophila, is expressed in a subset of muscle fibers and acts as a guidance receptor for Slit produced by the tendon cells[103]. Robo interacts also with a Leucin-rich tendon-specific protein (LRT) that promotes muscle-tendon targeting[104]. Slit and LTR expressions are induced by Stripe[104,105]. Kon-tiki, an other factor identified in Drosophila, is a transmembrane protein located at myotube tips that signals through the intracellular adaptor complex Dgrip/Echinoid[106,107]. This protein complex is required for myotubes to recognize their tendon cell targets and to establish a stable connection[106]. It has been shown very recently that, in Drosophila, the secreted WNT5 protein and the Ryk transmembrane receptor family members, DRL and DNT are essential for guidance of a subset of embryonic body wall muscle fibers to their tendon cells[108] (Fig. 4).

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The basement membrane, the indispensable link between muscle and tendon

Muscle basement membrane forms first at the site of MTJ formation, before appearing elsewhere around the muscle cell. The basement membrane is a thin scaffold of specific extracellular protein networks that separates epithelial, muscle and nervous cells from the underlying ECM of connective tissues. Laminins and collagen IV are the major structural components of basement membranes. They form independent networks that are connected by nidogens and the large proteoglycan perlecan. Laminins are heterotrimers formed by the association of three polypeptidic chains α, β and γ. In mammals 15 laminin isoforms have been described that are made up of varying combinations of five α (α1-5), three β (β1-3), and three γ (γ1-3) chains[109,110]. In zebrafish, laminins have been reported to be the major components of the notochordal basement membrane[111] and the analysis of the phenotypes of the mutants *bashfull* (laminin α1), *grumpy* (laminin β1), and *sleepy* (laminin γ1) mutants, revealed that these chains are all essential for the differentiation of chordamesoderm in notochord[112]. Pedrosa-Domellöf et al.[113,114] have determined the distribution of laminins in developing MTJ. The results showed that laminins α1 and α5 are early basement membrane markers of the forming MTJ. Later in development, laminin α5 is found around the entire muscle fiber, whereas laminin α1 remains restricted to the MTJ. The exclusive presence of the laminin α1 at the MTJ during the period of muscle morphogenesis suggests that this isoform may play an important role in myotube attachment to tendon. On the basis of double expression pattern analysis, the first deposited laminin isoform should be laminin 111 (α1β1γ1). This isoform is progressively replaced by laminin 123 (α1β2γ3) suggesting that the MTJ is undergoing important structural changes during development. Laminin α2 seems also to play an important role in the stability of vertebrate muscle attachments as observed in the zebrafish *candyfloss* (laminin α2) mutant that phenocopies the most common form of human congenital muscular dystrophy (MDC1A)[24]. In contrast to the zebrafish *sapje* (dystrophin) mutant[115], loss of laminin α2 results in mechanically-induced muscle detachment without sarcolemma rupture.

Laminin polymerization at the MTJ requires the nicotinamide riboside kinase 2b (Nrk2b)[116], which is well conserved from yeast to humans. In vertebrates, Nrk2b is a critical regulator to muscle growth and development. It phosphorylates the nicotinamide riboside protein and generates NAD+ (nicotinamide adenine dinucleotide) for which the role in morphogenesis is unknown. In Nrk2b-deficient zebrafish embryos, laminin 111 is not properly assembled and the MTJ is discontinuous resulting in abnormally elongation of muscle fibers. Nrk2 is required for the assembly of cell-matrix adhesion structures refered to as cell-matrix adhesion complexes (CMACs)[117]. During MTJ formation, Nrk2b is necessary for FAK and β-Dystroglycan assembly, and for the recruitment of paxillin at the junction. Hence, Nrk2b represents a key regulator of basement membrane assembly during early MTJ morphogenesis. How Nrk2b-mediated NAD+ production regulates CMAC assembly is unknown. Goody et al.[116] suggest that Nrk2b provides a local source of NAD+ used for the ADP-ribosylation of integrins.

An additional relevant ECM component of the MTJ is thrombospondin (TSP). Thrombospondins are a family of ECM proteins that mediate cell-cell and cell-matrix interactions by binding membranes receptors, ECM proteins and cytokines[118]. This ECM component is also an integral component of the basement membrane in a number of different tissues. In vertebrates, there are five genes encoding thrombospondins that are expressed in various tissues, including the brain, (*Tsp1* and *Tsp2*), bones (*Tsp5*) and tendons (*Tsp4*). Drosophila TSP is encoded by a single gene expresses only in tendon cells. Lack of TSP expression in Drosophila embryos leads to muscle cell mis-attachment to tendon cells[119]. How TSP participates to the formation of a fully functional MTJ is described below.

Integrin, an indispensable player in MTJ development

Laminins and thrombospondins bind to integrins that are the main ECM receptors. Integrins are transmembrane heterodimers of two different subunits, α and β, associated by non-covalent interactions. In humans, 18 different α and 8 different β subunits have been identified resulting in 24 different integrin isoforms[120]. The α7β1 integrin is the dominant laminin-binding integrin in skeletal and cardiac muscles. However, contrary to the α7 subunit, the β1 subunit associates with other different subunits and is expressed throughout the body. The absence of α7 integrin subunit leads to muscular dystrophy suggesting that integrin α7 is indispensable to muscle fiber attachment[121]. In patients with Duchenne muscular dystrophy and in *mdx* (dystrophin -/-) mice, it has been shown that integrin α7β1 is up-regulated to compensate the deficient dystrophin-mediated linkage of muscle to basement membrane[122]. In laminin α2-deficient (*dy/dy*) mice, α7 integrin is significantly reduced indicating that laminin α2 regulates the α7 integrin gene expression[122]. In humans, different a7 integrin isoforms have been described that are differentially expressed during development[123,124]. Deficiency of α7 integrin isoforms seems to be a common feature in muscular dystrophy/myopathy[42]. During myogenic differentiation, the expression of α7 integrin is under the positive regulatory control of MyoD and myogenin and conversely, under the negative regulatory control of c-Myc. In myoblasts, in which c-Myc levels are relatively high and levels of myogenic factors are low, α7 integrin expression is about 20-fold less important than in differentiated muscle cells. As myoblasts undergo differentiation, they gradually loose expression of c-Myc and up-regulate expression of myogenic regulatory factors leading to a shift from loss of negative regulation to positive regulation of the *Itag*7 gene promoter. Hence, α7 integrin is expressed at high levels, as required for the formation of MTJ[125].

In Drosophila the muscle-specific integrin is the heterodimer αPS2βPS. In absence of the common αPS2 or βPS subunit, the initial interaction between muscle and tendon is formed, but muscle cells detach from tendon cells following muscle contractions[126]. Both laminin and TSP interact with αPS2 βPS and participate to the dynamic of MTJ formation. As proposed by Subramanian et al.[119], TSP is continuously secreted by tendon cells and binds to the muscle-specific αPS2βPS integrin receptors (Fig. 4). TSP expression is induced by the tendon-specific transcription factor stripe,

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in particular the isoform SrA. Drosophila thrombospondin forms pentamers which can associate with several integrin receptors leading to the accumulation of αPS2βPS integrin receptors at the myotube leading edge. In a second step, TSP might bind to the tendon surface through an unidentified ligand. An elevated level of TSP synthesis is maintained following the establishment of the muscle-tendon junction through a positive feedback loop implicating moleskin and vein (Fig. 4). As seen above, moleskin activates secretion of vein from muscle cells that, in turn, activates EGFR signalling pathway in tendon cells. Activated MAPK activates thrombospondin gene transcription via SrA[99].

A new Drosophila player in MTJ construction, slowdown, which is secreted by tendon cells, has been identified as a regulator of TSP binding to αPS2βPS integrin receptors[127]. In slow mutants, TSP prematurely accumulates at muscle end leading to the formation of an aberrant MTJ architecture. Slow is secreted in parallel to TSP with which it forms a complex that attenuates the thrombospondin-αPS2βPS integrin interactions[127]. Thus, slow could represent a molecular link between the arrest of muscular migration and the formation of the MTJ.

Progress in the understanding of the MTJ formation was made by determining the manner by which integrins are linked to cytoplasmic and cytoskeletal elements. The connection to actin cytoskeleton involves a complex of adaptor proteins, which bind to the cytoplasmic tail of integrins and mediate the link to the cytoskeleton. A number of these adaptor proteins have been identified. These include talin[128], the integrin-linked kinase ILK[129], PINCH[130], and tensin[131]. These proteins are essential for integrin function and act by stably linking clusters of ECM-linked integrins to the cytoskeleton. Talin is necessary for the initial formation of the integrin adhesion complex and for the recruitment of intracellular proteins such as ILK and tensin. In a search of proteins able to modulate integrin-mediated muscle attachment, several other adaptors have been identified in Drosophila, ZASP (Z-band alternatively spliced PDZ-motif protein), a member of the PDZ-LIM protein family[132,133], has been identified as a novel regulator of cell-matrix adhesion. Several pieces of evidence, such as the strong genetic interactions between Drosophila integrin and ZASP, and the defects in the MTJ formation observed after loss of ZASP expression, indicate that ZASP regulates or strengthens the integrin binding to actin cytoskeleton after the initial attachment of integrins to actin via talin[134]. ZASP also organizes the Z-line by recruiting α-actinin. In Drosophila, another transducing adaptor has been identified and named “wech”. The wech gene encodes a multidomain protein containing a B-box zinc-finger domain and a coiled-coil domain characteristic of the RBCC/TRIM protein family[135]. The protein is found in the sarcomeric Z-line and co-localizes with tensin, ILK and talin, with which it interacts with the head domain of talin to connect integrins to the cytoskeleton. Talin is required for wech localization and wech is required for proper ILK localization.

Integrin complexes are highly dynamic during cell migration. Integrins are constantly internalized and recycled to the membrane. The integrin turnover mechanism to maintain stable structure such as the MTJ over long periods of time, is not elucidated. However the analysis of the dynamics of the integrin adhesion complex in Drosophila MTJ shows that integrins turnover to allow normal tissue growth and maintenance is mediated by clathrin-dependent endocytosis and the small GTPase Rab5[136].

Conclusion

The present review demonstrates that the study of the MTJ has provided insights not only into important morphological changes that occur during MTJ development but also into molecular mechanisms implicated in this event, and more specifically into the cross-talk between muscle cells and tendon cells. Multiple factors are involved in the MTJ development and it is certain that a lot of them are not known yet. The use of different animal models, such as mouse, Drosophila, and zebrafish that harbour mutations in genes encoding proteins implicated in MTJ development provides a valuable resource for the next stages of investigation to enlighten the complexity of the structure and the formation mechanisms of the two transmembrane-linkage systems and to understand the pathologies associated with defects in the MTJ development.

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