Connecting muscle development, birth defects, and evolution: An essential role for muscle connective tissue

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Abstract

Skeletal muscle powers all movement of the vertebrate body and is distributed in multiple regions that have evolved distinct functions. Axial muscles are ancestral muscles essential for support and locomotion of the whole body. The evolution of the head was accompanied by development of cranial muscles essential for eye movement, feeding, vocalization, and facial expression. With the evolution of paired fins and limbs and their associated muscles, vertebrates gained increased locomotor agility, populated the land, and acquired fine motor skills. Finally, unique muscles with specialized functions have evolved in some groups, and the diaphragm which solely evolved in mammals to increase respiratory capacity is one such example. The function of all these muscles requires their integration with the other components of the musculoskeletal system: muscle connective tissue (MCT), tendons, bones as well as nerves and vasculature. MCT is muscle's closest anatomical and functional partner. Not only is MCT critical in the adult for muscle structure and function, but recently MCT in the embryo has been found to be crucial for muscle development. In this review, we examine the important role of the MCT in axial, head, limb, and diaphragm muscles for regulating normal muscle development, discuss how defects in MCT-muscle interactions during development underlie the etiology of a range of birth defects, and explore how changes in MCT development or communication with muscle may have led to the modification and acquisition of new muscles during vertebrate evolution.

1. Introduction

Skeletal muscle powers many critical vertebrate functions: locomotion, postural support, feeding, respiration, and communication. Accounting for 30–50% of vertebrate body mass (Romer & Parsons, 1986), skeletal muscle is distributed in multiple regions that have evolved distinct functions. A shared feature of chordates are the axial muscles, metameric muscles that stabilize and flex the notochord or vertebrae to enable support and locomotion of the body (Fig. 1; reviewed by Wotton, Schubert, & Dietrich, 2015). The evolution of the head in vertebrates has been accompanied by the
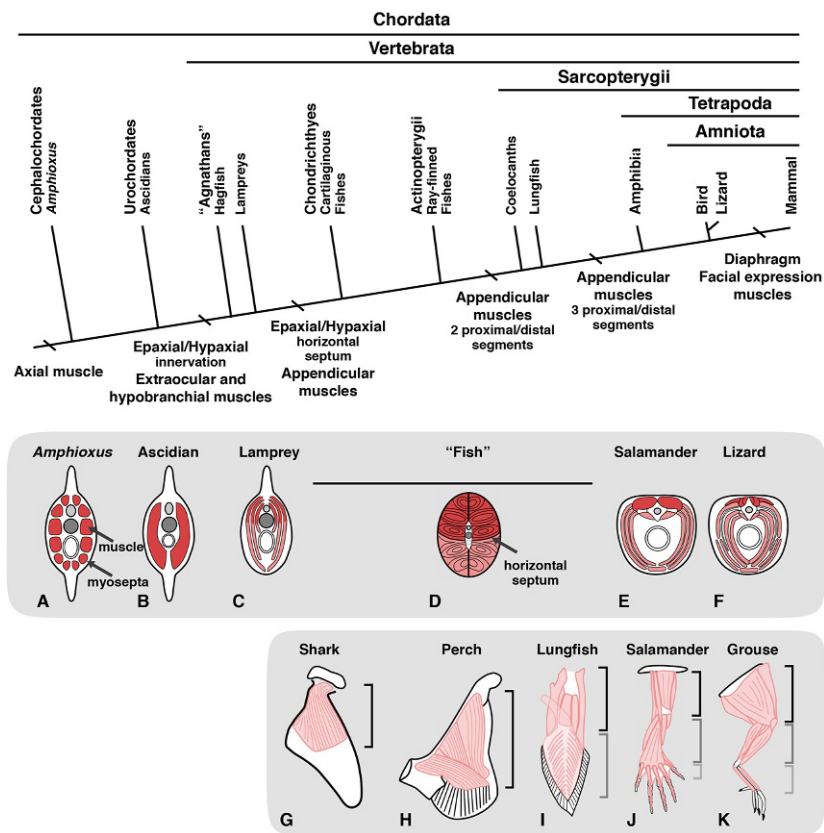

Fig. 1 During the course of chordate and vertebrate evolution, axial and limb muscles become increasingly complex. (A–B) Simple axial muscles in *Amphioxus* (A) and tail of ascidian free-swimming larva (B). (C) Lamprey myomeric axial muscle subdivided into epaxial muscle (red), innervated by dorsal roots, and hypaxial muscle (pink), innervated

development of muscles that allow for eye movement, feeding, vocalization, and facial expressions (Fig. 1). With the evolution of paired fins and limbs and their associated musculature, vertebrates have gained increased locomotor agility, populated the land, and been able to acquire fine motor skills, such as writing. In addition to axial, head, and limb muscles, different groups of vertebrates have evolved novel muscles that allow for specialized functions. Such novel muscles include the syringeal muscles which control song production in birds, facial muscles that allow for suckling in mammals, and the mammalian diaphragm that is essential for respiration and separating the thoracic and abdominal cavities. Development of all these different muscle groups has been crucial for the evolutionary success of vertebrates.

Muscle function requires its integration with the other components of the musculoskeletal system: bones, tendons, and muscle connective tissue as well as nerves and vasculature (Fig. 2A). The bones are the rigid part of the musculoskeletal system that support, and in response to muscle contraction, move the body. The tendons are the tensional links between the bones and muscles. The muscle connective tissue (MCT, sometimes called dense irregular connective tissue; Nassari, Duprez, & Fournier-Thibault, 2017) is the muscle's closest anatomical and functional partner (Fig. 2B). MCT is composed of three layers: the endomysium ensheathes individual myofibers, the perimysium

by ventral roots. (D). Myomeric axial muscle of Chondrichthyan, Actinopterygian, and Sarcopterygian fishes is subdivided into epaxial and hypaxial muscles innervated by dorsal and ventral roots, respectively, and separated by a horizontal septum. (E–F) Epaxial and hypaxial muscles of tetrapods, e.g., amphibians (E) and reptiles (F) are separated by vertebral transverse processes and subdivided into multiple muscles. (G) Simple shark ventral pectoral fin muscles extend from pectoral girdle to dermal rays. (H) Multiple ventral fin muscles of ray-finned perch extend pectoral girdle to bony rays. (I) Dorsal muscles of lungfish *Neoceratodus* pelvic fin are subdivided into two proximal-distal groups. (J) Dorsal muscles of salamander *Ambystoma* are subdivided into three proximal-distal groups. (K) Dorsal and ventral muscles of grouse *Dendragapus* are subdivided into three proximal-distal groups. *Panel (B and G) based on Walker, S. F. & Homberger, D. G. (1992).* Vertebrate dissection *(8th ed.). Fort Worth: Harcourt Brace College Publishers. Panel (C) based on Fetcho, J. R. (1987). A review of the organization and evolution of motoneurons innervating the axial musculature of vertebrates.* Brain Research, *434, 243–280. Panel (D–F) based on Romer, A. S., & Parsons, T.S. (1986). The vertebrate body. Fort Worth: Saunders College Publishing. Panel (H) based on Winterbottom, R. (1974). Descriptive synonymy of striated muscles of Teleostei.* Proceedings of the Academy of Natural Sciences of Philadelphia, *125, 225–317. Panel (I and J) after Diogo, R., Johnston, P., Molnar, J. L., & Esteve-Altava, B. (2016). Characteristic tetrapod musculoskeletal limb phenotype emerged more than 400 MYA in basal lobe-finned fishes.* Scientific Reports, *6, 37592. Panel (K) after Kardon, G. (1998). Muscle and tendon morphogenesis in the avian hind limb.* Development, *125, 4019–4032.*
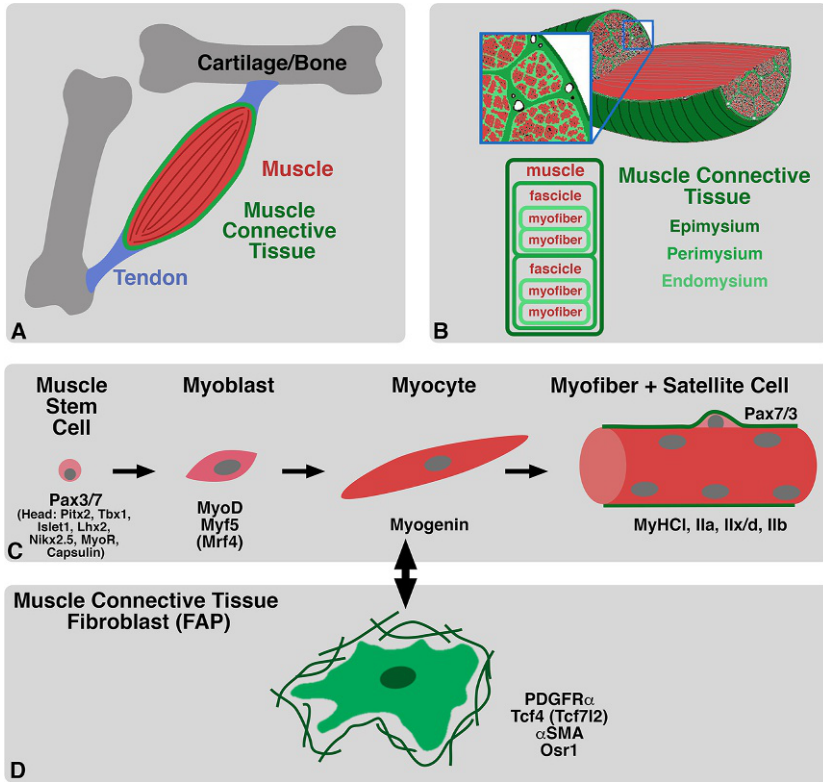
Fig. 2 Muscle and muscle connective tissue (MCT) structure, molecular markers, and development. (A–B) MCT surrounds myofibers, fascicles, and whole muscles to transmit contractile force of muscle to tendon and bone. (C) During myogenesis muscle progenitors/stem cells (which express Pax3/7 in axial and limb muscles and variety of transcription factors in the head) become committed MyoD/Myf5 + myoblasts, differentiate into Myogenin+ myocytes, which fuse into post-mitotic, multinucleate myofibers. (D) MCT fibroblasts (also known as FAPs in the adult) express Pdgfrα, Tcf7l22, and Osr1 and secrete ECM.

bundles myofibers into fascicles, and the epimysium surrounds anatomical muscles and links to the tendons at the myotendinous junctions (Sanes, 2004). All three MCT layers are composed primarily of extracellular matrix, largely collagens and proteoglycans, as well as the MCT fibroblasts that produce this matrix (Andrade & Brandan, 1991; Lipton, 1977; Sanes, 2004; Zou, Zhang, Sabatelli, Chu, & Bonnemann, 2008). The MCT is essential for maintaining muscle's structural integrity as well as transmitting muscle's contractile force to tendon and bone. In addition to MCT's important structural

and functional roles in the adult, recently MCT has been found to have critical roles in regulating muscle development.

Muscle development occurs via two interrelated processes, myogenesis and muscle morphogenesis. Myogenesis is the cellular process by which muscle progenitors differentiate into multinucleate myofibers (Fig. 2C). The first step in myogenesis is the establishment of muscle progenitors. Axial and limb muscle progenitors originate from the somites, transient epithelial blocks of paraxial mesoderm located bilateral to the neural tube, while head muscle progenitors largely come from cranial pre-chordal and pharyngeal mesoderm, lateral to the developing brain (Bryson-Richardson & Currie, 2008; Buckingham, 2006; Michailovici, Eigler, & Tzahor, 2015; Murphy & Kardon, 2011; Wotton et al., 2015). Reflecting their different embryonic origins, the axial and limb muscle progenitors express the transcription factors Pax3 and Pax7, while the head progenitors express a heterogeneous array of transcription factors, including Pitx2, Tbx1, Islet1, Lhx2, Nkx2.5, MyoR and Capsulin (Michailovici et al., 2015; Murphy & Kardon, 2011; Relaix, Rocancourt, Mansouri, & Buckingham, 2005; Sambasivan, Kuratani, & Tajbakhsh, 2011; Tzahor, 2009). Subsequently, in all regions of the body muscle progenitors become committed myoblasts that express the myogenic regulatory transcription factors MyoD, Myf5 and/or Mrf4. Finally, myoblasts terminally differentiate into committed post-mitotic myocytes that express Myogenin, synthesize muscle-specific proteins (e.g., myosin heavy chain), fuse into multinucleate myofibers (Abmayr & Pavlath, 2012; Sampath, Sampath, & Millay, 2018), and mature and diversify into myofibers that differ in their contractile and metabolic properties (Schiaffino, Sandri, & Murgia, 2007). Concurrent with myogenesis is muscle morphogenesis, the process by which individual myofibers are assembled into defined, unique muscles (e.g., biceps) and these muscles are arranged into a stereotyped spatial array. This process involves the migration of muscle progenitors to particular regions, regulation of the number of myoblasts, selective differentiation of muscle progenitors into myofibers, elongation and increase in size of myofibers via selective myoblast fusion, and the integration of myofibers with MCT, tendons, and bones.

Considerably less in known about the development of the MCT (Fig. 2D). MCT fibroblasts are the cellular source of the MCT extracellular matrix, and they originate from multiple different embryonic sources. The MCT fibroblasts associated with the axial muscles likely derive from the somites (Nowicki, Takimoto, & Burke, 2003). However, the MCT fibroblasts associated with limb muscles arise from the lateral plate mesoderm, the mesoderm

that will form the limb bud and give rise not only to the MCT, but the tendons, ligaments, and bones of the limb (Kardon, Harfe, & Tabin, 2003; Pearse et al., 2007). Finally, the MCT fibroblasts of head muscles originate from a third source, the neural crest cells (Le Lièvre & Le Douarin, 1975; Noden, 1983a; Olsson, Falck, Lopez, Cobb, & Hanken, 2001). Study of MCT fibroblasts had been hampered by the lack of molecular markers for these cells. Recently, several markers of embryonic and adult MCT fibroblasts have been identified, and these include Tcf4 (formally known as Tcf7l2), plate-derived growth factor receptor alpha (PDGRFRα), and alpha smooth muscle actin (αSMA) (Kardon et al., 2003; Mathew et al., 2011; Murphy, Lawson, Mathew, Hutcheson, & Kardon, 2011; Olson & Soriano, 2009; Tomasek, Gabbiani, Hinz, Chaponnier, & Brown, 2002). These proteins are expressed in MCT fibroblasts associated with muscles throughout the body. In addition, the transcription factor odd skipped-related 1 (Osr1) was recently found to identify a sub-population of embryonic limb MCT fibroblasts (Vallecillo-García et al., 2017). In the embryo Osr1 is expressed in limb MCT fibroblasts, with Osr1 and Tcf4 co-expressed in sub-populations of fibroblasts, and in the adult Osr1 is expressed in activated MCT fibroblasts following muscle injury (Stumm et al., 2018; Vallecillo-García et al., 2017). Interest in MCT fibroblasts in the adult has been accelerated by the finding of fluorescence-activated cell sorting strategies that allow MCT fibroblasts to be isolated from adult muscle and its associated MCT (Joe et al., 2010; Uezumi, Fukada, Yamamoto, Takeda, & Tsuchida, 2010). As these adult MCT fibroblasts can give rise to both fibroblasts and adipogenic cells, they have been called fibroadipogenic progenitors (FAPs; Joe et al., 2010; Uezumi et al., 2010). Since embryonic Osr1 + cells have been explicitly shown to give rise to FAPs (Vallecillo-García et al., 2017) and most adult MCT fibroblasts express Tcf4 and PDGFRα and activated FAPs express Osr1 (Murphy et al., 2011; Stumm et al., 2018; Uezumi et al., 2010), MCT fibroblasts and FAPs are likely the same cell population. However, molecular heterogeneity and sub-populations within these fibroblasts undoubtedly exist (also see discussions in Nassari et al., 2017; Wosczyna & Rando, 2018).

Development of skeletal muscle and its MCT is tightly linked and recent studies show that MCT fibroblasts are crucial regulators of muscle development throughout the vertebrate body. In this review we discuss and synthesize our current knowledge of the cellular and molecular mechanisms by which the MCT regulates myogenesis and muscle morphogenesis in four major types of vertebrate muscle: axial muscles, head muscles, limb muscles,

and the mammalian-specific diaphragm. Defects in MCT development or its communication with neighboring muscle have also emerged as a source of congenital birth defects in head and limb muscles and the diaphragm. We review these studies and explore how studies of normal muscle–MCT development interactions and genetic mouse models of limb and diaphragm defects together enhance and deepen our understanding of normal development and the etiology of birth defects. Finally, we review new studies that suggest that changes in MCT development may have been important developmental innovations that led to the modification or acquisition of new muscles during vertebrate evolution.

2. Axial muscle

Locomotion of chordates and their vertebrate descendants requires the axial muscles. Axial muscles lie in close proximity to the dorsal notochord, vertebrae, and the ventral trunk regions and served as the main propulsive force prior to the evolution of limbs (Romer & Parsons, 1986). The ancestral axial muscle was likely similar to that of the Cephalochordate, *Amphioxus*, and the swimming larvae of Urochordates, both close relatives of vertebrates (Fig. 1A and B). In *Amphioxus* axial muscle extends from the head to the tip of the tail and consists of a series of V-shaped myomeres, separated by connective tissue myosepta (Holland, 1996). These myomeres function as a series of separate contractile units that laterally flex the notochord and enable undulatory swimming and stabilize the back. With the evolution of jawless vertebrates (lampreys and hagfish), the axial muscle became subdivided into epaxial and hypaxial muscles (Fig. 1C; Fetcho, 1987). The epaxial muscles are the intrinsic muscles of the back that are closely associated with the vertebral column and innervated by the dorsal rami of the spinal cord ventral roots, while the hypaxial muscles lie ventral to the vertebrae and are innervated by the ventral rami (Fetcho, 1987). This division of axial muscle into two separately innervated muscles increased the range of motion, allowing for both lateral undulations and dorsal–ventral flexion in the earliest vertebrates (Ahmed et al., 2017). Subsequently in jawed vertebrates, epaxial and hypaxial muscles became further functionally separated, as a horizontal septum of connective tissue developed between these muscles (Fig. 1D; reviewed in Wotton et al., 2015). While in sharks, some fishes, and salamanders the epaxial muscle is primarily composed of one muscle (the dorsalis trunci; Fig. 1E), in many reptiles and mammals the epaxial muscle is subdivided into three muscles: the transversospinalis, longissimus dorsi, and iliocostalis

(Fig. 1F; Romer & Parsons, 1986). Mammals further elaborate epaxial muscles into a complex and regionally varied series of muscles that are important for dynamic and static stabilization, spinal extension, and lateral bending (Fig. 1F; Goodrich, 1958; Romer & Parsons, 1986; Schilling & Carrier, 2010; Webster, Hudson, & Channon, 2014). Similarly, the hypaxial muscles (e.g., intercostal and abdominal muscles) became progressively more elaborate and regionally varied during the evolution of tetrapods (Fig. 1E and F; Romer & Parsons, 1986). Interestingly, there are both epaxial and hypaxial muscles that lose their segmental arrangement and fuse to form sheets of muscle that are critical for locomotion (Carrier, 1990; Romer & Parsons, 1986).

Epaxial and hypaxial trunk muscles develop from the somites (Fig. 3; Bryson-Richardson & Currie, 2008; Buckingham, 2006; Wotton et al., 2015). The somites mature to form a dorsal epithelial layer, the dermomyotome, which gives rise to the overlying dermis and, via multiple waves of migrating muscle progenitors, the underlying primary myotome (Fig. 3A; Gros, Scaal, & Marcelle, 2004; Hollway & Currie, 2005; Kalcheim, Cinnamon, & Kahane, 1999; Venters & Ordahl, 2002). The myotome is a metameric series of muscles transiently present in all amniote embryos. Ultimately, the dorsomedial lip of the dermomyotome and the medial myotome gives rise to the epaxial muscles, while the ventrolateral lip of the dermomyotome and the lateral myotome give rise to the hypaxial muscles (Fig. 3A–C; Cinnamon, Kahane, & Kalcheim, 1999; Denetclaw, Christ, & Ordahl, 1997; Gros et al., 2004; Pu et al., 2013). On a molecular level, the transcription factor, Engrailed, appears to be essential for establishing the boundary between the epaxial and hypaxial muscle domains (Ahmed et al., 2017; Wotton et al., 2015).

The somites are also the embryonic source for the cartilages, bones, and tendons of the axial musculoskeletal system. As the somites mature, they not only form the dorsal epithelial dermomyotome and myotome but a ventromedial sclerotome, a region of mesenchymal cells (Fig. 3A; reviewed in Scaal, 2016). The ventral sclerotome gives rise to the vertebral column and also the ribs (Scaal, 2016). In addition, in response to signals from the overlying myotome, the dorsal cranial and caudal edges of the sclerotome form the syndetome, the region that gives rise to the tendons of the axial muscles (Fig. 3A; Brent, Braun, & Tabin, 2005; Brent, Schweitzer, & Tabin, 2003; Brent & Tabin, 2004). Thus developmental interactions between the muscle cells in the myotome and the cartilage progenitors in the sclerotome lead to the induction of the tendon progenitors that are ultimately needed to connect muscle to bone. Furthermore, the close spatial
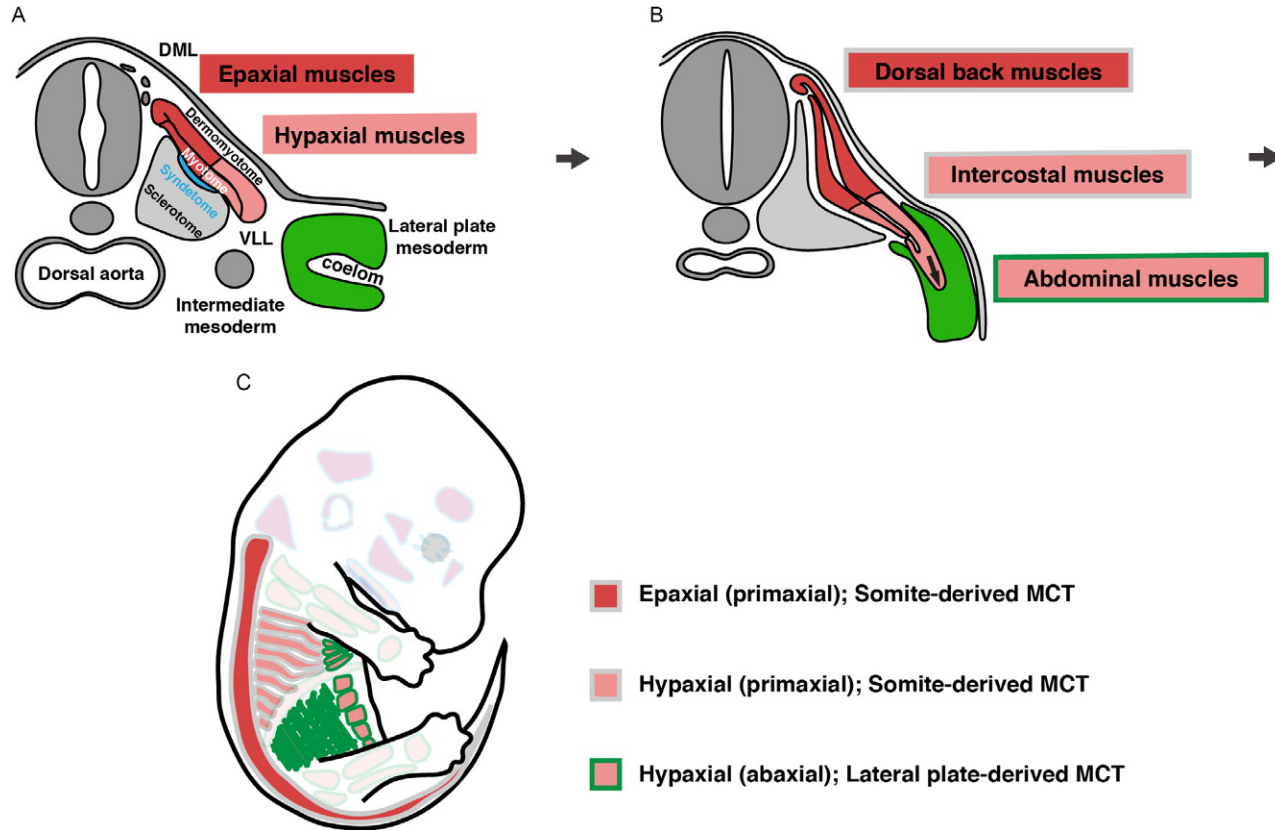
Fig. 3 Development of axial muscles in tetrapods. (A) Epaxial muscle derives from the dorsomedial region of the dermomyotome and myotome, while the hypaxial muscle derives from the ventrolateral region of the dermomyotome and myotome. DML, dorsomedial lip and VLL, ventro-lateral lip. (B–C) Dorsomedial dermomyotome and myotome give rise to epaxial back muscles with MCT presumably derived from the somite (shown as red muscles outlined in gray). Hypaxial dermomyotome and myotome give rise to primaxial hypaxial muscles (e.g., intercostal muscles, pink muscles outlined in gray) with MCT presumably derived from the somite and also abaxial hypaxial muscles (e.g., abdominal muscles, pink muscles outlined in green) with MCT derived from lateral plate mesoderm (green).

relationship between the myotome, syndetome, and sclerotome prefigures and ensures the future linkage of muscle, tendon, and bone.

The developmental origin of the MCT of the axial muscles is complex and less well known. Lineage studies using quail-chick chimeras or using the *Prx1Cre* transgene in mice to follow the fate of lateral plate mesoderm have determined that the MCT of a subset of hypaxial trunk muscles (distal inter-costals, pectoralis, and transverse, internal/external oblique, and rectus abdominal muscles) is derived from the lateral plate mesoderm (Fig. 3; Burke & Nowicki, 2003; Christ, Jacob, & Jacob, 1983; Durland, Sferlazzo, Logan, & Burke, 2008). This subset of hypaxial muscles with MCT derived from the lateral plate have been termed abaxial muscles (Burke & Nowicki, 2003). The MCT associated with the other hypaxial muscles (e.g., intercostals and longissimus dorsi) as well as the epaxial muscles are assumed to come from the somites and these muscles have been termed primaxial muscles (Burke & Nowicki, 2003). Thus there appears to be two different developmental sources for the MCT of the axial muscles: MCT of lateral plate origin that surrounds the abaxial muscles and MCT of somitic origin that surrounds the primaxial muscles (Nowicki et al., 2003). The boundary between the primaxial and abaxial muscles, with MCT of different developmental origins, has been termed the lateral somitic frontier. However, it should be noted that there is a dearth of formal evidence that the MCT of the primaxial muscles derives from the somites. Transplantation of quail somites into chick has shown that somitic cells can contribute to the MCT of some axial muscles (Saberi et al., 2017). In addition, analysis of a *ScxGFP* transgene that labels the syndetome and tendon cells also labels MCT fibroblasts in some epaxial muscles (Deries, Schweitzer, & Duxson, 2010). Together these data suggest that MCT fibroblasts of primaxial muscles may derive from the syndetome. However, a definitive test of the extent and particular location of somitic contribution to the MCT of primaxial muscles will require more extensive and detailed analyses of quail–chick chimeras or Cre-lineage experiments in mice in which particular regions of the somites are targeted.

The morphogenetic processes that transform the myotome and dermomyotome into the diverse array of epaxial and hypaxial muscles are just beginning to be elucidated. Via a detailed analysis of a developmental series of embryos labeled via whole mount immunofluorescence, Deries et al. (2010) have determined that the epaxial muscles develop gradually by the translocation, re-orientation, and elongation of the myotomal myocytes followed by cleavage of the myotomal masses. How the hypaxial muscles form has not been as fully detailed. Flank hypaxial muscles (both primaxial

and abaxial) arise from epithelial extensions of the dermomyotome and the myotome (Christ et al., 1983; Evans, 2003). The primaxial hypaxial muscles undergo morphogenesis into individual muscles in the context of presumably somite-derived MCT and those muscles in the thoracic region develop in close association with the sclerotome forming the ribs (Fig. 3; Burke & Nowicki, 2003; Chevallier, 1975; Durland et al., 2008; Evans, 2003). The abaxial hypaxial muscles derive from the lateral-most regions of the dermomyotome that extend into the lateral plate mesoderm, and their morphogenesis occurs in the context of lateral plate-derived MCT (Fig. 3; Burke & Nowicki, 2003; Durland et al., 2008).

Not only does the MCT surround epaxial and hypaxial muscles as they develop, but it is a source of signals regulating muscle development. The morphogenesis of the dermomyotome and myotome into epaxial muscles appears to be tightly correlated with changes in the extracellular matrix (Deries et al., 2012). The dermomyotome and myotome are surrounded by a laminin matrix that is remodeled at distinct stages of myotome development. When the myotome forms the epaxial muscle masses, the laminin matrix disassembles and the fibronectin matrix increases in density around the myocytes. This correlation suggests that the fibronectin matrix may regulate the morphogenesis of the epaxial muscles (Deries et al., 2012). As MCT fibroblasts are intercalated among the myogenic cells of the myotome (Deries et al., 2010), this suggests that these fibroblasts may be the source of fibronectin and thus regulate epaxial muscle morphogenesis.

Several studies have provided evidence that the MCT fibroblasts regulate the morphogenesis of abaxial hypaxial muscles. The first evidence that MCT fibroblasts play a role in hypaxial muscle morphogenesis comes from quail-chick chimera studies (Nowicki & Burke, 2000). As with most axial tissues, axial muscle expresses *Hox* genes appropriate to their anterior–posterior location. Interestingly, the *Hox* gene expression of transplanted somites and their abaxial muscle derivatives gets re-specified to match the *Hox* genes of the surrounding lateral plate mesoderm, which will give rise to MCT (Nowicki & Burke, 2000). This suggests that the MCT in the lateral plate mesoderm may be an important regulator, via *Hox* expression, of abaxial muscle morphogenesis. Subsequently, two other studies have specifically examined the role of the lateral plate mesoderm in regulating morphogenesis of abaxial muscles. Genetic deletion of the transcription factor *Pitx2* leads to the almost complete absence of abdominal abaxial muscles (external and internal obliques and transverse and rectus abdominus) (Eng et al., 2012). As *Pitx2* is most strongly expressed in the lateral plate mesoderm when

the muscle defects appear, the authors suggest that *Pitx2* in the lateral plate mesoderm, via its regulation of *Hox* genes, regulates abdominal muscle morphogenesis. A more direct test of the role of the lateral plate in muscle morphogenesis comes from an analysis of the development of the cutaneous maximus muscle, an abaxial muscle present in many mammals (but not humans) that functions to twitch the skin over the back (Pan et al., 2012). *Fat1* is an atypical Fat-like cadherin planar cell polarity molecule that is critical for development of the cutaneous maximus. While *Fat1* does have a cell–autonomous role controlling the polarity of muscle progenitor migration (Caruso et al., 2013), it also is essential in the surrounding lateral plate mesoderm for regulating spreading and differentiation of muscle progenitors (Helmbacher, 2018). Deletion of *Fat1* in the lateral plate mesoderm, via *Prx1Cre*, leads to hypoplasia of the ventral cutaneous maximus with disorganized myofibers and a reduction in the number of muscle progenitors (Helmbacher, 2018). Thus this study explicitly demonstrates that the lateral plate mesoderm (presumably the MCT fibroblasts) controls the morphogenesis of an abaxial hypaxial muscle.

In summary, during chordate and vertebrate evolution axial muscle becomes progressively more complex and allows for a greater range of motion. The muscle, tendon, and bone of the axial musculoskeletal system all derive from different regions of the somite and their close spatial relationship allows for their coordinated development. In contrast, the MCT of axial muscle arises from two different developmental sources: the somites and the lateral plate mesoderm. Similar to other regions of the embryo (see sections below), the MCT is likely a key source of patterning information that regulates axial muscle morphogenesis.

3. Cranial muscle

The evolution of the head has been a major innovation in vertebrates that concentrated the sense organs, neural tissues, and structures for feeding, breathing, and communication in the rostral region of the animal. Cranial muscles are essential for many of these functions, including feeding, breathing, talking and facial expressions (Edgeworth, 1935; Haas, 2001; Holliday & Witmer, 2007). Craniofacial muscles can be broadly divided into three groups: (1) extraocular muscles, which move and rotate the eye; (2) muscles developmentally associated with the pharyngeal arches (bilateral swellings on either side of the pharynx) that control the jaw, facial expressions, pharynx; and (3) tongue muscles (Fig. 4). Development of these
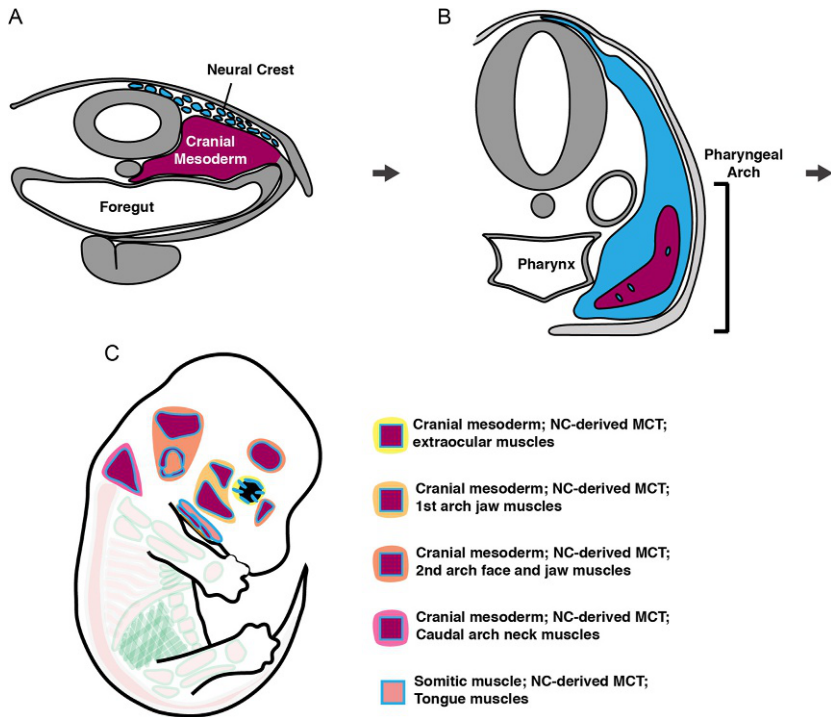
Fig. 4 Development of cranial muscle in tetrapods. (A) Transverse section through early head showing cranial neural crest migration and cranial mesoderm. (B) Transverse section through later developing head showing pharyngeal arch with a cranial mesodermal core surrounded by neural crest that has begun to infiltrate the mesoderm. (C) Groups of cranial muscles with their neural crest-derived MCT.

muscles involves the complex interplay of different embryonic tissues, and defects in these interactions are a source of cranial birth defects. Changes in development have also led to major evolutionary innovations in cranial muscles; for example, duplication of jaw muscles to increase the predation capabilities of Tetraodontoid fishes (Friel & Wainwright, 1997), evolution and diversification of facial expression muscles to allow increased modes of feeding and communication (Burrows, 2008), and the expansion of laryngeal muscles in humans to increase vocalization abilities (Diogo, Abdala, Lonergan, & Wood, 2008). Interactions between the MCT and muscle are critical for morphogenesis of cranial muscle and developmental changes in these interactions may be important for the etiology of birth defects and the evolutionary origin and modification of cranial muscles.

The head muscles and their MCT derive from several embryonic sources (Fig. 4). Most of the head muscles derive from the cranial mesoderm, mesoderm lying on either side of the neural tube and extending rostrally from the prosencephalon and caudally toward the somites. Unlike the paraxial mesoderm that segments into somites in the trunk, the cranial mesoderm is unsegmented, but does contain different regions that give rise to different cranial muscles (reviewed in Michailovici et al., 2015; Ziermann, Diogo, & Noden, 2018). The most rostral region (also termed the pre-chordal mesoderm) gives rise to the extraocular muscles. Pharyngeal mesoderm gives rise to the masticatory muscles (associated with pharyngeal arch 1), facial muscles (associated with pharyngeal arch 2), and the pharyngeal and laryngeal muscles (associated with more caudal arches). Interestingly, the pharyngeal cranial mesoderm region does not give rise exclusively to head muscles, but has also been found to give rise to major components of the heart (Lescroart et al., 2015, 2010). The tongue muscles derive from anterior somites, from which muscle progenitors migrate to the tongue region (reviewed in Diogo et al., 2015; Ziermann et al., 2018). The MCT comes from a third embryonic tissue, the neural crest. The neural crest is a migratory population derived from the dorsal neural tube and not only gives rise to the head's MCT, but also the tendons, facial bones, and many bones of the skull (Couly, Coltey, & Le Douarin, 1993; Noden, 1983b; Olsson et al., 2001; Piekarski, Gross, & Hanken, 2014). Unlike the true head muscles, the neck muscles and MCT represent a transitional region in which muscle derives from somites and cranial mesoderm and MCT derives from neural crest and the lateral plate mesoderm (Durland et al., 2008; Heude et al., 2018; Lescroart et al., 2015; Matsuoka et al., 2005; Piekarski & Olsson, 2007; Sefton, Bhullar, Mohaddes, & Hanken, 2016; Theis et al., 2010).

The development of the head muscles begins with the concurrent movement of muscle progenitors and neural crest cells and with the specification of muscle cells (Fig. 4). Muscle progenitors move into locations surrounding the eye, pharyngeal arches, and regions associated with the pharynx, larynx, and tongue. Concurrently, cranial neural crest cells collectively migrate in stereotyped streams to these same regions (Horigome et al., 1999; Landacre, 1921; Noden, 1975). In the pharyngeal region, muscle progenitors form the core of each arch, and neural crest cells circumscribe and infiltrate the muscle progenitors, leading to extensive mixing of neural crest cells and muscle progenitors (Grenier, Teillet, Grifone, Kelly, & Duprez, 2009; Hacker & Guthrie, 1998; Noden, 1983a; Trainor, Tan, & Tam, 1994). During this time, muscle progenitors begin the process of myogenesis and

are specified into myoblasts. Unlike axial or limb muscles, Pax3 and Pax7 are not the initial key regulators of myogenesis, but instead distinct regulatory networks of multiple transcription factors including Pitx2, Tbx1, Islet1, Lhx2, Nkx2.5, MyoR and Capsulin are required, but then converge on the requirement of MyoD and Myf5 for myoblast specification and Myogenin for myocyte differentiation (reviewed by Diogo et al., 2015; Harel et al., 2012; Michailovici et al., 2015; Tzahor, 2009; Ziermann et al., 2018). The initial steps of myogenesis leading up to the specification of MyoD + myoblasts are independent of signals from the neural crest (Tzahor et al., 2003).

The neural crest plays a key role in regulating the morphogenesis and pattern of cranial muscles (reviewed by Noden & Francis-West, 2006; Noden & Trainor, 2005; Ziermann et al., 2018). Initial evidence for the important role of the neural crest came from embryological transplantation experiments. When grafted in birds or amphibians to different anteroposterior levels, neural crest cells are able to dictate the pattern of muscles and skeletal structures that develop (Hörstadius & Sellman, 1946; Noden, 1983b). In contrast, prior to the emigration of cranial neural crest, all cranial mesoderm is equivalent in their potential to form different head muscles (Trainor et al., 1994; von Scheven, Alvares, Mootoosamy, & Dietrich, 2006). Extirpation of neural crest in axolotls reveals that the formation of head muscle with properly organized myofibers and origins and insertions depends on the neural crest (Ericsson, Cerny, Falck, & Olsson, 2004; Olsson et al., 2001). Experimental manipulations of chick embryos and analysis of mouse mutants further reveal that while neural crest regulates muscle morphogenesis, it is not essential for the initial steps of myogenesis. In the absence of neural crest, *MyoD+* and *Myf5+* myoblasts are specified (Rinon et al., 2007; Tzahor et al., 2003). However, neural crest via inhibition of WNT and BMP signaling regulates the proliferation, differentiation, and position of myoblasts and this, in turn, regulates the pattern of muscles formed (Rinon et al., 2007; Tzahor et al., 2003). Thus, initiation of myogenesis is independent of cranial neural crest, but subsequent patterning and proliferation rely on neural crest-derived signals.

Analyses of mouse mutants in which genes are deleted in neural crest have explicitly demonstrated that the neural crest regulates muscle morphogenesis in the head and also suggests that the MCT, which derives from the neural crest, may play a key role. First, analysis of the transcription factor *Pitx2*, which is required for development of extraocular muscles, has provided insights into neural crest function. Germline null mutations in *Pitx2*

lead to the complete loss of extraocular muscles (Gage, Suh, & Camper, 1999; Kitamura et al., 1999; Lu, Pressman, Dyer, Johnson, & Martin, 1999), but the expression of *Pitx2* in both the cranial mesoderm and the neural crest (Gage, Rhoades, Prucka, & Hjalt, 2005) made it unclear in which tissue *Pitx2* was required. Subsequently, tissue-specific deletion of *Pitx2* determined that while *Pitx2* is required in cranial mesoderm cell-autonomously for survival of muscle primordia and activation of *Myf5* and *MyoD* in extraocular muscles (Zacharias, Lewandoski, Rudnicki, & Gage, 2011), *Pitx2* is also important in the neural crest (Evans & Gage, 2005). Neural crest specific deletion of *Pitx2* via *Wnt1Cre* demonstrated that *Pitx2* is required in neural crest to regulate the orientation of extraocular muscles relative to the optic cup. In a second series of experiments, the transcription factors *Dlx5/6* were shown to be critical in neural crest cells for development of the masseter muscle. By analyzing mice null for *Dlx5/6* or its regulator *Endra*, Heude et al. (2010) determined that *Dlx5/6* in non–skeletal neural crest cells is critical for formation of this jaw muscle. Furthermore, the expression of *Dx5/6* in neural crest-derived cells closely associated with the masseter suggested that *Dlx5/6*+ MCT in particular may be critical for masseter morphogenesis. Finally, multiple studies have shown that signals from the neural crest are critical for several aspects of tongue muscle development (reviewed by Cobourne et al., 2018). The first step in tongue development is the migration of muscle progenitors following the migration of neural crest cells to the tongue bud (Han et al., 2012). Conditional deletion experiments have determined that cilia-dependent GLI processing in the neural crest is required for survival and migration of muscle progenitors into the bud (Millington et al., 2017). Once in the tongue bud, conditional mutagenesis experiments have established that reception of TGFβ signaling either through *Tgfβr2* or *Alk5* in the neural crest is essential for regulating proliferation and differentiation of tongue muscle cells (Han, Zhao, Li, Pelikan, & Chai, 2014; Hosokawa et al., 2010; Iwata, Suzuki, Pelikan, Ho, & Chai, 2013). The disorganized collagen and elastin fibers associated with the aberrant tongue muscle in *Tgfβr2* mutants further suggest that *Tgfβr2* is acting within the MCT to affect muscle morphogenesis (Iwata et al., 2013). Altogether these experiments demonstrate that the neural crest regulates the proliferation and differentiation of myoblasts and ultimately the pattern of head muscles that develop and suggests that signaling from the MCT may be key for this regulation.

Of babies born with birth defects, approximately one-third exhibit craniofacial defects (Gorlin, Cohen, & Blackburn, 1990) and usually these

defects are attributed to problems in neural crest (Trainor, 2010). Several of these birth defects include aberrant cranial muscles and comparison of these human birth defects with mouse genetic experiments suggest that defects in signaling between neural crest and muscle progenitors may underlie the etiology of these defects. Human patients with hemifacial macrosomia have unilateral underdevelopment of facial structures that develop from pharyngeal arches 1 and 2 (Heude, Rivals, Couly, & Levi, 2011). In addition to underdevelopment of bones, they also exhibit poorly developed masticatory muscles. The finding of patients with masseter muscle defects but normal jaw bones (Heude et al., 2011), coupled with the mouse genetic data on the development of the masseter (Heude et al., 2010), suggests that defects in the MCT associated with the masseter may be the cause of the defects in these patients. Another interesting example of cranial muscle defects is Axenfeld-Rieger syndrome, an autosomal dominant disorder characterized by defects in the eye (Lines et al., 2004; Meyer-Marcotty, Weisschuh, Dressler, Hartmann, & Stellzig-Eisenhauer, 2008; Semina et al., 1996), and although extraocular muscles are present, they may be smaller or have anomalous insertions (Bhate & Martin, 2012; Park, Kim, Heo, & Park, 2009). *PITX2* mutations are frequently associated with this syndrome and based on the mouse genetic analysis of the importance of *Pitx2* in the neural crest in patterning extraocular muscle insertions (Evans & Gage, 2005), it is possible that defects in the neural crest are responsible for this phenotype.

The head muscles have undergone substantial changes during vertebrate evolution and developmental modifications in neural crest are likely to be important drivers of these morphological changes. Comparative studies and interspecific transplantation experiments have demonstrated that species-specific muscle patterning information resides within the neural crest. Studies in amphibians provided the first evidence that the neural crest were the source of species-specific information in the head musculoskeleton; neural crest transplants from a toad into a newt resulted in a donor-specific cartilage as well as the induction of a host-derived, but toad-specific muscle (Wagner, 1959). Subsequent data largely come from the study of bird development. Quails and ducks have distinctly different jaw skeletal and muscle morphologies, associated with their different feeding habits (Tokita & Schneider, 2009). Transplantation of quail cranial neural crest into a duck host resulted in the production of quail-derived skeleton and MCT that transformed duck-derived muscle to acquire a quail-like shape and attachment sites (Tokita & Schneider, 2009). This establishes that, similar to amphibians, species-specific patterning information resides within the neural crest. Careful analysis of

this transformation process revealed that the expression levels and location of the MCT marker, *Tcf4*, and tendon marker, *Scx*, reflected the donor neural crest program. Furthermore, analysis of cranial myogenesis and muscle morphogenesis showed that the neither muscle specification nor differentiation was changed by the presence of quail–derived neural crest, but the shape and attachment sites of muscle were transformed. This suggests that the neural crest-derived MCT and tendons determine the species–specific shape and attachment of head muscles. Subsequently, developmental studies of parrots, which have acquired two anatomically unique jaw muscles to allow for feeding on hard-shelled nuts and seeds, suggest that changes in the neural crest-derived MCT and tendon may also lead to the evolution of entirely new muscles (Tokita, Nakayama, Schneider, & Agata, 2013). Altogether these studies strongly implicate an important role for the neural crest in determining the species-specific pattern of cranial muscles and suggest that changes in neural crest development may be the underlying driver of the evolutionary modification and generation of new cranial muscles.

4. Limb muscle

The origin and diversification of paired appendages have played critical roles in vertebrate evolution (Fig. 1G–K). Muscle is a crucial component of fins and limbs, as the number, arrangement, and anatomy of muscles has been modified to enable activities such as swimming, running, flying, and manipulating tools. Tetrapod limbs contain over 40 distinct muscles and each muscle extends over one or two joints, attaching to bone via tendons that originate proximally and insert distally (Fig. 1J and K). Muscles are organized into three proximal–distal groups associated with the upper arm/thigh, forearm/shank, and hand/foot, and each of these groups has a dorsal subdivision that extends or ventral subdivision that flexes the limb (Fig. 5). Compared with other regions of the body, the morphogenesis of limb muscles has been the most intensively studied because of the accessibility of the limb to surgical manipulation in chick embryos, identification of genetic reagents in mice to manipulate components of the limb musculoskeleton, and the nonessential requirement of the limb for viability. These studies have revealed that an assemblage of transcription factors expressed in the MCT is critical for limb muscle morphogenesis. In addition, recent evidence suggests that mutations in these transcription factors are a cause of congenital limb abnormalities that include muscle defects and changes
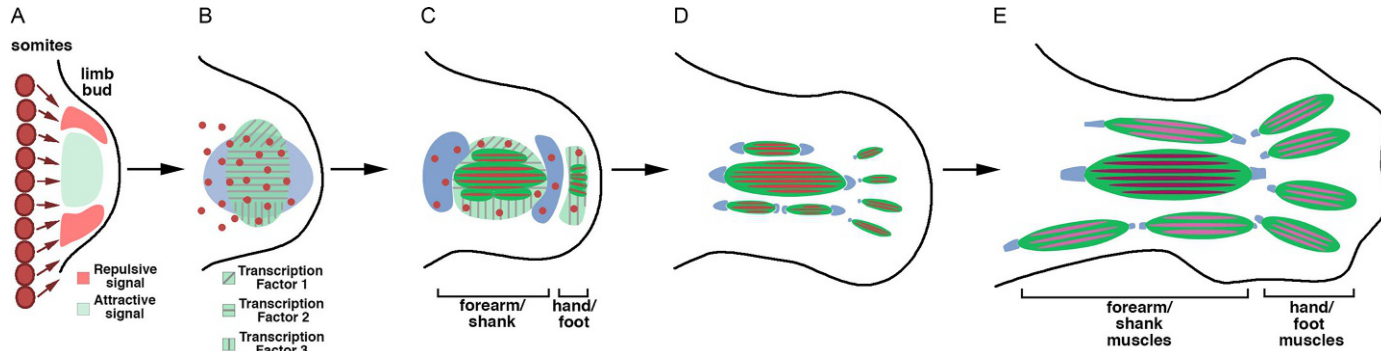
Fig. 5 Development of limb muscles in tetrapods. (A) Muscle progenitors (red) migrate from limb-level somites into the core of the limb, which contains attractive HGF and SDF signals, and avoids peripheral regions, which contains repulsive EphrinA5 signals. (B) Muscle progenitors populate MCT (green) regions that express an array of transcription factors (Tbx3–5, Tcf4, Osr1, Hoxa11/d11) and tendon (blue) regions. (C) Muscle progenitors that migrate into tendon regions do not differentiate, while those that migrate into MCT regions do differentiate. (D–E) In MCT regions muscle progenitors differentiate, and so the MCT pattern prefigures the future pattern of anatomical muscles. Tendons develop at the origin and insertion ends of forming muscles and their associated MCT.

in the expression of these factors can lead to the evolution of novel modifications of limb muscles.

The limb's muscle and MCT originate from two embryonic sources that are each accessible to surgical manipulation in chick and Cre-mediated manipulation in mouse (Fig. 5). Limb muscle derives from the ventrolateral dermomyotome of somites lying adjacent to the developing limb buds (Chevallier, Kieny, & Mauger, 1977; Christ, Jacob, & Jacob, 1977; Ordahl & Le Douarin, 1992). Quail-chick transplantations and chick viral lineage studies have provided a detailed map of which somites contribute to particular muscles in the fore and hind limbs (Beresford, 1983; Kardon, Campbell, & Tabin, 2002; Lance-Jones, 1988). Quail-chick studies have also determined that the lateral plate mesoderm proliferates to form the limb bud and the MCT, tendons, ligaments, and bones of the limb (Chevallier et al., 1977; Christ et al., 1977). In addition, more recent viral lineage studies have determined that the limb mesoderm gets progressively specified into the various cells types, with cartilage and perichondral cells coming from a common lineage and MCT and tendon deriving from another lineage (Pearse et al., 2007). The different embryonic sources of muscle and MCT have allowed each of these tissues to be individually genetically manipulated. $Pax3^{Cre}$ and $Pax7^{Cre}$ (and tamoxifen-inducible $Pax7^{CreERT2}$) have been used to lineage trace and manipulate muscle progenitors and their derivatives (Engleka et al., 2005; Keller, Hansen, Coffin, & Capecchi, 2004; Lepper, Partridge, & Fan, 2011; Murphy et al., 2011); $Pax3^{Cre}$ recombines in the earliest muscle progenitors, but also recombines in somitically-derived endothelial cells that populate the limb, while $Pax7^{Cre}$ and $Pax7^{CreERT2}$ recombine slightly later, but exclusively in muscle progenitors (Hutcheson, Zhao, Merrell, Haldar, & Kardon, 2009). The $Prx1Cre$ transgene (and tamoxifen-inducible $Prx1CreERT2$) recombines in the lateral plate-derived mesoderm of the limb and so has been a useful reagent to manipulate the limb's MCT, tendons, and bones (Hasson et al., 2010; Logan et al., 2002).

The first step in the development of limb muscles is the emigration of muscle progenitors from the somites into the limb, and attractive and repulsive signals from the limb bud mesoderm are critical for this process (Fig. 5A and B). Two signaling molecules have been found to act as chemoattractants for muscle progenitors. The ligand Hepatocyte Growth Factor (HGF) is strongly expressed in the limb mesoderm, while the migrating muscle progenitors express the receptor tyrosine kinase MET (Adachi, Pascual-Anaya, Hirai, Higuchi, & Kuratani, 2018). Genetic loss-of-function experiments in mice and HGF bead experiments in chick demonstrate that HGF/MET

signaling is necessary and sufficient to guide muscle progenitors into the limb (Bladt, Riethmacher, Isenmann, Aguzzi, & Birchmeier, 1995; Brand-Saberi, Müller, Wilting, Christ, & Birchmeier, 1996; Dietrich et al., 1999; Heymann, Koudrova, Arnold, Köster, & Braun, 1996). A second chemokine ligand Stromal Cell–Derived Factor 1 (SDF1 or Cxcl12) expressed in the limb bud mesoderm and its receptor Cxcr4 expressed in muscle progenitors also regulate muscle migration (Vasyutina et al., 2005). SDF1 bead experiments in chick and *Cxcr4* null mice show that SDF1/Cxcr4 signaling positively regulates the migration and survival of muscle progenitors into the limb. In addition to these positive regulators of migration, Ephrina5/Epha4 signaling negatively regulates limb muscle migration, as the ligand Ephrina5 expressed in the peripheral limb bud mesoderm repulses receptor Epha4-expressing muscle progenitors from populating these regions of the limb (Swartz, Eberhart, Pasquale, & Krull, 2001). Thus a combination of attractive HGF and SDF1 ligands expressed in the central limb and repulsive Ephrina5 ligand in the limb periphery determine where receptor Met+, Cxcr4+, Epha4+ muscle progenitors migrate to and ultimately reside in the limb (Fig. 5B). Although not yet explicitly tested, HGF and SDF1 are likely expressed in MCT fibroblasts or their precursors.

After entering the limb, muscle progenitors undergo a complex process of morphogenesis eventually resulting in the complex pattern of limb muscles. A detailed descriptive analysis of this process in developing chick hind limbs reveals that muscle progenitors initially form dorsal and ventral masses that gradually become subdivided into three dorsal and ventral muscle masses associated with the three proximal–distal limb regions and then these six masses subdivide into individual anatomical muscles (Fig. 5; Kardon, 1998). Early experiments in the chick suggested that the MCT may be an important regulator of this morphogenetic process. In particular, MCT forms in a muscle pre-pattern even in the absence of muscle, and MCT can organize even non-muscle cells to form muscle-like structures (Grim & Wachtler, 1991; Jacob & Christ, 1980; Lanser & Fallon, 1987). Subsequently, Tcf4 was identified as the first gene expressed in MCT of the limb in a muscle pre-pattern, independent of muscle (Kardon et al., 2003). Furthermore, virally expressed ectopic Tcf4 in chick limb non-muscle mesoderm led to induction of ectopic muscle and disruption of Tcf4 led to muscle mis-patterning (Kardon et al., 2003). These data suggested a model whereby the lateral plate-derived MCT establishes a pre-pattern in the limb that determines where muscle progenitors differentiate and ultimately the number and location of limb muscles (Fig. 5C–E).

Elucidation of the important role of MCT in regulating limb muscle morphogenesis has been facilitated by the identification in mouse of transcription factors that are expressed in the MCT and functionally important in establishing the pattern of limb muscles. Six transcription factors are expressed in different limb MCT regions. The T-box transcription factors Tbx5 and Tbx4 are expressed throughout the mesoderm of fore and hind limbs, respectively, including the MCT (Hasson, Del Buono, & Logan, 2007; Naiche & Papaioannou, 2007), while Tbx3 is expressed in the anterior and posterior MCT, posterior bones and a subset of bone eminences (eminences are the protusions on bones to which tendons attach; Colasanto et al., 2016). Hoxa11 is expressed in the MCT and also in the tendons and perichondrium of the forearm and shank regions of the fore and hind limbs (Swinehart, Schlientz, Quintanilla, Mortlock, & Wellik, 2013). The odd skipped-related 1, Osr1, transcription factor is expressed in multiple MCT regions of the fore and hind limb and also in the synovial joints (Stricker, Brieske, Haupt, & Mundlos, 2006; Vallecillo-García et al., 2017). Finally, analysis in mouse shows that Tcf4 is expressed in MCT throughout the limb (as well as some bones and bone eminences), but Tcf4 lineage studies show that it is most strongly expressed in MCT near the elbow and knee (Colasanto et al., 2016; Mathew et al., 2011). Genetic deletion of these genes leads to defects in the muscles in regions where these genes are expressed. Thus genetic deletion of *Tbx5* and *Tbx4* leads to mis-patterned fore and hind limb muscles, respectively (Hasson et al., 2010); compound mutants of *Hoxa11* and *Hoxd11* paralogue cause defects in forearm and shank muscles (Swinehart et al., 2013); mutations in *Tcf4* lead to muscle truncations near the knee (Mathew et al., 2011); and *Osr1* null mice have various muscle defects (Vallecillo-García et al., 2017). The phenotype of *Tbx3* mutants is most striking because two specific anterior muscles, the lateral triceps and brachialis, are absent in these mice (Colasanto et al., 2016). In addition to affecting the size and shape of muscles, Tcf4 also affects myofiber maturation and fiber type (Mathew et al., 2011). It is thought that all of these genes primarily function in the MCT, and at least in the case of Tbx3 and Tcf4 it has been demonstrated that these genes do not function cell-autonomously within muscle cells (via *Pax3^{Cre}* deletion; Colasanto et al., 2016; Mathew et al., 2011). However, it should be noted that none of these transcription factors are exclusively expressed in nor have been specifically deleted just in the MCT and so may function in other tissues (e.g., tendons, bones, or bone eminences) to regulate muscle patterning.

The array of transcription factors expressed in the MCT regulates limb muscle morphogenesis through several mechanisms. Studies of Tbx3, Tbx5, and Osr1 examined when loss of these genes affected muscle development and all found that defects appeared by E12.5, 2 days before the pattern of limb muscles is established at E14.5 (Colasanto et al., 2016; Hasson et al., 2010; Vallecillo-García et al., 2017). Interestingly, while Osr1 and Tbx5 cell non-autonomously regulate the number and pattern of MyoD + myoblasts at E12.5, Tbx3 does not regulate the number of myoblasts, but rather their differentiation into myofibers. There are at least three mechanisms by which these transcription factors may act within MCT fibroblasts to ultimately affect muscle morphogenesis. First, as found with Osr1, the transcription factors may regulate MCT fibroblast cell fate, and in the absence of *Osr1* lateral plate-derived limb mesodermal cells adopts a more chondrogenic or tendon-like fate (Vallecillo-García et al., 2017). Second, the transcription factors may regulate the number and/or pattern of MCT fibroblasts in particular limb regions, and in *Hoxa11/d11* mutants alterations in the pattern of Tcf4 + MCT fibroblasts are present (Swinehart et al., 2013). Finally, the transcription factors may regulate the MCT fibroblasts' secretion of signaling molecules or extracellular matrix that regulate the development of neighboring muscle cells; in *Tbx5* mutants MCT fibroblasts express less and more disorganized N-Cadherin and β-Catenin (Hasson et al., 2010) and OSR1 regulates a large number of secreted signals (e.g., SDF and Bmp4) and extracellular matrix proteins (e.g., Col6; Vallecillo-García et al., 2017).

Analysis of mutations of the transcription factors expressed in the MCT also provides insights into the relationships between the developing MCT and muscle with the developing tendons and bones. Analysis of *Hoxa11/d11*, *Tbx5*, and *Tbx3* mutants indicates that while muscle and MCT development are de-coupled from bone development, they are tightly linked with tendon and bone eminence development. In particular, in *Hoxa11/d11* compound mutants with one wild-type allele of either gene muscle and associated tendons in the forearm and shank are highly aberrant, but the bones appear normal (Swinehart et al., 2013). Careful analysis of the temporal requirements for *Tbx5* and *Tbx4* indicates that while these genes are required early (prior to E10.5–11.5) to regulate hind and fore limb bones, at later developmental times these genes coordinately regulate muscle and tendon morphogenesis without affecting bones (Hasson et al., 2010). However, a similar study of the temporal requirements of *Tbx3* indicates that while *Tbx3* is required early (prior to E9.25) for ulna and posterior digit development, the degree of development of the lateral triceps and brachialis

muscles is tightly linked with the degree of development of their bone eminence attachment sites (Colasanto et al., 2016). Together these data, as well as lineage studies in the limb (Pearse et al., 2007), suggest that the long bones of the limb are specified and patterned early, while the MCT, tendons, and bone eminences are specified and undergo coordinated morphogenesis later in limb development.

In summary, an array of transcription factors are expressed in the lateral-plate derived mesoderm in different regions that are critical for patterning the musculoskeletal system. Some of these factors (e.g., Tbx3, 4, and 5) are expressed and required early in the limb (prior to E11.5 in mouse) for proper development of the long bones. All of the factors are expressed in the MCT as well as other lateral plate derivatives, such as the tendons and bone eminences. The expression of these transcription factors in the MCT is critical for regulating the morphogenesis of neighboring muscles and the expression of single or overlapping sets of transcription factors may function as a molecular address specifying particular muscles. The specification of two specific muscles by Tbx3 supports such a hypothesis (Colasanto et al., 2016). Mechanistically, these transcription factors act either by regulating the specification of MCT fibroblasts, the number and pattern of these fibroblasts, and/or the signaling or extracellular matrix molecules secreted by these fibroblasts. The MCT fibroblasts, in turn, determine the pattern of muscles that forms via their regulation of the number, survival, and/or differentiation of muscle progenitors. Finally, the coordinate morphogenesis of MCT, with muscle, tendon, and bone eminences, ensures that a functional musculoskeleton develops.

The MCT is not only important for regulating the normal development of limb muscles, but defects in MCT likely underlie the abnormal muscles found in congenital limb abnormalities. Mutations in *TBX3* and *TBX5* have been identified as the cause of two congenital limb syndromes, Ulnar–Mammary syndrome and Holt-Oram syndrome (Bamshad et al., 1997; Basson et al., 1997; Li et al., 1997). Individuals with Ulnar–Mammary syndrome exhibit a variety of limb phenotypes, ranging from the absence of the forearm and hand, to posterior bone defects, including loss of the ulna and/or posterior digits (Bamshad et al., 1999, 1997). Individuals with Holt-Oram syndrome are characterized by deformities in the forelimb, ranging from malformations of carpal bones, thumb abnormalities or phocomelia (Basson et al., 1997). In addition to the obvious bone defects, muscle defects are also associated with both syndromes. Individuals with Holt-Oram syndrome have been found to have hypoplastic pectoralis major, deltoid, and shoulder muscles (Newbury-Ecob, Leanage, Raeburn, & Young, 1996), and recently at least one individual

with Ulnar–Mammary syndrome has completely lost the lateral triceps in one limb (Colasanto et al., 2016). Based on the genetic data from mouse mutants (Colasanto et al., 2016; Hasson et al., 2010), it is likely that loss of either *Tbx5* or *Tbx3* expression in the MCT is the cause of these muscle defects.

During vertebrate evolution fins and limbs have been subject to many evolutionary modifications, including changes in their musculature. One example of limb evolution is the striking variation in the feathering of feet in domestic pigeons, with some birds exhibiting many large feathers (Domyan et al., 2016). Intriguingly, the presence of feathered feet is also accompanied by changes in the leg musculature (Domyan et al., 2016). Underlying these morphological changes are genetic changes such that the presence of feathered feet and changed musculature results from a partial transformation of the hindlimb to a forelimb mediated by the decreased expression of the normally hindlimb-specific transcription factor *Pitx1* and the increased expression of the normally forelimb-specific *Tbx5*. Based on the data in mouse supporting a role of Tbx5 in MCT regulating forelimb muscle morphogenesis, it is likely that the increased expression of Tbx5 in the MCT during development of feathered footed pigeons is the cause of the morphological changes in their limb muscles. Potentially other changes in the genetic regulation of developing MCT underlie evolutionary changes in limb musculature.

5. Diaphragm

The diaphragm is a unique mammalian muscle that has been crucial for the evolutionary success of mammals. It is a domed muscle lying at the base of the thoracic cavity that is essential for the inspiratory phase of respiration and for separating the abdominal contents from the overlying thoracic cavity containing the heart and lungs (Merrell & Kardon, 2013). In humans, the common birth defect, Congenital Diaphragmatic Hernia (CDH), highlights the diaphragm's critical function. In CDH, the diaphragm fails to form properly and weaknesses in the diaphragm allow abdominal contents to herniate into the thoracic cavity and impede lung development. The resulting lung hypoplasia leads to 50% mortality and high morbidity and thus demonstrates the importance of proper diaphragm development (Kardon et al., 2017; Merrell & Kardon, 2013). The diaphragm is not only critical for humans, but also for all mammals. The evolution of mammals involved the appearance of tidally ventilated, alveolar lungs and increased aerobic requirements (Farmer, 2015; Perry, Similowski, Klein, & Codd, 2010). It is thought that

the evolution of the diaphragm in mammals was essential for obtaining maximal lung function, as the diaphragm both opens the thoracic cavity during inspiration (allowing for influx of air) and keeps the abdominal contents caudal. Thus understanding the development of the diaphragm has important implications for human health and the overall evolutionary success of mammals. Strikingly, recent studies show that the MCT is critical for the development of the diaphragm, an important cellular source of CDH, and may play a key role in the evolution of the diaphragm.

During development the diaphragm's muscle and MCT originate from two different embryonic sources. Similar to axial and limb muscles, the diaphragm's muscle arises from the somites and recently was shown to specifically come from cervical-level somites (Sefton, Gallardo, & Kardon, 2018). The diaphragm's MCT comes from the pleuroperitoneal folds (PPFs), a lateral plate-derived pyramidal tissue located between the thoracic (pleural) and abdominal (peritoneal) cavities (Fig. 6A). Histological, immunofluorescent, and genetic lineage studies in mice reveal that the PPFs expand dorsally and ventrally to give rise to the diaphragm's MCT and central tendon (Fig. 6; Babiuk, Zhang, Clugston, Allan, & Greer, 2003; Merrell et al., 2015; Sefton et al., 2018). The muscle progenitors migrate from the cervical somites to the PPFs, and as the PPFs spread the muscle cells are carried throughout the nascent diaphragm and differentiate into the radially oriented myofibers of the costal diaphragm in close association with the MCT (Fig. 6; Merrell et al., 2015; Sefton et al., 2018).

Multiple studies demonstrate that the PPFs, and likely the PPF-derived MCT, control development of the diaphragm and are the cellular source of CDH. The normal morphogenetic expansion of the PPFs ahead of muscle (and also nerve and vasculature; Merrell et al., 2015; Sefton et al., 2018), as well as their expansion in the absence of muscle (Merrell et al., 2015) suggested that the PPFs regulate the morphogenesis of the diaphragm's muscle. Further studies conditionally deleting the CDH-associated genes, *Gata4*, *WT1*, and *β-catenin*, in specific embryonic tissues have now definitively shown that mutations in the PPFs (or their associated mesothelium) lead to defective diaphragm development and hernias (Carmona, Cañete, Cano, Ariza, & Muñoz-Chápuli, 2016; Merrell et al., 2015; Paris, Coles, & Ackerman, 2015) and so establish that PPFs regulate normal diaphragm development and are a cellular source of CDH. Detailed analysis of *Gata4* additionally revealed that the PPFs regulate the proliferation and survival of adjacent muscle progenitors, and because the PPF cells closest to the muscle are PPF-derived MCT fibroblasts, this suggests that these fibroblasts are the
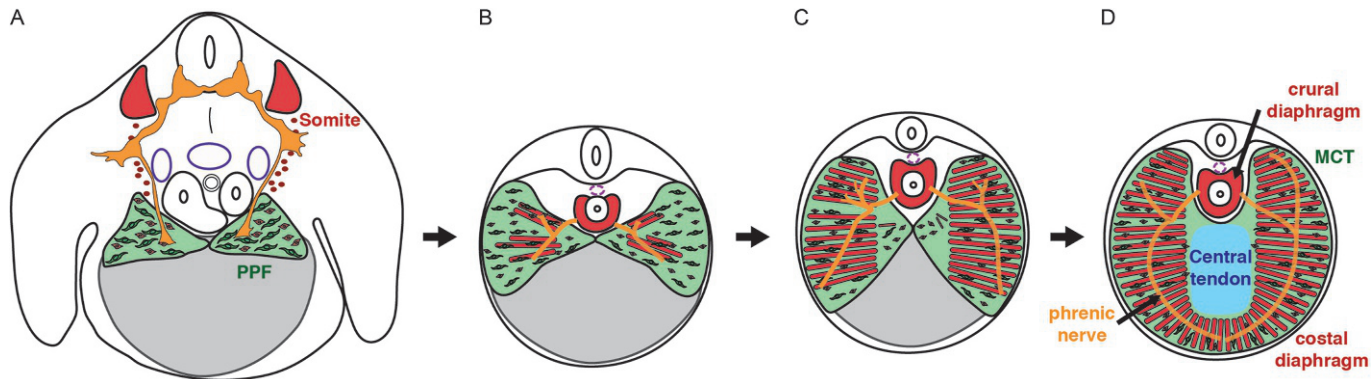
Fig. 6 Development of the mammalian diaphragm. (A) Muscle progenitors (red) migrate from cervical somites to the pleuroperitoneal folds (PPFs, green). The PPFs are also the target for the axons of the phrenic nerve (orange). (B–C) As PPFs spread dorsally and ventrally they carry the muscle progenitors, which differentiate into radially oriented myofibers. Outgrowth of phrenic nerves is also regulated by PPF expansion. (D) PPFs give rise to the MCT guiding expansion and differentiation of muscle and ultimately surrounding myofibers of the costal diaphragm.

critical regulators of the diaphragm's muscle (Merrell et al., 2015). Thus PPF–derived MCT fibroblasts guide normal diaphragm muscle development and defects in MCT development or MCT–muscle communication lead to CDH. What precisely is the molecular communication between the MCT fibroblasts and the muscle progenitors remains an open question.

How the diaphragm evolved at the origin of mammals during the Permian era is a major unanswered question (Farmer, 2015; Perry et al., 2010), but studies of diaphragm development in mouse suggest that the PPFs may be critical for diaphragm evolution. One way to elucidate the evolutionary origin of the diaphragm is to compare the developmental innovations present in mammals, but absent from birds and reptiles, which do not have a muscularized diaphragm. While cervical-level somites give rise to neck and limb muscles in birds, reptiles, and mammals, it is only in mammals that muscle progenitors migrate to the nascent diaphragm (Hirasawa, Fujimoto, & Kuratani, 2015; Hirasawa & Kuratani, 2013; Sefton et al., 2018). Thus recruitment of muscle progenitors from cervical-level somites to this region is a crucial event uniquely present in mammals. In mammals, the PPFs not only are the target of the migrating muscle progenitors, but they subsequently guide muscle morphogenesis (Babiuk et al., 2003; Hitachi et al., 2009; Merrell et al., 2015; Sefton et al., 2018). This suggests that the PPFs, which give rise to the diaphragm's MCT, may be the developmental innovation that allowed for the formation of a muscularized diaphragm. Critical to determine will be whether mammals are unique in their possession of PPFs or whether PPFs are present more broadly in amniotes, but only in mammals are the PPFs able to recruit muscle progenitors and guide their morphogenesis.

6. Conclusion

Muscles in different regions of the vertebrate body have evolved distinct anatomical structures and functions, but in all regions muscle connective tissue plays a central role in regulating muscle morphogenesis and determining the pattern of muscles that develop. The muscles in the trunk, head, limb, and diaphragm are composed of similar multinucleate post-mitotic myofibers, but they differ in the contractile and metabolic properties of their myofibers, the size and organization of myofibers, and the overall shape and attachment to tendons and bones. The generation of myofibers by the process of myogenesis is largely similar in axial, cranial, limb, and diaphragm muscles; following a heterogeneous network of genes regulating the development of muscle progenitors, the molecular and cellular processes of myoblast specification,

differentiation, and fusion into myofibers are similar in all muscles (Fig. 2C). However, it is during the process of muscle morphogenesis that the distinctive pattern and anatomy of different muscles emerge, and MCT plays a crucial role in this developmental process. The MCT of the trunk, head, limbs, and diaphragm arises from different embryonic tissues—somites, neural crest, lateral plate mesoderm, and pleuroperitoneal folds—but nevertheless they function equivalently. In all regions, MCT does not regulate the initial development of muscle progenitors or their specification into myoblasts. Instead, MCT regulates several aspects of muscle morphogenesis. First, in the head, limbs, and diaphragm, MCT guides migration of muscle progenitors to target regions likely, as indicated by limb experiments (Bladt et al., 1995; Brand-Saberi et al., 1996; Dietrich et al., 1999; Heymann et al., 1996; Swartz, Nguyen, Mccarthy, & Eberhart, 2012; Vasyutina et al., 2005), by a combination of attractive (e.g., HGF, SDF), repulsive (e.g., Ephrin/Eph), and potentially permissive signals. Once muscle progenitors populate target regions, the MCT then locally promotes the proliferation, survival, and differentiation of neighboring myoblasts into myofibers. These localized regions of differentiated myofibers form the nucleus of anatomical muscles. Thus an attractive model is that the MCT serves as a mesodermal pre-pattern that determines the sites of myofiber differentiation and ultimately determines the number, location, size, and shape of muscles. Consistent with such a model, development of the MCT in the limb and diaphragm is independent of muscle development (Kardon et al., 2003; Merrell et al., 2015; Vallecillo–García et al., 2017). The data in the limb suggest that spatially complex and overlapping sets of transcription factors expressed in MCT fibroblasts in different regions may function as molecular addresses that specify particular muscles. The specification of two specific muscles by the transcription factor Tbx3 in the forelimb supports such a notion (Colasanto et al., 2016). How such genes expressed in MCT fibroblasts ultimately regulate muscle morphogenesis is unclear. These genes may regulate MCT cell fate (e.g., Osr1; Vallecillo-García et al., 2017), regulate the number and pattern of MCT fibroblasts, and/or the secretion by MCT fibroblasts of signaling molecules or extracellular matrix proteins (e.g., Hasson et al., 2010; Iwata et al., 2013) that regulate myoblast proliferation, survival, and/or differentiation. The experiments on MCT regulation of muscle morphogenesis also shed light on how the different components of the musculoskeletal system are assembled. Experiments in the limb and head (Colasanto et al., 2016; Hasson et al., 2010; Heude et al., 2010) suggest that specification of bones precedes and their development can be de-coupled from the rest of the musculoskeleton, while the development

of the muscles, MCT, tendons, and bone eminences is tightly linked. The tight developmental linkage between muscles, MCT, tendons, and their bone attachment sites ensures that a functional musculoskeletal system develops.

While much progress has been made in our understanding of muscle morphogenesis, particularly with reference to limb muscles, many outstanding questions remain. First, the array of transcription factors in the limb suggests that overlapping sets of genes expressed in the MCT in different domains of the vertebrate body may be critical for specifying the pattern of muscles that develop. However, our current understanding of gene expression in MCT fibroblasts and their regulation of muscle patterning does not explain the complex array of axial, head, and limb muscles present. Second, our understanding of how MCT fibroblasts regulate the proliferation, survival, and differentiation of myoblasts is limited. For instance, the very first myofibers to differentiate develop in an orientation that prefigures the position of the future muscle (Kardon, 1998), and so this initial myofiber orientation determines the origin and insertion and therefore the function of the muscle that develops. Yet, we have little understanding of the molecular or cellular processes that determine where myofibers differentiate and their orientation. Finally, the coupled development of muscle, MCT, tendons, and bones is critical for development of a functional musculoskeleton. While signals from the MCT are critical for coupling muscle and MCT development, it is unclear how MCT, tendons, and bones are specified from a common embryonic source (e.g., somites in the trunk, neural crest in the head, or lateral plate in the limb) and then their development subsequently coordinated. In fact, one of the major impediments to studying MCT has been the lack of molecular markers and genetic reagents that are specific for MCT. To date, all MCT markers and reagents label MCT as well as at least one other component of the musculoskeleton, e.g., tendon or bone eminence, and this likely reflects their close lineage relationship. Finding unique molecular markers and creating more specific MCT Cre lines is a challenge for future research.

Birth defects in the development of the head, trunk, limbs, and diaphragm commonly arise in humans. Defects in development of the diaphragm muscle have long been recognized since CDH is both common and has major health consequences (Lally, 2016). In contrast, while birth defects in bone development have been readily recognized in the head and limbs, it is only more recently that abnormalities in cranial and limb muscle development have been identified (Colasanto et al., 2016; Heude et al., 2011; Newbury-Ecob et al., 1996). Strikingly, mouse models of CDH and cranial and limb birth defects

suggest that MCT fibroblasts are an important cellular source for these muscle defects (Colasanto et al., 2016; Hasson et al., 2010; Heude et al., 2010; Merrell et al., 2015). Thus increasing our understanding of the normal role of MCT in regulating muscle morphogenesis will be essential for elucidating the molecular and cellular processes underlying the etiology of these birth defects.

Finally, the progressive modification of the form and function of different muscles and the acquisition of new muscles have been instrumental to the evolutionary success of vertebrates. How muscles have been modified or new muscles originate during evolution has been an outstanding question. Recent comparative studies in birds suggest that developmental modifications of the MCT have been key to evolutionary changes in their jaw musculature and have allowed birds to exploit new food sources (Tokita et al., 2013; Tokita & Schneider, 2009). Developmental innovations in the MCT may also underlie the evolution of novel muscles, such as the syringeal muscles in songbirds and the diaphragm in mammals. Future studies of the development of their MCT and muscle should provide insights into the mechanisms underlying the evolutionary origin of these important muscles.

In summary, the MCT is essential for regulating morphogenesis of muscles throughout the body; defects in MCT development have emerged as a source of congenital birth defects in head, limb, and diaphragm muscles; and developmental changes in MCT may have been key developmental innovations that enabled the evolution of the vertebrate musculature. Therefore the MCT serves as a critical link for understanding muscle development, birth defects, and evolution.

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