


Ca²⁺ as a coordinator of skeletal muscle differentiation, fusion and contraction

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Muscle regeneration is essential for vertebrate muscle homeostasis and recovery after injury. During regeneration, muscle stem cells differentiate into myocytes, which then fuse with pre-existing muscle fibres. Hence, differentiation, fusion and contraction must be tightly regulated during regeneration to avoid the disastrous consequences of premature fusion of myocytes to actively contracting fibres. Cytosolic calcium (Ca²⁺), which is coupled to both induction of myogenic differentiation and contraction, has more recently been implicated in the regulation of myocyte-to-myotube fusion. In this viewpoint, we propose that Ca²⁺-mediated coordination of differentiation, fusion and contraction is a feature selected in the amniotes to facilitate muscle regeneration.

Introduction

The formation of multinucleated muscle fibres is essential for myogenesis from flies to humans. Myogenesis initiates with the activation and asymmetric cell division of muscle stem cells to generate both committed myoblasts and muscle progenitors to maintain the stem cell pool [1,2]. Myoblasts subsequently differentiate into post-mitotic bipolar mononucleated myocytes that fuse, forming multinucleated myotubes [3–8]. Myotubes mature into myofibers through the assembly of the contractile apparatus (myofibrillogenesis) [9–11] (Fig. 1; Box 1).

After embryonic myogenesis, the muscle progenitors localize between the plasma membrane (sarcolemma) and basement membrane of the myofibers and are, therefore, called satellite cells (SCs) [1,2,20]. In response

to muscle injury, SCs activate and divide asymmetrically to maintain the SC pool and expand a population of myoblasts that will differentiate into myocytes and repair the pre-existing fibres [18,21,22]. Myocytes either fuse with each other (primary myogenesis) or with the pre-existing fibres [3,5,6,8]. Distinct stages of myogenesis are defined by the expression of specific transcription factors and myogenic regulatory factors (MRFs) in SCs, myoblasts and myocytes (Fig. 1).

SC activation, myogenic differentiation and fusion

Activated SCs downregulate Sprouty1 (Spry1), a receptor tyrosine kinase signalling inhibitor, and divide

Abbreviations

CRGs, calcium-responsive genes; DysF, dysferlin; EC, excitation–contraction; ER, endoplasmic reticulum; IGF1, insulin growth factor 1; Mef2C, myocyte enhancer factor 2C; MRF, myogenic regulatory factors; MYMK, Myomaker; MYMX, Myomixer; MyoD, myogenic determination 1; MyoF, myoferlin; MyoG, myogenin; MyoM1/2/3, myomesin; MyoZ1/2/3, myozenin; NFAT, nuclear factor of activated T cells; PAX7, paired box 7; PM, plasma membrane; RyR, ryanodine receptor; SC, satellite cell; SOCE, store-operated Ca²⁺ entry; SR, sarcoplasmic reticulum; STIM, stromal interaction molecule.

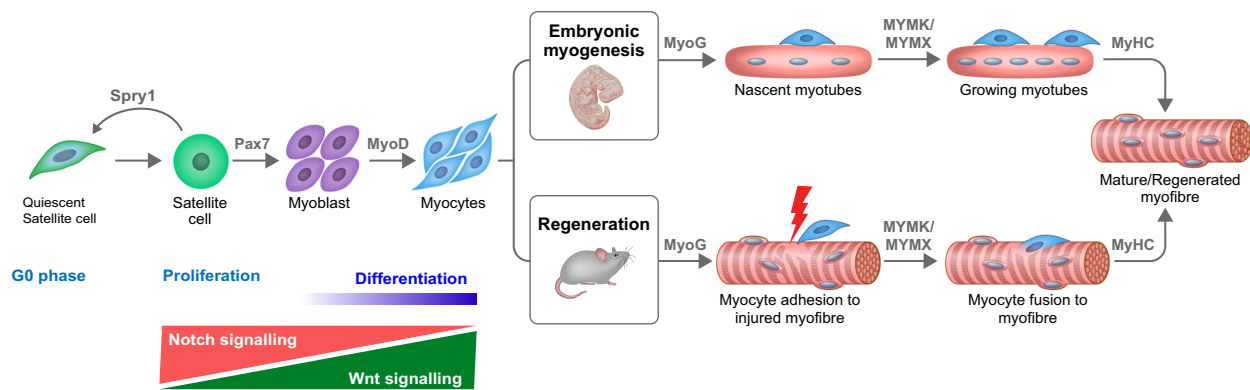


Fig. 1. Schematics of the role of myoblast fusion during embryonic myogenesis and regeneration across vertebrates. The figure shows distinct stages of myogenesis along with stage-specific marker proteins. As activated SCs enter the differentiation programme, they undergo significant morphological changes to generate myotubes that eventually mature into myofibers. Activated SCs divide asymmetrically to generate both quiescent and activated SCs. The upregulation of Wnt signalling and downregulation of notch signalling pathways contribute to the exit from senescence and the initiation of differentiation in activated SCs. Activated SCs generate MyoD-expressing myoblasts that form terminally differentiated myocytes. Myocytes fuse with other myocytes during embryonic myogenesis and with pre-existing myofibers during regeneration. The multinucleated myotubes then mature to contractile myofibers via myofibrillogenesis.

Box 1. Myotube maturation

During maturation, the myotubes express MyHC and α -actin (Acta2) [12]. The filaments of actin and myosin are arranged in parallel to form the myofilaments of each myofibril. The formation of aligned myofibrils within myotubes leads to their maturation into contractile myofibers [9–11]. Each myofiber is covered by an excitable membrane called the sarcolemma, and its cytoplasm is called sarcoplasm [13]. While myosin forms the thick filament, actin forms the thin filament and originates at the Z-disk. The area between the two consecutive Z-disks is called a sarcomere, the basic contractile unit of a myofiber. The M-band is the transverse structure at the centre of the sarcomere that is formed by antiparallel dimers of Myomesin. Myomesin facilitates myosin filaments crosslinking, which is necessary for withstanding the stronger tension during muscle contraction [14–16]. Within a sarcomere, the alignment of actin and myosin is maintained by regulatory proteins Titin and Nebulin [9,12,17]. During contraction, the Z-disks move towards each other, and actin and myosin filaments slide over each other [12]. During the later stages of maturation, the sarcolemma invaginates around the sarcomeres as the transverse tubular system (T-tubules) and ER align itself into specific domains [sarcoplasmic reticulum (SR)] around the sarcomeres [9–11]. The regions of SR that contact the T-tubules contact the SR are called terminal cisternae (TC), and the regions between TCs, which are largely tubular, are called the longitudinal SR. Calcium (Ca²⁺) is stored in the SR, which also contains voltage-gated Ca²⁺ channels and pumps on its membrane for muscle contraction. Ca²⁺-binding proteins such as troponin and tropomyosin regulate muscle contraction [9,10,18,19].

asymmetrically to generate activated and quiescent SCs [23]. The quiescent SCs, characterized by expression of Pax7 and myogenic regulatory factor 5 (Myf5), upregulate Spry1 and enter a G (alert) state, priming them for rapid entry into the cell cycle [23,24]. Activated SCs committed towards myogenic differentiation express MyoD along with Myf5 and Pax7, and proliferate to generate myoblasts. Activation of Wnt and repression of Notch signalling drives SC exit from senescence and initiation of myogenesis [25]. Myoblasts downregulate Pax7 and begin expressing MyoD to differentiate into myocytes, which subsequently

express myogenin (MyoG) and downregulate MyoD [26–32]. The appearance of MyoG expression marks the exit of myoblasts from the proliferation phase and their entry into the differentiation phase [33]. The terminally differentiated MyoG-positive myocytes express the fusion proteins Myomaker (MYMK) and Myomixer (MYMX), align, adhere and fuse with each other to form nascent myotubes with two to three nuclei (primary fusion) [18,34,35]. Large multinucleated myotubes are formed during and after embryogenesis in mammals by additional rounds of myocyte fusion to the nascent myotubes (secondary fusion/

hypertrophic muscle growth). Myotubes express myosin heavy chain (MyHC) and undergo maturation to form contractile myofibres via myofibrillogenesis (Box 1) [9–11].

Calcium (Ca²⁺) signalling plays an instrumental role in myogenic differentiation, fusion and contraction in amniotes

The endoplasmic reticulum (ER) functions as the cell's central Ca²⁺ storage facility in the vertebrates. The release of Ca²⁺ from ER to the cytosol by Ca²⁺ channels and pumps triggers a decline in ER Ca²⁺ stores. Ca²⁺ acts as a secondary messenger within the cytosol and as a cofactor for various enzymes, triggering a wide range of specific signalling pathways that fine-tune cell physiology [36]. Ca²⁺ signalling is also linked to the maintenance and activation of stem cells in various tissues, including SCs [34,37].

Store-operated Ca²⁺ entry (SOCE) is a process where the depletion of Ca²⁺ from the ER triggers an influx of extracellular Ca²⁺ across the plasma membrane (PM) to replenish the ER's Ca²⁺ stores [38]. A ubiquitous, central mechanism in cellular Ca²⁺ signalling, SOCE, relies on the function of two proteins: stromal interaction molecule (STIM), an ER-Ca²⁺ sensor, and Orai, a highly selective Ca²⁺ release-activated Ca²⁺ channel that resides at the plasma membrane. STIM senses ER Ca²⁺ levels through a low-affinity EF-hand Ca²⁺-binding motif. In all cell types, upon sensing a decline in ER calcium levels, STIM undergoes activation and translocates to ER subdomains, where it physically interacts with Orai to form ER-PM membrane contact sites and activates Orai resulting in an influx of Ca²⁺ into the ER lumen directly from the extracellular milieu [39,40].

Ca²⁺ signalling in myogenic differentiation

Ca²⁺ signalling plays a central role in myogenic differentiation [34,41,42]. STIM1 is required in myoblasts for neonatal muscle growth and differentiation [43]. STIM1- and Orai1-mediated SOCE have also been shown to regulate post-natal myoblast differentiation in humans [44]. Ca²⁺ efflux from the ER to the cytosol occurs via ryanodine receptor 3 (RyR3), which activates the Ca²⁺ calmodulin-dependent phosphatase calcineurin. Calcineurin triggers early myogenic differentiation by activating MyoD and Myocyte Enhancer Factor 2C (Mef2C) [45]. Consistently, lowering cytosolic Ca²⁺ levels inhibit differentiation in the

C2C12 cell line [46], and blocking RyR activity inhibits fetal myoblast differentiation in mice [47]. In C2C12 myoblasts, insulin growth factor 1 (IGF1) triggers Ca²⁺ release from ER to the cytosol via the inositol receptor (IPR₃). Within the cytosol, Ca²⁺ binds to a Ca²⁺-responsive transcription factor nuclear factor of activated T cells (NFATC3) that translocates to the nucleus to regulate the transcription of target genes involved in myogenic differentiation (e.g. genes involved in PI3K/AKT signalling). In parallel, the NFATC3 transcriptionally represses myostatin, a negative regulator of myogenic differentiation [48,49]. The release of Ca²⁺ from the ER during early myocyte differentiation generates ER stress that leads to ER fragmentation [50–53].

Ca²⁺ signalling in muscle contraction

In addition to its involvement in myogenic differentiation, Ca²⁺ is well known for its role as a secondary messenger in skeletal muscle contraction [19]. Muscle contraction initiates with the depolarization of the sarcolemma, followed by Ca²⁺ transport from the extracellular milieu into the cytosol. The transport of Ca²⁺ from extracellular space to cytosol occurs through Ca²⁺ channels localized at membrane contact sites between the SR and plasma membrane. Ca²⁺ is subsequently pumped into the SR and mitochondria [51,52,54–57]. In mammals, during skeletal muscle excitation–contraction (EC) coupling, membrane depolarization activates the sarcolemma L-type voltage-dependent Ca²⁺ channel (Ca_{v1.1}), which is located in the T-tubules of muscle cells. Ca_{v1.1}, in turn, associates with the sarcoplasmic Ca²⁺ release channel ryanodine receptor 1 (RyR1), located on the TC of the SR and triggers its opening. Activation of RyR1 mediates the release of Ca²⁺ from the SR to the sarcoplasm [51,52,55,56]. In the sarcoplasm, Ca²⁺ subsequently binds the troponin C subunit of the troponin protein complex, thereby mediating the conformational changes to the myofibrillar structures that lead to contraction [19]. During muscle relaxation, the P-type ATPase pump SERCA takes up cytosolic Ca²⁺ back into the SR [51,52,55,56]. In the sarcoplasm of maturing myotubes, SR to cytosolic Ca²⁺ release from RyR1 activates calcineurin that leads to the accumulation of contractile proteins, suggesting an active role in myotube maturation [58–60]. Many more Ca²⁺-responsive genes (CRGs) such as myomesins (Myom1/2/3) and myozenins (MyoZ1/2/3) are activated by calcineurin-regulated muscle contraction by contributing to myotube maturation. Myomesins contribute to the formation of M-bands in myofibers, and myozenins (a.k.a

Calsarcins) are involved in linking Z-disk proteins (e.g. alpha-actinin, gamma-filamin, TCAP/telethonin and LDB3/ZASP) and in localizing calcineurin signalling to the sarcomere [14–16,61–63]. On the other hand, calcineurin is activated in mature myofibers under the increased influx of Ca²⁺ from T-tubules to sarcoplasm, which is associated with muscle fibre-type conversion. Calcineurin in mature myofibers is related to converting fast fibres to slow ones via transcriptional activation of slow muscle fibre-type proteins via NFATC1 [58–60]. The underlying importance of Ca²⁺ signalling in mammals is further highlighted in several pathological conditions, including Brody's disease and malignant hyperthermia [53,64,65] and muscle dystrophies associated with impaired Ca²⁺ homeostasis [66,67].

Interestingly, Ca_{V1.1} in zebrafish (*Danio rerio*, teleost fish; *DrCa_{V1.1}*) acts only as a voltage sensor and not as a voltage-gated Ca²⁺ channel, suggesting that in fish, as opposed to mammals, the influx of extracellular Ca²⁺ via Ca_{V1.1} is not essential in triggering EC coupling during muscle contraction [68]. *DrCa_{V1.1}* interacts with RyR1 and RyR3 in slow and fast muscle fibres, respectively, to trigger Ca²⁺ release from ER to the cytosol. These observations suggest the emergence of EC coupling via Ca_{V1.1}-mediated Ca²⁺ influx for contraction of skeletal muscles in amniotes [69–72].

Calcium (Ca²⁺) signalling in myocyte-to-myotube fusion

In addition to its role in myogenic differentiation and muscle contraction, Ca²⁺ has recently been implicated in myoblast fusion in C2C12 cells and human and mice primary myoblasts [73,74]. In regenerating myotubes, the intracellular Ca²⁺ influx via the mechanosensitive channel PIEZO1 at the plasma membrane inhibits myotube-to-myotube fusion to ensure polarized growth of the existing myotubes through the addition of myocytes [73]. In another recent study, PIEZO1 knockdown suppressed myoblast fusion during myotube formation and maturation. PIEZO1 knockdown was accompanied by downregulation of TMEM8C/Myomaker (MYMK) and a reduction in Ca²⁺ influx after stretch stimuli [74]. Both studies suggest a role for PIEZO1-mediated Ca²⁺ influx in myotube formation and maturation via modulation of MYMK expression. These studies are consistent with previous studies wherein depletion of Ca²⁺ in media did not affect proliferation or the generation of nascent myotubes but strongly inhibited the formation of large multinucleated myotubes, suggesting that Ca²⁺ signalling is specifically required to initiate secondary fusion [34]. The specific role of cytosolic Ca²⁺ in

triggering secondary fusion is also supported by the activation and nuclear translocation of Ca²⁺-responsive transcription factor NFATC2 in nascent myotubes. Consistently, mice lacking NFATC2 exhibit myofibers with fewer myonuclei and diminished regenerative capacity *in vivo* [75].

Hypothesis: Ca²⁺-signalling regulates fusion and contraction, specifically in amniotes

We recently demonstrated that elevated cytosolic Ca²⁺ and the subsequent activation of CaM-kinase-II (CamKII) in myotubes are essential for myocyte-to-myotube fusion in cultured primary mouse and chick myoblasts [76]. We found that the same Ca²⁺ pumps that mediate muscle contraction (RyRs, SERCA) are also essential for myocyte-to-myotube fusion. During myocyte-to-myotube fusion, RyRs and SERCA2 (ATP2A2) contribute to ER-to-cytosol Ca²⁺ release, while SERCA1 (ATP2A1) contributes to the reuptake of Ca²⁺ from the cytosol back into the ER. Consistently, treatment of myoblasts with RyR inhibitor blocked secondary fusion but not myogenic differentiation and primary fusion. We further demonstrated that the release of Ca²⁺ via RyRs and SERCA from ER to the cytosol of the nascent myotubes activates CamKII, resulting in myocyte recruitment and preferential fusion with multinucleated myotubes. We also showed that p-CamKII regulates MYMK. These findings directly link the Ca²⁺ pumps that mediate muscle contraction to myocyte–myotube fusion during growth and regeneration [76]. A shared role of membrane-bound CRGs in fusion and contraction was also recently demonstrated by a study on Ferlin family members myoferlin (MyoF) and dysferlin (DysF), showing that they regulate myoblast fusion [77,78]. Interestingly, mice myofibers lacking either of these proteins also show aberrant Ca²⁺ handling and differences in the RyR expression pattern [79]. These studies suggest that both cytosolic Ca²⁺ and the responsive CRGs regulate both fusion and contraction in at least two distant vertebrate species within the amniotic lineage. Hence, we hypothesize that while cytosolic Ca²⁺ regulates myogenic differentiation, and contraction in all the vertebrate lineages, in addition to these processes, it also regulates myoblast fusion specifically in amniotic skeletal muscles.

The hypothesis that Ca²⁺ signalling is involved in coordinating fusion and contraction in amniotes is supported by the observation that CRGs implicated in muscle contraction are induced earlier during mouse myotube formation and are deregulated in human

muscle dystrophies [66,67,80,81]. In contrast, induction of CRGs appears absent during muscle formation in fish [82,83]. The expression of CRGs positively correlates only with robust swimming in fish, which is consistent with a shared role for CRGs in muscle contraction and not in muscle differentiation [84]. Additionally, Ca²⁺ has recently been implicated in the proliferation of myogenic progenitor cells during spinal cord and skeletal muscle regeneration in frogs [85], which resembles Ca²⁺-induced MyoD activation during myogenic differentiation in mammals. However, the involvement of CRGs in myoblast fusion has not been reported in amphibians, suggesting that the induction of CRGs during late myogenic differentiation and fusion is a feature unique to amniotes. This is consistent with the involvement of Ca²⁺ signalling in MyoG-driven late myogenic differentiation and muscle contraction in the organisms of this lineage [34,41,46,51,52,55]. The active involvement of CRGs in secondary fusion in amniotes and their absence during myocyte fusion in fish and *Drosophila* also support the hypothesis that Ca²⁺ signalling plays a role in myoblast fusion, specifically in amniotes [5,74,76,80,82,86,87]. Taken together with the role of Ca²⁺ in regulating secondary fusion, it stands to reason that Ca²⁺ signalling in amniotes serves as both a master regulator of differentiation and a 'switch' between muscle contraction and fusion.

Why might coordination of fusion and contraction be necessary for amniotes?

It is tempting to speculate that myocyte fusion and muscle contraction need to be coordinated in post-natal amniotes to avoid the detrimental consequences of myocytes fusing with contracting myotubes during regeneration. While the fluidic nature of membranes has been speculated to be critical for myoblast fusion [35], the structurally complex nature of myofibre membrane would pose challenges for the fusion of myocyte to contractile myofiber. Therefore, myocyte-myofiber fusion may involve complex membrane remodeling at the site of fusion possibly by stalling contraction at the site of fusion. Moreover, while the ER is fragmented in myocytes and sarcomeric in myofibers [9,51], the opposing ER morphologies between the two cell may stall myofibers contractions locally so as to allow for organelle remodeling at the fusion synapse. Therefore, while Ca²⁺ regulates fusion and contraction in amniotes, we speculate that it temporally regulates these processes to prevent their co-occurrence to avoid detrimental consequences to the fusing cells. We

assume that this feature is evolutionarily selected in amniotes due to their larger body sizes than fish. We predict that amniote myofibers switch between a fusion- and contraction-competent state to ensure structural rearrangements like nuclei and organelle repositioning could occur during the integration of mononucleated cells into damaged myofibers. These notions are consistent with the recently reported Ca²⁺-triggered sarcomere rearrangements and nuclear movements towards the injury site in mice [88] and with the mechanistic differences in muscle formation and growth between the amniotes and anamniotes.

Divergent mechanisms of muscle formation and growth between amniotes and anamniotes

Muscle formation

Muscles arise from the somites, paired blocks of mesodermal cells located paraxially to the notochord [89–92]. During embryogenesis, as the somites develop (somitogenesis), they differentiate to form the myotome, sclerotome and dermomyotome cells, which give rise to muscle, skeleton (bone and cartilage) and dermis (skin) respectively. While in the anamniotes, the myotome differentiates at a very early stage of embryogenesis and forms the bulk of the early somite, in amniotes, the sclerotome differentiates first [89–92].

All vertebrates contain two populations of muscle fibres: slow- and fast-twitching muscle fibres. In amniotes, the same myoblast pool generates slow and fast muscle fibres. While the slow fibres are generated during embryogenesis via the fusion of mononucleated myocytes, the fast fibres are formed by the fusion of myocytes with pre-existing primary fibres [72,81,83,93]. In contrast, in zebrafish, the two fibre types are formed from spatially separate pools of myoblasts that give rise to the two muscle types in distinct phases of embryonic myogenesis [72,81,83,93]. The slow, mononucleated muscle fibres are formed in the initial phase of myoblast differentiation and the fast, multinucleated muscle fibres later on, in the second phase.

In zebrafish, slow muscle fibres remain mononucleated during embryonic development and grow by multinucleation only after birth [82,94–97]. The fast, multinucleated muscle fibres are formed from late-differentiating somite cells that undergo a 90° whole-somite rotation and then migrate towards the top of the basement membrane, where they fuse. While these myoblasts form multinucleated myofibers in teleost fish, they form multinucleated muscle lamellae in lungfish that only later undergo longitudinal division to

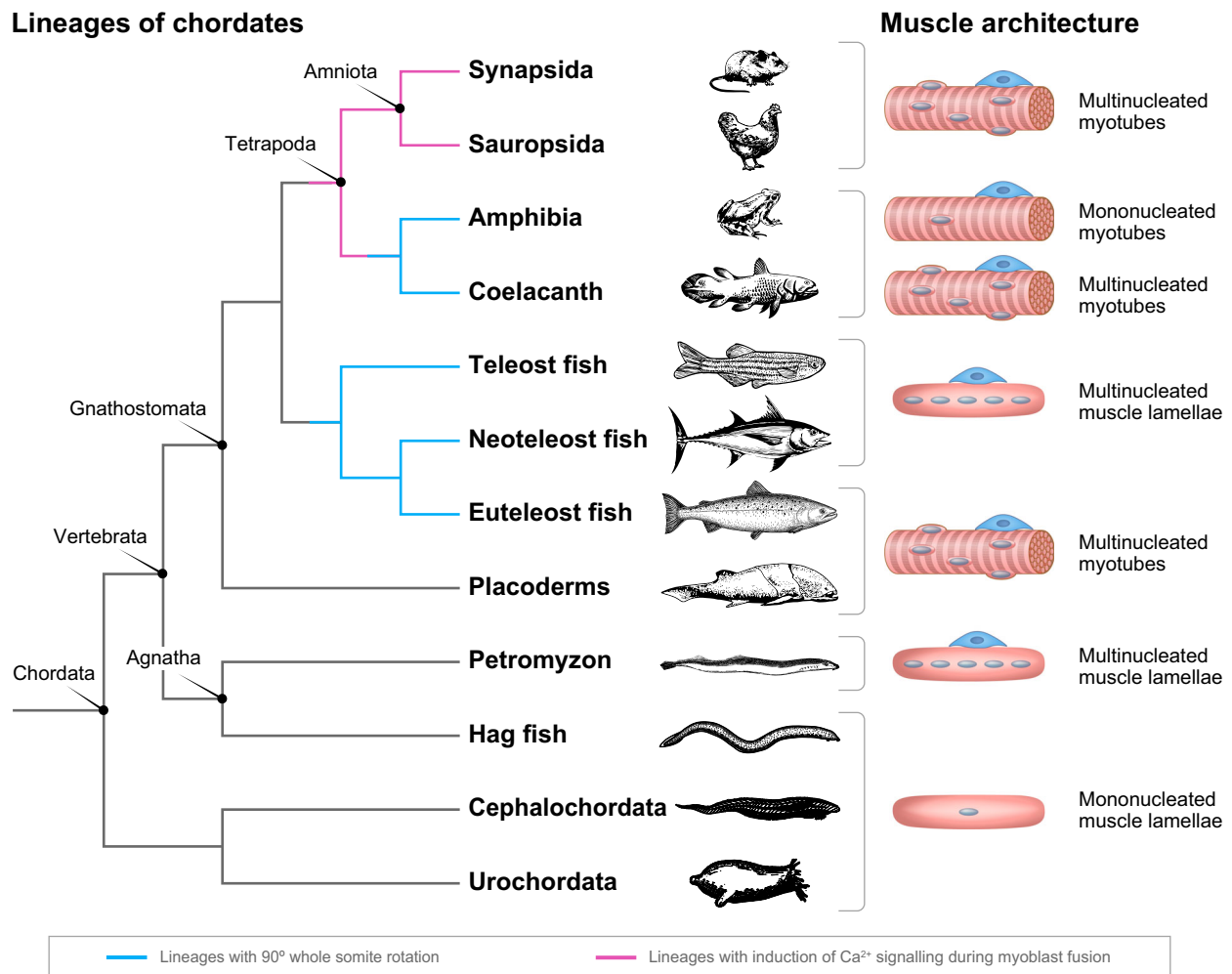


Fig. 2. Illustration summarizing the evolutionary changes in muscle formation during embryonic myogenesis across chordates. The lineage (left) and the corresponding muscle structure (right) are shown. In all vertebrates, the muscle forms after somitogenesis. In the anamniotes (fish and amphibians; blue lines), the somite undergoes a 90° rotation during embryonic myogenesis and later differentiates to form muscle progenitor cells. This feature of whole somite rotation is specific to anamniotes. Myoblasts in amniotes and lower amphibians bypass fusion during embryonic myogenesis and differentiate directly into mononucleated myotubes. Later, the SCs fuse with mononucleated myotubes and form multinucleated myotubes that later mature to form myofibers. Different lineages of fish differ in the process of muscle formation. For example, in teleost fish, myoblast fuse to form multinucleated myotubes during embryonic myogenesis, and these grow further in adulthood by adding mononucleated myocytes derived from SC differentiation. However, in Neoteleost and Euteleost fish lineages, myoblasts fuse to form multinucleated muscle lamellae that undergo longitudinal splitting to form cylindrical muscle fibres. Therefore, while all vertebrates possess multinucleated myotubes, the process of their formation varies across the vertebrates. Unlike vertebrates, the non-vertebrates contain mononucleated muscle lamellae instead of multinucleated muscle fibres. While EC coupling for muscle contraction emerged in the vertebrate lineage within chordates, EC coupling with Ca²⁺ influx for muscle contraction appeared for the first time in amniotes (shown in pink lines in the figure). The vertebrates also mark the first emergence of fusion proteins MYMK and MYMX and CRGs such as RyRs. Thus, the amniote-specific involvement of cytosolic Ca²⁺ and its responsive CRGs in myoblast fusion and muscle contraction appears to align with the changes in skeletal muscle architecture and with the emergence of EC coupling with Ca²⁺ influx in muscles in the organisms of this lineage.

form cylindrical myofibers [98] (Fig. 2). Only the fast myoblast fraction fuse to generate the multinucleated myotubes during embryonic development in the fish lineage [78,95,99,100]. These observations suggest that zebrafish fusion mechanisms differ between embryonic

and post-embryonic development in contrast to mice. The 90° rotation of whole somite and the presence of two kinds of myoblasts (slow and fast myoblasts) is a unifying feature of anamniotes, contrary to amniote embryogenesis [78,95,99,100].

Amphibian myoblasts either differentiate directly into mononucleated myofibers (bypassing fusion) or fuse to form multinucleated myofibers (Fig. 2). While mammals (amniotes) use SCs-derived myocytes for fusion, amphibians derive mononucleated cells by dedifferentiating injured myofibers to myocytes that fuse with injured myofibers (dedifferentiation model for muscle regeneration) [85,101–103].

Muscle growth

Amniotes and anamniotes also differ in the aspects of muscle growth in adulthood. In amniotes, the number of muscle fibres in adults is determined during embryogenesis, and the addition of new fibres to pre-existing ones stops after birth. Muscle fibres in adult amniotes are only regenerated by hypertrophy [104,105]. In contrast to amniotes, fish muscles continue to grow until adulthood by adding new muscle fibres (hyperplasia) to the pre-existing ones. The type of muscle growth in fish also depends on body size. While the fish with smaller body sizes continue to grow muscles by hypertrophy, the larger fish (> 22 cm) grow muscles by hyperplasia [98,106,107]. Given the differences in muscle formation and growth between amniotes and anamniotes, it is presumable that the genes involved in these processes (including the CRGs) also vary across the two lineages. Despite the differences in the genes involved in myoblast fusion and muscle formation and growth differences between amniotes and fish, the marker proteins and the signalling pathways involved in SC activation, myogenic differentiation, and myotube maturation are conserved between the two lineages [30,72,83]. Moreover, in both the lineages during myogenesis, the myotubes only fuse with SCs and not between themselves [72]. Therefore, the basic scheme of myogenesis has only been fine-tuned as per habitat and growth requirements between the two lineages.

Divergent fusion machinery between amniotes and anamniotes

The fusion proteins employed by vertebrates also vary considerably. Studies aimed at understanding myocyte fusion in vertebrates have identified several muscle-specific myogenic genes that have first appeared in vertebrates [48,72,77,78,108–110]. These include MYMK (221aa in humans) and Myomerger/Minion/Myomixer (MYMX), which are essential and together sufficient for myoblast fusion in vertebrates [111–114]. While MYMK and MYMX are present in all vertebrates, their sequences vary between the amniote and anamniote lineages [112,115,116]. As opposed to other vertebrates,

including the teleost fish, the MYMK orthologs in neoteleost and euteleost fish such as Protacanthopterygian fish (salmonids) are longer (~ 434aa) with a functionally important additional C-terminal 224aa region [115]. The specific differences in the MYMK protein sequences between fish and amniotes indicate the neofunctionalization of MYMK in the fish lineage, possibly to regulate primary and secondary myogenesis. This may imply that CamKII does not interact with MYMK in fish muscles, probably because: (a) fish rely on hyperplasia for muscle growth [98,106,107], and (b) fish muscles do not require Ca²⁺ and CRGs for fusion [83,95,117,118]. Hence, it would be interesting to study the deregulation of Ca²⁺ and CRGs like CamKII in the context of fusion in fish. These lineage-specific protein-sequence differences also appear in MYMX sequences across vertebrates [113,114,116].

A recent study has suggested that MYMK and MYMX have coevolved in the vertebrates [119]. These observations indicate that MYMK and MYMX evolved together for myoblast fusion, parallel to the emergence of sarcomeric muscle and EC coupling in this lineage [120]. The emergence of fast- and slow-twitching muscle fibres in the vertebrates appears to align with differences in the mechanism of myoblast fusion and the sequence divergence in MYMK and MYMX paralogs. A similar appearance and subsequent divergence of genes within chordates were previously reported for the molecular evolution of other myopathy-associated CRGs such as the RyRs and ferlin family members, suggesting that MYMK, MYMX and the CRGs coevolved in the chordate lineage [121–123]. In addition, Kirrel and the receptor–ligand pair Jam-b/Jam-c, which are essential for myoblast fusion in zebrafish [5,83,115,124], do not exhibit an apparent muscle phenotype in knockout mice [125–127].

Taken together, the difference in the involvement of Ca²⁺ and its responsive CRGs in myoblast fusion between the amniotes and the anamniotes (fish and amphibians) aligns well with the molecular and morphological differences in muscle formation and growth and with variation in the identity of fusion proteins between the two lineages. Hence, the amniote-specific involvement of CRGs in fusion and contraction appears to be a lineage-specific adaptation in line with muscle regeneration in this lineage.

Concluding remarks

In summary, in this viewpoint article, we propose that Ca²⁺ signalling is involved in myogenic differentiation, fusion and contraction in amniotes. We hypothesize that Ca²⁺ was selected during evolution to coordinate

these processes in amniote myofibers to support robust regeneration. If this hypothesis is correct, it follows that regenerating muscle fibres do not fully contract upon excitation, suggesting that muscle innervation could regulate regeneration. Hence, identifying additional Ca²⁺-regulated effector proteins with a dual role in fusion and contraction would help elucidate the mechanisms that shape amniote regeneration and develop novel avenues for muscle therapy [128].

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

SS and OA conceived the viewpoint. SS performed the analysis and interpretation. SS and OA wrote the manuscript with contributions from YE-A. OA secured funding and supervised the study.

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