IN VITRO DEVELOPMENT OF SKELETAL MUSCLE FIBER

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ABSTRACT

The present review will discuss new insights of myogenesis that begins when embryonic mononucleated progenitor cells become committed to myogenic lineage and subsequently proliferate and fuse to form multinucleated contracting skeletal muscle fibers, responsible for generation of force and movement. This review will focus mostly on the influence of specific myogenic transcription factors on skeletal muscle differentiation and on the compartmentalized expression of nicotinic acetylcholine receptors and acetylcholinesterase at specific muscle fiber microdomains. Experiments using cultured muscle model obtained from embryonic or adult animals, have provided detailed information on myogenesis. Differentiated cultured skeletal muscle fibers contract spontaneously and preserve several properties of *in vivo* multinucleated muscle fiber, including the expression of specific myogenic transcription factors as well as the compartmentalized synthesis and expression of neuromuscular synaptic proteins around individual nuclei. Besides, cultured muscle cells express multiple receptors coupled to G protein, including muscarinic acetylcholine receptors. Considering that many aspects of the present knowledge about the development and differentiation of muscle fiber and formation of the neuromuscular synapse were established in studies using muscles cultures, protocols of primary tissue-cultured skeletal muscle obtained either from embryonic myoblasts or adult satellite cells will be presented.

Key words: Acetylcholinesterase, acetylcholine receptors, myogenic transcription factors, myogenesis, satellite cells

INTRODUCTION

The multinucleated skeletal muscle fibers are highly specialized cells functioning primarily to generate force and movement through the synchronized shortening of their sarcomeres. Together with the motor neuron terminal, muscle fiber forms one of the most studied synapses - the skeletal neuromuscular junction (NMJ) [39,96]. This classical cholinergic synapse is characterized by a precise alignment of pre- and postsynaptic apparatuses, respectively responsible for release and detection of neurotransmitter acetylcholine (ACh).

In spite of large mammal muscle fiber length, the nerve-muscle contact invariably occurs on its central region, occupying approximately 0.1% of the cell surface [93]. Therefore, the efficiency of excitation-contraction coupling must be guaranteed by a regionalized specialization in protein synthesis and in expression of specific genes, including those of nicotinic acetylcholine receptor (nAChR) subunits and the enzyme acetylcholinesterase (AChE), responsible for ACh hydrolysis.

The present review is intended to discuss new insights of myogenesis that begins when embryonic mononucleated progenitor cells become committed to myogenic lineage and subsequently proliferate and fuse to form multinucleated muscle fiber. This review will also focus on the influence of specific transcription factors on muscle fiber development and differentiation that culminates with synapse formation and regionalized expression of proteins involved in excitation contraction coupling: nAChRs and AChE. Finally, considering that many aspects of the actual knowledge about the development and differentiation of muscle fiber and formation of the neuromuscular synapse were established in studies using muscles cultures, protocols of primary tissue-cultured skeletal muscle obtained either from

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embryonic myoblasts or adult satellite cells will be presented.

Development and differentiation of muscle fiber

During early vertebrate embryogenesis, proliferating progenitor cells, derived from the embryonic mesenchyme of the somite (segmented clusters of mesoderm that form on either side of the neural tube) undergo a programmed maturation giving rise to a layer of non-proliferating myoblasts that form the primary myotome. Over this foundation, proliferating myogenic cells will give rise to the vertebrate skeletal muscle, under influence of permissive/repressive signals and sequential expression of specific genes, as described below.

Transcription factors and development of skeletal muscle cells

Many myogenic regulatory factors (MRFs) expressed at distinct embryonic and fetal stages are involved in the development and differentiation of muscle fiber, including members of paired box (Pax) family proteins, defined by the presence of the paired domain, a 128-amino acid DNA-binding domain, that makes sequence-specific contacts with DNA [17]. Pax3 is the earliest myogenic regulatory protein expressed in muscle progenitor cells, playing an important role in the determination of myogenic cell fate. Pax3 positive (Pax3$^+$) cells give rise to hypaxial muscle precursors that migrate to the ventral body wall, the limbs, the diaphragm and the tongue.

Although primary myotome is constituted by non-proliferating myoblasts, other Pax3$^+$ progenitor cells migrate from the central domain of dermomyotome into the myotome, constituting the major source of skeletal muscle. According to Relaix and co-workers [80], at embryonic day (E) 10.5, Pax3 is expressed throughout different mice somite levels (97% of cells). At this stage, 87% of skeletal muscle Pax3$^+$ progenitor cells also express Pax7, a distinct molecular marker of adult myogenic cells known as satellite cells that remain associated with the differentiated muscle fiber.

Whereas Pax3 is important during embryogenesis, its close homolog Pax7, expressed in satellite cells, plays a special role in postnatal muscle fiber growth, regeneration and self-renewal (see below). The great majority of Pax3$^+$ (96%) and Pax3$^+$/Pax7$^+$ (81%) are co-expressed with the mitotic marker cyclin A, indicating that these markers are expressed in a proliferating population of cells. The relevance of Pax3$^+$/Pax7$^+$ cells in the myogenesis is demonstrated by total loss of skeletal muscle or adoption of non-muscular fates when these cells fail to express both Pax3 and Pax7 [80].

At the following embryonic stages, the success of skeletal muscle differentiation depends on the appropriated expression of other MRFs, via binding to consensus CANNTG sites (E boxes), which are present in the promoters and enhancers of muscle-specific genes [4,97]. At least four MRFs belonging to the family of basic helix-loop-helix (bHLH) nuclear phosphoproteins are important for myogenic commitment and formation of myotube: Myf5 [6], MyoD [22], myogenin [110] and MRF4 [82]. Regardless some overlap in expression of MRFs, they can be associated to distinct phases of muscle development.

A significant progress in understanding the contribution of MRFs on molecular control of myogenesis came from studies using MRF deficient mice and cell cultures. The first evidence of the involvement of MRFs on myogenesis was the demonstration that ectopic expression of MyoD in 10T1/2 fibroblast transforms these cells in a myogenic lineage [53]. In fact, either Myf5 or MyoD are required for determination of myoblasts during embryonic development. Whereas double MyoD/Myf5 knockout mice lack skeletal muscle tissue dying at birth, single MyoD or Myf5-null mice display basically normal skeletal muscle and the absence of either MyoD or Myf5 is compatible with life. Interestingly, MyoD null mice show an increased Myf5 transcript level [89], indicating that the loss of one transcription factor may be compensated by the expression of the other [48,90]. To assess which genes were common targets to MyoD and Myf5, Ishibashi and co-workers infected clonal double-null MyoD$^-$/Myf5$^-$ embryonic fibroblasts lines with retrovirus expressing MyoD and/or Myf5. Reintroduction of MyoD increased the expression of 47 genes, whereas 17 genes were increased by Myf5 and 11 were upregulated by both transcription factors; see [47] for details. The majority of MyoD-regulated genes are markers of differentiated skeletal muscle (e.g, myogenin), indicating a major role of MyoD in controlling myogenic events. It is important to emphasize that other signaling molecules, such as members of Wnt family of secreted glycosylated

factors and sonic hedge-hog (Shh), also contribute to muscle determination by inducing expression of MyoD and Myf5, (reviewed in [10]).

Whereas MyoD and Myf5 are important for myogenic determination, myogenin (also known as Myf-4) is required for terminal differentiation of myoblast. Inactivation of myogenin gene induces major defects in skeletal muscle resulting in perinatal death [78,90]. The impairment on muscle differentiation includes disorganization of muscle fibers, disruption of myogenic lineage and disappearance of myogenic cells, depending on the region of the body analyzed [69]. The 4th myogenic factor MRF4 is expressed transiently in the mouse myotome from E9.0 to E11.5, returning at E16.0. Different lines of evidences indicate the restricted involvement of MRF4 on terminal differentiation of myoblasts [48,76]. However, its complex temporal expression pattern, at distinct myogenic phases, suggests an additional role of MRF4 in the early stages of myogenesis. Besides, recent gene targeting studies have revealed that MRF4 also confers myogenic identity to embryonic multipotent cells, indicating an hierarchical relationships between MRF4 and myogenin [48].

In summary, the myogenic pathway might be divided into three stages according to expression patterns of specific transcription factors, as illustrated in Figure 1: premyogenic phase, characterized by expression of Pax3, early myogenic phase, associated to expression of "specification" factors MyoD/Myf5 and/or MRF4 and terminally differentiation phase, when MRF4/myogenin are expressed.

Myoblast fusion

The fusion of myoblasts begins immediately after the cell cycle arrest. This process involves elongation of the round myoblast in both directions with the establishment of a long cell axis. The subsequent steps include cell-cell interaction, recognition and adhesion, followed by membrane coalescence that culminates in merging of competent-myoblasts to form the multinucleated myotube; (for review see [98]).

Many proteins located at the myoblast surface (e.g., β1 integrin, NCAM and M-cadherin) have been involved in muscle cell fusion [99,104]. One of the most studied is M-cadherin, a member of the transmembrane calcium-dependent cell adhesion molecule family, found predominantly in developing skeletal muscles during secondary myogenesis and in adult satellite cells [49,113]. It has been recently shown in C2C12 myoblasts that active RhoA, a member of Rho GTPase family, leads to degradation of M-cadherin through a lysosomal-dependent pathway [15]. Thus, RhoA activity must be down-regulated to allow appropriate function of M-cadherin and myoblast fusion. In the adult mammals, the expression of M-cadherin is drastically increased upon muscle damage [46] indicating a conceivable role of this protein during postnatal muscle repair.

Another protein involved in myoblast fusion is meltrin α, also known as ADAM 12 [111]. It may also contribute to muscle repair since it is expressed in satellite cells of fusing muscle but absent in normal adult muscle [5]. As a member of transmembrane protein that contains extracellular disintegrin and metalloprotease domains, ADAM 12 has both cell adhesion and protease activities. According to Brzoska *et al.* [8], in fusing myoblasts, the metalloproteinase domain of ADAM12 is proteolyzed allowing the interaction of ADAM12 disintegrin domain to integrins, (eg., integrin α3).

Genetic studies in *Drosophila* embryos have provided new insights into the process of myoblast fusion. In a genetic screen for regulators of muscle development in *Drosophila*, Eric Olson´s group found a gene encoding a guanine nucleotide exchange factor, called *loner.* The encoded protein Loner is expressed at subcellular sites of fusion and controls fly myoblast fusion by recruiting the small GTPase ARF6 and promoting its guanine nucleotide exchange [16]. Since proteins highly homologous to Drosophila's Loner were already found in mouse

Cell Type	Phenotype	Markers
Progenitor		Pax3; Pax7
Myoblasts		MyoD; Myf5
		MRF4; Myogenin
Myotube		Muscle creatine kinase; α-Actin; Myosin heavy chain; Other contractile proteins
Differentiated Muscle fiber		
Satellite cell		Pax7

Figure 1. Sequential phases of myogenesis, showing cell types and corresponding phenotypes and molecular markers.

and human cells [101], it is possible that homologs of *loner* gene involved in Drosophila myoblast fusion might play similar roles in mammalian skeletal muscle development.

After the initial myoblast-myoblast fusion, cellular growth requires the fusion of other myoblast to pre-existing myotubes. This process involves calcineurin, a calcium-activated serine/threonine phosphatase and some members of a transcription factor family known as nuclear factor of activated T cells (NFATs). Once activated, calcineurin dephosphorylate specific NFAT isoforms (C1-C3) allowing their translocation into the nucleus. The relevance of this signaling pathway on the muscle fiber growth is demonstrated in NFATC2 and NFATC3 deficient mice which display decreased myofiber size and myofiber number, respectively [43]. Although the initial fusion of NFATC2 deficient myoblasts is normal, the subsequent fusion of newly formed myotube with myoblast is impaired [73]. Interestingly, the proinflammatory cytokine interleukin-4 (IL-4) has been referred as a downstream target of NFATC2 signaling in muscle cells. The sequence of events begins when NFATC2 induces the secretion of IL-4 by newly formed myotubes, allowing its interaction with IL-4 receptors expressed on surrounding myoblasts, promoting their fusion to existing myotubes [44].

The coalescence of myoblast membranes will also depend on membrane lipid constitution that will ultimately affect its fluidity. For example, a decrease in membrane cholesterol has been associated to an improvement of myoblast fusion [42,64]. Although a large number of macromolecules that contribute to myoblast fusion have been identified, the precise molecular mechanisms responsible for this process are just beginning to be understood.

Differentiation of multinucleated skeletal muscle fiber and developmental regulation of nAChRs and AChE

Once the multinucleated myotube is formed, cellular differentiation continues as centrally placed nuclei migrate to the periphery, assuming a relatively constant distribution along the entire fiber. At this stage, muscle specific genes such as myosin heavy chain (MHC) and muscle creatine kinase (MCK) are expressed and muscle fiber acquires mature contractile apparatus. Simultaneously, the expression of nAChRs and AChE increases and becomes compartmentalized around individual nuclei; for review, (see [93,96]).

The expression of nAChRs initiates during late myogenesis and functional receptors able to respond to ACh or analogs can be detected in proliferating myoblasts [24,27,50]. Both embryonic and adult nAChRs are transmembrane ligand-gated channels formed by four homologous subunits assembled into a pentameric macromolecule: $\alpha_2\beta\gamma\delta$ or $\alpha_2\beta\varepsilon\delta$, respectively [67] with distinct conductances and mean open time [7,54].

Prior to muscle innervation, $\alpha_2\beta\gamma\delta$ nAChRs are expressed throughout the myotube surface (~100/ μm^2) and most, if not all, nuclei in the myotube express mRNA for α, β, γ and δ subunits. During this stage, the expression patterns of nAChR subunit genes are influenced by members of MRFs. According to Charbonnier *et al.* [13], Myf5, MyoD, myogenin and MRF4 induce the expression of α nAChR subunit gene in *Xenopus laevis* embryo. Among the MRFs, Myf5 specifically induces the expression of the δ-subunit gene whereas MyoD and MRF4 activates γ-subunit gene expression but are unable to activate δ-subunit gene expression [78]. However, after the NMJ has been formed, the nAChR density at the synaptic site increases from $100/\mu m^2$ to 10,000 molecules/μm^2, whereas it drops to less than 10 molecules/μm^2 at extrajunctional domains [52,93,94]. The perinatal upregulation of nAChRs occurs in parallel to the replacement of immature nAChR containing γ subunit ($\alpha_2\beta\gamma\delta$) by nAChR containing ε subunit ($\alpha_2\beta\varepsilon\delta$) [67] which, in turn, depends on signals from motoneuron, that maintain appropriate levels of synthesis at the adult neuromuscular synapse but repress the transcription of nAChR subunits in nonsynaptic nuclei; for review see [25].

The reduced synthesis of extrasynaptic nAChR is triggered by the neurotransmitter ACh itself and involves membrane depolarization and calcium-dependent phosphorylation of the muscle transcription factor myogenin [58,62]. Classical experiments performed in primary cultures of chicken myotubes have shown that blockage of membrane electrical activity with the Na^+ channel blocker tetrodotoxin (TTX) increases the number of nAChRs on the cell surface [3] and their transcripts [29]. This phenomenon was correlated to the limited binding of phosphorylated myogenin to target E-box sequences that regulate nAChR promoter activity, which results in reduced nAChR expression [45,58,62,63].

Upon innervation, a comparable redistribution of AChE is observed with restricted accumulation of the enzyme underneath the nerve terminal. In vertebrate NMJ, AChE exists in multiple oligomeric forms distinguished by their number of catalytic subunits and by their hydrodynamic properties. Synthesized as a monomeric globular glycoprotein (G_1), it can form dimers (G_2) and tetramers (G_4). Part of them become attached to the collagen-like tail forming the asymmetric AChE, which consist of one (A_4), two (A_8) or three (A_{12}) tetramers covalently linked to a three stranded collagen-like tail (ColQ); reviewed in [59,60,88]. The highly specific localization of ColQ-AChE forms at adult NMJ prominence them as markers for nerve muscle interaction. Whereas ColQ anchors AChE to the postsynaptic basal lamina through the heparan sulfate proteoglycan perlecan [87], a proline rich membrane anchor (PRiMA) organizes and anchors most membrane-bound G_4 AChE in brain and muscle [75]. Since AChE is detected as both membrane-bound and secreted forms, it is suggested that individual forms play distinct physiological roles in different cellular locations. As observed *in vivo*, aneural cultured muscle synthesizes globular and asymmetric AChE molecular forms and ColQ and PRiMA anchors (Fig. 2). In this system, nAChRs are concentrated at discreet clusters on the cell surface in perfect apposition to ColQ-AChE clusters at basal lamina [38,83]. Analysis of distribution of AChE clusters on the fiber surface of quail skeletal muscle showed that approximately 95% of the enzyme is localized within a 20 µm radius of nucleus, while the distance between nuclei is 65 µm on average [83]. Moreover, it was demonstrated that translation and assembly of the AChE molecules occur on the rough endoplasmic reticulum surrounding the nucleus encoding that particular transcript [86]. Then, the AChE subunits encoded by different nuclei do not associate randomly and are expressed exclusively in the region surrounding the original nucleus.

The regulatory effects of motor neuron

Motor innervation plays a critical organizational role on muscle differentiation and on the maintenance of normal adult fiber type (fast or slow contracting), number and levels of glycolytic or oxidative enzyme systems. As observed with nAChRs, the nerve-evoked membrane electrical activity also modulates the expression of postsynaptic AChE. Experiments performed in avian cultured myotubes showed that depolarization of cells with veratridine increases the AChE clusters at cells surface whereas TTX inhibition of spontaneous contraction reduces the AChE clusters [84]. In addition, nerve-derived substances released on synaptic cleft are able to influence subsynaptic myonuclei, strategically anchored underneath the site of nerve-muscle contact, stimulating the local expression of nAChRs and/or AChE. For example, the EGF-like polypeptide ARIA (acetylcholine receptor inducing activity, also know as neuregulin β) and the heparan sulfate proteoglycan agrin upregulate the nAChR genes at neuromuscular junction by activating their respective tyrosine kinase receptors: ErbBs and MuSK (muscle specific kinase) [12].

MuSK activation, upon either autoactivation or the binding of neural agrin, triggers the clustering of AChRs [56] and other postsynaptic proteins involved in synaptic transmission (e.g., 43K/rapsyn, AChE, voltage-gated sodium channels). The MuSK-dependent accumulation of high densities of nAChRs at postsynaptic membrane occurs in membrane micro-domains, enriched in sphingolipids and cholesterol, known as lipid rafts. Campagna *et al.* [11] showed that lipid rafts are involved in agrin-induced AChR aggregation. While AChR and MuSK translocate into lipid rafts after agrin stimulation, disruption of lipid raft integrity reduces nAChR accumulation. In addition, the interaction of the AChR with rapsyn, an intracellular protein necessary for AChR clustering also requires lipid rafts [114]. Muscle fiber also synthesizes agrin but it is 1,000 times less efficient than neural agrin in inducing aggregation of nAChRs [79].

Other neuronal or systemic signaling molecules are also able to influence the expression of nAChR and AChE [23,34,36,102], some of them acting through specific G protein-coupled receptors [18,21,33,65,68,71,105]. The most studied example is the calcitonin gene related peptide (CGRP), a neuropeptide released from motor nerve terminal upon action potential. CGRP modulates the neuromuscular transmission by stimulating the synthesis and insertion of nAChR into postsynaptic membrane [30,71]. Since CGRP receptors are highly expressed at the endplate sarcolemmal membrane [77], the neuropeptide provides a localized signal that selectively influences the subsynaptic nuclei, through activation of Gs protein and cyclic AMP-dependent mechanisms. Activation of CGRP

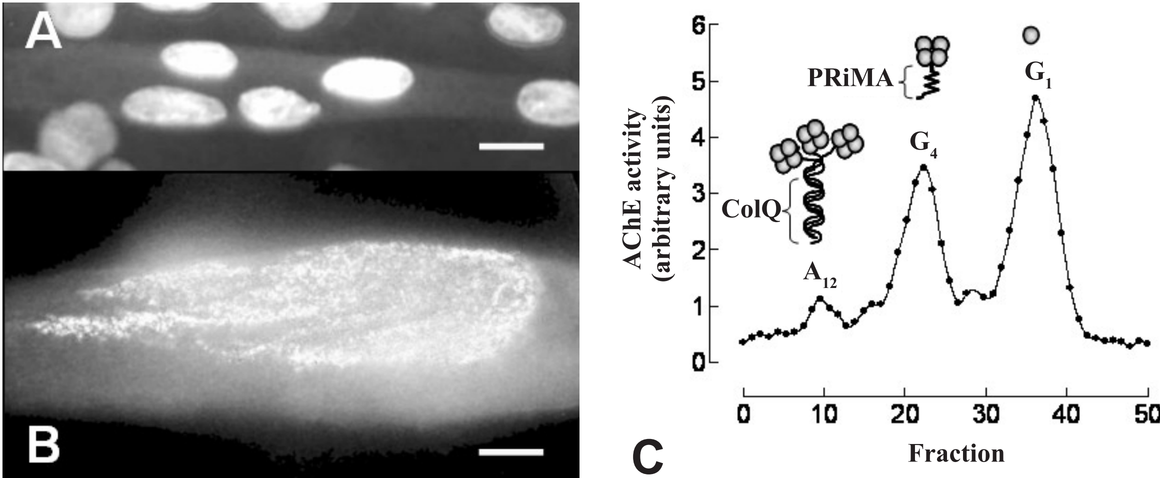

Figure 2. Rat cultured skeletal muscle fiber. Myoblasts/satellite cells from newborn rat were seeded in 35 mm collagen-coated culture dishes. On day 7, the cells were labeled with Hoescht dye 33258 or tetramethylrhodamine α-bungarotoxin to evidence the nuclei (**A**, bar = 20 μm) and the clusters of nAChRs (**B**, bar = 10 μm), respectively. At this stage the cultured fibers are able to synthesize asymmetric (A_{12}) and globular (G_1 and G_4) isoforms of AChE (**C**). AChE from cultured fibers was extracted using 500 μl of borate extraction buffer (20 mM borate buffer, pH 9.0, 1 M NaCl, 5 mM EDTA, 0.5% Triton X-100, 5 mg/ml bovine serum albumin, 2 mM benzamidine, 5 mM *N*-ethyl maleimide and 0.7 mM bacitracin). After a 30 min centrifugation at 12,000 rpm, 200 μl of supernatant were loaded on 5-20% sucrose gradients in 20 mM borate buffer and centrifuged during 18 h at 36000 rpm at 4°C. The fractions were assayed for AChE activity using ^{3}H-ACh (0.1 μCi, 24 mM; specific activity = 55.2 μCi/mmol) as substrate, in the presence of 10^{-4} M iso-OMPA as previously described [19].

receptors also modulates the synthesis of AChE [19,28,71,85], but the final effect of CGRP depends on the duration of the stimulus [21].

In fact, the differential compartmentalization of organelles (e.g., nuclei) [1,91] and signaling molecules (e.g., receptors, protein kinases and kinase anchoring proteins [74] at subsynaptic site amplify the chemical signals from the neuron and circulating hormones. Conversely, the compartmentalization of adult synapses seems to depend on anterograde signals from motor neurons during the formation of neuromuscular synapse that initiate the postsynaptic specialization and trigger the synthesis of synapse-specific genes, including those components of the G-protein signaling cascade [31].

Trophic influences of ACh on developing skeletal muscle

In addition to essential role of ACh at adult NMJ triggering muscle contraction, many evidences indicate that it plays an important role during myogenesis. It have been shown that *in vitro* application of ACh induces the fusion of myoblasts into myotubes [26,51]. It appears that ACh acting through fetal nAChRs ($\alpha_2\beta\gamma\delta$) ensures the orderly innervation pattern of skeletal muscle [50]. Other studies using mutant mice lacking the acetylcholine-synthesizing enzyme (choline acetyltransferase) also revealed an incomplete development of skeletal muscle tissue due to an impaired cell fusion, indicating that ACh synthesized and secreted by the motor nerve *in vivo* [66], contribute to the differentiation of muscle fiber. Interestingly, during skeletal muscle development, muscle cells by themselves could represent a source of cholinergic agonists. Recently, Bandi *et al.* [2] showed an autocrine activation of nAChR channels by compounds released by the myotubes giving rise to intracellular calcium spikes and contractions.

Although the ACh effects have been related to activation of nAChRs [20], the possible contribution of muscarinic acetylcholine receptors (mAChRs) cannot be disregarded. Studies from different labs have shown that avian and rodent cultured skeletal muscle express mAChRs [33,35,57,81]. Activation of these metabotropic receptors stimulates phospholipase C (PLC) [35,81] and adenylyl cyclase (AC) [33] in response to ACh, carbachol or selective

mAChR agonist oxotremorine-M. The expression of these receptors seems to be a characteristic of non-innervated skeletal muscle cell since they are detected in newborn rat myoblasts/satellite cells, aneural cultured muscle fibers and in denervated adult muscle [31]. Our results suggest that activation of both PLC and AC on cultured muscle fibers are mediated by M_1 and/or M_3 mAChRs subtypes [31]. Interestingly, in rat cultured muscle mAChRs agonists increase the synthesis of AChE via activation of G protein/PLC signaling cascade [31]. Furthermore, functional studies showed that carbachol and oxotremorine-M are able to induce membrane hyperpolarization in the denervated rat diaphragm, an effect inhibited by muscarinic antagonists atropine and pirenzepine [106]. Taken together, these results indicate that mAChRs may be responsible for trophic influences of ACh during either formation of neuromuscular synapse or regeneration of adult neuromuscular synapse.

Tissue-cultured skeletal muscle

Several details of the skeletal muscle development, described above, were achieved in studies using cultured skeletal muscles. Despite the lack of synaptic contact, differentiated primary cultured skeletal muscle preserve several properties of *in vivo* multinucleated muscle fiber, including the expression of specific biochemical markers such as myogenin, MyoD and Myf5 [6,55,97]; reviewed in [92], and compartmentalized synthesis of synaptic proteins, such as nAChRs and AChE, around individual muscle fiber nuclei [38,86]. Moreover, differentiated cultured muscle fibers contract spontaneously [108] and express enzymatic and structural sarcomeric muscle proteins, such as myosin heavy chain (MHC), α-actin, troponin and tropomyosin organized in basic longitudinally repeated units to form the mature myofibrils [95].

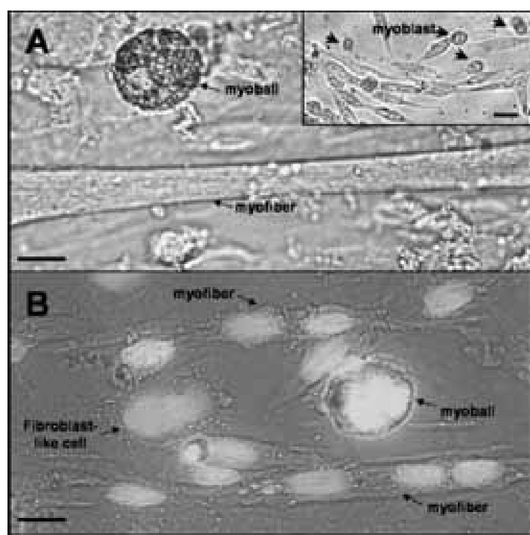

Figure 3. Rat cultured skeletal muscle fiber. Myoblasts from newborn rats were seeded in 35 mm collagen-coated culture dishes. On day 5, the cells were labeled with Hoescht dye 33258 and photographed under brighfield (**A**) and epifluorescence microscopy (**B**). Inset shows one-day-old cultured skeletal muscle cells. Bar: 10 µm for all panels.

Besides, a valuable way to simulate the synaptogenesis *in vitro* include the development of co-cultured systems using myogenic and neuronal cells [9,70,103]. In addition, fruitful use of these *in vitro* techniques has allowed the analysis of interference of endogenous substances (e.g., growth factors, neurotransmitters and hormones) or drugs on muscle differentiation, synapse formation and regulation of specific cholinergic proteins at developing skeletal muscle cells.

It is important to emphasize that the plentiful differentiation of muscle fiber *in vitro* will depend on numerous variables, such as the source of progenitor cells (e.g., embryo, neonatal or adult animals), cell contamination (e.g., fibroblasts), supplementation of the culture medium (e.g., different serum and antibiotics) and the tissue-culture substratum, which is critical to support contractile activity of differentiated fiber.

Primary skeletal muscle cultures can be easily obtained from embryonic myogenic cells or satellite cells from newborn or adult animals. Currently, a wide variety of experimental procedures are available in the literature to obtain skeletal muscle cultures from different animal species. The following protocols describe the standardized procedures used in our lab to obtain differentiated avian or rodent muscle cultures [21,23,32,37,72]. The protocols given here describe basic steps to obtain aneural skeletal muscle cultures and should be used as a general guide.

Primary skeletal muscle cultures from newborn rats

1. Under CO_2 anesthesia, decapitate 6-8 newborn rats (0 to 12 hour-old) and transfer into a beaker containing 70% ethanol. All the following steps must be performed under sterile environment using a laminar flow hood. Pipettes, bottle necks, screw caps, scissors and other instruments should

be flamed before use and the surgical instruments should be kept in a beaker containing 70% alcohol.

2. Under laminar flow, add 5 ml of ice cold Hanks balanced salt solution (HBBS, free of calcium) in three 60 mm petri dishes. Place the first decapitated rat in a sterile petri dish and remove the skin of the lower abdomen and limbs. This step is important to avoid fibroblast contamination. The limb muscles appear like an undifferentiated tissue mass.

3. The hindlimb should be transferred to a 60 mm petri dish containing HBSS.

4. Carefully remove the muscle tissue from the bone, transferring them to the second 60 mm petri dish. At this step, the researcher should avoid incidental transference of small pieces of bone, since it might result in contamination of muscle tissue with hematopoietic stem cells.

5. Remove all connective tissue and blood vessels from the muscle fragments and transfer the muscle tissue to the 3rd petri dish. This procedure can be performed using a stereoscope previously cleaned with 70% ethanol.

6. Place all the muscle fragments on a dry sterile petri dish, mince muscle tissue into about 1-2 mm^3 pieces and incubate them in 10 to 15 ml of Hank's balanced salt solution containing collagenase (200 U/ml) at 37°C.

7. After 1-2 h, gently pipette the cell suspension (5-10 times) and centrifuge the cells in a 15 ml conical tube at 500 x g for 5 min.

8. Resuspend the resulting pellet in 15 ml of Dulbecco's Modified Eagle's Medium (D-MEM) supplemented with 5-10% horse serum (HS) and 2% fetal calf serum (FCS) and 40 mg/l gentamicin (complete medium). The concentration of HS should be previously standardized since the characteristics of HS might change depending on the origin or even on different lots of the same Company. Usually a low concentration of FCS (~ 2%) (mitogen-poor media) induces the differentiation of mononucleated myogenic cells that fuse to form multinucleate myotubes. On the other hand, a high concentration of HS (~10%) improves the attachment of cells to the substratum and reduces the proliferation of fibroblasts.

9. Filter the cells in a 100 μm mesh nylon filter. If necessary, a second filtration should be performed using 20-40 μm mesh filter.

10. To reduce fibroblast contamination, pre-incubate the cells in culture flasks at 37°C for 30 min. This step is optional. Normally, the fibroblasts attach to the flask surface and the myogenic cells are kept in suspension.

11. Take an aliquot of cell suspension, dilute it in the same volume of sterile 0.4% trypan blue solution. Add one drop of the mixture on a clean hemocytometer slide and count the viable (unstained).

12. Then, seed the viable cells (1-2 x 10^5 cells/ml) in 35 mm (2 ml) or 100 mm (8 ml) collagen-coated culture dishes and incubate them in 5% CO_2 humidified atmosphere (day zero) at 37°C. Plating the cells at high concentration will reduce the fibroblast proliferation.

13. Renew the medium after 24 h and every 48 h with complete medium to induce the muscle fiber differentiation. If the contamination of fibroblast is still high, on day 3 add to the medium 10 μM arabinofuranosylcytosine (AraC), for 24-48 h.

In 2-3 days, the cells will start to fuse to form multinucleated myotubes. On day 5-6, it is possible to observe some contracting muscle fibers (Figs. 2 and 3).

Skeletal muscle culture obtained from avian embryonic myoblasts

Avian cultured skeletal muscle is usually obtained from 10 to 11-day embryonated eggs, as described bellow.

1. Clean the eggs with 70% alcohol and place it in a small beaker with the blunt end upward. Carefully puncture the top of the egg with a sterile sharp scissor and cut away a circle of the shell (~2-3 cm diameter). This procedure will expose the underlying membrane (the chorioallantois), that should be peeled off with a sterile forceps.

2. Carefully remove the embryo by gently grasping the neck, using a sterile curved forceps.

3. Place the embryo in a 100 mm petri dish. If necessary, clean the embryo with HBSS.

4. Remove the head, and carefully peel off the chest skin.

5. Using sterile forceps take the chest muscle mass, placing it in a sterile petri dish.

6. Mince the muscle tissue with a small scissor and transfer it to a conical tube (15 mL) containing

HBSS free of calcium (~1.0-1.5 ml/egg) and incubate it at 37°C. The use of collagenase or other enzyme is not necessary.

7. After 30 min, vortex the sample and centrifuge the cells for 5 min at 500 x g.

8. Remove the supernatant and resuspend the cells in sterile DMEM containing 10% HS, 2% chicken embryo extract and 40 mg/l gentamicin. The chick embryo extract can be replaced by fetal calf serum (FCS).

9. Filter the cells consecutively in 100 μm and 20-40 μm mesh nylon filters.

10. Count and seed the cells as described above.

Tissue from one egg is sufficient to prepare 10 to 15 x 35 mm dishes. This protocol can be used with other avian embryos at same age (e.g., quail).

Skeletal muscle culture obtained from adult rat satellite cells

It is well known that at adult multinucleated fiber, mitosis and DNA synthesis arrest almost completely and little turnover of nuclei is observed. On the other hand, upon severe damage, vertebrate skeletal muscles are able to initiate a rapid and extensive regeneration process that involves activation of quiescent myogenic cells known as satellite cells. First described by Alexander Mauro [61] in 1961, these cells, located underneath the basal lamina of individual postnatal and adult myofibers, can be activated under special conditions, e.g. exercise and trauma, leading to muscle hypertrophy and regeneration. Recent studies indicate a common somitic origin for embryonic muscle progenitors and satellite cells. According to Gros and co-workers [40], chick satellite cells derive from the same dermomyotome cell population ($Pax3^+/Pax7^+$ proliferating cells) that originates the skeletal muscle cells during embryonic and fetal life, but are positioned under the basal lamina and in close proximity to the muscle fiber [80]. Once activated, those cells proliferate and fuse to form new myofibers, morphologically and functionally indistinguishable from the undamaged ones [41,112]. These cells are present in all phases of animal postnatal development but the number of satellite cells varies according to the animal age. In mice, ~30% of nuclei in the newborn animal are from satellite cells, whereas in the adult life it represents ~5% of total nuclei. In fact, satellite cells may be considered an adult stem cell population committed to the myogenic lineage. At quiescent state, satellite cells express CD34 and Pax7. Once activated, other macromolecules involved in differentiation of satellite cells into myoblasts (e.g., M-cadherin, MyoD, and Myf-5) are detected [14,100,107].

We have used satellite cells from adult rat muscles to obtain primary skeletal muscle cultures. However, taking into account that quiescent satellite cells are firmly attached to muscle fiber sharing a common extracellular matrix, additional procedures are necessary to activate and dissociate them from muscle surface. The following protocol describes the procedure for isolating satellite cells from extensor digitorum longus muscle (EDL) of 2-month-old adult male Wistar rats.

1. Under deeply anesthesia, place an adult rat (2-3 month-old) on its back and spray the hindlimbs with 70% alcohol. Perform the animal exsanguination by sectioning the abdominal aorta.

2. Using sterile instruments, remove left and right EDLs, trimming off fat and connective tissue.

3. Transfer the muscles to a sterile vessel (e.g., 15 ml conical tube or 60 mm culture dish) containing ice cold HBSS plus 40 mg/l gentamicin.

4. Transfer the muscle to a 100 mm or 60 mm petri dish with fresh HBBS and dissect the muscles in a laminar flow hood, carefully removing the tendons.

5. Transfer the muscle to another petri dish, mince the muscle into pieces about 1-2 mm^3 with an sterile scissor and incubate for 2 h at 37°C with, 200 U/ml type IA collagenase.

6. After gently mechanical dissociation, centrifuge the cells for 5 min at 500 x g.

7. Resuspend the resulting pellet in DMEM (10 ml/100 mg of muscle) supplemented with 15% FCS and 40 mg/l gentamicin and incubate them in a sterile culture flask for 48 h under a 5% CO_2 humidified atmosphere at 37°C. This procedure is necessary to induce satellite cell activation and to detach them from the muscle fiber.

8. After 24 h (day 1), disperse the tissue with a Pasteur pipette. On day 2, repeat the dispersion procedure and centrifuge the tissue 5 min at 500 x g.

9. Resuspend the cells in DMEM containing 10% HS and 2% FCS and follow the steps 10-14, described in the previous protocol (for isolation of avian muscle cells).

Tissue from one EDL muscle is sufficient to prepare at least 6 x 35 mm dishes.

CONCLUSIONS AND PROSPECTS

In the past 40 years, the use of primary skeletal muscle culture has contributed to a better understanding of myogenesis and formation of neuromuscular synapse. Because of its accessibility to pharmacological agents, it is still an excellent *in vitro* model for evaluation of drugs or endogenous substances direct on the muscle fiber.

On the other hand, several recent studies have addressed the potential use of progenitor skeletal muscle cells and satellite cells for regenerative medicine, e.g. in cardiac tissue repair [109], functioning as a cellular delivery system able to carry therapeutic genes. Skeletal muscle myoblasts and satellite cells from skeletal muscle have been successfully engrafted into cardiac tissue of different animal species and clinical trial are underway in many countries [109], including Brazil. The success of clinical application of these cells, and/or other skeletal muscle-derived stem cells, will depend on appropriate methods to induce cell expansion *in vitro* and a better comprehension of the mechanisms involved in the determination of their phenotype fate. In face of these challenges, cell culture system will certainly remain as a useful model to investigating molecular mechanism of cell differentiation and to establishing the *ex-vivo* procedures for cellular therapy.

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