Hindawi Publishing Corporation
ISRN Developmental Biology
Volume 2014, Article ID 713631, 15 pages
http://dx.doi.org/10.1155/2014/713631

Review Article

Cytoskeleton and Adhesion in Myogenesis

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Received 27 January 2014; Accepted 2 March 2014; Published 15 April 2014

Academic Editors: M. Behra, A. Grimaldi, and J. R. Jessen

The function of muscle is to contract, which means to exert force on a substrate. The adaptations required for skeletal muscle differentiation, from a prototypic cell, involve specialization of housekeeping cytoskeletal contracting and supporting systems into crystalline arrays of proteins. Here I discuss the changes that all three cytoskeletal systems (microfilaments, intermediate filaments, and microtubules) undergo through myogenesis. I also discuss their interaction, through the membrane, to extracellular matrix and to other cells, where force will be exerted during contraction. The three cytoskeletal systems are necessary for the muscle cell and must exert complementary roles in the cell. Muscle is a responsive system, where structure and function are integrated: the structural adaptations it undergoes depend on force production. In this way, the muscle cytoskeleton is a portrait of its physiology. I review the cytoskeletal proteins and structures involved in muscle function and focus particularly on their role in myogenesis, the process by which this incredible muscle machine is made. Although the focus is on skeletal muscle, some of the discussion is applicable to cardiac and smooth muscle.

1. Introduction: Myogenesis

When a muscle contracts it can shorten or develop force. To accomplish its physiological role of moving us around, it must do both—and for this, muscle cells undergo a dramatic structural and physiological change during development, from a single cell 20 μm long into a multinucleated muscle fiber 30 cm in length. The contractile apparatus itself is based on cytoskeletal structures that exist in all cells and includes microfilaments and intermediate filaments. In striated muscle, these filaments are organized in a crystalline fashion and contract synchronously after a nerve impulse. Moreover, all the cytoskeletal components, including microtubules, have to change during myogenesis to accommodate the physiological muscular adaptation. Since muscle needs a substrate to develop tension, important adaptations in cell adhesion also occur during muscle differentiation.

In order to contextualize the review, it is convenient to define the major steps in myogenesis as determination and differentiation (Figure 1). Skeletal muscle determination begins with molecules secreted from the neural tube and notochord, such as wingless (Wnt) and sonic hedgehog (Shh) [1]. These molecules in turn induce the expression of muscle regulatory genes such as myoD and myf, which control all the features of the muscle differentiation program, including cell cycle withdrawal, alignment and fusion between myoblasts, and changes in reticulum and mitochondria. These master switches control the expression of muscle-specific cytoskeletal and adhesion proteins, either directly or through the regulation of muscle differentiation genes such as mgn and MRF4. Many of the myogenic processes require specific cytoskeletal, membrane, and adhesion proteins. For instance, the assembly of the contractile apparatus is based on the regulated expression of muscle-specific actin and myosin (with the help of chaperones like HSP90). The changes in cell shape are caused by the rearrangement of microtubules. Alignment depends on extracellular clues and fusion is a consequence of interactions between membrane proteins.

In this review, I will focus on molecules involved in two aspects of myogenesis: the changes that the three major cytoskeletal systems undergo as muscle cells assume their mature form and function and the related changes in cell and matrix adhesion. These changes are related to both structural and physiological (regulatory) functions of the cytoskeleton in muscle. While contraction is actin-based, and therefore actin is arguably the major functional cytoskeletal

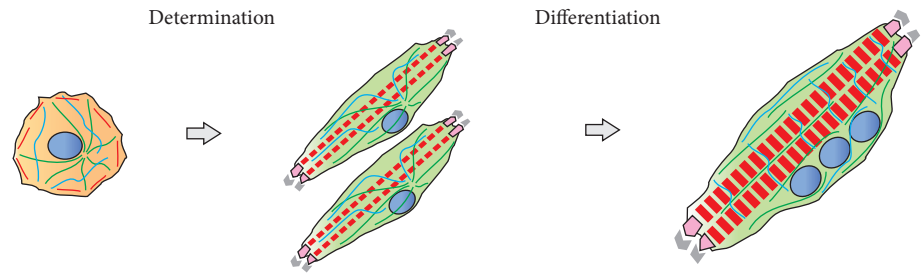


Figure 1: Schematic view of skeletal myogenic steps, focusing on the cytoskeleton. A round, mononucleated cell has short actin filaments in the cortex, dispersed intermediate filaments, and radial microtubules. After replication, myoblasts align with each other, and their actin filament begins to form the myofibrils, anchored in adhesion regions that connect to the extracellular matrix. After fusion, the differentiated myotubes are multinucleated and have strong myofibrils. The intermediate filaments are present around Z-lines (among other regions), and microtubules have no single organizing center. For color scheme, see Figure 4.

component in muscle, myofibrillogenesis is a target of intense investigation and has been dealt with in several reviews [2, 3]. Therefore, I will not emphasize it in this review.

2. Cytoskeleton

The cytoskeleton is composed of a polymeric arrangement of proteins organized in specific ways: microfilaments are double helices of a single actin isoform, microtubules are hollow tubes composed of 13 protofilaments of alpha and beta tubulin, and intermediate filaments result from the lateral association of different, although similar, proteins of the same family. Most of the physiological roles of the filaments are related either to changes in their length and/or organization, modifying the cell shape for instance, or to associated motor proteins that slide along them. In fact, the control of all cytoskeletal functions and structures depends on associated proteins, such as myosin and dynein/kinesin (movement-transducing ATPases), or alpha-actinin and microtubule-associated proteins (MAPs) (which regulate the actin and tubulin networks). Not only do the cytoskeletal proteins act as the bones and muscles of the cell, but also they are sensitive: they relay signals through associated protein kinases. Thus adhesion sites are places where the cytoskeleton interacts mechanically with the extracellular matrix and where the cell senses the substrate through focal adhesion kinases (FAKs).

During myogenesis, the precursor cells must somehow discard their "generic" cytoskeleton and organize a muscle-specific, highly specialized contractile cytoskeleton. During this process, housekeeping isoforms of cytoskeletal proteins will be replaced by muscle-specific isoforms. I discuss the changes during myogenesis in each cytoskeletal compartment: microfilaments, which will give rise to myofibrils, intermediate filaments, and microtubules. Although I analyze each compartment individually, it is important to keep in mind that the three cytoskeletal systems are highly integrated, and I will discuss the role of this integration in myogenesis.

2.1. Microfilaments. Actin filaments are present in all cells and in all eukaryotes, since they are essential for fundamental cellular processes such as cytokinesis. Actin is the most cortically distributed of the cytoskeletal components, usually being involved in cell adhesion. But actin is also an important component of the nucleoskeleton, although in the nucleus it probably forms shorter filaments than in the cytoplasm. In the nucleus, actin is involved in gene regulation [4]. The filament networks are formed by polymerization of actin in conjunction with several actin-associated proteins, which regulate all aspects of actin structure and function.

In precursor muscle cells, actin is distributed close to the membrane, and it changes its distribution to form myofibrils that will occupy most of the cell cytoplasm in the mature cell (Figure 2).

2.1.1. Actin. Together with myosin, actin was one of the first cytoskeletal molecules to be purified from whole muscle, initially in the form of actomyosin. Human actin is a 43 kDa protein, with a highly globular structure. In fact, its three-dimensional structure depends on compaction by chaperonins [5]. Actin is an old and ubiquitous gene present in all eukaryotes. The bacterial MreB proteins, found in prokaryotes, have structures similar to actin but with only 15% gene homology [6]. Actin is a conserved gene; the human genome has 6 actin genes and 2 pseudogenes [7]: smooth muscle alpha actin, cardiac alpha actin, skeletal alpha actin, cytoplasmic beta actin, cytoplasmic gamma actin, and enteric gamma actin. Although actin isoforms in different tissues are very similar, the relationship between isoform and function is still being unraveled. Initial experiments showed that fluorescent labeled gamma-actin can substitute for alpha-actin without noticeable effects [8]. More recent experiments have shown that the induced expression of human beta-actin in insect flight muscle causes structural disturbances [9]. It now appears that certain isoforms have specific roles, such as the regulation of cell spreading by beta-actin [10].

The binding of ATP in the interior of the actin molecule changes its shape and is associated with the polymerization state: usually monomeric (G, "globular") actin is bound to ATP while polymerized (F, "filamentous") actin is bound to ADP. In adequate conditions, in the presence of ATP, Ca^{2+}, and Mg^{2+}, actin polymerization is dependent on time and monomer concentration: initially, there has to be the assembly of a filament "core" of a few molecules. After the core reaches a certain size, in a specific range of concentrations,

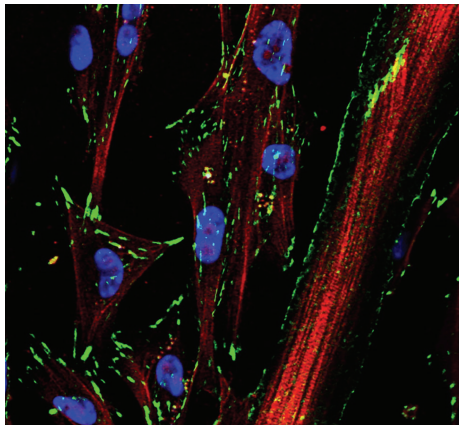


Figure 2: Actin filaments in a chicken primary culture. In this laser confocal image, actin filaments are stained with red phalloidin, paxillin with green antibodies, and nuclei with Dapi. A single large myotube is seen on the right, while mononucleated fibroblasts and/or myoblasts appear on the left. While the more cortical actin filaments terminate in paxillin adhesion regions around which the mononucleated cells attach, several striated myofibrils run along the myotube, and only a single, large paxillin region can be seen. The myotube nuclei are out of focus and thus not visible in this single confocal slice.

one of the filament ends (called "minus") will tend to lose monomers, while the other end (called "plus") will tend to gain monomers. This will cause movement of monomers along the filament without a net increase in its length. This treadmilling has been observed both *in vitro* and *in vivo* [11].

A large variety of actin structures exist in all cell types, and there are usually several microfilament organizations in the same cell. One interesting demonstration that cells have different "actin compartments" is revealed by treatment of muscle cells with cytochalasin D. Cytochalasin D is an actin-depolymerizing drug, but in certain concentrations it is capable of disassembling cortical actin without affecting sarcomeric actin (data not shown). Structurally and physiologically independent actin compartments can be assembled because, although actin itself is a conserved protein, these structures are organized by several actin-associated proteins, which can be very specific for each cell compartment.

In sarcomeres, the length of the actin filaments is regulated by proteins that bind to both extremities (CapZ and tropomodulin) and by nebulin, a giant protein that binds along the whole actin filament, spanning half the length of the sarcomere [12]. It is noteworthy that the turnover of actin molecules in myofibrils is slower than in other cell structures [13], possibly due to the large number of actin-associated proteins in myofibrils. In addition to these partners, actin filaments in sarcomeres are bound to several proteins, such as tropomyosin and troponin, which control their stability and their interaction with myosin. These partners make it possible for contraction to be regulated by the release of Ca^{2+} stored in the sarcoplasmic reticulum. This release is induced by ryanodine receptors in the membrane of the reticulum, which in turn are induced by dihydropyridine receptors, reacting to the cell membrane depolarization in the T tubule. Calcium binds to the subunit troponin C ("calcium-binding subunit") in the myofibrils. Troponin C is related to the ubiquitous protein calmodulin: both have four calcium-binding sites, and they undergo a radical change in structure when bound to calcium. Troponin C in turn binds to troponin I ("inhibitory subunit"), which blocks the interaction between actin and myosin through its binding to troponin T ("tropomyosin-binding subunit"). In the presence of excess Ca^{2+}, troponin T displaces tropomyosin from the myosin-binding domain on the actin filament, allowing the interaction between actin and myosin, which leads to muscle contraction. Tropomyosin both regulates contraction and is an actin-stabilizing molecule. Its long shape allows it to bind to 7 actin monomers at once along the filament. Similar to other myofibrillar proteins, tropomyosin switches isoforms during development, and several isoforms (muscular and nonmuscular) are generated from different genes by alternative splicing [14]. The tropomyosin isoform switch has physiological implications, and specific isoform alterations lead to characteristic disease phenotypes.

2.1.2. Myosin. In muscle, movement is generated by the sliding of the ATPase myosin over actin filaments. Multiple isoforms of myosin have been identified in a variety of cell types and organisms, sharing a conserved ATPase head but with varying size tail, which also varies in its ability to carry different cargoes in the cell [15]. Thus myosin-V transports cargo vesicles in the brain [16] and myosin XVIII attaches actin filaments to the plasma membrane in ear cells [17]. The muscle isoform is myosin II, which has several tissue and development specific variants: fetal myosin, fast and slow skeletal myosin, and cardiac myosin. Experimentally, myosin can be cut by trypsin and yields two fragments: heavy meromyosin and light meromyosin, which can be used to decorate actin filaments, revealing their polarity. Myosin II can spontaneously assemble into filaments 1 μm long and 16 nm in diameter, where the long tails are twisted together while the heads project to the outside of the filament. While highly detailed models of the force-producing mechanisms involved in actin-myosin interactions have been proposed, it is still not clear exactly how the chemical energy is transformed into movement or force, through atomic displacements in the myosin.

2.1.3. Other Sarcomeric Proteins. The basic unit of myofibrils is the sarcomere, an interdigitating crystal of actin and myosin. In the polarizing microscope, myosin filaments are anisotropic and form the A-band, while actin filaments form the isotropic I-band and part of the A-band, where it overlaps with myosin. Dedicated structures link the filaments laterally: the Z-line (from Zwischenscheibe—"disc between the bands") for actin and the M-line (from Mittelscheibe—"disc in the middle of the bands") for myosin. These structures are formed by specific proteins, among them alpha-actinin (and indirectly desmin) in the Z-line and myomesin in the M-line. Alpha-actinin is a versatile protein, capable of linking actin filaments to the membrane and to each other.

Myomesin is an elastic protein, involved in the production of force during an isometric contraction [18]. The actin-capping protein CapZ binds the actin filament to alpha-actinin in the Z-line.

Z-lines are linked to M-lines through the giant protein titin. Titin is the largest polypeptide synthesized by our genome, spanning almost 1.5 μm. Titin has a structural role in the alignment of muscle but has also a regulatory role, of transmitting information to the myofibril [19].

2.1.4. Myofibrillogenesis. The formation of a myofibril is a remarkable process, since it is the construction of a moving structure that has to function while it is being assembled. In fact, inhibition of contraction blocks myofibril assembly [20]. Myofibrils are grown over a preexisting scaffold of cytoskeletal structures, such as Stress Fiber-Like Structures (SFLS), which are made of nonmuscle isoforms of proteins. These isoforms have to be replaced by their muscle counterparts again, while muscle is contracting.

These myofibril precursors are already attached to the membrane, through proteins such as alpha-actinin and integrin. Therefore, alpha-actinin is present in myofibrils both in the membrane region and periodically along the filament. During myogenesis, SFLS associate laterally, aligned by alpha-actinin-decorated regions (Z-bodies), which will be the precursors of Z-lines. These premyofibrils have minisarcomeres, since the periodicity of their Z-bodies is smaller than the actual sarcomeric Z-line distance [21]. The Z-line is organized before the M-line: therefore, antibodies directed against Z-line epitopes of the titin molecule will stain periodically before M-line epitopes [22]. The M-line is organized while the myosin isoform is switching from nonmuscle to muscle. The periodicity of the M-line, like that of Z-lines, also increases during development [23]. This has been confirmed recently by two-photon and second harmonic microscopy [24].

The substitution of cytoskeletal isoforms during myogenesis is gradual and precise: first there are nonmuscle beta and gamma actin, then smooth muscle alpha actin, then cardiac alpha-actin, and then skeletal alpha-actin [25].

Several other structural proteins that are involved in myofibril formation and function such as troponin, actin, myosin, and alpha-actinin also undergo isoform substitution during myogenesis. Other proteins that are not part of the myofibrils themselves also participate in myofibrillogenesis, such as the chaperones Hsp90 and Unc45b [26]. For instance, obscurin, a giant protein like titin and nebulin, has been shown to bind to Z- and M-protein clusters and to participate in myofibrillogenesis [27].

2.2. Intermediate Filaments. Filaments with approximate diameter of 10 nm have been observed in several tissues and have been called intermediate filaments (IF) by Holtzer (Ishikawa et al. [28]). The purification of IF proteins revealed their varied composition, specific foreach cell type. While there can be several intermediate filament proteins expressed in a given cell type, forming copolymers, they are always expressed in a regulated form in specific cell types: this specificity led to the use of intermediate filament identification in cancer diagnosis. In our genome, there are apparently 63 genes for IF proteins [29]. IFs are not present in all cell types or in all eukaryotic species, although the nuclear lamina, a supporting structure under the internal nuclear membrane, is formed by the IF proteins called lamins. There are reports of IF-like proteins in insects and protozoans, but they are typical of vertebrates. Despite their variety, all IF proteins share a common rod core with heptad repeats and have globular extremities of varying sizes. Contrary to actin monomer, IF proteins align laterally, in an antiparallel way; therefore they form a strong attachment yielding a stable filament that is not polarized. The phosphorylation of IF proteins leads to depolymerization of the filament. They are classified according to their ability to copolymerize and their structural homologies. Type I IFs (acidic cytokeratins) always polymerize with type II (basic cytokeratins). Type III includes desmin, vimentin, glial acidic fibrillary protein (GFAP), and others.

A variety of functions have been attributed to intermediate filaments, such as maintenance of the cell shape and signal transduction. Intermediate filaments are involved in diseases such as epidermolysis bullosa, caused by mutations in cytokeratin genes. The fragility of the affected epithelium suggests that the IF network has the structural role of linking the cytoskeletons of adjacent cells through their adhesion foci. In muscle, they are assumed to participate in the integration of structure and function, because of their stability and the fact that they link myofibrils, mitochondria, nuclei, and the plasma membrane. Muscle cells may express the IF proteins desmin, nestin, vimentin, synemin, syncoilin, cytokeratin, and lamin.

2.2.1. Desmin. The IF protein of skeletal muscle cells was purified simultaneously by Lazarides, who named it desmin [30], and by Small, who called it skeletin [31]. Desmin is expressed in all muscle types and in endothelial cells. It is an important marker of muscular origin, used for instance to prove the muscular origin of the electric organs of the electric eel [32]. Desmin is the first muscle structural protein to be expressed during differentiation and is retained, usually upregulated, in muscle dedifferentiation models, such as electric organs, TPA-induced myosacs, and heart Purkinje fibers [29]. Desmin, like any IF protein, can be phosphorylated, and changes in its phosphorylation pattern have been related to physiological conditions, such as heart hypertrophy or electrical discharge pattern. Desmin distribution also changes along development: initially it is expressed around the nuclei; then it accumulates around the myofibrils, associating with the Z-line and becoming striated. Later, it also accumulates around some adhesion structures, such as myotendinous junctions. It is also present in costameres and around mitochondria.

The function of desmin is not clear: desmin is not essential for muscle formation or functioning, since desmin knockouts exhibit only a smaller resistance to fatigue than normal mice [33]. The KO handicap could be due to mislocalization of their mitochondria. On the other hand, desminopathies have been characterized in humans,

caused by mutations in the desmin gene [34]. Interestingly, desminopathies can also be caused by mutations in the alpha-B-crystallin gene. Alpha-B-crystallin is a small heat-shock protein that is distributed along the IF network and participates in its organization. Mutated alpha-B-crystallin proteins can disrupt a preexisting intermediate filament network.

Desmin may have structural or signaling functions, since it can interact with muscle gene regulatory elements such as myoD. It has also been proposed that changes in the polymerization state of individual filaments are an interesting way of sending signals to the interior of the cell [29].

2.2.2. Nestin. Nestin ("NEuroepithelial STem cell protein") is a type VI IF protein that is mostly expressed during development in proliferative muscle and nerve cells before differentiation, although it is also expressed in other tissues such as kidney. Not only is the expression of nestin downregulated upon differentiation but also it can be reinduced upon injury and regeneration [35]. The relationship between nestin and cell division led to the use of nestin as a marker of stem cells in the brain and as a marker of muscle regeneration. Nestin is phosphorylated by Cdk5, which is related to the cyclin-dependent kinases but which is actually regulated by p35. The switch from conventional Cdks to Cdk5, a nonmitotic kinase, correlates with muscle differentiation and with an increase in nestin phosphorylation. Although nestin has the central rod domain typical of intermediate filaments, essential for their antiparallel alignment and polymerization, their N-terminal domain is short, which may be related to the fact that nestin is commonly found in heteropolymers. From structural studies, it is thought that nestin interacts with microtubules and microfilaments [36]. In mature muscle, nestin expression is mostly restricted to neuromuscular and myotendinous junctions. Nestin knockout mice show structurally normal muscles but with more neuromuscular junctions than wild-type mice, which led to impaired muscle performance in some tests [37]. Interestingly, Cdk5 and p35 expression are required for proper muscle development, since both Cdk5 knockdown and p35 overexpression interfere with myogenesis [38]. Both nestin and Cdk5 have been implicated in several diseases, including Alzheimer's, muscular dystrophy, and cancer [39].

2.2.3. Vimentin. Vimentin is expressed in several mesenchymal cells and is downregulated during muscle differentiation. While there is some controversy regarding the levels of vimentin in adult muscle tissue, its transcriptional downregulation during myogenesis has been well characterized [40]. Although the role of vimentin is not clear, it is speculated that both nestin and vimentin are expressed before differentiation and/or as a back-up IF system. Vimentin knockout mice are viable, with subtle phenotypes. Since vimentin is coexpressed with several other IFs, vimentin KO mice show defects in the corpus callosum and cerebellum, probably because of its interaction with the intermediate filament protein of glial cells, GFAP [41]. Since no muscle-specific vimentin function seems to have been demonstrated up to now, the presence of vimentin in muscle could be just a remnant of its embryological origins. On the other hand, vimentin has been demonstrated to be part of adhesion structures in endothelial cells that are similar to hemidesmosomes, because they contain plectin and integrin, but they also contain vinculin and actin [42].

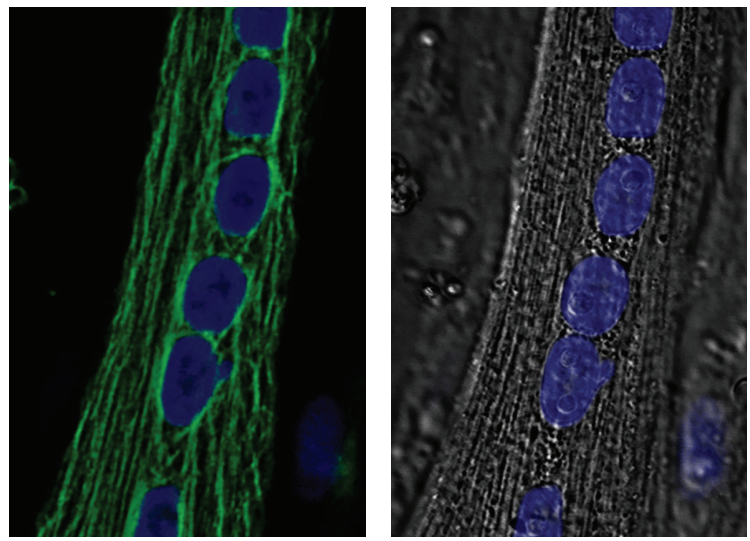
2.2.4. Syncoilin. Syncoilin was identified in a two-hybrid system for its interaction with alpha-dystrobrevin, a component of the dystrophin-associated protein complex (DAPC) [43]. In normal skeletal muscle, syncoilin is concentrated at the neuromuscular junction, together with alpha-dystrobrevin, but is also present close to Z-lines and costameres, where it binds to desmin [44]. Syncoilin is mostly expressed in skeletal muscle and heart but can also be present in lung and testes. The distribution of syncoilin is affected in muscle diseases, such as muscular dystrophy, central core diseases, and neurogenic muscle disorders [45]. Studies with syncoilin knockout mice showed that it is not required for skeletal and cardiac myogenesis but is important in the production of maximal force, possibly through its interaction with desmin [46]. Syncoilin seems to be unable to form filaments by itself, it has to form heteropolymers with desmin and nestin.

Both syncoilin and synemin have been shown to bind to alpha-dystrobrevin and to desmin, and they characterize a link between the IF network and the DAPC [47]. This association may be particularly important in dystrophies and desmin myopathies and in NMJ and costameres. On the other hand, the fact that syncoilin seems to be upregulated in some diseases may mean that it is also involved in regeneration [48].

2.2.5. Cytokeratin. Skeletal and cardiac muscle cells express cytokeratins K8 and K19 [49]. These are also found in skin and at lower levels in other tissues; in striated muscles they are distributed in costameres and Z-lines. Cytokeratin K19 knockout mice have altered costameres and mitochondrial distribution and develop a mild myopathy [50]. Cytokeratins may reinforce the connection between myofibrils and the plasma membrane through IFs, and, in the absence of this link, mitochondria can redistribute.

Cytokeratin K19 has been shown to interact with dystrophin through immunoprecipitation, and cytokeratins are displaced in the dystrophin null, *mdx* mice. Desmin and cytokeratin double knockouts display a more altered muscle phenotype in some respects than single desmin or cytokeratin knockouts, such as creatine kinase levels, contractile torque after injury, and costamere disruption [51]. Curiously, some defects are less pronounced in the double knockout, such as membrane gaps and mitochondria accumulation, suggesting that the interaction between desmin and cytokeratin can be quite complex.

2.2.6. Lamin. Lamins are intermediate-filament proteins that form the nuclear lamina, the structural scaffold of the nuclear envelope. While all metazoan cells express lamins, it is interesting to note that mutations in lamins or in lamin-associated proteins (LAPs) have a large impact on muscle



(a) (b)

Figure 3: Longitudinal microtubules in a chicken primary culture multinucleated myotube. In the left panel, microtubules are visualized in this laser confocal section with specific antibodies and compared to nuclei, stained with Dapi. In the right panel, the same cultured myotube is visualized with differential interference contrast optics, which shows the myofibrils and the nuclei (again compared with the Dapi stain). Note that there are no conspicuous microtubule-organizing centers, although it is possible to see an accumulation of microtubules around the nuclei.

fibers and cause muscle diseases. Mutations in the lamin-associated protein emerin have been identified as the cause of Emery-Dreifuss muscular dystrophy (EDMD). Although emerin is present in all cell types, it is not clear why they cause mainly muscle deficiency. In one hypothesis, the mechanical stress that is imposed on muscle cells makes their nuclei more susceptible to injury, while another hypothesis is based on the role of emerin on gene expression [52]. Indeed, several functions for lamins and LAPs have been described, including intranuclear roles such as regulation of transcription and extranuclear roles such as nuclei anchorage. The LAPs SUN1 and SUN2 have been shown to participate in positioning of nuclei through farnesylation of the lamin precursor protein prelamin A [53]. Accordingly, in EDMD muscle fibers, the position of muscle nuclei is disturbed and prelamin A and SUN1 are reduced.

Recently, the nucleoskeleton has been related to the cell membrane, and Wnt receptors have been shown to translocate to the nuclei, where they form foci and are involved in the expression of synaptic proteins [54]. A direct relationship between the nucleoskeleton and extracellular adhesion has been shown; it involves interactions between beta-dystroglycan and lamin and emerin, and knockdown of beta-dystroglycan affects nuclear morphology [55].

2.2.7. Other IF Proteins: Synemin—Paranemin, Transitin, and Desmuslin. Several other IF proteins have been identified in muscles of different species. Some of these proteins are not capable of forming filaments on their own and have been grouped as type VI IF proteins [56].

Synemin was first described by Lazarides as an IF-associated protein [57], because it was copurified with desmin and vimentin from muscle cells. Now it is considered to be an IF protein, even though it is always forming heteropolymers with other IF proteins and is not capable of forming a homopolymer by itself. The synemin gene can yield several variants based on alternative splicing. In nonmuscle tissues, synemin is distributed along the leading edge of migrating cells, and it participates in cell migration [58]. In cardiac muscle cells, while alpha-synemin has been shown to be distributed at cell junctions, beta-synemin is preferentially present close to the Z-line [59]. It binds to alpha-actinin, vinculin, and dystrophin.

Paranemin has also been isolated as an IF-associated protein [60]. Transitin is a splice variant of paranemin, expressed in birds. Tanabin is another IF protein, expressed transiently in amphibians.

The IF protein desmuslin has been described mainly in heart and skeletal muscle cells and binds to alpha-dystrobrevin, a protein component of the dystrophin-associated adhesion complex, and therefore it links the extracellular matrix to Z-discs [61]. Desmuslin is probably the human ortholog of synemin.

2.3. Microtubules. During myogenesis, microtubules are extensively remodeled, and their radial distribution in myoblasts is replaced by a longitudinal distribution along the elongated myotubes (Figure 3).

Microtubules have several roles in myogenesis. It was shown a long time ago that they are important in determining the overall shape of the cell, a property that is related to their radial distribution. During myogenesis, myoblasts undergo an extensive cell shape change from round to bipolar and afterwards fuse into myotubes. Interfering with microtubule

integrity in myoblasts using the drug taxol, which blocks depolymerization, induces the formation of star-shaped cells [62]. More recently, it was shown that microtubules participate in cell shape change during myogenesis through the microtubule-associated protein EB1, since the knockdown of EB1 via shRNA blocks elongation. Zhang et al. [63] have also shown that, during development, EB1 is required for fusion, microtubule stabilization and cadherin, and catenin accumulation at the plasma membrane.

Posttranslational modification can interfere with microtubule polymerization rates. Removal of the C-terminal tyrosine of alpha-tubulin monomers, exposing a glutamine residue, renders microtubules more stable. Conversely, tyrosinated microtubules are more dynamic than detyrosinated microtubules. Mian et al. have shown that microtubules in myoblasts pass through a "mostly unstable" phase before fusion and elongation, as shown by staining with isoform-specific antibodies [64]. Moreover, this destabilization is dependent on the protein kinase LKB1, since RNAi against LKB1 blocks myogenesis.

Microtubules are used for the distribution of organelles such as mitochondria and nuclei. The distribution of mitochondria in muscle cells is conditioned by its energetic requirements. During maturation of specific muscle types, the content and distribution of mitochondria depend on the functional adaptation for fast or slow contraction: slow muscles ("red meat") are more oxidative and have more myoglobin and more mitochondria, while fast muscles are more anaerobic, with less myoglobin and fewer mitochondria. Curiously, since desmin knockouts have displaced mitochondria, intermediate filaments must be involved in mitochondrial distribution, yet the distribution of microtubules is not critical for mitochondria redistribution during myogenesis [65]. On the other hand, microtubules are important for repositioning of nuclei during myogenesis. More specifically, knockdown of the microtubule associated protein MAP7 or the motor protein kinesin Kif5b induces the nuclei to stay centrally localized, as is characteristic of several myopathies. Microtubules also seem to be involved in myofibrillogenesis, according to time-lapse studies with tagged myosin and EB1 [66]. Disruption of microtubules with nocodazole destabilizes nascent myofibrils but does not affect mature sarcomeres. The direct participation of microtubules in myofibrillogenesis has been challenged, however, by several groups. For instance, treatment of myoblasts with myoseverin, a drug that depolymerizes microtubules, does affect both myofibrils and microtubules, but upon removal of myoseverin, myofibrils could be formed even in the absence of microtubules [67]. In the conditions used, treatment with nocodazole disassembled microtubules but did not interfere with myofibrils.

Microtubules are related to adhesion. It has been shown that microtubules associate with dystrophin and that they are related to Ca^{2+} influx and production of reactive oxygen species [68]. Microtubules also bind to cadherin and are therefore involved in alignment and fusion during myogenesis [69]. Finally, microtubules bind to dysferlin, a transmembrane protein involved in repair, and it is speculated that microtubules may participate in dysferlin trafficking to the plasmamembrane [70].

2.4. Cytoskeletal Integration. Several proteins link the cytoskeletal filaments with each other and with other structures, such as adhesion complexes. Among the major integrator proteins are those of the plakin family, which bind IF to microtubules and to actin filaments. Some plakins are components of adhesion complexes, such as desmoplakin and gamma-catenin. Several plakins are expressed in skeletal muscle, such as plectin, microtubule-actin crosslinking factor (ACF7/MACF1) and bullous pemphigoid antigen 1 (dystonin/BPAG1) [71].

Plectin is expressed in muscle in several isoforms and associates with the dystrophin-associated adhesion complex. A mutation in the plectin gene causes both epidermolysis bullosa and muscular dystrophy [72]. Plectin isoforms are selectively expressed during myogenesis [73].

Another member of the plakin family expressed in muscle is dystonin/BPAG1. Initially identified with a gene that causes neuropathy in the mouse mutant dystonia musculorum, it was later shown that the same gene codes for the protein bullous pemphigoid antigen 1, against which the autoimmune disease pemphigus reacts [74]. It has been assumed that Bpag1 has a linker role, connecting the cytoskeleton to the cell membrane. In muscle, it binds to desmin.

Another member of the plakin family, shortstop, binds through EB1 to microtubules [75]. Shortstop is the ortholog of MACF1, which may participate in the organization of the neuromuscular junction.

The protein MURF-2 (muscle-specific RING finger-2) also associates myofibrils with microtubules. Knockdown of MURF-2 in chicken muscle cultures disturbs fusion and myogenesis [76].

Cytoskeletal dynamics are controlled by the small GTPases Rho, Rac, and Cdc42. These proteins are related to the Ras protein and mediate the signaling between several membrane receptors, such as tyrosine kinase and integrins, and downstream targets, such as mitogen-activated protein kinases and Rock. Since these proteins have been shown to control the formation of filopodia, pseudopods, and ruffled membranes and they interfere with both microfilaments and microtubules, it was reasonable to expect that they are involved in myogenesis. Indeed, inactivation of RhoA with dominant-negative mutants inhibits fusion, and mice deficient in Trio (a downstream molecule in the Rho pathway) exhibit skeletal muscle deformities [77].

The relationships among cytoskeletal components in a muscle cell are depicted in Figure 4.

3. Adhesion Complexes

Some cell types do not attach to any substrate and keep circulating in the body, like blood cells. Other cells need to attach to the substrate to function in specific locations. Yet muscle cells also need to be able to exert tension on specific locations in the body, and thus they are connected to bones through tendons. During development, muscle cells migrate

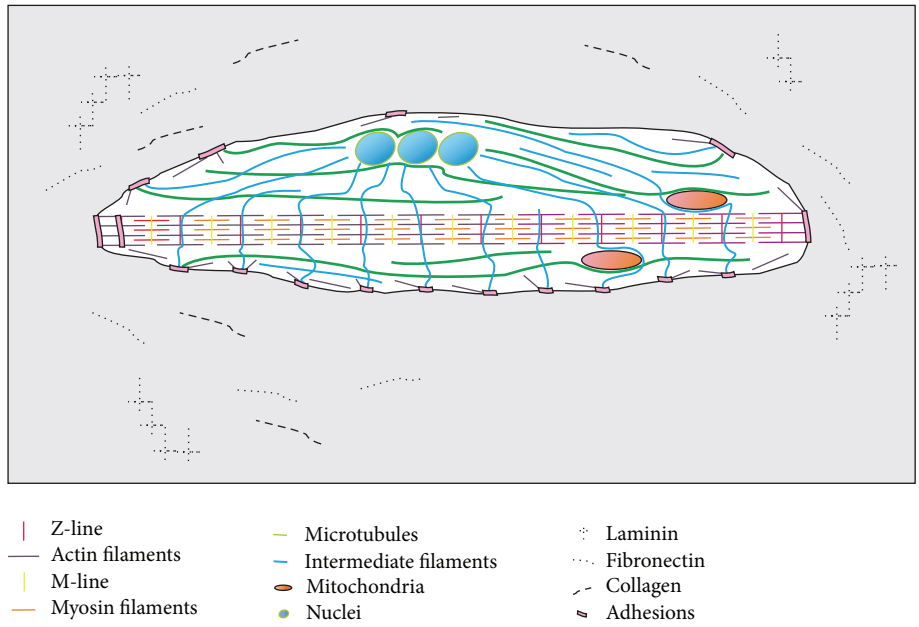


Figure 4: Scheme showing the distribution of the major cytoskeletal components in a muscle fiber. Actin filaments (thin, purple) appear either in myofibrils or close to the sarcolemma, interacting with adhesion sites (pink). Intermediate filaments (blue) are distributed all over the cell, particularly around Z-lines, around the nuclei and mitochondria, and sometimes terminate in adhesion regions. Microtubules (green) are parallel to the major cell axis and have no conspicuous organizing center. Z-lines (red) alternate with M lines (yellow) and are aligned in separated myofibrils. Nuclei (blue) are aligned in the cell periphery, while mitochondria (orange) remain close to the myofibrils. Adhesion sites appear at the extremities of the myofibrils and periodically along the Z-line, forming costameres. Extracellular matrix molecules (black) are indicated vaguely, as they interact with several types of adhesion proteins. The nerve (checkered) contacts the muscle at a specialized locus, the neuromuscular junction.

and transiently express specific attachment proteins, while later on, upon reaching their final destinations, they built strong adhesions and express definitive adhesion proteins. These adhesion proteins have both structural and signaling roles.

3.1. Cadherin-Based Adhesions. The major cell-to-cell adhesion protein is cadherin, which keeps cells together in layers. Cadherins are transmembrane proteins with extracellular calcium-binding domains through which they bind to other cadherins. There are several tissue-specific cadherin isoforms: E (epithelial), N (neuronal), M (muscular), and others. They can form heterophilic interactions, and each combination of cadherins has a specific adhesive property.

During myogenesis, cells switch their adhesion protein from N- to M-cadherin. This is particularly interesting in zebrafish, because cells migrate from the interior of the embryo, close to the notochord, to the surface, while they switch their cadherin isoforms [78]. These authors suggest that the differential adhesion caused by the different isoforms is involved with the cell migration. It is clear that M-cadherin is required for fusion, since when peptides corresponding to the binding sequence of M-cadherin are applied to a cell culture, they block fusion [79]. On the other hand, satellite cells, which are quiescent muscle stem cells, express M-cadherin even before they undergo replication and differentiate into myotubes. Moreover, M-cadherin knockout mice have normal muscles [80]. M-cadherin was shown to regulate the GTPase Rac1 and interfere with myoblast fusion [81].

Cadherin adheres to alpha-, beta-, and gamma-catenin (also known as plakoglobin). Beta-catenins are versatile molecules that have a role as a stable, localized connection between cadherin and actin and also as a soluble factor that can migrate to the nucleus and regulate gene expression. Free cytoplasmic beta-catenin is usually phosphorylated by GSK3beta, recognized by E3 ligases and degraded by proteasomes, but upon Wnt binding to frizzled receptors, GSK3beta is inhibited and catenin is not degraded and can enter the nucleus, where it binds to LEF/TCF factors and activates genes involved in survival, proliferation, and differentiation, among others. Conditional inactivation of beta-catenin in mouse muscle was shown to inhibit muscle fiber growth [82].

3.2. Integrin-Based Adhesions. Integrin is one of the major cell-matrix proteins, although it can sometimes participate in cell-cell adhesions. Integrins are always assembled as a dimer of alpha and beta integrins. There are up to now 18 alpha and 8 beta integrin isoforms in humans, expressed in a cell-type specific manner: muscle cells express initially alpha1beta1, later on alpha6beta1, and upon terminal differentiation, alpha7beta1 [83]. Integrins can be further modified posttranslationally, and in muscle alpha7 integrin is ADP-ribosylated. Integrins bind externally to specific substrates, depending on their subunit composition: laminin,

fibronectin, and collagen. Integrin can bind to selectins and to IgCAMs presented by other cells. In muscle, integrin binds mostly to collagen IV. Internally, integrin binds to vinculin, talin, and alpha-actinin, and indirectly to FAKs. Integrin has been demonstrated to be a regulator of gene expression in muscle [84]. During myogenesis, several combinations of integrins are expressed, and interfering with the integrin expression pattern affects myogenesis.

Vinculin is a linker protein that undergoes a dramatic structural transition from a closed, inactive form to an open, active form. It binds to several molecules, depending on phosphorylation. Because of its role in adhesion, vinculin has been related to cancer and metastasis, and it has been shown that mutations in the phosphorylation region of vinculin are involved in cancer.

Talin is another adapter protein that binds to integrin, and talin-1 knockout mice have muscle defects, although their myoblasts fuse normally into muscle cells and assembly of costameres and myotendinous junctions is also normal [85]. This is consistent with the fact that talin-1 is present in focal adhesions while talin-2 is present in costameres. Senetar et al. suggest that talin-1 has a transient, myogenic role, while talin-2 is expressed in mature muscle cells [86].

A role for integrin in myogenesis has been shown through its association with kindlin-2 [87]. The expression of kindlin-2 is upregulated during muscle differentiation, and it concentrates close to integrin. Knockdown of kindlin blocks elongation and fusion.

Integrin-based adhesion signals through the enzyme FAK (focal adhesion kinase). Besides integrin, molecules such as growth factor receptors are capable of activating FAK, which in turn can phosphorylate several substrates, such as paxillin, talin, cytoskeletal GTPases, and cadherins. FAK itself is regulated through phosphorylation, and its importance in myogenesis is clearly visible in zebrafish, where there are complementary gradients of FAK and phosphorylated FAK [88]. FAK has been proposed to act also as a mechanotransducer, relating contraction to gene regulation [89].

3.3. Dystrophin-Associated Complexes. The dystrophin gene was identified by chromosome mapping of muscular dystrophy carrier families. It is the largest gene in our genome, spanning 2.4 kb, with 89 introns. Different protein products are generated through several tissue-specific promoters in the brain, Purkinje neurons, and muscle [90]. Dystrophin has an N-terminal actin-binding domain, a long repeated central rod domain, and a membrane-binding domain. While absence of the end regions of dystrophin is sufficient to cause the fatal Duchenne dystrophy, absence of parts of the central region only causes the milder Becker dystrophy. Although a lot has been learned about dystrophin and its associated molecules, the etiology of the disease is still debated: current hypotheses include a mechanical role for cytoskeletal-extracellular connection, a role in Ca^{2+} homeostasis, a gene-regulation model, based on dystrophin as a mechanotransducer, and an inflammatory model, explaining the effects of corticosteroid treatments [91].

Since the discovery of dystrophin in 1987, several related molecules have been isolated and shown to form the dystrophin-associated protein complex (DAC). Dystroglycan is the transmembrane protein that links the actin cytoskeleton, through dystrophin, with the extracellular protein laminin 2. There are four sarcoglycans: alpha, beta, gamma, and delta, and the absence of each one causes a variation of limb-girdle muscular dystrophy. Other proteins supposedly are linked to DAC: nitric oxide synthase, caveolin, and syntrophin. Another member of the complex is utrophin, which is quite similar to dystrophin. The dystrophin-deficient mouse mdx exhibits some characteristics of the human disease but not its lethality. In mdx mice, utrophin is upregulated, and it may compensate for the lack of dystrophin. In fact, this is the basis of a human gene therapy protocol, intended to interfere with regulatory sequences in the utrophin gene [92]. Recently, dystroglycan has been shown to translocate to the nucleus in myoblasts, and knockdown of dystroglycan disturbed the nuclear envelope and the formation of centrosomes [55]. The DAC is concentrated at neuromuscular junctions, and it has been shown to participate in stabilization of acetylcholine receptor clusters; dystroglycan-negative cells do not form proper synapses [93].

3.4. Costameres. Costameres are sites of lateral interaction between myofibrils and the sarcolemma. Since the nonsliding parts of the sarcomere are the Z- and M-line, there are proteins connecting both structures to adhesion sites in this membrane. A large number of proteins have been identified as components of costameres, including vinculin, desmin, and Na^+, K^+-ATPase. An important protein in costameres is FAK, and Quach and Rando demonstrated that a dominant-negative form of FAK blocks the formation of costameres and myofibrillogenesis [94]. They further showed that molecules that block contraction and disrupt myofibrillogenesis also block the formation of costameres, suggesting that both structures are linked in myogenesis.

3.5. Neuromuscular Junctions. Neuromuscular junctions (NMJ) are sites of connections between muscle cells and neurons that release the mediator acetylcholine. These areas show invaginations, an adaptation for increasing their surface area, studdedwith acetylcholine receptors, ligand-activated sodium channels, and acetylcholinesterase (which degrades acetylcholine and turns off signaling). The fact that these regions are specialized areas of the membrane is illustrated by experiments in which sectioned nerves grow back to the same region [95]. During myogenesis, the clustering of acetylcholine (ACh) receptors is a late event, dependent on both microfilaments and microtubules [96]. The formation of the NMJ is based on the nerve proteoglycan agrin and its muscle tyrosine kinase receptor MuSK, which activate the downstream proteins rapsyn and Dok-7 to induce the clustering of ACh. Although with different phenotypes, all these components have been shown to be essential in knockout mice [97]. Another essential NMJ constituent is laminin, which may act by an agrin-independent pathway

[98]. Interestingly, it has been shown that Wnt is involved in the clustering of ACh receptors during myogenesis [99].

The electric organs of the electric eel, which are a model for muscle dedifferentiation, are formed by disassembly of the sarcomeric cytoskeleton, increase in expression of desmin, changes in cell geometry, and accumulation of NMJ, which form a large surface capable of simultaneous membrane polarity reversal. The concurrent polarity reversal of parallel cells leads to a high-voltage discharge.

4. Membrane

The muscle cell plasma membrane, or sarcolemma, has several characteristic components. Like the neuronal plasma membrane, the sarcolemma is excitable, due to the presence of voltage-gated Na^+ and K^+ channels. During contraction, the electrical impulse travels deep into the muscle cell, close to the myofibrils, due to T (transverse)-tubules, plasma membrane invaginations that are aligned with the intracellular membranes, or sarcoplasmic reticulum. Proteins connect the T-tubules to the reticulum complex ("dyads" or "triads"), transmitting the depolarization and inducing the opening of Ca^{2+} channels in the reticulum. In a sense, contraction is a membrane-mediated action.

Muscle is a highly endocytic tissue, which may be related to the fact that these cells have multiple caveolae and a specific form of caveolin, caveolin-3. Muscle cells fuse to form myotubes, and therefore the sarcolemma has specific fusion-related molecules. These include phosphatidyl serine (PS) and cholesterol.

4.1. Phosphatidyl Serine. Phosphatidyl serine is involved in several membrane signaling and fusion processes, probably due to its negative charge. Usually PS is restricted to the inner membrane leaflet, but it can be exposed by events such as apoptosis. The cells that express PS can then be recognized and eliminated by phagocytic cells. Exposure of PS also occurs during egg-sperm fusion.The relationship between apoptosis and cell fusion is demonstrated by the fact that blocking apoptosis also blocks myoblast fusion [100]. Expression of phosphatidyl serine in myoblasts is a requirement for fusion [101]. In fact, knockout mice for the phosphatidylserine ligand BAI1 have smaller muscles, although in this case the cells that expose PS are not the ones involved in fusion [100].

4.2. Cholesterol. Cholesterol is present in varying quantities in the plasma membranes of different cell types. Since it is a more rigid molecule than phospholipids, a large amount of cholesterol in a membrane renders it less fluid. On the other hand, a membrane containing only phospholipids will be more organized, and less fluid, than a membrane containing phospholipids and small amounts of cholesterol. High cholesterol is an indicator of heart disease, which is one of the most common causes of mortality in the developed world. In fact, although cholesterol levels correlate well with heart-disease prognosis, the mechanisms are still being debated. Even the relationship between cholesterol intake and blood levels can be misleading: besides collecting cholesterol in the form of low density lipoprotein (LDL), cells can make their own cholesterol. To complicate the matter, cholesterol is a precursor for steroid hormones, which have profound effects on cell behavior.

Since fusion, an important myogenic step, is a membrane event and cholesterol is a regulator of membrane fluidity, it would not be surprising if cholesterol were related to myogenesis. Earlier studies have shown that inhibition of cholesterol synthesis blocks fusion [102]. More recently, cholesterol was identified as the defining molecule of lipid rafts, either planar or caveolar, and research has focused on the roles of lipid microdomains in myogenesis.

4.3. Microdomains: Reggie and Caveolin. While the current membrane model is still one of a fluid mosaic of lipids and proteins, the heterogeneity in its composition in some areas has been clearly characterized. Membrane microdomains are less fluid regions with particular compositions of cholesterol, phospholipids (usually more saturated), and proteins (usually with a longer hydrophobic domain). These regions have a protein scaffold composed of either reggie/flotillin, in planar rafts, or caveolin, in caveolar rafts. Both proteins have self-polymerization domains and hydrophobic domains that interact with the membrane core.

Lipid rafts interact with the cytoskeleton either directly, binding to actin, or indirectly, activating Rho, which in turn controls the cytoskeleton. Interfering with rafts using methyl-beta-cyclodextrin, a cholesterol-removing agent, blocks myogenesis in chicken myoblast cultures [103].

5. Extracellular Matrix

The extracellular matrix is both a mechanical support and an active signaling environment. The connective tissue surrounding skeletal muscles has been shown to participate in muscle development and innervation, controlling proliferation and growth [104]. Among the protein filament systems involved in myogenesis are laminin, fibronectin, and collagen.

5.1. Laminin. There are several laminin structures, formed by specific isoforms and by specific polymerization conditions, including their interactions with collagen. Integrins and dystroglycans bind to laminin. The monomeric molecule has a characteristic cross shape and is composed of alpha, beta, and gamma laminin. Besides the variations in isoforms, laminin undergoes posttranslational modifications such as glycosylation.

During myogenesis, a laminin network is organized by selective binding to myoblasts, which in turn will nucleate the organization of the cytoskeleton through integrin and its associated proteins [105].

The protein netrin is related to laminin and associates with extracellular matrix and cell membranes. Netrin was initially shown to guide neurons, but Kang and coworkers have shown that the levels of neogenin, the netrin receptor,

regulate myogenesis [106]. Later it was shown that neogenin works by regulating FAK and ERK [107].

5.2. Fibronectin. Fibronectin forms filaments when bound to integrins on the cell membrane. Fibronectin isoforms generated by alternative splicing are selectively expressed during development, but several isoforms are expressed in muscle at different times. Fibronectin expression increases in muscle regeneration.

Fibronectin increases myogenesis in *in vitro* cultures and this effect can be blocked by the addition of RGD peptide (a tripeptide composed of L-arginine, glycine, and L-aspartic acid), which competes with the fibronectin-cell attachment domain [108].

5.3. Collagen. Collagen is the very resistant protein of tendons. In humans, it is synthesized by several combinations of 42 genes that produce monomers capable of polymerizing in characteristic ways, originating fibers with different properties. The typical collagen fiber is formed by a triple helix of highly helical protofilaments.

One of the important collagen forms in muscle is collagen-VI. Mutations in the genes Col6A1, Col6A2, and Col6A3 cause a range of myopathies, with the more severe phenotype called Ullrich congenital muscular dystrophy and the less severe Bethlem myopathy. Collagen-VI links fibrous components of the matrix, such as collagen-IV, to the cells [109]. Another isoform involved in myogenesis is collagen-XV. It is expressed mostly in heart and skeletal muscle, and its inactivation by morpholinos (synthetic interference oligonucleotides) in zebrafish has demonstrated that it is essential for notochord differentiation and muscle development [110]. Both collagen-IV (associated with laminin in the basal lamina) and collagen-XIII have been implicated in the formation and maintenance of the NMJ [111]. Collagen-XIII knockout mice, although viable, show defects in NMJ.

5.4. Other Matrix Components. The aforementioned fibrous proteins are not the only matrix molecules involved in myogenesis. Proteoglycans also influence muscle differentiation, and knockdown of syndecan-4, glypican-1, and decorin all increase myogenesis [112].

Metalloproteases are involved in degradation and turnover of several matrix components, and they also degrade cadherins. They have a role in myoblast migration during myogenesis, as well as in growth and repair [113].

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The author thanks Drs. Cláudia Mermelstein and Martha Sorenson (Universidade Federal do Rio de Janeiro, RJ, Brazil) for reviewing the paper. The author apologizes to the many authors whose work has not been cited or cited indirectly through other review articles. The author thanks the members of the Laboratorio de Diferenciação Muscular e Citoesqueleto, Universidade Federal do Rio de Janeiro for their collaboration. His research is supported by FAPERJ and CNPq.

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