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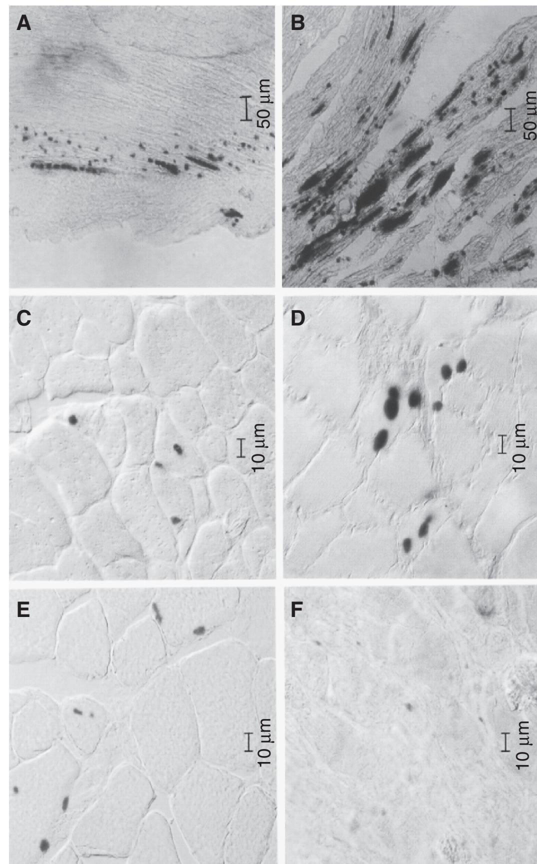
SKELETAL MUSCLE REGENERATIVE ENGINEERING



Formation of muscular cells from transplanted bone marrow cells. The images show nuclear lacZ expression in whole-mount dissected muscle fibers (A,B) or cryostat sections (C–F) of regenerating muscles of scid/bg mice. Mice were injected with unfractionated (A,C), adherent (E), or nonadherent (F) bone marrow cells, or with control satellite cells (B,D), from C57/MlacZ transgenic mice. (A,B) Brightfield; scale bars: 50 μm. (C–F) Nomarski optics; scale bars: 10 μm. (Reprinted with permission from Giuliana Ferrari G et al: Muscle regeneration by bone marrow-derived myogenic progenitors, *Science* 279:1528–30, copyright 1998 AAAS.) See color insert.

Bioregenerative Engineering: Principles and Applications, by Shu Q. Liu
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ANATOMY AND PHYSIOLOGY OF THE SKELETAL MUSCLE SYSTEM

Structure [21.1]

The skeletal muscle system is composed of muscle cells, connective tissue, blood vessels, and nerve fibers. Skeletal muscle cells are organized into bundles, which are attached to the bone via the tendon. The primary functions of the skeletal muscle system are contraction, force generation, and induction of skeletal movement. A skeletal muscle cell is a fiber-like cell and is also called a muscle fiber. Each muscle cell contains a number of nuclei, contractile myofibrils, sarcoplasmic reticulum, and common subcellular organelles, including mitochondria, glycogen granules, endosomes, and Golgi apparatus. The myofibrils are thread-like structures that are aligned in the axial direction of the cell and are responsible for cell contraction and force generation. Each muscle cell contains a large number of myofibrils. Each myofibril is composed of contractile filaments, regulatory proteins, and supporting structures. There are two types of contractile filament: actin filaments and myosin filaments. Since the actin filaments (~8 nm in diameter) appear thinner than the myosin filaments (~12 nm), the actin filaments are referred to as *thin filaments*, while the myosin filaments are referred to as *thick filaments*.

An *actin filament* is composed of two filamentous actin strands, known as F actin, which are organized into a double helix (Fig. 21.1). Each F actin strand is polymerized from a large number of globular actin monomers, known as the G actin. Each G actin monomer is capable of interacting with a myosin molecule at an active myosin-binding site. The double helical actin strands are associated with several proteins, including tropomyosin and troponin. *Tropomyosin* is a fiber-like protein that is aligned along the groove of the helical F actin filaments. *Troponin* is a protein complex, which is responsible for binding to actin, tropomyosin, and calcium ions. Tropomyosin and troponin play critical roles in regulating the interaction between the active sites of actin and myosin filaments, an essential process for muscular contraction.

A *myosin filament* consists of a large number of rod-shaped myosin molecules. Each myosin molecule is composed of two heavy chains, each of which consists of a head and a tail. The tails of the two heavy chains are organized into a helical myosin rod. Each heavy chain is associated with two light chains, which are located at the hinge region between the myosin head and tail. The myosin light chains regulate the activity of the myosin heavy-chains and the interaction of myosin with actin. The myosin molecules are organized into myosin filaments with the myosin heavy-chain heads arranged at both ends of each myosin filament. The heavy-chain head plays a critical role in mediating myosin–actin interaction. It can bind to the active site of actin and can bend at the hinge region between the head and the tail, causing the actin filament to slide along myosin filaments and thus inducing muscular contraction. The heavy-chain head possesses ATPase activity, providing energy for contractile activities.

The actin and myosin filaments are assembled into a structure with highly ordered organization. Each myosin filament is surrounded by six parallel, equally spaced actin filaments. Each actin filament is aligned with a row of myosin heavy-chain heads, which can physically interact with the active sites of the actin filaments. The actin and myosin filaments are organized into functional units called *sarcomeres*, which appear as consecutive short segments along a myofibril. Under an electron microscope, there appear several structures within each sarcomere. These include two Z-disks (located at the two ends of

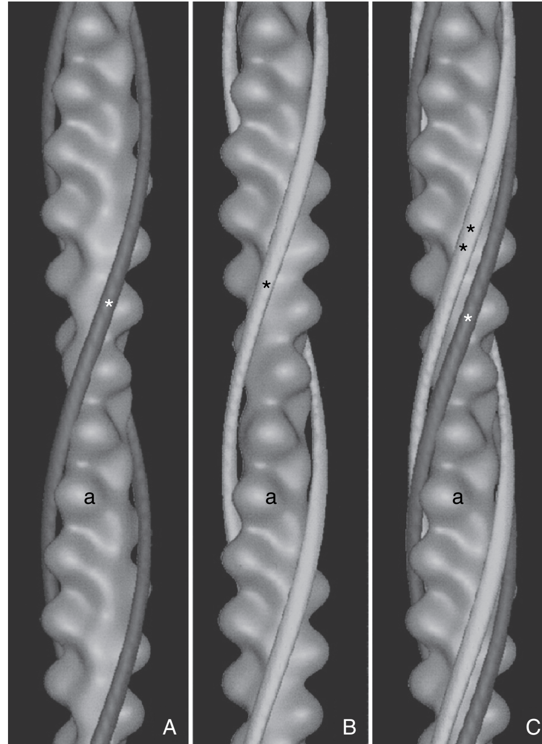


Figure 21.1. Surface views of reconstructions of thin actin filaments (a) showing the positions of tropomyosin strands (*) superimposed on actin in the presence of EGTA (A) and Ca^{2+} (B). In panel C tropomyosin strands associated with both EGTA and Ca^{2+} are superimposed on actin for comparison. Reconstructions show characteristic bilobed actin (a) and continuous tropomyosin strands. In EGTA, tropomyosin (*) occupies a position on the inner edge of the outer domain of actin, whereas in Ca^{2+}, tropomyosin (**) lies along the outer edge of the inner domain. Surface rendering was carried out by superimposing tropomyosin strand densities obtained by difference analysis on the maps of pure F-actin. (Reprinted with permission from Xu C et al: *Biophys J* 77:985–92, copyright 1999.)

a sarcomere and shared by adjacent sarcomeres), a middle band known as the A-band (anisotropic band, defined based on optical properties), a H-zone in the middle of the A-band, an M-line in the middle of the H-zone, and an I-band (isotropic band) between each end of the A band and the Z-line. The formation of these structures is based on the arrangement of actin and myosin filaments. The *Z-disk* is a filamentous network for the anchorage of the actin filaments; the *A-band* covers the entire length of the myosin filaments; the *H-zone* is the region where myosin filaments do not overlap with the actin filaments; the *M-line* is a filamentous network for the anchorage of the myosin filaments; and the *I-band* is the region with actin filaments only. Such a arrangement of actin and myosin filaments gives a striated appearance for muscular cells under an optical or electron microscope.

In addition to contractile elements, the sarcomere contains supporting protein structures. Two well known proteins are titin and nebulin. *Titin* is a filamentous protein and is anchored to the Z-disk at one end and to the M-line at the other end. This molecular structure provides anchorage to the myosin filaments, contributing to the structural integrity and stability of the myosin filaments. Furthermore, titin contributes to the elasticity of the sarcomeres and muscle cells. *Nebulin* is a filamentous protein that is distributed with the actin filaments and provides structural and mechanical supports to the actin filaments.

Mechanisms of Muscle Contraction [21.1]

Based on a hypothetical model, muscle contraction is induced by actin filament sliding against myosin filaments. The myosin filaments are organized with the heavy-chain heads localized symmetrically to both ends of the myosin filaments. The actin filaments are distributed symmetrically with respect to the myosin filaments, so that the actin filaments can interact with the myosin heavy chain heads simultaneously at both ends of the myosin filaments. Thus, the interaction of myosin heads with the actin filaments induces sliding of the actin filaments toward the center of the myosin filaments or the M-line, resulting in the shortening of the sarcomeres and the contraction of the muscle cells.

The contraction of the muscle cells is a highly regulated process, which involves complicated regulatory mechanisms and numbers of regulatory molecules. A contractile process is initiated and controlled by signals from the motor centers of the central nervous system, including the brain and spinal cord. The skeletal muscle system is innervated with nerve fibers originated from these nerve centers. The central motor-controlling neurons generate electric signals called *action potentials*, which can be transmitted from the neurons to the peripheral muscle cells via nerve axons, initiating muscle contraction. The generation of action potentials is dependent on the resting *membrane potential*, which is defined as the voltage difference across the cell membrane at the resting state (without actin potentials). In a normal cell, the cytoplasmic surface of the cell membrane is negatively charged, whereas the extracellular surface is positively charged. The resting potential difference in almost all cell types ranges from −70 to −90 mV under physiological conditions. Such a resting potential difference results from the gradients of ion concentrations across the cell membrane. The concentration of ions, such as Na^+, K^+, Ca^{2+}, and Cl^-, differs from the cytoplasm to extracellular space with the degree of difference depending on the type of the ion. Ion channels specific to each ion type in the cell membrane are responsible for the generation and maintenance of the ion difference. The formation of the resting membrane potential is often called *polarization*. A cell with resting membrane potentials is said to be polarized.

The resting membrane potential can be reversed from a cytoplasmic negative to a positive value in response to various types of stimulation. Such a process is known as *depolarization* and the resulting potential is defined as *action potential*. The stimulation of resting cells induces the opening of gated Na^+ channels, resulting in Na^+ fluxes from the extracellular space to the cytoplasm (note that the Na^+ concentration in the extracellular space is higher than that in the cytoplasm) and a reduction in the membrane potential. When the membrane potential is reduced to a threshold level, a rapid depolarization process is triggered, leading to the generation of an action potential. Shortly after the generation of the action potentials, the Na^+ channels are closed, while the K^+ channels

are open, resulting in the termination of inward Na^+ flux and the beginning of outward K^+ flux (note that the K^+ concentration in the cytoplasm is higher than that in the extracellular space). The outward K^+ flux results in the reestablishment of the resting membrane potential. Such a process is defined as *repolarization*.

There are two characteristics for the action potential. First, an action potential is initiated only when the resting membrane potential is reduced to the threshold level in response to a stimulus. Any change in the resting membrane potential that does not reach the threshold will not initiate action potentials. Once an action potential is initiated, the amplitude of the action potential remains constant. The level of stimulation does not influence the amplitude of the action potential. This phenomenon is known as the *all-or-none phenomenon*. Second, an action potential can propagate through the cell membrane. A cell is initially depolarized within a small area of plasma membrane. A local action potential can stimulate the cell membrane near the depolarized area, inducing the spreading of the action potential. This is a basic process for the propagation of action potentials.

The contractile activity of the skeletal muscle system is controlled by the action potentials that are initiated in the neurons of the motor control centers and transmitted to the peripheral muscle cells via the nerve axons. Each axon develops multiple levels of branches, which project toward peripheral muscle cells. The end of each axon branch enlarges to form a terminal structure, which interacts with the plasma membrane of a muscle cell. The axon terminal and the local area of the muscle cell that interacts with the axon terminal are together called *synapse*. The axon terminal is referred to as the *presynaptic terminal*, the plasma membrane of the muscle cell is called the *postsynaptic membrane*, and the gap between the two types of membrane is known as the *synaptic cleft*. Each presynaptic terminal is composed of plasma membrane vesicles, referred to as *synaptic vesicles*. These vesicles contain a substance known as acetylcholine, a neurotransmitter that can be released into the synaptic cleft and stimulates the initiation of action potentials in the muscle cells.

When an action potential is transmitted from a motor neuron to the presynaptic terminal, the actin potential induces the opening of voltage-gated calcium channels, resulting in calcium flux into the presynaptic terminal. Increased calcium concentration in turn induces the release of acetylcholine from the synaptic vesicles to the synaptic cleft. Released acetylcholine interacts with and activates the acetylcholine receptors located in the postsynaptic membrane of the muscle cell. The activation of the acetylcholine receptors induces the opening of voltage-gated sodium channels, resulting in sodium flux into the muscle cell. The inward sodium flux causes depolarization of the muscle cell and initiation of action potentials.

The action potentials in the plasma membrane of the muscle cell can initiates muscle contraction via a process known as *excitation–contraction coupling*. In this process, the action potentials can be transmitted from the synapse to a tubular network called *T-tubules*, which are distributed continuously around the sarcoplasmic reticulum and the sarcomeres (note that the sarcoplasmic reticulum contains a high concentration of calcium). The action potentials in the T-tubules stimulate the sarcoplasmic reticulum, inducing the opening of the voltage-gated calcium channels. This action results in calcium release from the sarcoplasmic reticulum to the sarcoplasm, where the contractile myofibrils are located. Calcium ions bind to troponin, inducing a conformational change in the troponin–tropomyosin complex and the exposure of the active binding sites of the actin filaments. The active sites in turn interact with the myosin heads and induce the movement of the

myosin heads. The actin–myosin interaction results in the sliding of the actin filaments against the myosin filaments and thus contraction of the muscle cells. After the sliding process, the myosin heads are released from the actin filaments and are prepared for another cycle of contraction. These activities require energy from ATPs. The myosin heads are composed of ATPases, which hydrolyze ATP molecules and generate energy. The energy is stored in the myosin heads and used for the release of the myosin heads from the actin filaments. Following the contraction process, the calcium ions are actively transported from the sarcoplasm to the sarcoplasmic reticulum. The decrease of the calcium concentration in the sarcoplasm induces muscle relaxation.

DISORDERS OF THE SKELETAL MUSCLE SYSTEM

Muscular Dystrophies

Pathogenesis, Pathology, and Clinical Features [21.2]. *Muscular dystrophies* are a group of hereditary disorders characterized by progressive loss of muscle mass and function, resulting in the disorder of the skeletal muscle system. Gene mutation-induced protein alterations and deficiency are primary causes for muscular dystrophies. Genetic studies have identified more than 30 forms of muscular dystrophy. Among these forms of muscular dystrophies, several forms are commonly seen, including the Duchenne's, Becker's, myotonic, and facioscapulohumeral muscular dystrophies. The clinical manifestations and pathological changes of these types are briefly discussed as follows.

Duchenne's muscular dystrophy is a recessive genetic disorder found in male patients. The disorder is induced by mutation of the dystrophin gene (*dys*), which encodes the dystrophin protein (see Table 21.1). Dystrophin is a constituent of the sarcolemma of muscle cells. This protein is capable of binding to actin filaments at the *N*-terminus, binding to syntrophin at the *C*-terminus, and binding to β-dystroglycan at a cysteine-rich domain. These molecular links provide a structural basis for the interaction of the actin cytoskeleton with extracellular matrix. In Duchenne's muscular dystrophy, dystrophin deficiency is often found, suggesting a role for dystrophin deficiency in the initiation and development of muscular dystrophy. Molecular analyses have demonstrated that dystrophin deficiency is often a result of dystrophin (*dys*) gene mutation. In most patients with muscular dystrophy, dystrophin gene mutation is induced by large fragment deletion, insertion, or point mutation that causes frameshifting of gene codons. These genetic alterations result in the deficiency or modulation (primarily in the cysteine-rich domain) of the dystrophin protein. The deficiency or modulation of dystrophin influences the interaction of the actin filaments with extracellular matrix, resulting in instability of the sarcolemmal structure and reducing cell adhesion and survival capabilities. All these changes promote cell degeneration, eventually leading to cell apoptosis and muscular dystrophy.

Duchenne's muscular dystrophy is the most common type of muscular dystrophy. The incidence of this disorder is about 0.01–0.03%. Signs of muscular dystrophy, such as muscle weakness and movement disorders, are usually found at the age of 5. Gait analyses often provide useful information for the diagnosis of the disorder. The patients eventually lose muscle strength and the ability of movements. The weakening of the

TABLE 21.1. Characteristics of Selected Muscular Dystrophy-Related Proteins*

Proteins	Alternative Names	Amino Acids	Molecular Weight (kDa)	Gene Locus	Expression	Functions
Dystrophin	DMD, apo-dystrophin 1	3685	427	Xp21.2	Skeletal muscle, heart, brain	Regulating the integrity and stability of skeletal and cardiac muscles
Syntrophin 1	SNT1, syntrophin α1, dystrophin-associated protein A1, Pro TGF α-cytoplasmic domain interacting protein, 159-kDa dystrophin-associated protein A1	505	54	20q11.2	Heart, skeletal muscle, brain, lung, liver, kidney, pancreas	A membrane protein associated with dystrophin, playing a role in regulating the integrity and stability of skeletal and cardiac muscles
Dystroglycan	Dystrophin-associated glycoprotein 1 (DAG), dystroglycan α, agrin receptor, dystroglycan β	895	98	3p21	Skeletal muscle, heart, brain, lung, liver, kidney	Forming complexes with dystrophin, regulating the integrity and stability of skeletal and cardiac muscles, and serving as a dual receptor for agrin and laminin-2 in the Schwann cell membrane

*Based on bibliography 21.2.

respiratory muscle system influences the function of the lung. Most patients with muscular dystrophy die of respiratory failure. Pulmonary infection often occurs because of food aspiration resulting from the loss of muscular strength responsible for swallowing. A unique feature of Duchenne's muscular dystrophy is the elevation of the serum c reatine kinase, although the level of the serum creatine kinase is normal at birth. Most patients show a significant increase in serum creatine kinase when muscular weakening occurs. Pathological examinations often demonstrate muscular necrosis, a reduction in the mass of skeletal muscle cells, and an increase in adipocytes and extracellular matrix.

Duchenne's muscular dystrophy is often associated with cardiac disorders, such as cardiomyopathy, which induces cardiac dilation and a reduction in cardiac contractility. Cardiomyopathy-induced heart failure is the second leading cause of fatality in Duchenne's muscular dystrophy (note that the first leading cause of death in this disorder is respiratory failure). Mutation or deletion of the dystrophin gene is responsible for the cardiac disorders. Cardiomyopathy is usually induced by truncated or reduced level of dystrophin, which may not cause noticeable skeletal muscle dystrophy. In dystrophin-deficient mice, the cardiomyocytes exhibit reduced compliance and enhanced cell contracture, presumably due to increased cell susceptibility to stretch-mediated calcium overload. Prolonged cardiac myocyte contracture induces cell death, a critical mechanism for the development of cardiomyopathy.

Characteristics of muscular dystrophy-related proteins are listed in Table 21.1.

Becker's muscular dystrophy is a disorder similar to Duchenne's muscular dystrophy in pathogenesis, pathology, and clinical features. Becker's muscular dystrophy is induced by mutation of the dystrophin gene. Compared to the Duchenne's muscular dystrophy, which is induced by complete dystrophin deficiency or mutation of the cysteine-rich domain of the dystrophin gene, Becker's muscular dystrophy is not associated with complete dystrophin deficiency or mutation of the cysteine-rich domain of the dystrophin gene. Thus, Becker's muscular dystrophy is not as severe as and progresses slower than the Duchenne's muscular dystrophy. The Becker's type of muscular disorder is also referred to as the "benign" form of Duchenne's muscular dystrophy. Clinical manifestations are usually found after the age of 15. Death due to muscular dystrophy occurs after the age of 40, which is much later than that due to Duchenne's muscular dystrophy.

Myotonic dystrophy is an autosomal dominant genetic disorder induced by gene mutation in chromosome 19. This disorder is characterized by progressive muscle weakening and the association of disorders in other systems, including cardiac disorders, cataract, intellectual impairment, gastrointestinal disorders, and respiratory failure. Muscle weakening is usually found in the distal extremities at the age of 10–30. Some patients may not show apparent signs or symptoms for a long time. Compared to the Duchenne and Becker muscular dystrophies, the level of serum creatine kinase may remain normal. Pathological examinations often show muscular atrophy or a reduction in the muscle mass. In the heart, conduction system is often involved with a high incidence of conduction block. In the lung, weakening of the respiratory muscles may result in ventilation disorder, hypoxia, cor pulmonale, and respiratory failure.

Facioscapulohumeral muscular dystrophy is a disorder characterized by progressive weakening of the facial, shoulder girdle, and arm muscles. This disorder is induced by autosomal dominant gene mutation and is found in both males and females at any age with a high incidence from age 30 to 40. Some patients may not show any signs of disorder.

Unlike the Duchenne and Becker muscular dystrophies, other systems are usually not involved. The serum creatine kinase level is normal or slightly increased.

Transgenic Models of Dystrophin Deficiency [21.3]. The dystrophin gene (dys) is a large gene with approximately 2.25 million base pairs, which contains 79 exons and a large fraction of introns (99.4%). In a large population of patients with muscular dystrophy (about 50%), dystrophin deficiency is caused by gene deletion- or insertion-induced frameshifting. A mouse *mdx* dystrophin-deficient model has been established for the study of human muscular dystrophy. The mouse dystrophin gene is basically homologous to that of the human. The genetic mechanisms of dystrophin gene mutations are also similar between mice and humans. Thus, the mouse *mdx* dystrophin-deficient model is commonly used for the study of the human disorder. In this mouse model, a C $\rightarrow$ T transition is induced in exon 23 of the dystrophin gene, resulting in frameshifting and dystrophin deficiency. Mice with dystrophin deficiency show increased degeneration and death of skeletal muscle cells and reduced muscle cell density (Fig. 21.2), in association with a reduction in the contractile strength (Fig. 21.3), which resemble pathological and functional alterations found in human muscular dystrophies. The overexpression of a dystrophin gene in the *mdx* model significantly reduces muscle cell degeneration and death, and improves the contractility of the skeletal muscle system. It was found from the mouse model that dystrophin is a necessary protein for maintaining the stability of the skeletal muscles and for preventing muscular dystrophy. The dystrophin deficiency model has also been established in dogs and cats, but the mouse *mdx* model is more popularly used for investigating the pathogenic mechanisms of human muscular dystrophy.

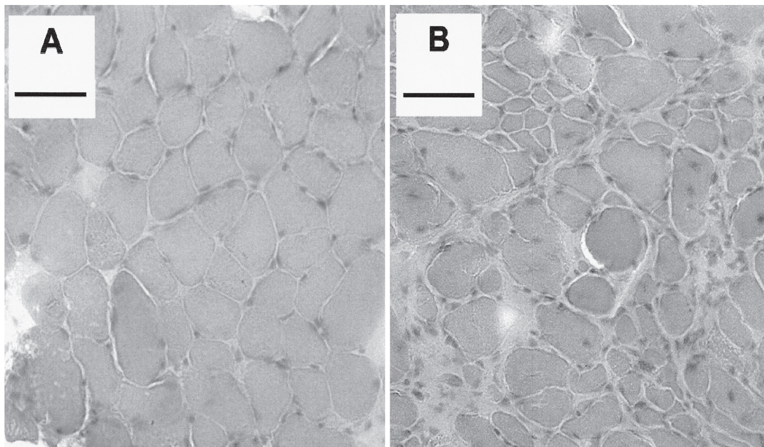


Figure 21.2. Photomicrographs of H&E-stained transverse sections of diaphragm muscles of control mice (A) and *mdx* mice, a model for Duchenne muscular dystrophy (B). Scale bars: 50 μm. (Reprinted with permission from Gregorevic P et al: Improved contractile function of the mdx dystrophic mouse diaphragm muscle after insulin-like growth factor-I administration, *Am J Pathol* 161:2263–72, copyright 2002.)

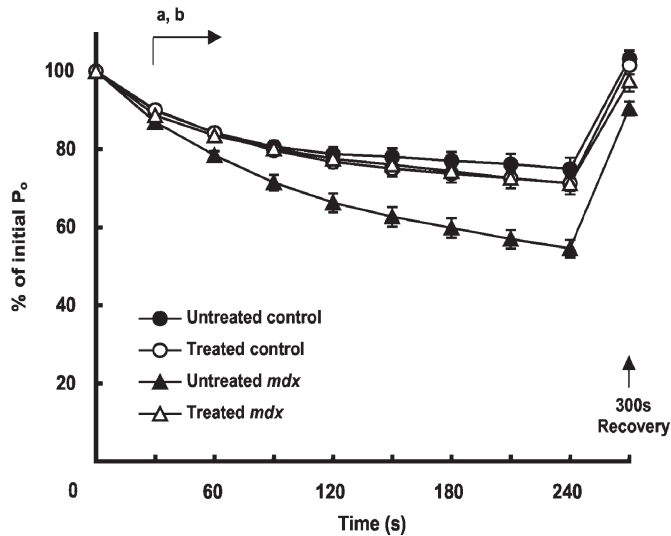


Figure 21.3. Maximum force output during and 300 s after a repeated stimulation fatigue protocol for diaphragm muscle preparations from control and *mdx* mice (an animal model for Duchenne muscular dystrophy) with and without insulin-like growth factor. Note the comparatively greater reduction in force throughout time in the diaphragm muscles of untreated dystrophic mice compared with untreated control mice (panel A, $P < 0.05$), and the enhanced resistance of muscles from treated dystrophic mice compared with the untreated dystrophic muscles (panel B, $P < 0.05$) present from 30 s after commencement of stimulation, to the cessation of stimulation. (Reprinted with permission from Gregorevic P et al: Improved contractile function of the mdx dystrophic mouse diaphragm muscle after insulin-like growth factor-I administration, *Am J Pathol* 161:2263–72, copyright 2002.)

Molecular Treatment of Muscular Dystrophy [21.4]. The various types of muscular dystrophy as described above are inherited disorders induced by gene deletion or mutation. To date, there are few effective approaches which can be used to treat these disorders. Recent studies have demonstrated that molecular regenerative therapies may be potentially used to treat muscular dystrophy and cardiomyopathy. Numerous genes that are deleted or mutated in muscular dystrophy, contributing to the development of the disorder. The repair and replacement of these genes are potential approaches for the treatment of muscular dystrophy. There are two basic molecular approaches: transfer genes that encode proteins directly responsible for the disorder, and transfer "booster" genes that encode proteins responsible for the survival of muscle cells and the prevention of cell apoptosis. In this section, Duchenne's muscular dystrophy is used as an examples to demonstrate the principles of molecular therapy for muscular dystrophy.

Transfer of Wildtype Dystrophin Gene [21.5]. For Duchenne's muscular dystrophy, the primary cause is the deletion or mutation of the dystrophin gene. Thus, direct transfer of a functional wildtype dystrophin gene into the muscular cells is a potential approach for the treatment of the disorder. This gene has been tested in a transgenic animal model, the dystrophin-deficient *mdx* mouse, which resembles the Duchenne's muscular dystrophy in

humans. A number of approaches have been developed and used for the delivery of the dystrophin gene, including virus-, liposome-, and electroporation-mediated gene deliveries. Among these approaches, the virus-mediated gene delivery is the most effective approach. The dystrophin gene can be integrated into modified viral vectors and used to transfer into target muscular cells. Experimental investigations have demonstrated the effectiveness of such an approach. In particular, the transfer of the full-length dystrophin gene with a muscle-specific gene promoter (muscle creatine kinase promoter) has been shown to effectively prevent the progression of muscular dystrophy in the *mdx* mouse model of muscular dystrophy. The use of the muscle-specific gene promoter can increase the efficiency of gene transfer. However, there are potential problems. These include limited efficiency of gene transfer, immune responses provoked by the expression of the transferred gene and corresponding protein products, temporary gene expression, and poor cell survival. These problems remain to be resolved.

Given the problems with the transfer of the wildtype dystrophin gene, several alternative strategies have been developed and used for the molecular treatment of muscular dystrophy. These include the construction and delivery of truncated dystrophin gene, mutant gene correction by small fragment homologous replacement, correction of mutant genes by chimeraplasty, removal of mutant gene fragments by exon skipping, and compensation for the lost function of mutant dystrophin. These approaches are discussed as follows.

Delivery of Truncated Dystrophin Genes or Microdystrophin Gene Constructs [21.6]. The dystrophin gene is composed of a large number of base pairs, which complicate the preparation and manipulation of the dystrophin gene. Genetic and functional analyses has demonstrated that not all gene sequences are necessary for the production of functional dystrophin. Indeed, selected regions of the dystrophin gene can be deleted and the remaining regions can be recombined to generate a minidystrophin gene. A minimal requirement is that the reconstructed gene must contain critical domains responsible for regulating the structural stability and function of the sarcolemma of muscular cells. The reconstructed minidystrophin gene can be highly functional. When delivered into the mouse *mdx* muscular dystrophy model, pathological alterations of muscular dystrophy can be significantly prevented and the contractility of the skeletal muscles can be improved (Fig. 21.4). Furthermore, such an approach can be used to reduce muscular cell degeneration in the *mdx* mouse model of muscular dystrophy. However, it is still debating whether the delivery of the truncated dystrophin gene is more advantageous than that of the full-length dystrophin gene.

Mutant Gene Correction by Small Fragment Homologous Replacement (SFHR) [21.7]. Small fragment homologous replacement is a technique used for constructing and inserting PCR-generated DNA amplicons into the host genome to correct mutant genes. PCR can generate large DNA fragments up to several hundred base pairs. Designed corrective DNA fragments can be constructed and transferred into target cells. When integrated into the genome, these fragments can replace and correct mutant genes, resulting in the generation of functional genes. Such a technique has been applied to cells from the *mdx* mouse model of muscular dystrophy to correct mutant dystrophin gene. While the mechanisms of gene correction remain poorly understood, preliminary investigations have

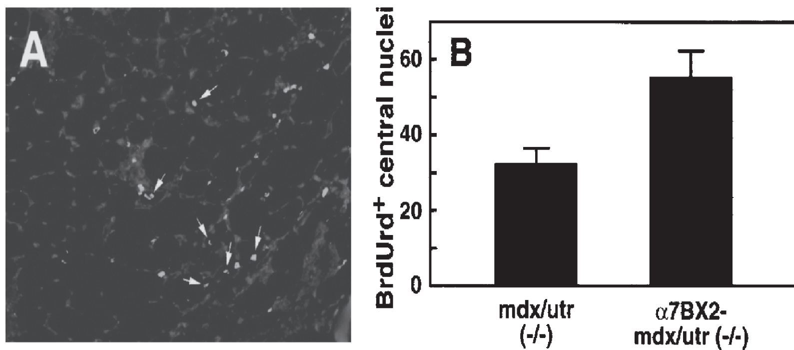


Figure 21.4. Increased integrin promotes the proliferation of satellite cells *mdx/utr*$^{-/-}$ mice (a animal model of muscular dystrophy) and *α7BX2-mdx/utr*$^{-/-}$ mice (a muscular dystrophy model with enhanced expression of the $\alpha7\beta1$ integrin). Mice were injected with BrdU to label replicating cells. Muscle specimens were collected and analyzed by immunohistochemistry for BrdU incorporation into DNA. Nuclei are stained with DAPI. BrdUrd-labeled central nuclei (arrows in panel A) in 50 random fields were scored for each animal. Mean numbers (±SEM) are given for 11 animals for each genotype. (B) Increased integrin expression enhanced the proliferation of satellite cells and the regenerative capacity of dystrophic muscle. (Reprinted with permission from Burkin DJ et al: Transgenic expression of alpha7beta1 integrin maintains muscle integrity, increases regenerative capacity, promotes hypertrophy, and reduces cardiomyopathy in dystrophic mice, *Am J Pathol* 166:253–63, copyright 2005.)

provided promising results for this technique. As shown in a study with cultured muscular cells, the delivery of a PCR amplicon into muscular cells with dystrophin deficiency results in the correction of the dystrophin gene in about 20% cells. However, a higher efficiency may be needed to achieve therapeutic effects.

Correction of Mutant Genes by Chimeraplasty [21.8]. Chimeraplasty is a technique used for correcting mutant genes with chimeric RNA–DNA oligonucleotides, also known as chimeraplasts. Short chimeric genetic structures can be constructed by hybridizing complementary 2′-O-methyl ribonucleotide analogues to desired DNA fragments. Such a chimeric complex protects the DNA fragments from exonucleolytic digestion. When the chimeraplasts are delivered to the cell nucleus, the DNA and RNA fragments can anneal to the target site during gene transcription. The chimeraplasts can repair or replace base-pair mismatches, if any, resulting in the correction of gene mutation, although the exact mechanisms remain poorly understood. Experimental investigations with in vitro models have demonstrated a correction efficiency about 30%. The efficiency may be further improved when the mechanisms of chimeraplasty are fully understood. Chimeraplasty has been applied to the *mdx* mouse model of muscular dystrophy. The delivery of chimeraplasts for the dystrophin gene into the skeletal muscle cells results in the correction of mutant dystrophin gene in about 10% cells. Such a manipulation induces an increase in the expression of functional dystrophin gene and a reduction in the symptoms of muscular dystrophy.

Removal of Mutant Gene Fragments by Exon Skipping [21.9]. Exon skipping is a technique used to target selected mutant gene fragments and block the transcription of the

targeted fragments by introducing specific antisense 2′-*O*-methyl ribonucleotide analogs to cell nuclei. The antisense ribonucleotides can bind to and block specific homologous DNA exons or sequences in the genome during transcription. Such a process induces a transcription-skip over the ribonucleotide-blocked exons. In other words, the blocked exons can no longer be transcribed. When a ribonucleotide sequence is designed and delivered to target a specific mutant gene fragment, the mutant fragment cannot be expressed and the function of the generated protein may be improved.

For the treatment of experimental muscular dystrophy in the *mdx* mouse model, a 2′-*O*-methyl ribonucleotide analogue sequence can be designed to target the exon 23 (at the junction with intron 22), which contains a mutant fragment responsible for the development of muscular dystrophy. The delivery of this ribonucleotide analogue into the *mdx* mouse model stops the transcription of the exon 23. Such a manipulation results in the generation of a dystrophin form similar to that found in Becker's muscular dystrophy, which is significantly less severe than Duchenne's muscular dystrophy. Although the approach does not provide a complete cure of the muscular dystrophy, the pathological changes are reduced and the contractility of the muscle system is improved.

Compensation for Lost Function of Dystrophin [21.10]. There exist proteins that potentially compensate for the function of dystrophin. One of such compensating factors is utrophin, also known as *dystrophin-like protein* and dystrophin related protein 1. Utrophin is a protein of 3422 amino acid residues and about 395 kDa in molecular weight. This protein is similar to dystrophin in structure and function. As dystrophin, utrophin is expressed in skeletal muscle cells and is localized to the sarcolemma and acetylcholine receptors at the neuromuscular synapses and myotendinous junctions, where it regulates the function of the postsynaptic membrane and, especially, the activity of the acetylcholine receptors. Utrophin is also expressed in the heart, brain, lung, kidney, liver, intestine, and testis. Utrophin can interact with dystrophin at the *C*-terminus. The suppression or loss of the utrophin activity exacerbates pathological changes of muscular dystrophy in the *mdx* mouse model of muscular dystrophy. The upregulation of the utrophin gene has been shown to reduce pathological alterations and compensates for functional abnormalities due to dystrophin deficiency in the *mdx* mouse model of muscular dystrophy. Growth factors, interleukin-6, l-arginine, and nitric oxide can enhance the expression of the utrophin gene promoter.

Transfer of Dystrophin "Booster" Genes [21.11]. In addition to the dystrophin gene, a number of "booster" genes have been discovered and used for the molecular treatment of muscular dystrophy. These genes encode proteins that mediate the survival and enhance the function of the of striated muscular cells. Common "booster" genes include integrin α7β1, ADAM12, calpastatin, nitric oxide synthase, insulin-like growth factor (IGF)I, myostatin, and mini-agrin.

INTEGRIN α7β1. Cell adhesion to extracellular matrix is critical to cell survival. The impairment of muscle cell adhesion to extracellular matrix may induce cell apoptosis, contributing to muscular dystrophy. In skeletal muscle cells, there are two major types of cell–matrix interaction-mediating molecules, including the dystrophin-associated

glycoproteins (see Table 21.1 chapter for dystroglycan, a major dystrophin-associated glycoprotein) and integrin $\alpha7\beta1$. These two molecules coordinately regulate muscular cell attachment to extracellular matrix. In the case of dystrophin deficiency, cell attachment mediated by the dystrophin-associated glycoproteins is impaired. The overexpression of the integrin $\alpha7\beta1$ gene can partially rescue the functional loss of dystrophin (Fig. 21.5). Thus, the transfer of the integrin $\alpha7\beta1$ gene into target muscular cells is a potential approach for the treatment of muscular dystrophy.

Characteristics of several therapeutic molecules for muscular dystrophy are presented in Table 21.2.

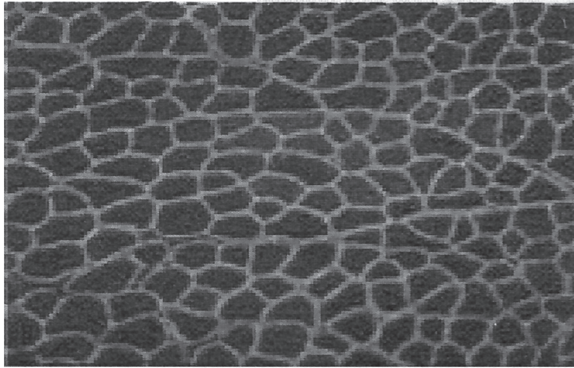
ADAM12. ADAM (A disintegrin and metalloprotease or meltrin)12 is a molecule that possesses integrin-binding and metalloproteinase activities. This molecule is expressed in skeletal muscle cells during development and regeneration, and plays a critical role in the regulation of muscular formation and morphogenesis. Furthermore, ADAM12 enhances cell attachment to extracellular matrix through interacting with syndecans and promotes cell spreading via binding to $\beta1$ integrin-containing complexes. In the *mdx* mouse model of muscular dystrophy, the overexpression of the ADAM12 gene results in a reduction in pathological changes found in muscular dystrophy and enhancement of muscular cell regeneration. Such effects may be related to the function of ADAM12 in regulating cell adhesion via interacting with integrins and syndecans.

CALPASTATIN. Calpastatin is a protein that inhibits the activity of calpain, a calcium-dependent protease that induces autoproteolysis and cell death. Calpain may participate in the regulation of cell degeneration in muscular dystrophy. In the transgenic *mdx* mouse model of muscular dystrophy, the transfer of the calpastatin gene into the target skeletal muscle cells results in the suppression of the activity of calpain in association with a reduction in muscle cell death and degeneration.

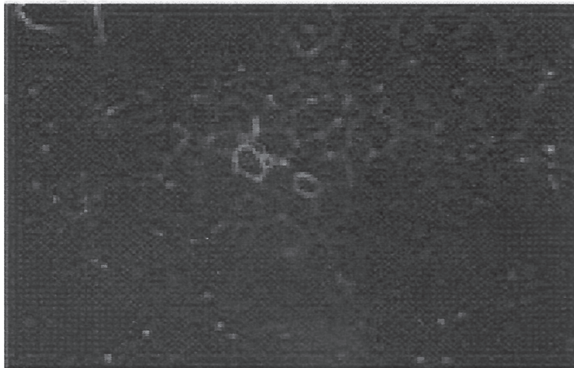
NITRIC OXIDE SYNTHASE. Nitric oxide synthase is an enzyme that catalyzes the formation of nitric oxide from L-arginine. Nitric oxide has been shown to exert an inhibitory effect on inflammatory reactions in various systems. Skeletal muscle cells express nitric oxide synthase, which is localized to the cell membrane. In muscular dystrophy, the expression of the nitric oxide synthase gene is impaired and translocation of nitric oxide synthase occurs, resulting in a reduction in the production of nitric oxide. These changes are associated with profound inflammatory reactions in the skeletal muscles, which are thought to contribute to the development of muscular dystrophy. The overexpression of the nitric oxide synthase gene in the skeletal muscle cells of the *mdx* mouse muscular dystrophy model induces an increase in the level of nitric oxide as well as a reduction in inflammatory reactions and muscle cell death (Fig. 21.6).

INSULIN-LIKE GROWTH FACTOR. Cell degeneration is a critical process that leads to muscular dystrophy. One treatment strategy for muscular dystrophy is to enhance cell regeneration. Insulin-like growth factor is a molecule that stimulates such a process. Experimental investigations have shown that the overexpression of the insulin-like growth factor gene in the mouse *mdx* muscular dystrophy model results in a reduction in muscular cell death and improvement of muscular cell survival and regeneration. The insulin-like growth

Wild type



mdx



T mdx

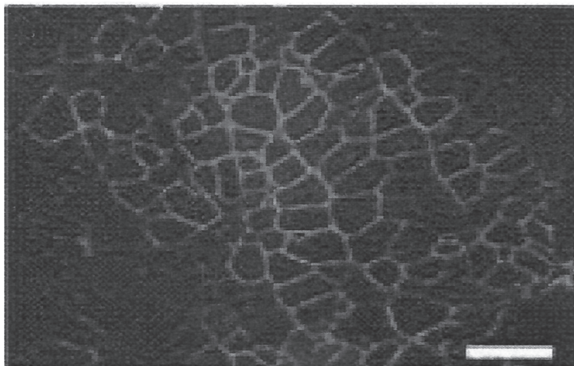


Figure 21.5. Systemic delivery of microdystrophin to dystrophic mice. (A) Antidystrophin immunofluorescence microscopy of tibialis anterior muscles from treated *mdx* mice (T*mdx*) administered 1×10^{12} vector genomes of rAAV6–CK6–microdystrophin and 10 µg VEGF, compared with wildtype and untreated mdx mice, a model of muscular dystrophy. Dystrophin expression is increased in the muscles of treated compared with untreated *mdx* mice, but remains mosaic compared with wildtype mice. Scale bars: 100 mm. (Reprinted by permission from Macmillan Publishers Ltd.: Gregorevic P et al: Systemic delivery of genes to striated muscles using adeno-associated viral vectors, *Nature Med* 10:828–34, copyright 2004.)

TABLE 21.2. Characteristics of Selected Therapeutic Proteins for Muscular Dystrophy*

Proteins	Alternative Names	Amino Acids	Molecular Weight (kDa)	Expression	Functions
Integrin $\alpha7$	ITGA7	1137	124	Heart, skeletal muscle, nervous system, lung, intestine, ovary, prostate gland	Joining with integrin $\beta1$ to form an integrin complex, which is a major integrin complex expressed in differentiated muscular cells (note that all integrins are heterodimeric integral membrane proteins composed of an α chain and a β chain), binding to the extracellular matrix protein laminin-1, and regulating cell attachment to extracellular matrix
Integrin $\beta1$	ITGB1, fibronectin receptor β subunit (FNRB), fibronectin receptor β subunit-like, very late activation protein β polypeptide (VLA β)	825	92	Heart, nervous system, skeletal muscle, lymphocytes, liver, bone, cartilage, skin	Joining with an integrin α subunit to form integrin complexes, regulating cell adhesion to extracellular matrix, and regulating various cellular activities, including embryogenesis, cell proliferation and migration, immune responses, and metastasis of tumor cells

ADAM12	A disintegrin and metalloproteinase domain 12, meltrin α	909	100	Heart, skeletal muscle, placenta, intestine, stomach, uterus, urinary bladder	A membrane-anchored protein that regulates cell–cell and cell–matrix interactions, and mediates other cellular processes including fertilization, skeletal muscle development, and regeneration, as well as neurogenesis
Calpastatin	Calpain inhibitor	708	77	Red blood cells, leukocytes, intestine, skeletal muscle, testis, prostate gland, spleen, intestine	Serving as an inhibitor for endogenous calpain (calcium-dependent cysteine protease), regulating membrane fusion events, such as neural vesicle exocytosis and platelet and red cell aggregation
Myostatin	MSTN, growth differentiation factor 8 (GDF8)	375	43	Heart, skeletal muscle	A member of the bone morphogenetic protein (BMP) family and the TGFβ family, negatively regulating skeletal muscle growth
Agrin	AGRN	2045	215	Ubiquitous	Inducing the aggregation of acetylcholine receptors and other postsynaptic proteins on muscle fibers; also regulating the formation of neuromuscular junction

*Based on bibliography 21.11.

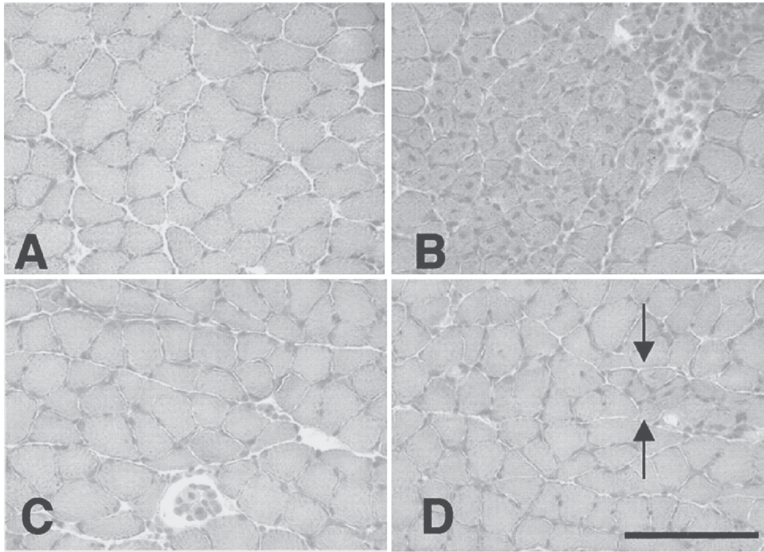


Figure 21.6. Influence of NOS expression on the morphology of skeletal muscle cells: (A) C57 control muscle showing fibers of uniform diameter, no central nucleation, and no clusters of inflammatory cells between adjacent fibers; (B) mdx muscle showing a typical focus of muscle pathology characterized by fiber populations of variable diameters and central nucleation (note that transgenic mdx mice are null mutants for dystrophin). Dark staining nuclei of inflammatory cells appear between adjacent fibers; (C) NOS Tg/mdx muscle showing typical histology, where fiber diameter is more uniform than age-matched mdx muscle, and there is little inflammation or central nucleation (note that NOS Tg/mdx is a transgenic mouse model with deficient dystrophin but with the expression of the NOS transgene); (D) NOS Tg/mdx muscle showing an example of the relatively small lesions that appear in NOS Tg/mdx muscle (between arrows) where there are small clusters of small-diameter, central-nucleated fibers. All micrographs are at the same magnification. Scale bar: 250 μm. (Reprinted with permission from Wehling M et al: Clonal isolation of muscle-derived cells capable of enhancing muscle regeneration and bone healing, *J Cell Biol* 155:123–32, copyright 2001.)

factor gene can serve as a candidate gene for the molecular treatment of muscular dystrophy.

MYOSTATIN. Myostatin is a protein that negatively regulates the development and regeneration of skeletal muscles. In animal models with myostatin gene mutation, hyperplasia and hypertrophy occur in the skeletal muscle system. The overexpression of the myostatin gene results in the degeneration of the skeletal muscle cells. Thus, it is conceivable that the suppression of the activity of the myostatin gene is beneficial for the treatment of muscular dystrophy. Such a goal can be achieved by delivering antisense oligonucleotides or small interfering RNA specific to the myostatin mRNA to compromise the translation of the myosin protein. In addition, local delivery of antimyostatin antibody and myostatin inhibitors can achieve the same goal.

MINIAGRIN. Miniagrin is a fragment of a large protein called *agrin*, which is known to induce the aggregation of acetylcholine receptors and other postsynaptic proteins in muscular cells and regulate the formation of the neuromuscular junction. Agrin also interacts with laminin and dystroglycan, enhancing cell adhesion and survival. There are two important domains within the agrin protein: the *N*-terminal domain (responsible for binding to laminin) and the *C*-terminal domain (responsible for binding to dystroglycan). A miniagrin gene has been constructed with the *N*- and *C*-terminal domains. The delivery of the constructed miniagrin gene into target skeletal muscle cells in a laminin-2-deficient model (associated with impairment of cell adhesion and muscle weakness) results in upregulation of laminin and crosslink of laminin with dystroglycan. These activities are associated with enhanced cell adhesion, reduced muscle degeneration and dystrophic symptoms, and improved muscular contractility.

Cellular Regenerative Engineering for Muscular Dystrophy. Cell transplantation is a potential approach for the treatment of muscular dystrophy. There are two potential cell types, including muscular progenitor cells and stem cells, which can be used for cell transplantation. Cell transplantation may elicit two possible therapeutic effects: (1) transplanted cells can differentiate into muscular cells, replacing cells with muscular dystrophy; and (2) transplanted cells can serve as carriers for the delivery of therapeutic genes such as the dystrophin gene and "booster" genes.

Muscular Progenitor Cells [21.12]. The skeletal muscle system contains muscular progenitor cells, also known as *skeletal myoblasts*, *myogenic cells*, and *satellite cells*, which are capable of differentiating to mature muscular cells. Experimental investigations have identified muscular progenitor cells based on stem cell- and progenitor cell-specific markers. The muscular progenitor cells express a cell surface molecule specific for stem-like cells, known as *stem cell antigen1* (Sca1). In addition, these cells may coexpress other cell surface markers, including CD34, myf5, and m-cadherin. The muscular progenitor cells can be identified by immunochemical labeling of specific surface markers in conjunction with a cell sorting approach, such as magnetic bead-assisted cell sorting (tagging iron beads with a specific antibody and enriching antibody-labeled cells by magnetization) and fluorescence-activated cell sorting or FACS (labeling cells with a fluorescent antibody and enriching antibody-labeled cells by fluorescence-based cell sorting). Once muscular progenitor cells are identified and enriched, the cells can be used for transplantation into target muscular cells. In the mouse *mdx* muscular dystrophy model, the transplanted muscular progenitor cells are capable of differentiating into mature muscular cells, replacing dystrophic cells, and reducing pathological changes and symptoms of muscular dystrophy (Fig. 21.7). Furthermore, the muscular progenitor cells can be transfected with therapeutic genes for muscular dystrophy, such as the dystrophin gene and "booster" genes, and used as gene delivery carriers. When the progenitor cells are transplanted into target muscular cells, the proteins produced by the transplanted cells can elicit a therapeutic effect on the host dystrophic cells.

Stem Cells [21.13]. As discussed on Chapter 9, there are several types of stem cells, including embryonic, fetal, and adult stem cells. These stem cell types can be potentially

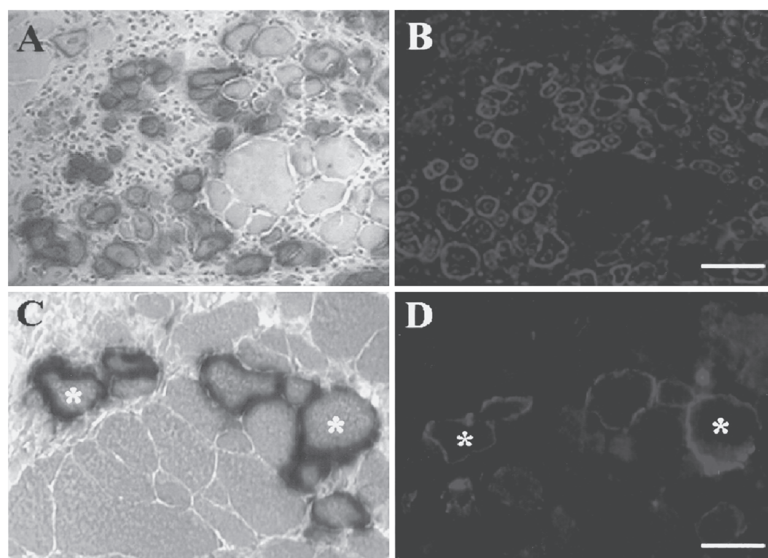


Figure 21.7. In vivo differentiation of mc13 cells into myogenic lineage after intramuscular (IM) and intravenous (IV) injection (note that mc13 cells are muscle-derived stem cells and are capable of differentiating into myogenic and osteogenic lineage in vitro and in vivo). The mc13 cells were stably transfected with a plasmid DNA construct encoding LacZ, dystrophin, and neomycin resistance genes and injected intramuscularly into hind limbs of *mdx* mice. After 7 days, and hind-limb musculature was isolated for histology. Many LacZ-positive myofibers (A) were found at the injected site that colocalized with dystrophin-positive myofibers (B). Some LacZ (C,*) and dystrophin positive myofibers (D,*) were also found in the hind limb muscle of mdx mice after IV injection of mc13. Scale bars: 100 μm (A,B); 50 μm (C,D). (Reprinted with permission from Lee JY et al: *J Cell Biol* 150:1085–100, copyright 2000.)

used for the treatment of muscular dystrophy. A desired type of stem cells can be identified, enriched, and transplanted into target muscular cells. These procedures are technically similar to those described above for the transplantation of muscular progenitor cells. In experimental investigations, multipotent embryonic stem cells have been used for the treatment of muscular dystrophy in the mouse *mdx* dystrophin-deficient model. These investigations have shown that embryonic stem cell transplantation is an effective approach for the prevention of pathological changes and the relief of the symptoms of muscular dystrophy. In addition, extensive investigations have been conducted to demonstrate the possibility of using adult stem cells for the treatment of muscular dystrophy. Bone marrow stem cells have been identified, enriched, and delivered into the circulation of the *mdx* mouse with dystrophin deficiency. The delivered cells can integrate into the skeletal muscle system and express dystrophin, improving the function of the dystrophic muscular cells. Other types of adult stem cells, such as the liver adipocytes, have also been used for transplantation into dystrophic muscular cells. Such an approach results in beneficial effects for the treatment of dystrophin deficiency-induced muscular dystrophy.

Potential Limitations [21.14]. There are several potential problems for the cellular treatment of muscular dystrophy. First, in patients with dystrophin deficiency and muscular dystrophy, the muscular progenitor cells or other types of stem cells are unlikely capable of expressing dystrophin. Thus, therapeutic cells can only be collected from an allogenic source or a donor individual. The transplantation of living allogenic cells induces acute immune rejection responses, resulting in rapid death of the transplanted cells. Second, it is impossible to deliver therapeutic cells to all dystrophic muscular cells over the entire body. The effects of cellular therapy are often limited to a small area around the cell delivery site. Although cells can be delivered through the blood circulation, the rate of cell integration into the skeletal muscle system is very low. Further investigations are needed to resolve these problems.

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