

1 Growth of Muscle from the Myoblast to Whole Muscle

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Introduction

Better understanding of the growth and development of skeletal muscle, and to a lesser extent, adipose tissue, is an important endeavor in meat science. This goal is driven by the need for the meat industry to consistently satisfy consumer demand for nutritious, high-quality, lean products in as efficient a manner as possible. Importantly, meat products are primarily derived from the skeletal muscle and associated fat of livestock.

Muscle growth, composition, and metabolism are integrally linked to meat quality through effects on yield, tenderness, and color. Typically, meat-producing animals are grown until an optimal balance between muscle mass and fattening is achieved. Upon slaughter, livestock carcasses are dressed leaving only the bones and edible muscles. Dressed carcasses are then aged in a temperature-controlled environment where biochemical processes such as glycolysis and protein degradation contribute to optimal meat quality.

Undoubtedly, advances in our understanding of factors that regulate the growth and development of muscle and the conversion of whole muscle to meat will lead to strategies that enhance meat quality. With these goals in view, this chapter will focus upon the growth of muscle from the myoblast precursor to whole muscle and upon cell culture techniques that allow these processes to be studied.

Overview of Skeletal Muscle Development

The growth of skeletal muscle can be meaningfully divided into stages by key developmental milestones. Landmarks such as conception, the maturation of the embryo (spanning the eight-cell stage through implantation), parturition, and finally, postnatal growth largely frame periods where specific mechanisms of growth contribute uniquely to muscle development. Thus, such landmarks represent useful points of reference that form a roadmap for better understanding of skeletal muscle development.

Viewed through this paradigm, skeletal muscle development can be divided roughly into two general phases of growth by parturition. Prenatal muscle development occurs primarily through increases in muscle fiber number (hyperplasia). Whereas postnatal growth of muscle is accomplished by increases in the size of preexisting muscle fibers (hypertrophy). In absence of injury, fiber number is essentially maintained during this period as very little new muscle fiber growth occurs after birth.

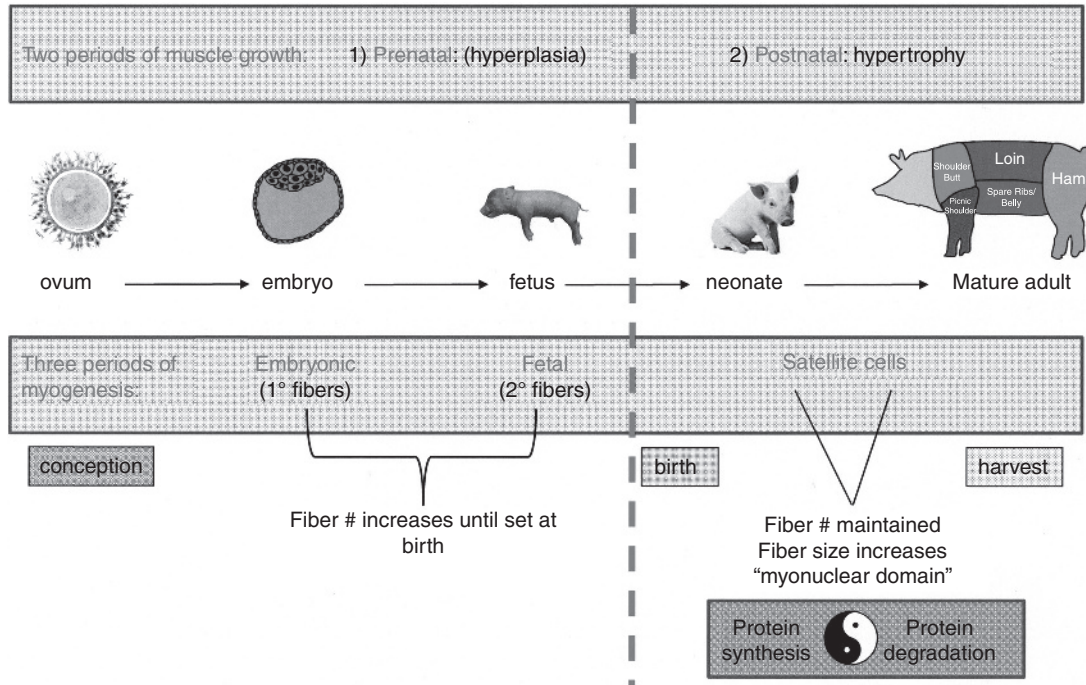


Figure 1.1 Roadmap for better understanding the skeletal muscle development based upon key developmental milestones. Such landmarks break development into discreet periods that are defined by unique mechanisms of muscle growth.

Alternatively, skeletal muscle development can be broken temporally into three phases of myogenesis. During early gestation, fiber number is increased via embryonic myogenesis (primary fiber formation). A second wave of fetal myogenesis (secondary fiber formation) then occurs during mid- to late-gestation. These two waves of prenatal myogenesis essentially dictate muscle fiber number in the adult. A third wave, satellite cell-related myogenesis mediates the postnatal increase in muscle fiber size that occurs in growing animals. Satellite cell fusion is also responsible for the maintenance of fiber number in the adult by facilitating the regeneration of damaged muscle fibers. Thus, an understanding of the regulation of myogenesis can largely inform all stages of skeletal muscle development (Fig. 1.1).

Types of Muscle

Three types of muscles can be distinguished structurally and physiologically in livestock. Smooth muscle is found in the walls of blood vessels, the lining of the gastrointestinal tract, uterine walls, and walls of respiratory passages. This type of muscle is innervated by the autonomic nervous system, thus its contraction is characterized by slow, but sustained contractile velocity that occurs without conscious thought. A second type of muscle, cardiac muscle, is innervated with an intrinsic nervous system unique to the heart that is specialized for generating highly controlled rhythmic contractions. Finally, skeletal muscle comprises the bulk of muscle in the body and its contraction is controlled by nerves emanating from the spinal cord. Importantly, skeletal muscle represents the primary source of meat from the carcass.

The unique and highly organized structure of skeletal muscle facilitates locomotion, a primary function of this muscle. Skeletal muscle appears striated due to the abundant expression of contractile apparatus proteins and, as discussed later, this muscle can appear reddish or whitish depending upon its fiber composition. Regardless of the anatomical location, skeletal muscles originate on a bone and terminate across the joint of another bone further away from the body's axis in such a way as to allow bones to rotate about the joint and move upon muscle contraction (Engel and Franzini-Armstrong, 2004). Muscles attach either via a tendon or upon a thin sheet of connective tissue (fascia).

Structure of Muscle

It is necessary to first appreciate the structural organization of skeletal muscle in order to understand why its development occurs as it does. The ultra-structure of the muscle cell and components of the contractile apparatus will be discussed in great detail in subsequent chapters. For now, we will focus upon the organization of the myofibril network and how this network interacts with the specialized membrane system of muscle fibers as these interactions are important for both prenatal and postnatal growth.

The basic structural unit of skeletal muscle is the muscle fiber (also termed muscle cell, myocyte, or myofiber). Muscle fibers are single, multinucleated cells that have peripherally located nuclei and a highly ordered and densely packed intracellular network of myofibrils, the organelle of contraction. Individual muscles are composed of a variable number of fibers that run parallel to one another. Individual fibers can be 10–100 μM in diameter and often reach many centimeters in length in the adult. Muscle fibers generally stretch from the origin to insertion in whole muscle though some individual muscle fibers terminate intrafascicularly (do not extend this entire length). Thus, intrafascicular fibers can confound efforts to quantify fiber number. Their size, multinucleated nature, specialized contractile apparatus, and unique membrane system distinguish muscle fibers from other animal cells (Fig. 1.2).

Myofibrils are specialized organelles that constitute the contractile apparatus of the muscle fiber. The basic unit of the myofibril is a repeating structure called the sarcomere. Each sarcomere comprises adjacent bundles of actin and myosin filaments which are capped on either end by a dense, proteinaceous Z-disc. Thin actin filaments extending from the Z-discs run in parallel to thick myosin filaments that appear to be centrally located in the sarcomere. Contraction occurs as these filaments interact and slide along one another thus drawing opposing Z-discs closer together.

In keeping with its unique structure, the muscle fiber is defined by a specialized, semi-permeable plasma membrane called the sarcolemma. The large number of channels and pores dispersed throughout the sarcolemma facilitate synaptic transmission and action potential propagation in response to acetylcholine release by motor neurons at neuromuscular junctions (Engel and Franzini-Armstrong, 2004). Thus, the sarcolemma is integral to proper contractile function in addition to performing the normal processes associated with a plasma membrane.

Immediately surrounding the sarcolemma is an extracellular matrix whose components are secreted by the muscle fiber proper. This thin layer of basal lamina and interwoven network of reticular fibers is directly attached to the sarcolemma and functions to maintain muscle integrity. Growth factors that bind to proteoglycans such as heparin sulfates in the basal lamina have been shown to regulate myogenesis and postnatal hypertrophy of muscle cells (Campbell and Stull, 2003). Furthermore, observed links between the basement membrane and sarcomeres of the myofibril suggest that the basal lamina may also function in contraction and myofibrillogenesis though the mechanisms are

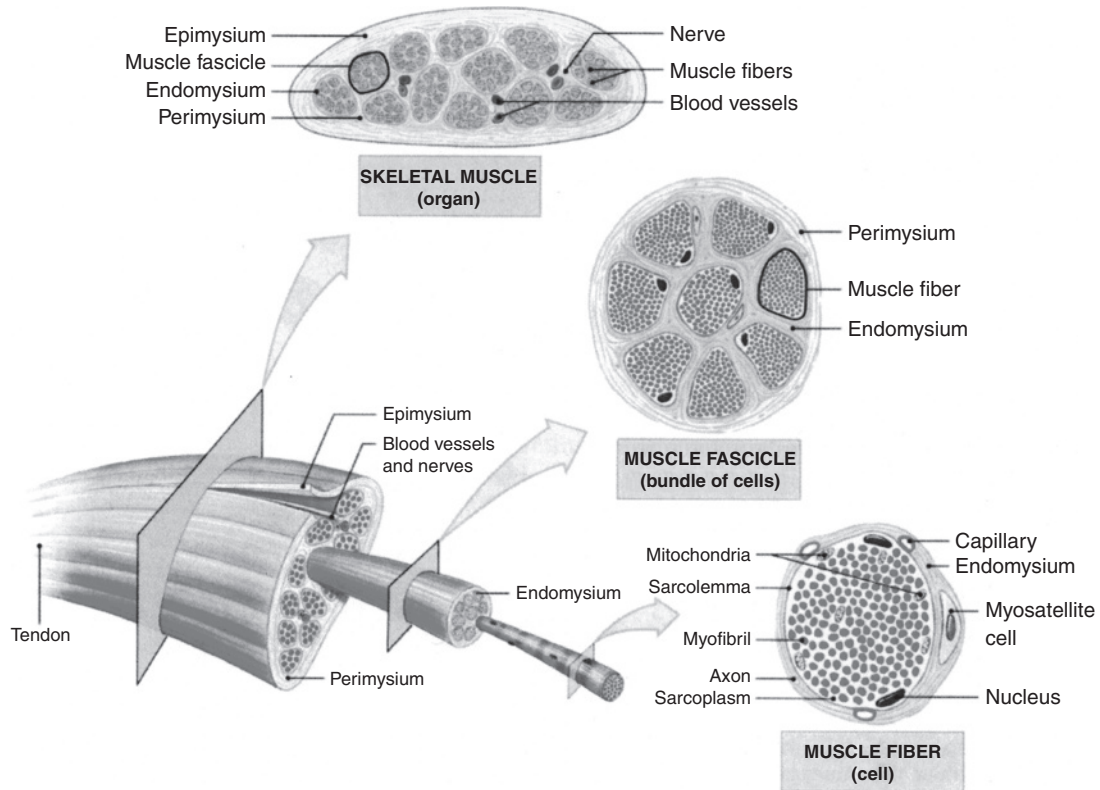


Figure 1.2 The organization of skeletal muscle. The muscle fiber is the basic structural unit of skeletal muscle. Fibers are further bundled into fascicles by the perimysium. Fascicles are then grouped into whole muscle by the epimysium. These connective tissue layers are highly organized and their function has implications for meat quality and muscle development. Modified from Wood et al. (2010).

poorly understood (Street, 1983; Danowski et al., 1992; Ervasti, 2003). Importantly, the basement membrane also facilitates connection of the muscle to tendons.

The endomysium, a final layer of connective tissue primarily composed of reticular fibers, encapsulates the muscle fiber proper and surrounding basement membrane. The endomysium is attached to the sarcolemma by multiple interactions with the basal lamina and functions as a sheath that allows capillaries, nerves, and lymphatics to reside in close proximity to the muscle fiber (Gerrard and Grant, 2003). Importantly, the endomysium is thought to influence meat tenderness (Swatland, 1975a, 1975b, 1975c; Lawrie, 1991).

Organization of Whole Muscle

Just as muscle fibers are multilayered structural units comprising of specialized membranes and connective tissue, whole muscle is organized by an elaborate, ordered system of connective tissue layers that can affect muscle function and meat quality. Among these connective tissue sheaths are the perimysium and the epimysium. The perimysium organizes individual fibers into bundles

called muscle fascicles. The number of individual fibers encased within the perimysium can vary. Furthermore, fascicular number and size varies with the function and anatomical location of the muscle. Importantly, intramuscular fat develops both within the perimysium and between bundles so it is possible that local signals emanating from the perimysium may affect the development of marbling through influencing either fat cell number or lipid filling. The epimysium encases multiple muscle fascicles effectively bundling them together to form the whole muscle. The epimysium is continuous around a muscle and is contiguous with the perimysium and endomysium ultimately thickening at junctions with tendons where muscle connects.

These individual layers intercalate to form a rich network that in addition to providing structural integrity also influences muscle growth both through direct and indirect effects. Physical links between the connective tissue layers and muscle fibers are thought to play important roles in regulating myofibrillogenesis and myogenesis (Menko and Boettiger, 1987; Rosen et al., 1992). Indirect effects can also be manifested by the ability of these layers to act as a reservoir for growth factors that are sequestered by proteoglycans in these rich networks (Timpl and Brown, 1996).

Although the muscle fiber is the basic structural unit of skeletal muscle, whole muscle is in fact heterogeneous with regard to cell types found within the tissue. Specialized mononucleated pre-muscle cells called satellite cells can reside between the endomysium and sarcolemma of the muscle fiber. Satellite cells play an important role in muscle hypertrophy serving as a local source of DNA that is essential to support postnatal muscle growth. Additionally, blood vessels, nerves, and lymphatics are interspersed between the perimysium and epimysium throughout the muscle. Motor neurons serve to activate a group of muscle cells which results in contraction of the fibers and thus facilitates movement. This relationship between a motor neuron and the downstream muscle fibers it stimulates is called a motor unit.

Fiber-Type Development

Heterogeneity is also a characteristic intrinsic to skeletal muscle fibers (Swatland, 1975a, 1975b, 1975c). Muscle fibers can be grouped as either slow oxidative, fast oxidative, or fast glycolytic based upon their speed of contraction and the predominant type of metabolism carried out by the fiber. Thus, muscle fibers can be distinguished by their functional properties through measuring the types of myosin heavy chain isoforms and mitochondrial oxidative enzymes expressed by the fiber. Fiber-type distribution within a muscle is related to the functional demands of the muscle, although generally within a given muscle, there will be a mosaic of fiber types with their distribution appearing more or less random (Swatland, 1975a).

Fiber-type distribution within a muscle can also affect the muscle's appearance. A muscle made up of predominantly oxidative muscle fibers will tend to be red in color, while a muscle consisting of predominantly glycolytic fibers will tend to appear white. This is because oxidative fibers have a high demand for oxygen to support their metabolism and thus contain a high myoglobin concentration. Myoglobin is a red pigment that functions to transfer oxygen from hemoglobin in the capillaries to the interior of the cell. However, there is a continuum in myosin heavy chain and mitochondrial enzyme expression in myofibers such that the rigid classification of fibers into three classes is not always possible.

Importantly, muscle fibers within a given muscle retain the ability to switch between types in response to environmental, physiological, or local signals thus allowing muscle to respond to changing demands but also complicating fiber typing. This is potentially significant because it suggests a mechanism whereby muscle mass can be altered independent of the need to alter fiber

number. Generally, glycolytic fibers are larger than oxidative fibers whose size may be limited by the need to diffuse oxygen efficiently. Glycolytic fibers also have a lower rate of protein turnover as well. Thus, white muscles are larger and more efficient. However, such fibers can be associated with poorer meat quality due to their color and metabolic properties. Therefore, the fiber-type composition of skeletal muscle as a whole can significantly impact meat quality. Genetic selection for rapid growth and leanness has generally resulted in a shift toward more glycolytic fibers in improved lines (Brocks et al., 2000).

The biology of fiber types and their impact on meat quality will be discussed in greater detail in later chapters but for now it is important to realize how the various fiber types arise during development. Primary muscle fibers initially form in the embryo and serve as scaffolds for secondary fiber formation. These initial fibers uniformly contain slow myosins regardless of the eventual presumptive muscle type they will give rise to in the fetus. As secondary myotubes form on the primary fiber scaffold, they become mixed in their myosin content. Although the etiology of fiber-type distribution in muscles of the developing fetus is still poorly understood, it is thought that innervations of the developing muscle by motor neurons dictates fiber type through orchestrating divergent calcium profiles in motor units (Beermann and Cassens, 1977a, 1977b; Beermann et al., 1977, 1978). Motor neurons excite skeletal muscle fibers by releasing the neurotransmitter, acetylcholine, into neuromuscular junctions. This in turn stimulates calcium release from the sarcoplasmic reticulum resulting in activation of the contractile apparatus. Chronic motor stimulation promotes a persistent, though low calcium level in the fiber that signals constant contraction, a phenotype consistent with oxidative fibers. On the other hand, intermittent stimulation signals spikes in intracellular concentrations of calcium resulting in rapid contraction, a phenotype characteristic of glycolytic fibers. The regulation of fiber type is currently an intense field of study given that manipulating the distribution of fiber types within muscle tissue has great potential to impact growth efficiency and quality.

Hyperplasia (Prenatal Muscle Development)

Overview

Skeletal muscle development is characterized by marked hyperplasia during gestation as muscle fiber number increases dramatically until birth. This increase in fiber number represents the primary mechanism of muscle growth during this period. Myogenesis is defined as the generation of muscle cell precursors and the subsequent fusion of such precursors to either form new fibers or to contribute DNA to existing fibers. As such, myogenesis drives the hyperplastic growth of prenatal muscle development. This complex process encompasses the recruitment of totipotent embryonic stem cells to the mesodermal lineage (determination) and the subsequent formation and maturation of muscle fibers. Importantly, myogenesis also extends beyond birth to include the postnatal addition of muscle cell precursor nuclei to mature fibers. Based upon this definition, skeletal muscle development can be broken temporally into three phases of myogenesis where fiber number is dictated by the extent of embryonic and fetal myogenesis and fiber size is determined by the degree of postnatal satellite cell fusion.

By influencing the number and type of myofibers that are present within whole muscle, factors that regulate myogenesis ultimately determine the potential mass of adult muscle tissue. Therefore, it is important to understand where muscle cell precursors originate and how they become functioning muscle fibers.

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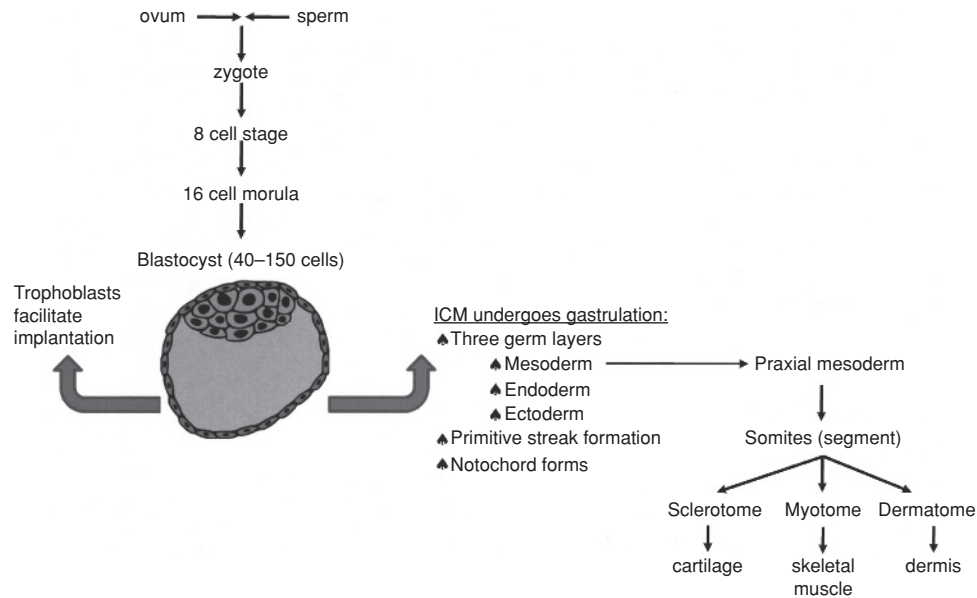


Figure 1.3 Development of the embryo and fates of cell populations found in the blastocyst. Cells of the inner cell mass give rise to the three germ layers subsequently forming the embryo proper. Trophoblast cells of the outer layer form the placenta.

Embryogenesis of Muscle

Skeletal muscle develops from a structural derivative of mesodermal somites of the early embryo identified as the myotome (Gilbert, 2006). Given that populations of muscle cell precursors arise during embryonic development, the discussion of muscle growth and development must begin with focus upon the development of the embryo.

Development begins at conception. During estrous, oocytes are released from the ovaries into the fallopian tube of the dam. Fertilization of the egg by the male sperm following copulation triggers activation of the oocyte and induces meiosis. The expulsion of polar bodies ensures the newly formed zygote contains a single set of male and female pronuclei (Fig. 1.3).

The events of early embryogenesis are very similar across mammalian species. Changes in gene expression dictate changes in cell function and fate. Initially, the two-cell zygote undergoes a period of rapid cell division characterized by an increase in cell number but a decrease in cell size. During this period, cells become tightly bound to one another causing the early embryo to assume a sphere-like shape. At this eight-cell stage, each cell of the embryo is totipotent, meaning they are completely unspecialized and capable of giving rise to any structure of the adult. However, beginning with the 16-cell morula, embryonic cells begin differentially expressing transcription factors via a poorly understood mechanism. Unique gene expression profiles drive cell specialization thus restricting the developmental fate of a given cell. For instance, cells in the outermost layer of the embryo at this stage begin to function as trophoblasts leading to the formation of a fluid-filled cavity that will become the blastocoel.

By the 40- to 150-cell stage, depending upon species, the developing embryo becomes a blastocyst that is capable of implanting into the endometrium of the uterine wall. At this point, the zona pellucida that surrounded the ovum has completely degraded and the embryo comprises two distinct

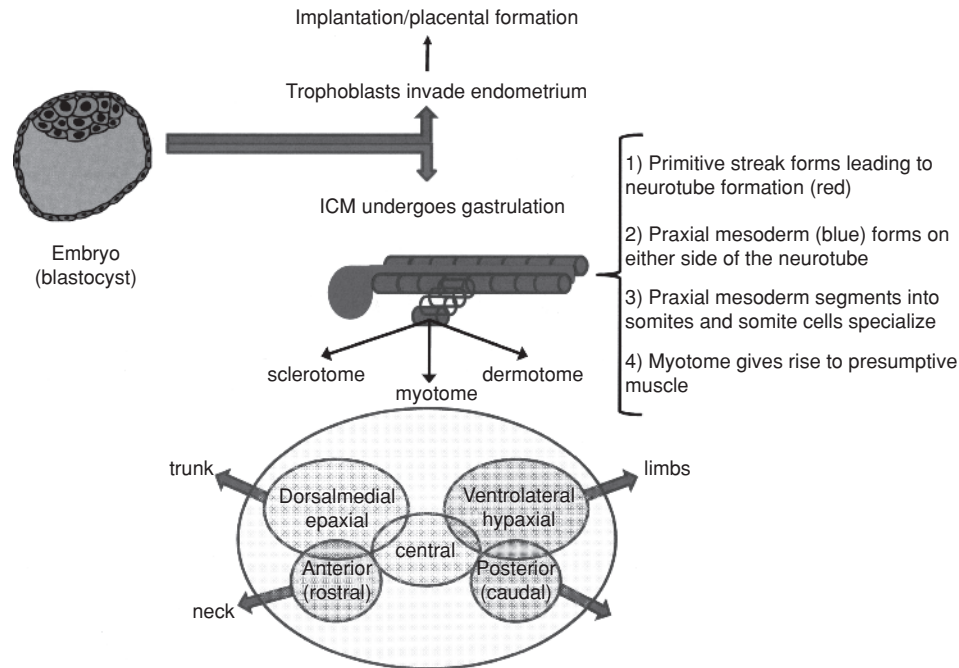


Figure 1.4 The derivation of the myotome and presumptive muscle.

cell populations, an outer layer of trophoblasts surrounding the well-formed blastocoel and a cluster of embryonic stem cells called the inner cell mass. The cells of the inner cell mass retain significant developmental potential whereas the cells of the trophoblast layer are more highly specified with their fate determined to cell types that largely support placentation (Gilbert, 2006).

At the time of implantation, two separate processes occur simultaneously within the blastocyst. First, trophoblasts differentiate into distinct layers. The outer syncytiotrophoblastic layer subsequently implants into the endometrium eventually developing into placental tissues. Cells of the second layer support placental growth. Meanwhile, as gastrulation is initiated, stem cells within the inner cell mass undergo a highly coordinated series of changes that eventually give rise to the three germ layers. Thus, trophoblasts facilitate implantation of the embryo and placental development while cells within the inner cell mass give rise to the mesoderm, endoderm, and ectoderm, three groups of cells from which all structures of the adult body will ultimately form.

Once cells of the mesoderm are specified, the stage is set for the development of presumptive muscle as the myotome is derived from the mesodermal layer (Fig. 1.4). Gastrulation is a period characterized by highly organized and coordinated cell migration and differentiation. During this period, the embryonic axis is set as the neural tube forms, the three pluripotent germ layers of the embryo become specified, and cells within embryonic germ layers become properly positioned. Following implantation of the embryo, the onset of gastrulation is signaled by the formation of the primitive streak. This structure emanates from the posterior end of the embryo as thickening of the epiblastic layer of the inner cell mass progresses. The paraxial mesoderm is formed as mesodermal cells migrate through the primitive streak and become positioned along either side of the developing neural tube. The paraxial mesoderm then segments into structures called somites. Cells of the somite further organize into three distinct masses via a poorly understood mechanism. Each cell mass gives

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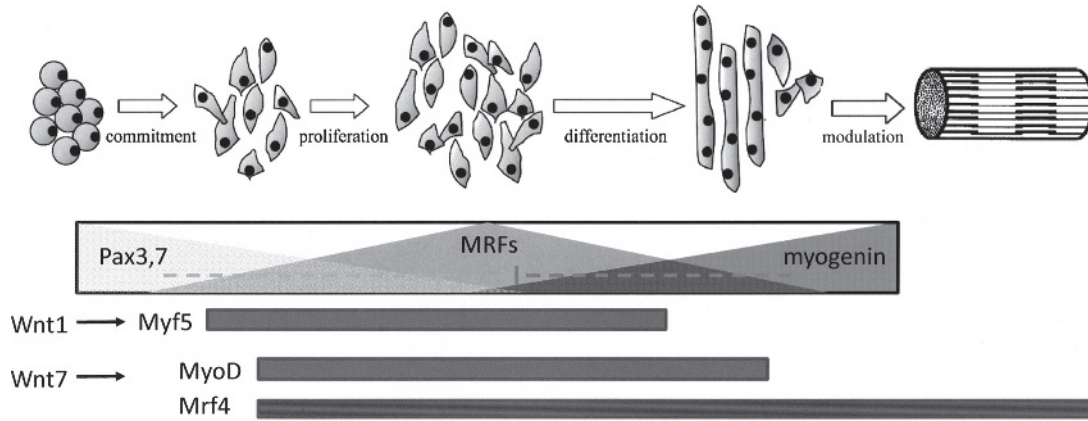


Figure 1.5 The basic events of myogenesis and the sequential changes in regulatory transcription factors that drive the process.

rise to distinct presumptive tissue. The sclerotome gives rise to cartilage. The dermatome gives rise to the dermis and the myotome gives rise to myoblasts (muscle precursor cells or muscle stem cells) that are capable of differentiating and fusing to become muscle fibers.

Myogenesis

Myogenesis is a complex multistage process. However, for the purposes of understanding, this process can be viewed as discrete phases encompassing muscle stem cell determination to myoblasts, proliferation of myoblasts, the differentiation and fusion of myoblasts to form myotubes, and the maturation of myotubes into mature muscle fibers (Fig. 1.5). Local signals from nonmyogenic cells direct the determination of somatic stem cells to the myogenic fate and orchestrate the highly ordered migration of myoblasts from the myotome through mesodermally derived extracellular matrix to sites of presumptive muscle (Sambasivan and Tajbakhsh, 2007; Buckingham and Montarras, 2008). Extracellular signals further direct the proliferation, fusion, and differentiation of myoblasts into multinucleated primary myotubes. These multinucleated cells specialize as changes in the myogenic transcriptome give rise to proteins such as myosin, actin, tropomyosin, troponin, desmin, and *m*-isoform of creatine kinase that allow the newly formed myotube to function as a mature muscle fiber fully capable of contracting, metabolizing energy, and communicating with its surrounding environment (Buckingham and Montarras, 2008).

Sequential changes in gene expression drive the formation of skeletal muscle fibers from individual stem cells. Muscle stem cells are regulated by interactions between their intrinsic potential (as conferred by their genetic profile), their unique local environments, and distinct signaling pathways. The current paradigm for the regulation of muscle cell development indicates that myogenic signals converge upon three transcription factor networks to control entry into the myogenic lineage (Perry and Rudnick, 2000). These networks include the paired-box transcription factors (Pax), myogenic regulatory factors (MRFs), and myocyte-specific enhancer factors (MEFs). In this model, extracellular signals act on the stem cell to trigger signaling cascades which elicit changes in the expression of key transcription factors. These transcription factors, often acting as dimers, bind to *cis* elements in skeletal muscle-specific gene promoters allowing them to regulate the transcription rates of these

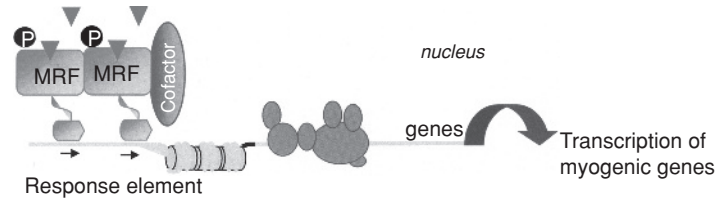


Figure 1.6 Muscle fiber formation occurs in a biphasic fashion from two separate populations of myoblasts during gestation. The precise timing of these events varies in a species-specific fashion.

genes (Fig. 1.6). Thus, altered transcription factor expression leads to changes in the expression of downstream genes resulting in altered cellular function.

Paired-box (Pax) genes are a family of transcription factors that regulate stem cell determination and tissue specification during early development of the embryo (Montarras et al., 2005). Nine family members have been identified to date based upon a conserved paired domain that dictates their DNA-binding specificity. Pax genes can further be grouped based upon their functional roles and tissue specificity. Importantly, expression of *Pax3* and *Pax7* (Pax group 3 genes) appears necessary for the emergence and survival of muscle stem cell precursors (Relaix et al., 2005; Relaix et al., 2006). Although it is now clear that Wnt signaling induces *Pax3/7* expression, the extracellular factors that regulate cell fate through *Pax3/7* are largely still unknown. *Pax7* is essential for the developmental specification of muscle satellite cells playing roles in their maintenance and activation (Smith et al., 2001; Montarras et al., 2005).

Two additional families of transcription factors, the MRFs and the MEFs, play key roles in regulating myogenesis at steps downstream of the specification of stem cells to the myogenic lineage. MRFs are proteins that contain basic helix loop helix (bHLH) domains which facilitate specific DNA binding and protein–protein interactions that allow MRFs to transactivate muscle-specific gene promoters containing E-box motifs (Lassar et al., 1994; Ma et al., 1994). Key MRFs include myogenic determination factor 1 (MyoD), myogenic factor 5 (Myf5), myogenin, and myogenic factor 6 (Myf6/MRF4). MEFs such as MEF2 are MADS box transcription factors that potentiate myogenesis by synergizing with MRFs (Black et al., 1998; Black and Olson, 1998).

The roles that these transcription factors play in myogenesis have largely been delineated using cell culture models and murine knockout models. Through these experimental approaches, MyoD has been identified as a master regulator of myogenesis (Davis et al., 1987; Tapscott et al., 1988; Rudnicki et al., 1993). Among its myriad actions, MyoD functions to increase muscle precursor cell number both by driving the commitment of somite-derived *Pax3/7*⁺ precursors to the myoblast lineage and by stimulating the proliferation of existing myoblasts (Davis et al., 1987; Thayer et al., 1989). Muscle cells appear to arise from at least two lineages in the somite, though in both lineages paracrine factors restrict development to the muscle fate by inducing MyoD expression. In hypaxial muscle, *Pax3* activates MyoD expression in the absence of inhibitory transcription factors. However, in epaxial muscle, expression of Myf5 induces MyoD expression. Regardless of the events leading to MyoD expression, MyoD appears to be a primary trigger signaling myogenic commitment. Although Myf5 has considerable functional redundancy with MyoD and Myf5 similarly stimulates myoblast proliferation, its precise role as a myogenic regulator remains controversial.

Other MRFs regulate myogenesis downstream of myogenic commitment. Myogenin regulates myoblast differentiation and fusion (Edmondson and Olson, 1989; Wright et al., 1989). Curiously, MRF4 has also been implicated in the differentiation and fusion of myoblasts during the formation of primary embryonic fibers but not during secondary fetal fiber myogenesis (Rhodes and Konieczny,

1989). MRF4 also plays a role in the maintenance of the myofiber transcriptome in adult muscle serving to induce many genes important for myofiber structure and function.

The regulation of myogenic determination is poorly understood and the literature addressing this issue is currently controversial. However, the regulation of myoblast differentiation is better characterized. Through studies examining myogenic gene expression during myogenesis, a regulatory cascade of MRF gene expression that is essential for myogenesis has emerged. As a result, Pax genes and MRFs also serve as muscle cell markers given their temporal pattern of expression plays a vital role in myogenesis. In this model, muscle stem cell progenitors express Pax3/7. Signals triggered by extracellular factors such as fibroblast growth factor (FGF) drive the determination of myoblasts by down-regulating the expression of Pax group 3 genes concomitant with the induction of MyoD and Myf5. Proliferating myoblasts express MyoD and Myf5 with increased myogenin expression only occurring immediately before myoblast differentiation. Finally, MRF4 expression is upregulated upon terminal differentiation of the myoblast. Myogenesis can be manipulated both *in vitro* and *in vivo* through altering these expression patterns either through knocking out or overexpressing individual MRFs. Thus, myogenesis is directed by the temporally controlled expression of MRFs which sequentially stimulate or repress muscle-specific gene promoters. This allows finite control of structural, contractile, and enzymatic protein expression that is essential for muscle cell function.

Somite-derived myoblasts are the only cell populations identified to date that can contribute to increased muscle fiber number. Importantly, myoblasts represent a pool of muscle cell precursors that can further expand in response to mitogenic signals. Myoblasts can either progress through the cell cycle when exposed to growth factors or they can remain replication-competent yet dormant. However, despite being committed to the muscle cell lineage, myoblasts remain unspecialized, meaning they do not yet express genes that are integral to conferring muscle function. Thus, while increased myoblast numbers favor greater muscle fiber formation, an increased number of myoblasts does not lead to increased fiber number *per se*.

In order to gain muscle function, myoblasts must first exit the cell cycle and differentiate before subsequently fusing to form myotubes. Importantly, as illustrated in Figure 1.5, these processes are distinct and controlled by different regulatory mechanisms. In the absence of mitogenic cues such as FGF, myoblasts can be directed to exit the cell cycle. Such myoblasts become intertwined in a network of connective tissue facilitated in part through their ability to secrete fibronectin to which integrin receptors anchor. This interaction between integrins and fibronectin appears necessary for myoblasts to differentiate (Menko and Boettiger, 1987; Rosen et al., 1992). Extracellular cues such as growth factors, hormones, cytokines, and components of the extracellular matrix drive the changes in gene expression that underlie these developmental changes (Hollis, 1993; Doumit et al., 1996). Thus, prenatal events that govern the extent of myoblast proliferation and the timing of differentiation ultimately determine the number of fibers a muscle will contain in postnatal life.

The fusion of myoblasts into multinucleated cells likewise depends upon extracellular factors. Differentiation-competent myoblasts align through cell surface-specific interactions mediated by cadherins and cell adhesion molecules expressed on the myoblast cell membrane (Mege et al., 1992). Once aligned, calcium ionophores allow an influx of calcium into the myoblast cytosol thus activating a set of metalloproteinases called meltrins which mediate membrane fusion in a poorly understood process. Concomitantly, myogenin is expressed leading to terminal differentiation of the myoblast through activation of a host of muscle-specific genes. The differentiated, fused myoblasts now comprise a multinucleated, immature muscle fiber termed a myotube. Further recruitment and fusion of myoblasts coupled with the expression of muscle proteins such as creatine kinase, myosin, and actin results in a mature, contraction-competent muscle fiber. Given that the primary function of muscle is contraction, myofibrillogenesis is an integral part of muscle fiber maturation. The regulation of myofibril development is poorly characterized at this time, however.

Biphasic Fiber Formation

Myoblasts are derived from the myotome and migrate from somites to sites of presumptive muscle where they proliferate, aggregate, align, and fuse into myotubes. Muscle fiber number increases in a biphasic fashion with an embryonic wave giving rise to primary fibers and a second fetal wave giving rise to secondary fibers. The timing of these waves of myogenesis occurs at species-specific gestational ages though ultimately total muscle fiber number is set by birth in most species.

Primary fibers first form from a population of embryonic myoblasts. The regulation of this process is poorly understood but it is thought that primary fiber number dictates the ultimate potential fiber number of the adult animal (Du et al., 2009). This is because these initial fibers serve as a scaffold that is critical for the alignment and fusion of secondary myoblasts. Thus, the number and cross-sectional area of primary fibers greatly impacts the potential for secondary fiber formation. Eventually, the innervation of primary fibers and their subsequent contraction breaks associations between primary and secondary fibers allowing individual muscle cells to become encapsulated by their own basal lamina (Fredette and Landmesser, 1991; Wilson and Harris, 1993). Although species-specific differences exist in the timing of these events and the number of secondary fibers that associates with each primary fiber, the biphasic nature of fiber development is conserved across species (Gerrard and Grant, 2003). In general, muscle fiber number is set by the time the fetus reaches the final third stage of gestation. Finally, not all myoblasts fuse to form new fibers, becoming instead satellite cells that are destined to fuse only with existing fibers in mature whole muscle (Fig. 1.7).

Myocytes, adipocytes, and fibroblasts all are derived from the mesoderm (Gilbert, 2006). Although the timing is species-specific, the formation of secondary fibers coincides with the onset of adipogenesis and fibrogenesis during mid- to late gestation. Thus, secondary fibers form during a time when populations of fibroblasts are beginning to secrete extracellular matrix components and presumptive adipose tissue is appearing. Given it is now known that adipocytes, muscle fibers, and fibroblasts secrete growth factors and cytokines, it seems likely that crosstalk between these emerging cell types may serve to coordinate the development of whole muscle by influencing fiber number, the development of the perimysium and epimysium layers, and influencing the potential to deposit intramuscular fat.

Given that increasing muscle mass is desirable from a profit standpoint, strategies which increased primary fiber number or size would be desirable. Unfortunately, primary fiber number has proven to be intractable to manipulations of the intrauterine environment (Russell and Oteruelo, 1981; Du et al., 2009). However, primary fiber number is significantly influenced by breed suggesting that it may be possible to select for increased primary fiber number (Stickland and Handel, 1986). Encouragingly, muscle mass can be manipulated within breed and several studies have now shown that primary fiber size can indeed be altered by manipulating the gestational environment as factors such as nutritional plane and exposure to factors such as IGF-1 can influence primary fiber hypertrophy (Du et al., 2009).

Hypertrophy (Postnatal Muscle Development)

As fiber number is set at birth in mammals, postnatal increases in muscle mass primarily occur through the hypertrophy of existing fibers. This growth is accomplished by increases in both the length and diameter of muscle fibers. Fiber size can also be modulated through changes in the fiber

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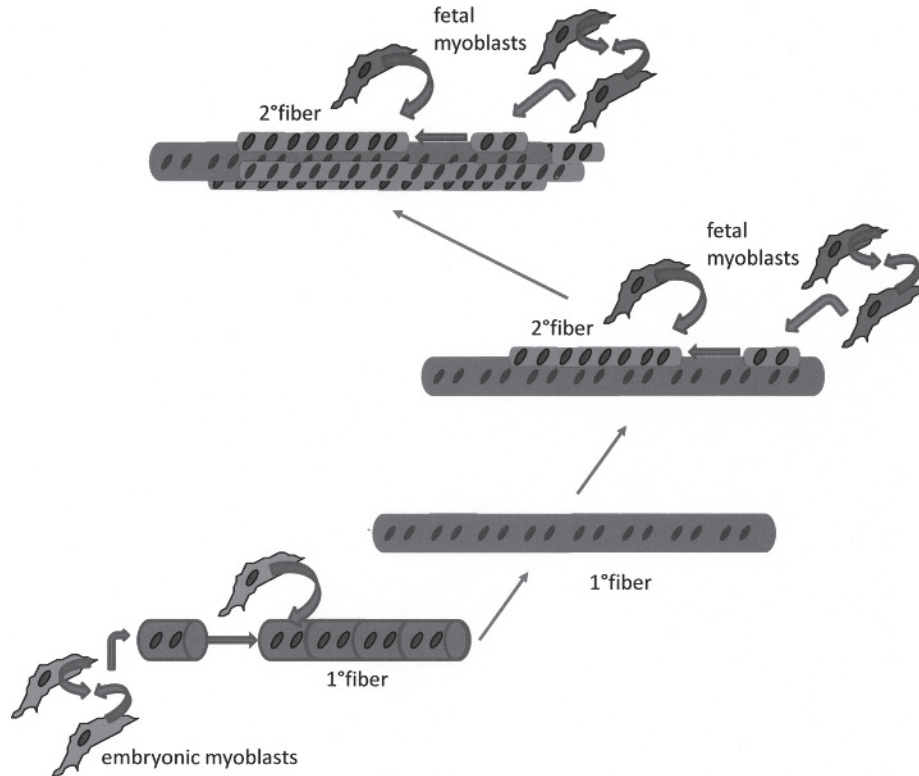


Figure 1.7 Mechanism for the transactivation of muscle-specific genes by MRFs. MRFs bind to specific response elements in the regulatory region of genes where they recruit other proteins forming a complex that promotes assembly of the transcriptional machinery leading to initiation of transcription. Signaling events can modulate this process through reversible phosphorylation of MRFs.

type composition of an individual muscle as, for instance, a switch from smaller oxidative fibers to the larger glycolytic fibers would be associated with an increase in muscle mass.

Regardless of the mechanism, increases in muscle mass are supported by dramatic reorganization of the contractile apparatus. Dynamic in nature, myofibrils can increase in size by the addition of sarcomeres at either ends of the myofibril and through the addition of thick and thin filaments to the periphery of the myofiber. Upon reaching a critical thickness, growing myofibrils can also split into new myofibrils via a poorly understood mechanism.

The characteristically large size and specialized contractile function of the muscle fiber imposes unique constraints upon its hypertrophy. Myofibrillar proteins comprise approximately 60% of total skeletal muscle protein (and may significantly impact meat texture). Consequently, the synthesis of myofibril regulatory proteins, the myofibril itself, and the maintenance of its integrity represents a tremendous burden upon the nuclei of the muscle cell. Thus, increases in muscle fiber size are only possible when rates of protein synthesis are significantly greater than rates of protein degradation. Therefore, the rate of postnatal muscle growth is predominantly a function of the balance between protein synthesis and degradation except for minor postnatal changes in fiber number associated with injury-stimulated fiber regeneration. Also, as the volume of sarcoplasm that can be supported by a single nucleus is limited by the rate of diffusion, increases in muscle fiber size are largely limited

by the ability of available nuclei to support myofibril protein synthesis. During postnatal growth, the number of nuclei in muscle cells increases substantially as evidenced by a 2- to 100-fold increase in the DNA content of the muscle cell (Gerrard and Grant, 2003). As the terminally differentiated myocyte is incapable of reentering the cell cycle, the potential for postnatal muscle fiber hypertrophy is dictated by the degree that satellite cells residing within the basal lamina contribute their nuclei through fusion with the growing muscle fiber (Montarras et al., 2005).

Satellite Cells

Satellite cells are a population of adult, mononucleated myoblasts that are sandwiched between the basal lamina and the sarcolemma of mature muscle fibers. Their primary functions are to act as a pool of nuclei that supports muscle fiber hypertrophy and facilitates muscle fiber regeneration following injury. There appear to be two populations of satellite cells in adult muscle (Montarras et al., 2005). One population resides within the basal lamina in close proximity to mature muscle fibers thus allowing their fusion. Even though satellite cells are replication competent, the presence of the basal lamina likely prevents them from forming new fibers by representing a physical barrier that prevents their aggregation and alignment. A second population of satellite cells is best characterized as a Wnt-activated muscle stem cell that differentiates in response to an injury-stimulated cascade (Montarras et al., 2005). Trauma to the fiber induces Wnt signaling in such cells resulting in the induction of Pax7 and ultimately leading to activation of MyoD family of genes. It is possible that subpopulations within these groups may underlie divergence in fiber type in the mature animal.

Generally, satellite cell number decreases with increasing muscle mass and animal age though satellite cell proliferation occurs throughout growth ceasing only when lipid deposition becomes appreciable in muscle (Trinkle et al., 1978). Satellite cells exist either as quiescent (reside in the G0 stage of the cell cycle) or active cells, and their behavior in culture mimics that of embryonic myoblasts with regard to an ability to proliferate, differentiate, and respond to mitogens and other known regulatory signals (Florini and Magri, 1989; Allen and Rankin, 1990).

It is currently unclear how satellite cell fusion is directed by the enlarging muscle fiber though it is clear that muscle fibers secrete cytokines and growth factors that are known to affect satellite cell activation, proliferation, and differentiation (Pedersen and Febbraio, 2008). *In vitro* experiments indicate that a myriad of growth factors regulate all phases of the satellite cell life cycle (Allen and Rankin, 1990). Importantly, since heparin sulfate and other proteoglycans abundantly expressed in the extracellular matrix associate with growth factors, the basal lamina represents a rich site of sequestered growth factors. Presumably, such growth factors can interact with satellite cells within the basal lamina and readily influence the satellite cell life cycle. Certainly, the location of satellite cells in close proximity with the sarcolemma suggests crosstalk between muscle and satellite cells is likely.

Protein Turnover

Proteins are constantly turned over in skeletal muscle by undergoing a continuous cycle of degradation and resynthesis. Although this cycling may seem wasteful and indeed represents a drag upon protein accretion rates in the growing animal, protein turnover allows muscle cells to rapidly regulate protein concentrations and remove improperly folded or damaged proteins. As a result, muscle cells are better able to adapt to changing external requirements.

Protein Synthesis

Significant amounts of protein synthesis are required to support increases in muscle fiber length and diameter. The central dogma describes the flow of biological information in cells leading to the expression of functional proteins. In this paradigm, messenger RNA (mRNA) is transcribed from genes specified by the nucleotide sequence of DNA. We have already seen how myogenic transcription factors direct the transcription of muscle-specific genes to mRNA during myogenesis through their interaction with the regulatory regions of these genes. Once transcribed and processed, mRNA is translocated from the nucleus to the cytosol where it interacts with ribosomes. The nucleotide sequence contained in the mRNA serves as a template specifying the specific amino acids that should be added to the growing peptide chain by interactions with small aminoacyl-tRNA. Finally, the newly synthesized peptide chain gets modified post-translationally.

Importantly, there are physical limits to the amount of cytosol a nucleus can efficiently supply with protein. The range is essentially set by the rate in which the nucleus can generate the mRNA species required to support the protein needs of the cell in light of the turnover rate of the given protein (Allen et al., 1999). As seen earlier, this problem is solved by the muscle cell being multinucleated with satellite cells serving as a pool of nuclei that can support increasing size of the muscle cell.

Protein translation, the conversion of the mRNA sequence to a growing peptide chain on the ribosome, is also a highly regulated process that occurs in three sequential stages (Pain, 1986). First, an initiation complex is formed through interaction of ribosomal subunits, the mRNA species being translated, and an initiator aminoacyl-tRNA. Second, the peptide is elongated as amino acids are added to the growing chain. Finally, the chain is terminated in response to a stop codon on the mRNA and the polypeptide chain gets processed and folded into the functional protein. Each step of translation represents a point of regulatory control and could be a rate-limiting step in the process of protein synthesis.

Protein Degradation

Protein degradation is the breakdown of proteins into smaller polypeptides and individual amino acids by proteolytic enzymes. If not counterbalanced by new synthesis, protein degradative pathways will decrease the amount of functional protein expressed by the cell. Given that proteins continually turnover or go through a reoccurring process of protein degradation and resynthesis, there is a constant need for new protein synthesis in order to maintain myofibril integrity and fiber size.

Traditionally, emphasis was placed on better understanding the regulation of protein synthesis, so this is a well-understood process. However, there are generally upper limits to which protein synthesis can occur in growing muscle. Therefore, much recent effort has been devoted to better understanding the regulation of protein degradation in the hopes of increasing protein accretion by limiting protein degradation rates.

Generally, protein turnover rates are much greater than protein accretion rates in a given muscle. Half lives for muscle proteins can range from 2 to 20 days depending upon the protein. Turnover rate is generally a function of the class of proteins being most rapid for sarcoplasmic proteins, intermediate for myofibrillar proteins, and slowest for stromal proteins (Gerrard and Grant, 2003).

There are currently three well-characterized proteolytic systems known in skeletal muscle. Lysosomal proteolysis is carried out in organelles that form a specialized cellular compartment equipped with proteases such as cathepsins that function optimally at low pH. Lysosomal proteolysis accounts for approximately 20–30% of the protein turnover in muscle fibers and specifically targets sarcoplasmic proteins (Lowell et al., 1986a, 1986b). The lower pH of the cytosol, presence of cytoplasmic cathepsin inhibitors such as cystatins, and the lack of evidence for myofibril transport into lysosomes suggests lysosomal proteolysis plays little role in the turnover of myofibrillar proteins.

A second pathway, the ubiquitin-proteasome proteolytic pathway, targets regulatory proteins, short-lived proteins, and sarcomeric proteins (Mitch and Goldberg, 1996). Multiple enzymes in this system function to attach ubiquitin to proteins that have become destined for degradation. Polyubiquitinated proteins are subsequently trafficked to 26S proteasomal complexes where they are broken down. Inhibiting the proteasome-ubiquitin pathway significantly lowers proteolytic breakdown suggesting that this pathway plays a significant role in protein degradation in muscle cells (Rock et al., 1994; Tawa et al., 1997).

Finally, a third system of proteases was discovered, the calpain system, due to the ability of these proteases to act directly on Z-discs from myofibrils (Bullard et al., 1990). Calpains are activated by intracellular calcium levels and were initially grouped based upon their calcium sensitivity *in vitro*. For instance, μ -calpain can be activated at micromolar concentrations of Ca while m-calpain requires millimolar amounts. The μ - and m-calpains are termed ubiquitous calpains because they are expressed in all tissues. A third calpain, p94 or skeletal muscle calpain, is specifically expressed in muscle. The activities of the ubiquitous calpains increase in conditions associated with muscle degradation. Although considerably less is known about muscle calpain, it seems to serve an opposite role by protecting the muscle from proteolysis (Gailly et al., 2007; Lamb, 2007). Calpastatin inhibits the activity of the ubiquitous calpains, it too being regulated by intracellular calcium levels. Finally, phosphorylation of calpains can modulate their requirement for calcium thus representing a further layer of regulation directing this important proteolytic system. Since calpains are integral in directing postmortem proteolysis, the calpain system is especially relevant to meat quality because of their influence on meat tenderness.

Muscle Cell Culture

During the past 20 years, cell culture techniques have taken on great importance for the study of muscle biology. Cell culture has facilitated the study of the transcriptional regulation of myogenesis, the hormonal regulation of protein turnover, and the control of nutrient uptake by muscle in ways that would otherwise be impossible *in vivo*. Importantly, cell culture systems facilitate mechanistic inquiry that can be useful for *in vivo* studies. Most recently, it has been revealed that muscle cells release cytokines and growth factors to a much greater extent than previously appreciated. Thus, myofibers may also participate in a peripheral endocrine axis between adipose tissue and skeletal muscle that modulates the effect of the hypothalamic–pituitary–gonadal axis on growth. Surely, skeletal muscle culture systems will be instrumental in further elucidating this emerging endocrine function of muscle fibers.

Essentially, all known mitogens and endocrine factors effecting myogenesis have been discovered through *in vitro* experiments. Gene expression studies using cell lines and primary cultures have revealed expression patterns that suggest regulatory roles. In many cases, these observations have been extended using gene overexpression and knockdown experiments. It was in this way that the original roles for MRFs were demonstrated using murine C3H 10T $\frac{1}{2}$ nonmuscle embryonic stem cells. Azacytidine is an analog of cytidine that incorporates into DNA and blocks methylation thus preventing gene silencing. It was observed that treating 10T $\frac{1}{2}$ cells with azacytidine could induce them to specify cartilage, fat, or muscle. Using this model, MyoD was subsequently discovered by a subtractive hybridization approach and it was demonstrated that the transfection of MyoD alone was sufficient to convert fibroblasts to myoblasts (Davis et al., 1987; Tapscott et al., 1988). These observations were followed up by similar overexpression studies which established myogenin, myf5, and MRF4 as important myogenic regulators and helped to elucidate the MRF transcriptional cascade discussed in Figure 1.5 (Edmondson and Olson, 1989; Rhodes and Konieczny, 1989; Wright

et al., 1989). The MRF family is unique in its ability to drive the transdifferentiation of nonmuscle cells to myoblasts. These data suggested its members are the master regulators of myogenesis and formed the basis for conducting more elaborate and costly experiments using transgenic animal models. As a result, there has been an explosion in our understanding of the regulation of myogenesis in the past decade.

A good deal of this chapter has been devoted to discussing the regulation of muscle cell hyperplasia and hypertrophy. We will next focus on the cell culture techniques that have allowed this regulation to be studied in great detail.

Basic Concepts of Cell Culture

Tissue or cell culture basically involves harvesting tissue or cells from the live animal and incubating them in a plastic dish. Thus, cell culture is synonymous with the term *in vitro* as it is the *in vitro* process of growing cells artificially in the laboratory. The definition of *in vitro* and *in vivo* research depends on the experimental model used. *In vivo* literally means within a living organism and refers to experimentation conducted in the intact organism. *In vitro* literally means “in glass” but generally refers to any artificial environment that is outside the body. For the purposes of cell culture, *in vivo* refers to experiments conducted in living animals and *in vitro* refers to experiments that use living cells propagated in an artificial environment outside of the animal. The purpose of this section is to provide a conversational knowledge of how researchers conduct cell culture.

The ultimate goal of cell culture is to mimic the *in vivo* situation by creating an *in vitro* environment such that the cells of interest model or maintain the behavior they would exhibit in the animal as closely as possible. This approach affords researchers significant advantages but they are not without important caveats.

Given the goal of cell culture is to create an environment that mimics the *in vivo* state, there are several critical requirements for successfully culturing cells *in vitro*. First, cells must be incubated in an environment that maintains the appropriate temperature and pH which in most cases is 37°C and a pH of 7.2. Cells must also be supplied a suitable growth medium that contains energy substrates, amino acids, and inorganic salts in sufficient quantities to support cell metabolism and growth. This medium can be provided as a liquid or semisolid depending upon the requirements of cell type being utilized. Growth regulators must also be provided as cells will not progress through the cell cycle without exposure to mitogens and growth factors. The specific requirements for sustained proliferation are often not well characterized for many cell types, so this criterion is most often satisfied by supplementing the base medium with serum from either bovine fetal or calf sources. One disadvantage of serum supplementation, however, is that serum is an undefined component that can display dramatic variation from one lot to the next so there is the potential that serum supplementation can confound experimental results. In many instances, serum-free conditions can be worked out to facilitate experiments that do not require cells to proliferate. Furthermore, several companies now make defined cocktails of mitogens and growth factors that may allow certain cultures to proliferate in serum-free conditions. Finally, it is vital that cell culture be conducted in aseptic (sterile) conditions. Microorganisms are virtually impossible to eradicate once in culture and they grow much more quickly than mammalian cells allowing them to quickly outcompete cells of interest for available resources. Ultimately, the need to meet these critical parameters will dictate how cell culture is performed in the laboratory.

There are two major classes of cell culture models—primary culture and continuous culture—that are defined by how the cells are derived and by their growth characteristics. Each system offers unique advantages and disadvantages. These are important to consider when determining the optimal

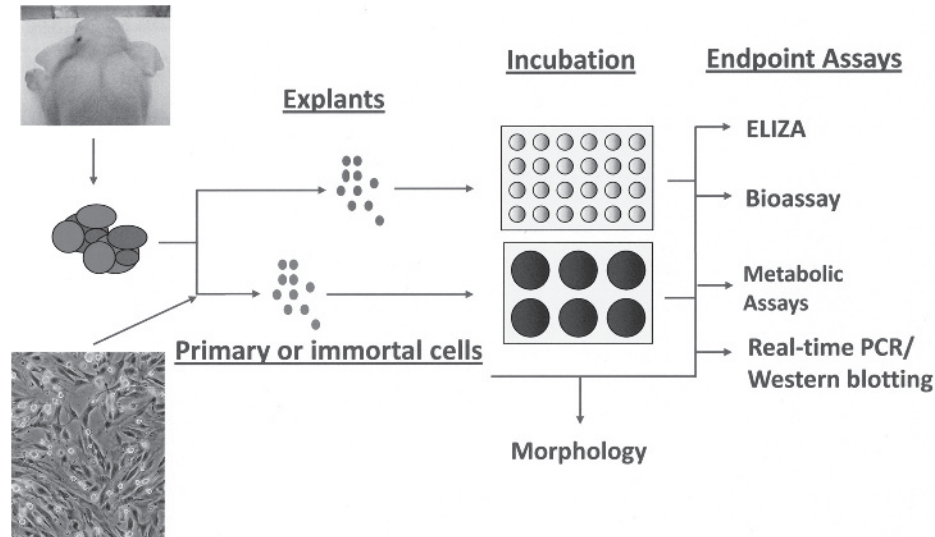


Figure 1.8 Generalized schematic for the use of cell culture in animal experimentation.

system to adopt and the degree to which data generated by such systems can be extrapolated to other models (Fig. 1.8).

The first model system is termed primary culture because cells are derived directly from excised, normal animal tissue. In this system, the culture can be incubated as either explants or as single-cell suspensions. Explants are essentially small chunks of tissue that maintain the cytoarchitecture of the original tissue. This may be advantageous depending upon the question being asked by the investigator. In contrast to explants, single-cell suspensions are derived by the dissociation of harvested tissue through the enzymatic digestion of connective tissue and extracellular matrix components (most often via collagenase). Cell suspensions allow the culture of specific cell types as opposed to the highly heterogeneous nature of explants. This allows the researcher to avoid confounding effects caused by the potential crosstalk between different cell types in explants. A major advantage of primary culture is that the cells usually retain the characteristics they display *in vivo* to a large degree once cultured *in vitro*. Thus, primary culture represents the most faithful system to model behavior in the animal. However, primary cultures can only be maintained for a limited time as primary cells senesce or exit the cell cycle after a limited number of cell divisions. Explants tend to undergo necrosis if chronically incubated. Furthermore, primary cultures often represent a heterogeneous population of cells that is largely undefined making mechanistic inquiries more difficult. Also, primary cultures will retain the genetic variation inherent in the animal from which they were harvested. Thus, primary culture necessitates the sacrifice of animals which are undesirable both from a cost standpoint and from the need to minimize animal suffering when conducting animal research.

The second major class of cell culture models is termed continuous culture because these cultures comprise immortalized cells. These cells have been cloned originally from an animal source but now are considered to exist as a homogeneous population that can be propagated indefinitely. Thus, continuous cultures are described as being cell lines. Cell lines are often derived from tumors, hence their immortal character. Alternatively, they can be stem cells or cells that have been immortalized chemically or via transfection of foreign DNA that disrupts the normal regulation of their cell cycle.

Cell lines offer several advantages in that they provide an endless supply of cells that should not vary greatly in their phenotype. Cell lines are often very easy to transfect facilitating mechanistic inquiry and overexpression/knockout studies. However, cell lines often have chromosomal abnormalities that arise from their continuous propagation. Thus, even with cell lines, phenotypic drift is a major concern. Furthermore, since they are often derived from tumors, the ability to extrapolate data back to the live animal may be limited.

Given the caveats associated with primary and continuous cultures, one might ask, why do cell culture at all? Despite the concerns associated with conducting *in vitro* experiments, cell culture offers many important advantages. First, cell culture systems allow growth biologists to conduct experiments that would otherwise be impossible *in vivo*. Furthermore, *in vitro* experimentation allows the investigator to control variation to a much greater degree thus making it more likely that the questions being asked will be answered more reliably. For instance, the investigator can control the physical and chemical environment, alter physiological conditions at will, as well as finely manipulate critical parameters such as concentration and time, all of which may be essential variables of an experiment. Importantly, cell culture can serve to limit the use of animals and the homogeneity of cell cultures can aid in interpreting experimental results as well as improving reproducibility.

It is paramount to practice proper aseptic technique in order to conduct successful cell culture experiments. While providing cells with a complete medium, a warm and humid environment, and a suitable pH is essential to maintain both their growth and a desirable phenotype; doing so also creates an environment that promotes the growth of undesirable organisms such as microbes, yeasts, and molds.

A primary tenant of sterile technique is that all work should be conducted in a sterile environment. This requires the use of a cell culture hood (alternatively called a biological safety cabinet) and chemical disinfectants the most common being a 70% alcohol solution. A class II laminar flow cabinet maintains a sterile environment by circulating air through a system of hepa filters such that the working environment is bathed in sterile air. It is important to appreciate the direction of airflow so that vents supplying the filtration system are not blocked. Furthermore, this knowledge allows motion to be minimized between the filter and the cultures. This is important as the primary source of contamination is in fact the user. Finally, the sash on the front of the hood serves to both minimize undesirable air currents created by movement outside of the hood as well as to protect the user from aerosols that are an unavoidable part of manipulating cultures and growth medium. Liberally spraying down work surfaces with a disinfectant can greatly mitigate the potential hazard posed by aerosols as well as prevent the growth of undesirable contaminants.

Density dependence is another important characteristic that must be considered as it dictates how cultures are handled. Cells can be grouped by morphology based upon whether they adhere to a surface and thus grow as a monolayer (as do most cells derived from tissue) or whether they float in culture and are thus grown in suspension (as do cells derived from the blood). Muscle cell cultures are adherent cultures thus their density can be described based upon the percent confluence of their monolayer. Percent confluence is determined roughly by the degree to which cells have achieved a state where they are in maximal contact with neighboring cells. Whether a particular culture has rigorous density dependence dictates the percent confluence the cultures can be allowed to reach. For instance, some cultures are not inhibited by cell-to-cell contact and thus will continue to divide regardless of whether confluence of the monolayer is achieved. Other cells will exit the cell cycle when cell-to-cell contact is achieved via a process called contact inhibition. Thus, allowing such cells to reach confluence could have dramatic consequences for the character of the cell culture being propagated.

As a result of their density dependence, the continuous propagation of adherent cell cultures generally requires that they be subcultured or “passed”. Passaging a culture simply involves disruption of the monolayer by protease treatment to create a cell suspension that can then be easily diluted. Fittingly, this process is termed a passage. In this way, cell density can be managed and cells can be obtained and reseeded for use in experiments. The most common protease used for passaging cultures is trypsin. Passage number is an important parameter to monitor as the character of primary cultures or a continuous cell line will inherently drift with increasing passage number. Thus, the behavior of cells can be altered greatly with time especially if cultures are not handled in a consistent manner.

The characteristic drift seen with the continuous propagation of cells in culture can be managed by the cryopreservation of large numbers of cell stocks that have been harvested at a low passage number. Cryopreservation involves the freezing and subsequent storage of cell stocks at ultralow temperatures. Storage is generally accomplished by maintaining cell stocks in the liquid nitrogen vapor phase within a cryogenic tank. Generally, cultures are frozen at an optimal cooling rate in the presence of serum and a cryoprotectant such as dimethyl sulfoxide (DMSO) or bovine serum albumin (BSA) that serves to prevent crystal formation and damage to the cell due to dehydration. By limiting the number of times a culture can be passaged before being terminated in favor of revitalizing a frozen stock, the character and phenotype of a continuous cell line can be maintained within narrow parameters thus allowing the maximal degree of repeatability.

Culture of Established Muscle Cell Lines—Murine C2C12 Myoblasts

The C2 myoblast cell line was originally isolated from C3H mice by causing trauma to their leg muscles in order to stimulate satellite cell proliferation (Yaffe and Saxel, 1977). The resultant cloned myoblasts maintain fibroblastic character in the presence of high levels of fetal calf serum. However, they can be induced to differentiate and fuse by withdrawing serum. Importantly, C2 myoblasts express MyoD, Myogenin, and Myf5 (Neville et al., 1997) making them an excellent model for studying myogenesis. The C2C12 mouse myoblast cell line is a diploid subclone of the C2 parent line selected on the basis of its ability to fuse rapidly to form contracting myotubes which highly express muscle-specific proteins (Blau et al., 1985a, 1985b). The C2C12 fusion is enhanced in confluent cultures by withdrawing fetal bovine serum from media and supplementing it instead with lower amounts of horse serum. Recently, it has been demonstrated that the overexpression of bone morphogenic protein 2 (BMP-2) induces C2C12 cultures to differentiate into osteoblasts suggesting the specialization of this cell line is not fixed solely to the myogenic lineage (Partridge et al., 2002).

Due to their growth properties, C2C12 myoblast cultures require constant maintenance. Myoblasts are spontaneously induced to differentiate by cell-to-cell contact. Since C2C12 myoblasts have a short doubling time (16 h) and tend to grow in clumps resembling the formation of colonies, it is necessary to monitor C2C12 cultures daily and to passage them often in order to avoid counterproductive selection pressures. Generally, cultures should not be allowed to grow to greater than 50–70% confluence. Allowing cell density to become too high selects for slower fusing myoblasts because fast-fusing ones will exit the cell cycle and drop out of culture as they differentiate. Similarly, due to their fast-fusing nature, cultures of C2C12 myoblasts are more susceptible to drift in their character with increasing passage number than most fibroblastic cell lines. This necessitates that large quantities of C2C12 myoblasts be cryopreserved at a low passage number so that fresh vials of cells closely resembling the character of the original culture can be thawed and seeded in order

to mitigate issues related to their continuous culture. Nonetheless, their rapid growth, fast-fusing character, ability to be easily transfected, and their robust expression of MRFs and muscle-specific genes makes the C2C12 myoblast cell line a valuable tool for studying myogenesis and muscle biology.

The procedures that follow have been adapted from the recommendations of American Tissue Type Collections for C2C12 myoblasts (ATCC[®] catalog number: CRL-1772) through the routine use of this cell line in the Brandebourg Laboratory. These protocols are meant to serve as a practical guide for the use of this cell line in teaching and research endeavors.

Propagation

Cultures are propagated at 37°C in a 5% CO₂ environment. Growth medium consists of Dulbecco's Modified Eagle's Medium (DMEM) formulated to contain 4.5 g/L glucose, 1 mM sodium pyruvate, 4 mM L-glutamine, 15 mM Na₂CO₃ (optimal for 5% CO₂ environment), a phenol pH indicator, and supplemented with fetal bovine serum to a final concentration of 10%.

Cultures should be observed everyday and evaluated for percent confluence, doubling rate, uneven distribution, and for signs of fusion. Growth medium should be changed every 2 days. Generally, C2C12 cultures seeded in a 75 cm² culture flask require 10 mL of medium per 48 h period in culture. This suggested volume to surface area is scalable to different-sized flasks. However, no more than 15 mL of medium should be added to a 75 cm² culture flask as too large a volume of medium can adversely affect cultures due to weight/pressure issues. Cultures should be subcultured (passaged) before the monolayer reaches no greater than 50–70% confluence.

Subculturing

The passage of cells that grow as a monolayer in culture requires the disruption of their attachments to one another and to the surface of the culture vessel in order to create a cell suspension that makes pipetting and diluting possible. It is important that cultures not be allowed to reach confluence. Multiple 75 cm² flasks can be used in order to achieve a sufficient yield of cells despite the strict passage of cultures when the monolayer reaches 50% confluence.

- (1) Aspirate and discard the conditioned growth medium using a mild vacuum.
- (2) Since the growth medium contains serum, rinse the culture flask with several milliliters of a balanced salt solution (BSS) that is congruent with the BSS used to make the trypsin solution (normally calcium-free Hanks).
- (3) Add 2 mL of a 0.25% (w/v) Trypsin-0.53 mM EDTA solution per 75 cm² of surface area in the culture flask and incubate the flask at 37°C for 3–5 minutes or until the monolayer is completely dissociated. Check to ensure that all cells have come off of the plate by examining the cell suspension under an inverted microscope. It may be necessary to gently tap the culture flask to encourage cells to dissociate from the flask surface but do not use excessive swirling as this promotes clumping of cells.
- (4) Once digestion is complete, add an equal volume of serum-containing growth medium to the culture flask and gently agitate the cell suspension with pipette action to allow trypsin inhibitors in the serum to inactivate the trypsin solution.
- (5) Sample an aliquot of the cell suspension in order to perform cell counting. Seed culture plates for experiments or new culture flasks by adding an appropriate volume of the cell suspension to the new plastic vessel in the presence of a sufficient volume of growth medium. Serum aids in cell attachment, so seeding medium will require the presence of serum regardless of whether subsequent experiments will be conducted under serum-free conditions. Avoid leaving a cell suspension on fresh plastic for any period of time without addition of growth medium as doing so promotes an uneven distribution of attached cells (Fig. 1.9).

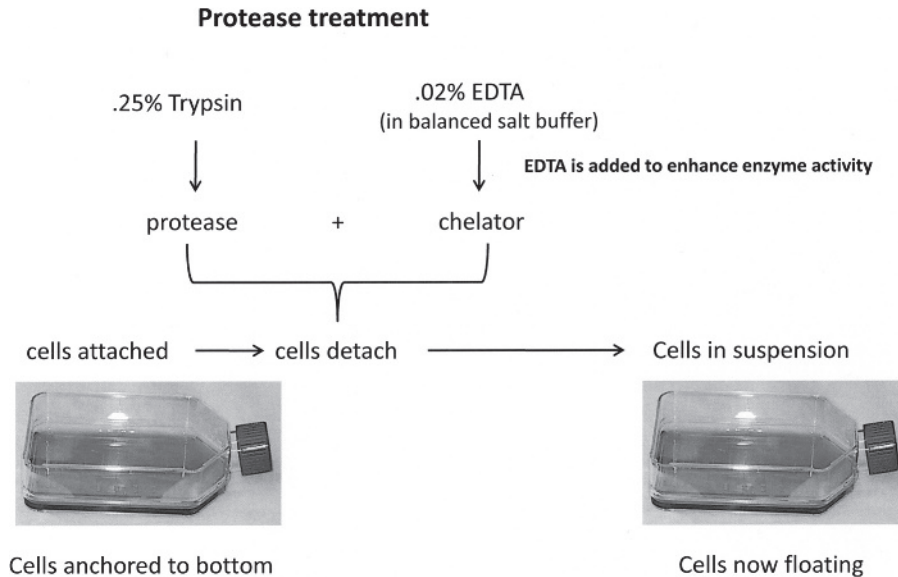


Figure 1.9 The subculture of adherent cell cultures. Trypsin is used to dissociate cells from a monolayer in order to produce a cell suspension that can more readily be diluted and handled.

Cell Quantification

It is important to use a consistent number of cells when freezing stocks or seeding flasks and experimental plates because consistency improves reproducibility by allowing the behavior of the cultures to be more readily maintained and monitored. C2C12 myoblasts will spontaneously fuse when coming into contact with neighboring cells. Thus, the character of the C2C12 culture can change dramatically if fast-fusing cells are allowed to differentiate and fall out of culture. Therefore, it is critical to maintain control over cell density in order to achieve the best experimental results.

- (1) While maintaining good sterile technique sample, an aliquot of a cell suspension is obtained as described in Step 5 of “Subculturing”.
- (2) To this aliquot, add an appropriate volume of Trypan Blue exclusion dye and gently mix. Nonviable cells will become stained blue.
- (3) Place a cover slip over the chamber of a hemacytometer and load 10 μ L of the sampled cell suspension per side of the chamber.
- (4) Place the hemacytometer under a microscope and count the number of viable and nonviable cells found in a defined portion of the grid. Take the mean counts for each cell population and calculate the percentage viability (number of viable cells/total cells) and the concentration of viable cells (number of viable cells \times dilution factor \times the correction factor based upon the area of the grid) (Fig. 1.10).

Induction of Differentiation and Fusion

The differentiation and fusion of C2C12 myoblasts occurs spontaneously upon the obtainment of cell-to-cell contact. Fusion can be induced by withdrawing serum. This is accomplished by removing the typical growth medium containing 10% fetal bovine serum and feeding the cultures growth medium supplemented with 5% Horse serum (Fig 1.11).

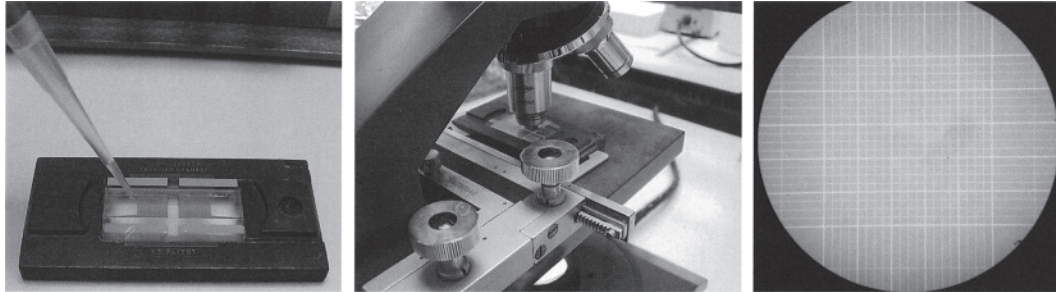


Figure 1.10 Cell quantification using a hemacytometer. The hemacytometer allows cells to be counted within a grid of known surface area. In this way, cell counts can be related to volume allowing the calculation of cell concentration.

Cryopreservation

Cultures selected for cryopreservation should be viable, they should display the expected growth rate and fast-fusing character of C2C12 myoblasts, and they should be free of contamination. Furthermore, preconfluent cultures should be utilized to ensure that they are in the log phase of growth and to maximize the number of fast-fusing myoblasts in the cultures. Freeze medium consists of DMEM supplemented with 20% fetal bovine serum and 5% DMSO (v/v) as the cryoprotectant.

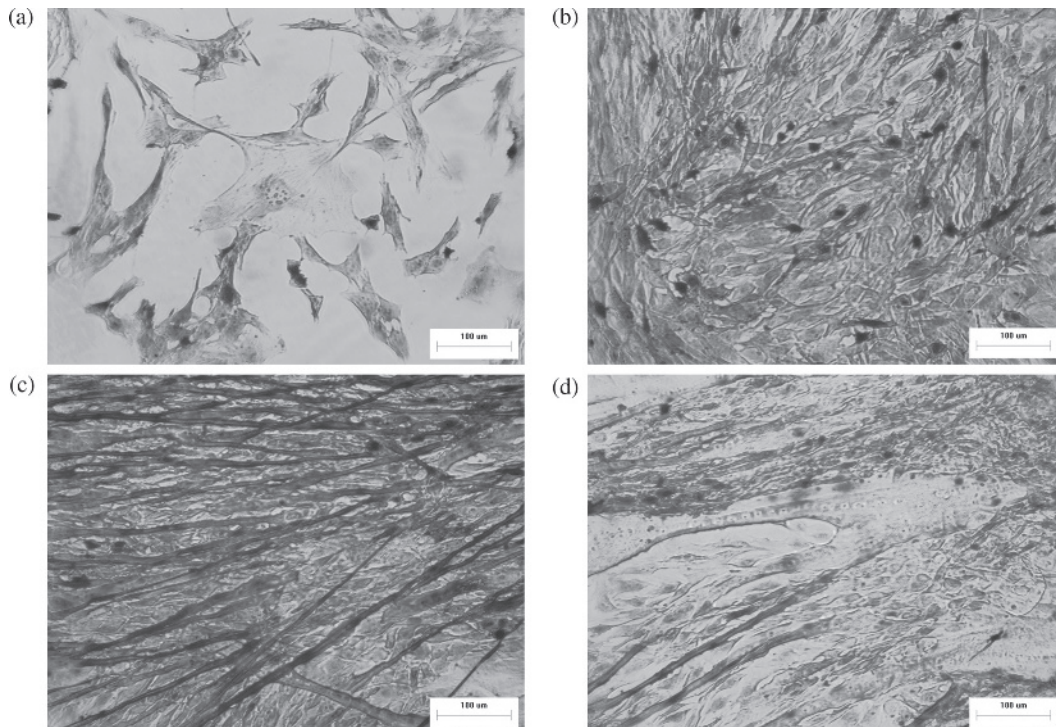


Figure 1.11 Myogenic differentiation of the C2C12 muscle cell line. (a) Subconfluent, proliferative myoblasts approaching 50% confluence. (b) Confluent monolayer of myoblasts. Some spontaneous fusion has given rise to immature myofibers exemplified by an elongated morphology. (c) C2C12 cultures 4 days after serum withdrawal characterized by fully differentiated myotubes. (d) C2C12 cultures 7 days after serum withdrawal. Note the multinucleated nature of mature myotubes.

Inclusion of DMSO prevents crystal formation and dehydration which could negatively impact cell viability. However, DMSO is toxic to C2C12 myoblasts when exposure is prolonged at high concentrations.

- (1) Dissociate cells from monolayer to obtain a cell suspension as described in Step 3 of “Subculturing”.
- (2) Sample a small aliquot to facilitate cell quantification.
- (3) Centrifuge the remaining cell suspension at 200 g for 5 min to pellet the cells.
- (4) Remove the supernatant and re-suspend cells at a concentration of $1-3 \times 10^6$ cells per milliliter in freeze medium.
- (5) Pipette 1 mL aliquots of cells into labeled cryogenic vials. Label should include cell line name, data, passage number, cell concentration, and the initials of the preparer so that vials can be cross-checked to the appropriate laboratory notebook.
- (6) During initial freezing, cultures should be cooled at a uniform rate of -1 to -3°C per minute. This is best accomplished by placing the vials in a Cryo-Safe cooler saturated with isopropanol and stepping down the temperature by placing it an ultra-freezer (-80°C) for several hours before transferring the vials to a cryogenic tank containing liquid nitrogen. Once appropriately frozen, temperature fluctuations should be avoided during storage. Furthermore, vials should be stored in the liquid nitrogen vapor phase to avoid over chilling.
- (7) Finally, the location, number, and content of the vials should be recorded in the storage log dedicated for the cryogenic tank being utilized. This allows vials to be quickly retrieved, thus preventing temperature fluctuations in the cryogenic tank.

Thawing Cryopreserved Stocks

Bringing cell stocks back up from cryopreservation is a fairly straightforward process. However, there are some factors to consider which can significantly affect cell viability of newly thawed cultures. When thawing preserved cultures, vials should be warmed to 37°C as quickly as possible by incubation in a water bath for 3–5 minutes. Given DMSO is toxic to C2C12 cells, steps should be taken to wash DMSO out of the cell suspension upon thawing.

- (1) Prepare a conical tube containing 10 mL of pre-warmed growth medium for each vial to be thawed.
- (2) Wear a laboratory coat and protective eyewear when thawing cells. Occasionally, the lids of cryovials will explode due to pressure differences that can occur during the thawing process. Thus, it is also advisable to place thawing vials in a sealed container during handling whenever possible.
- (3) Place vials in a 37°C water bath and thaw stocks as quickly as possible. A slow thaw can dramatically reduce cell viability. Furthermore, large amounts of DMSO can be toxic to C2C12 cells. So, it is necessary to remove the DMSO as quickly as possible.
- (4) Once thawed, transfer the vials to a sterile culture hood while thoroughly wiping down each vial with kemwipes saturated with 75% ethanol.
- (5) Carefully remove the cap from the vial being mindful to avoid aspirating the contents and to prevent the transfer of cells from one’s gloves to inappropriate surfaces. Transfer the cell suspension to the prewarmed growth media in a slow, drop-wise fashion. Then, gently invert the conical tube several times to mix the contents.
- (6) Centrifuge the cell suspension at 200 g for 5 min to pellet the cells and allow the removal of DMSO when the supernatant is removed.

- (7) Re-suspend the cells in 10 mL of growth medium. Provided the cells were frozen down at a concentration of $1-3 \times 10^4$ cells per milliliter, one vial should sufficiently seed a 75 cm² culture flask at a density less than 50% confluence assuming no cell loss. However, a 10–20% loss of viability should be expected. Cells require as much as 4–8 h to become attached. Furthermore, the longer cultures were stored frozen, the greater the expected loss of viability.

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