Chapter 2

Myogenic precursor cells

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Introduction

Formation of skeletal muscle

Skeletal muscle fibers (myofibers) are long syncytial cells with thousands of nuclei. Careful histological observation in the late 1800s clearly demonstrated a great capacity for new muscle formation in many species and it is now widely accepted that during development and in regenerating muscle new myonuclei result from proliferation of mononucleated muscle precursor cells (myoblasts) that fuse together to form multinucleated cells (myotubes) and these mature into myofibers (Figure 2.1).

Origin of myogenic precursor cells and different muscles during development

A detailed discussion of the origin and formation of skeletal muscle during embryogenesis (for reviews see [1, 2, 3]) falls beyond the scope of this chapter, which is focused largely on postnatal muscle. Skeletal muscle is distributed throughout the whole organism, and, when one considers spatial regulation, it is striking that different muscle groups are subjected during development to distinct signaling environments. Highlighting the complexity of understanding muscle formation, little is known about how muscle patterning is regulated. However, it is now widely accepted that all the skeletal muscle of the vertebrate trunk and limbs is derived from progenitor cells located in the somites, pairs of transient epithelial segments derived from paraxial mesoderm that form following an anterior–posterior progression on either side of the neural tube in birds and mouse embryos. Somites arise from the mesenchymal paraxial mesoderm in a regular sequence in an anteroposterior direction as pairs of epithelial spheres budding off on each side of the neural tube. This process is controlled by a segmentation clock involving the *Notch*, *Fgf* and *Wnt* signaling pathways [4]. The peripheral nervous system, which is the other component of the nerve–muscle motor unit, is formed at the same time from neural crest cells that migrate from the dorsal neural tube [5]. In response to environment cues, the somites differentiate into a ventral mesenchymal domain, the sclerotome, and a dorsal epithelium, the dermomyotome. While the sclerotome provides the tendons, cartilage, and bones of the axis (vertebral column and ribs), the latter gives rise to the dermis and the skeletal muscle of the trunk, limbs, pharyngeal and tongue, in addition to some blood vessels.

In brief, there are three major sources of different groups of skeletal muscles (reviewed in [1, 2, 3]). The somitic **myotome** gives rise to cells that develop into the epaxial trunk and back muscles whereas others in the lateral/ventral domain develop into hypaxial muscles, including body wall, intercostal, and abdominal muscles. While the embryological development of epaxial muscles has been well described, less is known about postnatal satellite cells and molecular signaling in these muscles, compared with limb muscles that have been the focus of much research using animal models. Yet disturbed function of the back muscles has many medical consequences, e.g., related to kyphosis and lower back problems. Some of the **hypaxial somites** (the cervical somites and somites facing the limbs) do not contribute to the myotome and body muscle masses but instead undergo long-range migration to form distant muscles, such as those of the limb, tongue, and diaphragm. The **paraxial head and prechordal mesoderm** give rise to craniofacial muscles including extraocular, branchial and laryngoglossal, and esophageal muscles. Strikingly little is currently known about the distinct genetic networks at work in the formation of facial and head muscles [1]. However, because certain diseases (e.g., oculopharyngeal muscular dystrophy) appear to target or spare specifically all, or groups of, head muscles (i.e., extraocular muscles are generally not affected in patients with Duchenne muscular dystrophy, DMD), understanding the developmental and molecular specificity of these muscles is of much interest.

The populations of muscle precursor cells that give rise to these disparate types of muscles will eventually contribute to the satellite cell pool (Figure 2.1) of postnatal myogenic precursors [6]. From embryonic day 16.5 in the mouse, satellite cells are formed from the Pax7-expressing fetal muscle progenitor cells that progressively become embedded under the basal lamina, in close contact with the myofibers [7]. Satellite
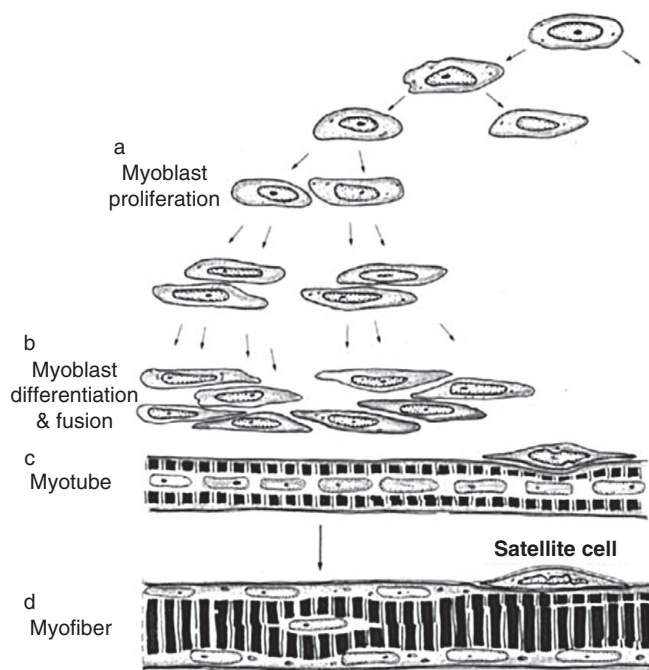
Figure 2.1. Formation of skeletal myofibers and satellite cells. Diagram of myogenesis. This simple diagram illustrates (a) the proliferation of mononucleated myogenic precursor cells called myoblasts; followed by (b) myoblast alignment associated with cessation of proliferation and onset of differentiation; (c) fusion of many myoblasts to form multinucleated young muscle fibers (myotubes) that (d) differentiate further to mature into functional myofibers (under the influence of innervation – not shown). A similar sequence of myogenic events occurs during both embryogenesis and regeneration of damaged adult muscles. The satellite cell is a resident quiescent mononucleated myogenic precursor cell located on the surface of the myofiber beneath the basal lamina.

cells cannot be identified until a basal lamina can be detected and this is around 10–15 weeks in utero in humans [8]. Genes and signaling pathways involved in the transition from a population of fetal muscle precursor cells to a self-renewing population of postnatal satellite cells have not yet been characterized.

Source of myoblasts in adult muscle

The source of the myoblasts in adult muscle has been widely debated since the 1800s. The four main possibilities are that myoblasts in adult muscle might originate from: (1) a nucleus within the myofiber, (2) a cell beneath the basal lamina (specialized extracellular matrix) on the surface of the myofiber, (3) local cells in the interstitial connective tissue, possibly perivascular or (4) non-local cells derived from the circulation. In myotubes and myofibers, the nuclei within the sarcoplasm (myonuclei) are generally considered to be postmitotic. In response to injury of adult muscle, the possibility that these postmitotic myonuclei might become sequestered (by new membrane to generate mononucleated cells) to form functional new myoblasts has received little support for mammalian muscle (although this certainly occurs in some other species) but is difficult to completely exclude (reviewed in [9]). Instead, it is now widely accepted that myoblasts are
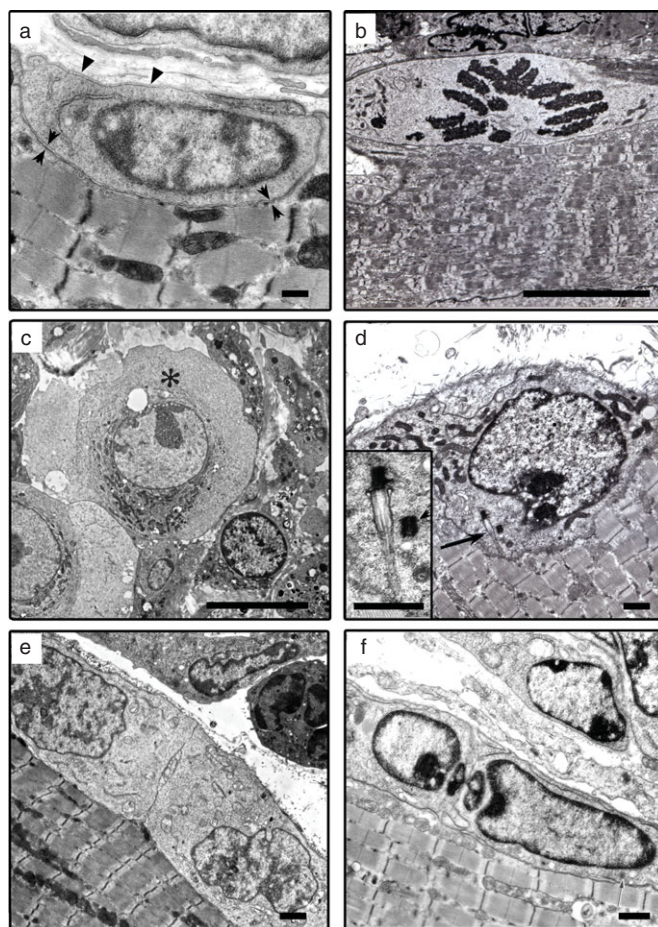

Figure 2.2. Satellite cells identified by electron microscopy. High magnification of satellite cells/myoblasts shown by transmission electron microscopy, in regenerating adult mouse muscle sampled up to 5 days after chemical injury. (a) Classical quiescent satellite cell: note the minimal cytoplasm, the cell membrane surrounding the satellite cell in close proximity to the sarcolemma of the underlying myofiber (short arrows) and the basal lamina of the myofiber enclosing the satellite cell (arrow heads). (b) Activated satellite cell undergoing mitosis; the sarcomere architecture is disturbed in this injured myofiber. Many activated satellite cells remain fusiform often with pseudopodial extensions, but some are spherical with organelles arranged concentrically around the nucleus, similar to (c) spherical myoblasts lying between myofibers: an electron lucent zone can be seen in one of the two cells (asterisk) and phagocytic cells are closely apposed. Cilia are relatively frequent in myoblasts located outside the myofiber although they are rare in satellite cells. (d) An activated satellite cell with cilium (long arrow) with a high power of the cilium and centriole (short arrow) in the insert; the cilium are presumably associated with motility. (e) Two daughter satellite cells following cell division. (f) Two macrophages located between the basal lamina and sarcolemma (distinguished by lysosomes in the cytoplasm – arrow), emphasizing the difficulty of precisely identifying satellite cells on the basis of position. Scale bar is 1 m in (a) and insert in (d); whereas it is 10 m for all other panels (b, c, d, e, f). All images are from the PhD thesis by Terry Robertson, 1996, the University of Western Australia.

derived mainly from a quiescent myogenic mononucleated precursor cell located on the surface of the myofiber beneath the basal lamina; this was described for frog muscle in 1961 and named the satellite cell purely on the basis of its anatomical position [10] (Figures 2.1 and 2.2). For an excellent description of satellite cells see [11] and for further historical perspectives of satellite cells see [12].

Myogenic cells (myoblasts) extracted from adult skeletal muscle can be grown in tissue culture where they proliferate and form myotubes: it is widely assumed that the myoblasts in such primary muscle cultures originate from satellite cells although it is difficult to exclude the contribution of other cells within the interstitial tissue (e.g., associated with blood vessels or circulating cells). Satellite cells are now widely held to be the main source of myoblasts and may have stem-cell-like properties in postnatal skeletal muscle. Recently, the revived idea that myoblasts can also arise from other sources of cells (points 3 and 4 above) has attracted intense interest as part of the stem cell debate as discussed below (see "Cell therapy: stem cells and other sources of myoblasts").

Postnatal muscle: satellite cells and their control

Genetic hierarchies operating in postnatal satellite cells

Unraveling the complex regulation of muscle formation is a challenging task. Despite recent progress in the field using large-scale genomic approaches, little is known about the genetic regulation that leads to the specificity of distinct myogenic programs, and how these programs are regulated by extracellular signaling pathways. Another issue awaiting elucidation at molecular and cellular levels is the observation that specific defects in genes expressed in all skeletal muscles can lead to phenotypes affecting only some groups of muscles (this has many clinical manifestations). Muscle progenitor cells depend upon Pax3 and Pax7 [7], while myogenesis and the formation of myofibers depend upon expression of the myogenic regulatory factors (MRFs), Myf5, Mrf4 and MyoD. Targeted disruption of *Myf5*, *Mrf4* and *MyoD* genes in the mouse (so that they are no longer expressed) suggests that these three MRFs independently determine muscle identity, since in triple mutant mice (where all three gene products are absent) myoblasts and skeletal muscles are missing at all myogenic sites and the progenitor cells remain undetermined [1, 13, 14]. While Pax3 is a specific marker for early and fetal embryonic muscle precursors [3, 7] nearly all postnatal quiescent satellite cells are identified by the presence of Pax7 protein [15]; *Pax3* expression, unlike that of *Pax7*, is not uniformly maintained in adult satellite cells [2]. Expression of the MRF proteins is not detected in quiescent satellite cells, however a Myf5-driven reporter labels almost all satellite cells, reflecting either the self-renewing mechanism of satellite cells (Figure 2.3) or that Myf5 can be expressed at a low level in quiescent satellite cells. During postnatal muscle growth and after injury, satellite cells are activated and proliferate. Activated satellite cells maintain the expression of the *Pax* genes, and show robust expression of Myf5 and MyoD. Myogenin and MRF4 are only detected in terminally differentiating satellite cells undergoing cell cycle exit. Studies performed ex vivo and in vivo have led to different models where activated satellite cells can undergo

asymmetric division, as a means of providing fate diversification allowing self-renewal as well as contributing to muscle repair or growth (Figure 2.3).

As observed during embryonic development, the interplay between the *Pax* and *MRF* genes is important for self-renewal and differentiation of satellite cells: genetic hierarchies at work during embryonic muscle formation are redeployed in adult myogenesis [2], with Pax7 regulating MyoD expression during satellite cell activation [2]. Furthermore, failure of downregulation of Pax7 as activated satellite cells undergo terminal differentiation leads to delayed myogenin expression [3]. While Pax7-deficient mice have a nearly normal content of satellite cells at birth, the population is progressively depleted as a result of increased apoptosis and cell cycle defects [2].

Cell fate decisions in the satellite cells are controlled by Notch signaling [16], as well as asymmetric distribution of Numb, an inhibitor of Notch [17] that segregates with Pax7. The link between Notch signaling and transcriptional regulation has yet to be made.

Markers for satellite cells

Satellite cells were classically identified by their anatomical position using electron microscopy (Figure 2.2). Even this can be difficult since pericytes can resemble satellite cells, and macrophages, neutrophils and other cells can infiltrate and lie beneath the basal lamina of myofibers [11] (Figure 2.2). Now, satellite cells can also be visualized on the surface of myofibers by light microscopy (aided by confocal microscopy) using combinations of specific antibodies to immunostain components of the basal lamina (e.g., laminin or collagen IV) and the sarcolemma (e.g., dystrophin or spectrin). Beyond this approach, quiescent satellite cells are very difficult to observe in tissue sections because they have little cytoplasm and relatively low levels of gene expression (it is difficult to detect small amounts of key proteins in vivo using routine immunohistochemistry, e.g., Myf5). Activated satellite cells can move out of this classical position beneath the basal lamina (into the extracellular matrix space) and, to further complicate the situation, it is now recognized that myoblasts may be derived from cells other than satellite cells, originating outside the myofiber [18]. In adult muscle, all mononucleated myogenic cells are often widely referred to as myoblasts, regardless of their origin.

One of the most reliable markers for quiescent satellite cells in mouse muscle is the cell surface marker M-cadherin that is located at the interface with the underlying myofiber, although mRNA expression appears to be very low. M-cadherin is also present on myoblasts in culture and on isolated myofibers, where most (but probably not all) mouse satellite cells are positive for M-cadherin protein and it appears that M-cadherin protein may be very low (or absent) in some satellite cells. For human muscle, antibodies to M-CAM (CD56), originally called Leu-19, are a useful marker to identify quiescent and activated satellite cells and give similar results to M-cadherin
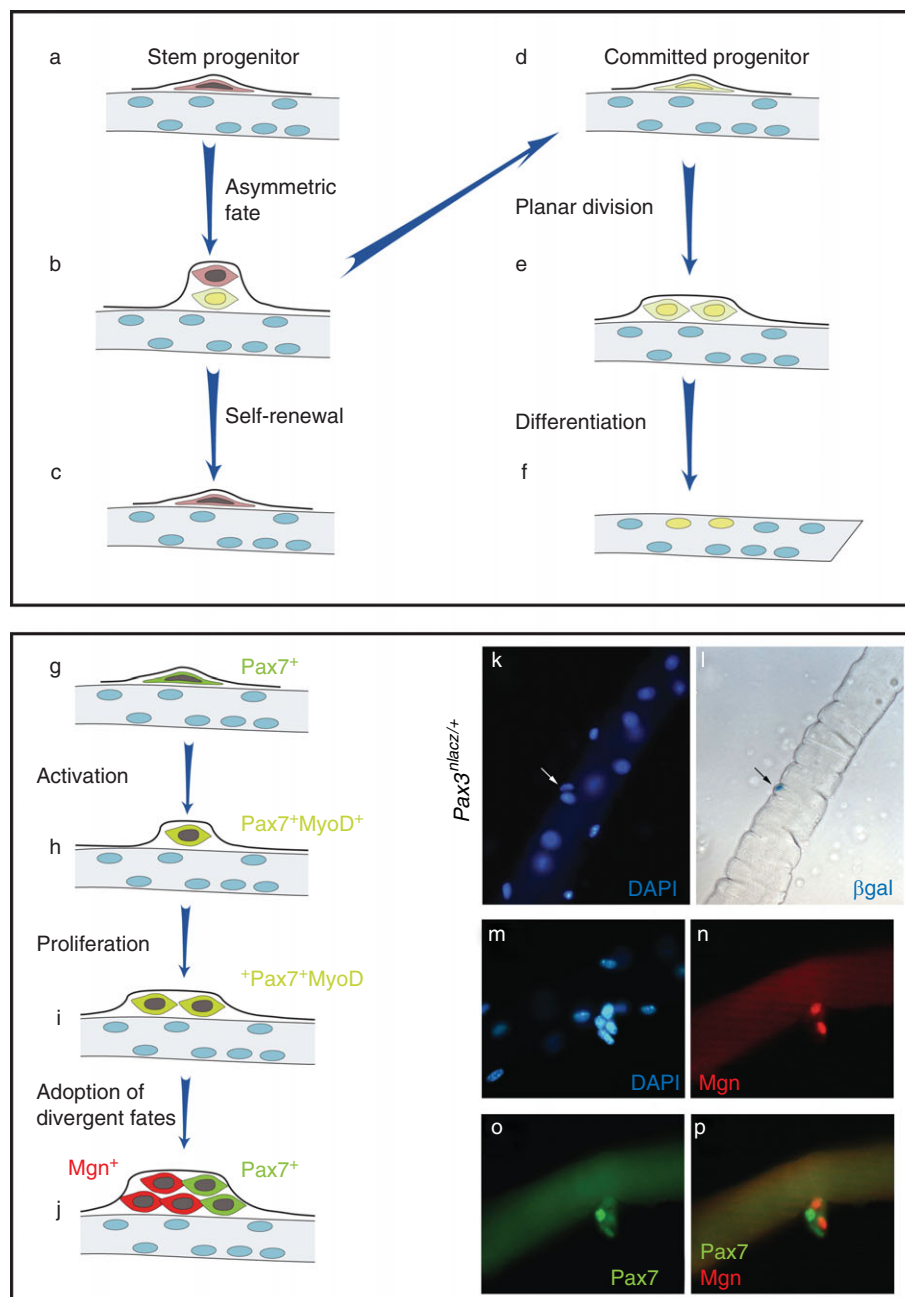
Figure 2.3. Models for satellite cell self-renewal and commitment. (a–f) Satellite cells located under the basal lamina (a) can adopt different fates through asymmetric division (b) by dividing in an apical-basal orientation, which allows self-renewal (c) and specification of committed progenitors (d). Both stem progenitor cells and committed progenitors can also proliferate through planar divisions (e) before differentiation (f) and fusion with the parent myofiber [28]. (g–j) Cultured single mouse myofibers with associated satellite cells allow the visualization of adoption of divergent fates [30]. Quiescent satellite cells are labelled by Pax7 (and Pax3). In floating cultures of intact myofibers, the satellite cells can undergo activation (expressing Pax7 and MyoD; h) before dividing (i). After 3 days of culture, in the clusters formed by the activated satellite cells, divergent fates can be observed: a subset of the cells activates myogenin (Mgn) and undergoes terminal myogenic differentiation while a subset returns to a quiescent-like stage and expresses Pax7. (k, l) Example of cultured single myofibers from $Pax3^{nlacZ/+}$ mice. The satellite cells are labelled by gal (l), and represent a subset of the DAPI-positive nuclei (k). (m–p). Example of a cluster of satellite cells on isolated myofibers after 3 days in culture, with asymmetric cell fates. DAPI labelling of the nuclei in shown in (m) (blue), myogenin in (n) (red), Pax7 in (o) (green), and complete absence of co-labeling between Pax7 and myogenin in (p): the images o and p correspond to the diagrams (i) and (j) respectively. (All images provided courtesy of Sonia Alonso-Martin & Relaix.)

antibody [19]. A plethora of molecular markers (cell surface and intracellular) have been described since the early 1990s to help identify satellite cells by light microscopy, using either highly specific antibodies or (in experimental studies) reporter genes such as beta-galactosidase (LacZ) or green fluorescent protein (GFP). Most of these markers are not exclusive to satellite cells (reviewed in [9, 18, 20]). Some that are found only in skeletal muscle cells, e.g., Myf5 and MyoD, are rapidly upregulated in activated satellite cells (Figure 2.3) but are also expressed by differentiated myoblasts and myonuclei (e.g., in denervated muscle). The cytoskeletal protein desmin is a very useful marker for identifying myoblasts [21] but levels are low in quiescent satellite cells and desmin is also expressed by smooth muscle cells of the vasculature and cardiomyocytes. Many other satellite cell markers are less specific since they are also expressed by a variety of other cell types (blood vessels, interstitial or circulating cells) within skeletal muscle tissue e. g., c-Met (the receptor for hepatocyte growth factor), syndecan-3 and syndecan-4 (proteoglycans that bind many growth factors), and CD34. Other important molecules expressed by satellite cells such as Pax3, Pax7, and nestin are also markers of cells in neural and other tissues. While Pax7 is generally an excellent marker of adult satellite cells (it does not recognize other cell types in skeletal muscle), it is not expressed by many

cells lying in the satellite cell position in very old muscle [12, 22]. Combinations of the above markers are often employed. While such molecular markers have been widely used to identify and study isolated cultured muscle precursor cells or satellite cells associated with single myofibers, they do not readily overcome the difficult problem of visualizing all satellite cells in tissue sections, especially of human muscle. Expression of many of these molecular markers has been visualized using reporter genes in experimental animal models. Unfortunately, specific antibodies suitable for immuno-histochemistry on sections of human (and mouse) muscle are not readily available for some of these myogenic markers, e.g., Myf5, and thus they cannot be exploited to identify satellite cells in this situation. However, with the development of new antibodies and due care in interpretation, these markers can be useful to identify in tissue sections some (if not all) satellite cells through their expression of Pax7 and nestin [23] and also Myf5. Quiescence is also associated with expression of the truncated form of the cell surface protein CD34 and the β isoform of the forkhead transcription factor MNF (myocyte nuclear factor/FoxK1). When satellite cells are activated (in tissue sections or in culture), Pax7 and nestin expression decreases, whereas levels of Myf5 and/or MyoD increase.

Sca-1 (stem cell antigen-1) is absent from quiescent myo-blasts but has been used as a marker of a subpopulation of activated myogenic (putative stem) cells that have a slower rate of proliferation. The expression of Sca-1 demonstrates the heterogeneity of satellite cell/myoblast populations [24] and modulation of Sca-1 by the micro-environment emphasizes the importance of extrinsic factors in the control of myogen-esis, a recurring theme throughout this discussion.

Isolated myogenic (or stem) cells extracted from skeletal muscle tissue by enzymatic digestion can be purified by fluorescent activated cell sorting (FACS) using a range of specific antibodies that bind to cell surface markers such as CD34 and CD133 (also known as AC133). It is noted that expression of such cell surface markers may rapidly change once the cells are removed from their normal in vivo envir-onment. Alternatively, side-populations of cells that do not stain with the nuclear dye Hoechst can be isolated and these are widely considered to represent stem cells. None of these markers are exclusive for satellite cells (as indicated above). However, they can be combined for more specific sorting with size and granulosity [25] and are very useful for collect-ing populations of myogenic (or stem) cells from skeletal muscle for tissue culture studies or for transplantation purposes.

Is there a stem cell subpopulation of satellite cells?

The idea of stem cells was largely motivated by mathematical logic, with little scientific verification for the distinction between stem and precursor cells. The properties of stem cells include longevity, asymmetric cell division, genetic fidelity (immortal DNA strand hypothesis), and plasticity, although many of these also apply to precursor cells.

The first criterion of longevity is clearly met by satellite cells since they are present even in very old muscle, with a capacity to proliferate extensively and to self-renew [22, 26]. The well documented heterogeneity of satellite cells might reflect the presence of a stem cell subpopulation. Asymmetric cell division is a feature of stem cells during embryogenesis, as is preservation of the original strand of DNA (immortal DNA strand hypothesis) throughout many cell divisions, thus pro-viding an unaltered original template for the generation of a replacement stem cell. There is good evidence (using BrdU-labeling of new DNA, as well as lineage studies using genetic-ally controlled reporters) for both the segregation of a template strand of DNA and asymmetric cell division of satellite cells both in tissue culture and in vivo [17, 27, 28, 29] (Figure 2.3). Asymmetric distribution of a range of proteins has been dem-onstrated in daughter cells after mitosis of satellite cells. One of these is Numb, which is an important marker of asymmetric cell division during development; it also inhibits the transcrip-tion factor Notch, which is required for activation of satellite cells in damaged adult muscle (discussed above under *Aging muscle – numbers and function of satellite celles*). Other mol-ecules with demonstrated asymmetric distribution into only one daughter satellite cell are Pax7 and Myf5 (Figure 2.3).

As shown in Figure 2.3, it has been proposed that where a satellite cell divides in a plane where the mitotic spindle is perpendicular to the myofiber (i.e., one daughter cell is in intimate contact with the sarcolemma whereas the other con-tacts only the basal lamina), this results in asymmetric division to generate one committed (Pax7$^+$/Myf5$^+$) myoblast (adjacent to the sarcolemma) and one self-renewing (Pax7$^+$/Myf5$^-$) stem cell (in contact with the basal lamina). In contrast, it is proposed that where the two daughter cells resulting from division of a satellite cell lie parallel to the myofiber (i.e., both have equal exposure to the sarcolemma and the basal lamina) this results in symmetric division with generation of two identical daughter cells (either committed progenitors or stem cells) [28, 29]. Studies performed using floating, isolated, single myofiber cultures have demonstrated that satellite cells can also undergo asymmetric cell fate choice within clusters [30] (Figure 2.3). There are technical issues associated with identi-fication of such asymmetric divisions (that appear to be rare), and the extent to which this might occur in vivo is unclear. That physical contact can determine the lineage fate of cells and generation of stem cells is well established for events during embryogenesis and there is certainly evidence that physical contact is required for non-myogenic cells to convert to a myogenic lineage [18]. One of the key molecules impli-cated in such myogenic lineage conversion is Notch signaling. Additional studies are required to determine the exact sequence of these events and the molecular pathways involved. Furthermore, the regeneration potential of the different satel-lite cell populations (i.e., possible stem versus committed

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progenitors, see Figure 2.3) remains essentially uncharacterized due to the lack of specific markers. In addition, the mechanism determining the consistency of numbers and distribution of satellite cells on different types of myofibers, especially during self-renewal, is not understood and is hardly explained by the current models (Figure 2.3).

With respect to plasticity, there is plenty of evidence that mesenchymal cells such as myoblasts can readily convert into different lineages (adipocytes, fibroblasts, chondrocytes), depending on the precise tissue culture conditions to which they are exposed. Whether this represents a true lineage conversion or a shifting of the molecular and biochemical program within a cell can be debated. Plasticity of satellite cells has clearly been demonstrated experimentally and the impact of a fibrogenic environment that can convert cells from a myogenic to a fibrogenic program in diseased and aged muscle is discussed later.

Overall, the satellite cell population manifests all the properties of stem cells but the question remains as to whether there is a dedicated stem cell subpopulation of satellite cells, or whether the heterogeneous nature of the population means that all satellite cells have the potential to manifest these properties.

Factors controlling satellite cell quiescence, activation, and proliferation in vivo

The factors that maintain the satellite cells in a quiescent state in normal skeletal muscle, as well as the conditions that activate satellite cells from this quiescent state, are not fully understood although there have been intensive studies in tissue culture to try to define the key molecular events involved (reviewed in [18]). With respect to maintenance of quiescence, it seems that the electrical activity (electrical potential) of the sarcolemma may play a role, since silencing neuromuscular transmission (by botulinum toxin or denervation) results in transient activation of satellite cells. However, the precise sequence of membrane-associated signals that results in such activation is not understood. Other situations that alter the status of the sarcolemma are: mechanical tension; growth and hypertrophy that increase myofiber size and stimulate satellite cell proliferation and fusion with the growing myofiber; and physical trauma or muscle diseases that damage the sarcolemma to result in myofiber necrosis that provokes regeneration (with associated inflammation, satellite cell activation, and new muscle formation). All of these situations probably change the response of satellite cells to growth factors (GFs). This involves many different events including modulation of membrane and extracellular matrix components, changes in the availability of GF stored in the extracellular matrix with conversion from inactive to bioactive forms, possible changes in binding proteins that affect the bioavailability of extracellular GFs, and altered expression of specific GFs and also of receptors for different GFs.

Cell membrane: Sphingolipids are important components of the plasma membrane and sphingolipid signaling may play a central role in maintaining quiescence and in the early events initiating satellite cell activation. Sphingomyelin is located in the inner leaflet of the lipid bilayer of the plasma membrane and, upon activation, is metabolized to form the bioactive sphingolipid, sphingosine-1-phosphate, which binds to a range of cell surface receptors and is mitogenic for many cell types including satellite cells [31]. Differential interactions between the surface of satellite cells and the sarcolemmal or the overlying basement membrane have been proposed as a determinant of asymmetric cell division (in a perpendicular plane compared with symmetric planar division), as a mechanism for possible self-renewal of a stem cell compartment of satellite cells [29].

Extracellular matrix: the cell membrane surface of satellite cells (and myofibers) is in intimate contact with the extracellular matrix (ECM), especially the specialized basement membrane that surrounds the myofiber, and even small changes in this environment will have an impact on the cells. The great complexity of molecular interactions in the ECM that affect satellite and other cells has been recently reviewed with respect to the many events that occur during skeletal muscle regeneration [32]. A brief outline of some of the key molecular interactions involved in normal homeostasis and for activation of satellite cells and all aspects of myogenesis (myoblast proliferation, differentiation, and fusion to form myotubes) follows.

Heparan sulfate (HS) proteoglycans and their modification by sulfation play a crucial role in GF regulation in all tissues. The HS proteoglycans bind to GFs to affect their stability and bioavailability and are also required for the binding of many GFs to their cell surface receptors, e.g., this is especially important for fibroblast growth factors (FGFs) and hepatocyte growth factor (HGF; also known as scatter factor). In skeletal muscle some of the important HS proteoglycans for modulating GF interactions at the satellite cell surface are biglycan, perlecan, syndecans and glycipan-1, with decorin in the interstitial connective tissue playing a role in sequestering GFs such as transforming growth factor beta (TGFβ) and myostatin. The ECM is constantly being modified by myriad enzymes including sulfatases and proteases and their inhibitors, and it is reasonable to conclude that these also play crucial roles in many aspects of myogenesis in muscle tissue. Other ECM molecules such as laminin (in the basement membrane), collagen, fibronectin, and hyaluronan affect different aspects of myogenesis in tissue culture studies, especially myotube formation and maturation, although relatively little is known for many of these regarding their specific importance for satellite cell quiescence, activation, proliferation, and fusion in muscle in vivo. The central importance of the ECM environment in determining the properties and response of satellite cells in vivo is discussed below with respect to the impact of fibrosis in dystrophic, denervated, and aging muscle.

Growth factors and their receptors: growth factors (GFs) are small protein molecules that influence cell behavior, and cytokines are GFs that are produced mainly by inflammatory blood-derived cells (although many are produced by a multitude of other cell types). Many GFs are produced locally to affect the same cell (autocrine action) or an adjacent cell (paracrine effect) but other GFs travel through the bloodstream to affect distant cells (endocrine). Multitudes of GFs play important roles in the complex in vivo milieu to influence many aspects of muscle progenitor behavior including chemotaxis, activation of satellite (and possibly other stem) cells, stimulation of myoblast proliferation and differentiation, and there may be overlapping functions and redundancy. The discussion below focuses on some GFs that are produced by muscle, act locally, and have been extensively studied in cultured muscle cells. The important role of many other GFs (e.g., platelet-derived growth factor, tumor necrosis factor, interleukins, vascular endothelial factor, and hormones) is too broad a subject to be addressed here. Much attention has focused on the role of GFs in all aspects of satellite cell myogenesis, with the vast majority of studies having been carried out using tissue culture and immortalized cell lines, primary cultures of skeletal muscle cells, and isolated myofibers (reviewed in [33]). It can be difficult to translate the results of tissue culture experiments to the in vivo situation due to many factors; for example, the extent to which some of the high doses of GFs used in tissue culture studies reflect normal physiological conditions. Crucial interactions of GFs with many ECM components as occurs in live muscles in vivo [32] also need to be considered, although until recently this was not a feature of most tissue culture studies; however, the importance of heparan sulfate proteoglycans (in the ECM) binding to members of the FGF family and to HGF is now widely recognized (as indicated above). Overall, the availability of the specific heparan sulfate proteoglycans combined with the specific GF receptor and bioavailable GF controls the response of satellite cells. The relative balance between availability and activity of different GFs (and their receptors) determines the final cellular response.

The quiescent state of satellite cells appears to be associated with high levels of the TGFβ superfamily, with decreased activity required for satellite cell activation. In brief, some of the most important GFs involved in the very early events of satellite cell activation and proliferation appear to be decreased levels of the TGFβ superfamily, combined with increased activity of various FGFs and HGF that are mitogenic for satellite cells (reviewed in [33, 34]).

The TGFβ superfamily has three typical TGFβs that are released as an inactive complex. They are stored in the ECM and have little biological activity until proteinase activity reveals the active domain. It is generally agreed that TGFβ1 suppresses myoblast differentiation and high levels of TGFβ are also strongly associated with fibrosis (the latter is of increasing importance in diseased and aging muscle). There is some dispute over the role of TGFβs in suppressing satellite cell activation and proliferation but this has been overshadowed by the discovery of **myostatin** (GDF8: growth differentiation factor), a member of the TGFβ superfamily that is highly expressed in skeletal muscle. Myostatin attracted huge attention in 1997 when a mouse deficient in myostatin was described with a striking phenotype of massive muscle growth. Such myostatin deficiency associated with "double muscling" has also been identified in cattle, dogs, and humans. It is proposed that myostatin has a negative influence on satellite cell proliferation and that a lack of myostatin leads to increased activation of satellite cells; although, evidence is now emerging that postnatal myostatin blockade results in myofiber hypertrophy unaccompanied by any evidence of increased satellite cell activity [35]. The pronounced increase in muscle mass in the absence of myostatin during development is considered to be due to sustained satellite cell proliferation, resulting in additional myofibers (hence the term "double muscling") in addition to myofiber hypertrophy: the relative roles of these two processes during development and in different postnatal muscles lacking myostatin are complex and controversial [36]. Tissue culture studies indicate that the potent effect of myostatin as a suppressor of satellite cell activation and proliferation is mediated by upregulation of p21 (and hence inhibition of cyclin-dependent kinase), which leads to reduced phosphorylation of the retinoblastoma (Rb) protein. Myostatin (like TGFβ1) also prevents myoblast differentiation due to inhibition of MyoD expression and activity: myostatin affects the MyoD promoter via activation of Smad3 signaling. Mighty is a recently characterized gene that appears to play a key role in the signaling cascade between extracellular myostatin and the transcription factors that govern myogenesis [37]. Effects of myostatin on adipocytes and adipogenesis are also of interest [36]. It is important to note that big is not always better: while muscle mass is greatly increased in the absence of myostatin, one group reported no increase in strength [35] and another showed that force production is compromised and muscles are weaker with reduced strength per cross-sectional area of the muscle (specific force) [38]. In addition, dystrophic mdx muscles in which myostatin was inhibited had reduced endurance to treadmill exercise [35]. Furthermore, although overall initial numbers of satellite cells per myofiber are increased in myostatin-null mice, the normal age-related decline in satellite cell numbers appears to occur [33]. There is much interest in the roles that myostatin may play in muscle atrophy and hypertrophy and thus much attention to possible clinical interventions in muscle wasting and disease [36].

The FGF family has over 20 members, which bind to receptors coded for by five different genes (FGFR-1–FGFR-5) with numerous splice forms of these gene products [33]. Of this large GF family, FGF-2 is well recognized as a potent mitogen for satellite cells and myoblasts. Administration of additional FGF-2 to damaged muscles in vivo does not increase myoblast proliferation or improve muscle regeneration, possibly because there is already sufficient FGF-2 available: instead the presence of the FGF-2 receptors and critical

HS proteoglycans may be a limiting factor in vivo. Tissue culture studies show that FGF-1, -2, -4, -6, -9 and HGF all enhance satellite cell proliferation to a similar extent, whereas other FGFs have no effect. High levels of expression of FGF-6 (that correlate with expression of the receptor FGF-4) in developing muscle and also in normal and damaged myofibers suggest that FGF-6 plays a particularly important role in myogenesis of developing and adult muscle. However, studies in FGF-6-null mice are conflicting and it seems likely that there is overlapping function between FGF-2 and FGF-6. Similarly there may be different and overlapping functions between the FGF receptors FGFR1 (present in many cell types) and FGFR4 (high in developing muscle). It is proposed that FGFR1 may maintain myoblast proliferation, whereas FGFR4 may be involved in the transition from proliferation to differentiation [33]. The additive beneficial effects of the FGFs and HGF appear to be critical for satellite cell activation and proliferation but their precise interactions and roles are yet to be fully defined in vivo.

Hepatocyte growth factor (HGF, also called scatter factor, SF) activates quiescent satellite cells and is a potent mitogen for myoblasts but not fibroblasts (in this way it differs to FGF-2), therefore making HGF a very attractive factor for preferentially stimulating myogenesis without fibrosis in vivo. HGF is present in two forms: the inactive monomer (single chain) pro-HGF is secreted and stored in the ECM, where it is cleaved by proteases to form the active heterodimer HGF that has a limited capacity to diffuse in vivo [33]. HGF protein is present in myotubes in vitro and adult myofibers in vivo and the mRNA is produced by myofibers and myoblasts. The receptor for HGF, c-Met, is expressed by quiescent satellite cells and is considered a marker for satellite cells, although c-Met is also expressed on other types of cells present in muscle tissue. Once activated, the satellite cells are kept proliferating and prevented from differentiating by HGF (combined with certain FGFs). The relative importance of HGF compared with FGFs in the early events of satellite cell activation and proliferation is slightly controversial. One very early event that may activate HGF in response to muscle stretch and injury is the release of the enzyme nitric oxide synthase from the basal lamina; this produces nitric oxide that then activates the metalloproteases in the ECM to cleave the pro-HGF to the active form. In addition to their multiple roles in activation, proliferation, and differentiation of muscle cells, both HGF and FGFs are also chemotactic and this may serve to attract satellite (and other) cells to the site of injury to facilitate regeneration. The complexity of in vivo administration of GFs is illustrated by experiments where intramuscular injection of HGF into injured muscles increased myoblast proliferation but did not improve regeneration, whereas sustained administration inhibited myoblast differentiation leading to impaired regeneration. Such studies emphasize the critical importance of the timing of various GF actions that normally occur throughout the process of regeneration, with each GF present in the right amount at the right time.

Insulin-like growth factor-1 (IGF-1) is another important GF that has attracted much attention with respect to skeletal muscle. It is well documented that the IGFs have potent effects on myoblast proliferation and differentiation, and they have recently attracted particular interest due to their anabolic effects which lead to muscle hypertrophy [39]. This has led to suggestions that IGF-I administration might prevent myofiber atrophy and loss of function resulting from aging, disuse, cachexia, and disease as well as reduce the necrosis of dystrophic myofibers (discussed below).

The principles outlined above indicate the complexity of regulating GF activity, with a wealth of different forms of GFs and their receptors, as well as crucial interactions with ECM molecules that determine their bioavailability and bioactivity. Whether administration of exogenous GFs as a therapeutic strategy can significantly enhance clinical muscle function or repair remains to be determined.

Postnatal muscle: response of satellite cells in clinical situations

Satellite cells during muscle regeneration (in response to trauma, disease or transplantation)

Minor trauma or certain stimuli may transiently activate satellite cells; however, if the required conditions are not present, the satellite cells may not proliferate extensively but instead lapse back into a quiescent state. Furthermore, since mature myofibers appear to be refractory to fusion, specific conditions are required to alter the status of the sarcolemma in order for new myoblasts to fuse with the mature parent myofiber: such conditions include significant sarcolemmal/myofiber damage or growth/hypertrophy.

Necrosis and regeneration

Where damage results in myofiber necrosis, the process of regeneration and new muscle formation is initiated. Regeneration involves key early events of inflammation and angiogenesis and then later innervation to restore full function, in addition to actual myogenesis to form the new muscle cells. Muscle damage that leads to necrosis will rapidly alter properties of the sarcolemma. In addition, damage stimulates the rapid accumulation of neutrophils (polymorphonuclear leukocytes) that exit the capillaries at the site of injury within minutes due to the release of cytokines from damaged cells and also from degranulated mast cells. In turn the neutrophils and the damaged myofiber release chemokines that attract macrophages (these predominate by 24 hours) and other cells including myoblasts to the site of injury. The inflammatory cells produce a wealth of proteases (that degrade the ECM) and cytokines, in addition to phagocytosing and removing the necrotic tissue. It is emphasized that the inflammatory cells are of central importance for muscle regeneration yet they are not present throughout myogenesis during development; thus different factors are involved in modulating myogenesis in

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these two situations, even though the cellular events of muscle formation may be very similar. Autoradiographic studies in vivo show that at least 18–24 hours elapse before satellite cells start to synthesize new DNA in response to muscle injury in mice [40]. Differentiation and fusion of the myoblasts occurs within 3 days, with myotubes first being apparent 2.5–3 days after injury. The wave of myoblast proliferation increases from day 1 to peak by about 3 days and greatly decreases thereafter and is essentially over by 5–6 days in response to cut (minor) or crush (severe) injury in mice [40]. The factors controlling the initiation of activation of satellite cells, and the prolifer-ation, differentiation, and fusion of myoblasts are briefly out-lined above although the precise sequence of combined factors controlling these events in vivo remains unclear. Fusion of new myotubes to the ends of the damaged myofibers is delayed until after about a week, further emphasizing the normally refractory nature of the adult myofiber to fusion [41]. It seems likely that similar events occur in human muscle with new muscle formation essentially completed within 1–2 weeks after injury.

While treatments such as low-energy laser irradiation, ultrasound, and hyperbaric (increased) oxygen can activate satellite cells, not all have benefits on skeletal muscle regener-ation. Low-energy laser irradiation (LELI) improves muscle regeneration in experimental animal models and these benefits are also demonstrated in tissue culture where LELI increases the survival and also the activation and proliferation of satellite cells via GF-related signaling pathways [42]. In contrast, ultra-sound does not seem to improve muscle regeneration as shown by animal experiments: in one study ultrasound produced a marked stimulation of satellite cell proliferation but no overall effect on myotube formation or regeneration [43] and more recent studies confirm no benefit of ultrasound treatment on muscle repair after contusion injury [44]. Hyperbaric oxygen is a therapeutic strategy to improve regeneration of ischemic muscle and it appears to act by increasing expression of FGF and HGF, which activate satellite cells (see above), as well as stimulating the formation of new blood vessels [45].

Fibrosis and impaired regeneration

Repeated cycles of myofiber necrosis occur in Duchenne mus-cular dystrophy (DMD) and the mdx mouse and dog models of this disease, due to fragility of the sarcolemma resulting from defects in dystrophin. Over time, new muscle formation fails and the muscle is replaced by fibrous fatty connective tissue (this is pronounced in dystrophic humans and dogs). Why does muscle regeneration fail? In part this may be due to different growth parameters and the size of different species [46]. Tissue culture studies of myoblasts from dystrophic mdx muscles report altered kinetics of myoblast proliferation and accelerated differentiation, and that this is influenced by the parent myofiber [47]. Early tissue culture studies which con-cluded that satellite cell numbers and myogenic capacity are low in dystrophic muscle proposed that this was due to exhaustion of the satellite (stem) cell population by the

repeated cycles of damage and regeneration; this is supported by decreased telomere lengths in muscles from DMD boys [48]. However, an alternative explanation that is now gathering favor proposes that an adverse fibrous ECM environment accounts for difficulties in extracting satellite cells for tissue culture studies and adversely affects the myogenic capacity of these cells [49]. Thus an altered ECM environment (that does not favor myogenesis) may be the main problem, rather than the demise or an impaired intrinsic capacity of the satellite (stem) cell population per se.

This explanation also accords with studies of aged (and denervated) muscle where increasing fibrosis in very old muscles (see below) is associated with lineage conversion of myogenic precursors into non-myogenic fibroblasts; both the age-related fibrosis and lineage conversion involve systemic factors and Wnt signaling [50]. With each cycle of myofiber necrosis and regeneration a small amount of fibrous connect-ive tissue (mainly collagens) is deposited around the myofibers and the increasing fibrous connective tissue alters the ECM composition. There is increasing evidence that fibrosis pre-sents unfavorable conditions for myogenesis, with altered gene expression in satellite cells and a lack of myogenic markers on satellite cells from myofibers isolated from old mdx mice, leading to impaired new muscle formation with loss of muscle and replacement by fibrous fatty tissue [49]. It is clearly critical to determine the underlying cellular reasons for the failure of muscle regeneration in DMD (i.e., altered environment versus loss of myogenic capacity), in order to design appropriate therapeutic strategies (e.g., drugs versus stem cells).

Muscle transplantation

Segments or intact whole muscles are transplanted routinely in clinical situations to treat conditions such as incontinence and facial palsy. In muscle that is regenerating after transplant-ation, a similar sequence of events occurs although here the timing is delayed initially by several days, because the blood vessels are severed during grafting and thus revascularization with formation of new vessels (angiogenesis) is needed (unless vessels are surgically anastomosed). The infiltration of inflam-matory cells precedes new vessel formation with macrophages releasing angiogenic factors that stimulate revascularization of the ischemic muscle graft. The importance of angiogenesis for muscle regeneration is emphasized in the situation of ischemic damage of the extremities [51]. Accordingly, administration of the potent angiogenic agent vascular endothelial growth factor (VEGF) accelerated new muscle formation in ischemic muscle grafts in mice [52]: such enhanced angiogenesis might signifi-cantly improve new muscle formation and reduce fibrosis in the center of large muscle grafts. Similar viral delivery of VEGF had striking benefits for the pathology of dystrophic muscle in mdx mice, due to effects on angiogenesis and also possible direct effects on satellite cell migration and myogenesis, or recruitment of stem cells into the myogenic lineage [53].

Satellite cell contribution to growing or hypertrophic muscle

In postnatal life, an increase in skeletal muscle mass, due mainly to increased size of the cross-sectional area of individual myofibers, occurs during the growth phase and in response to physical activity (loading). It is widely accepted that the number of myofibers is fixed during development. However, the interpretation of actual myofiber numbers can be complicated by the splitting or branching of (large) myofibers in hypertrophic and aging muscle. Regulation of muscle mass (size) depends on the balance between protein synthesis and degradation, with synthesis exceeding breakdown for mass to increase. Skeletal muscle growth and mass are controlled by nutritional, hormonal, and mechanical factors. While nutrition and hormones are essential during the growth phase, increased mass (hypertrophy) of adult skeletal muscle is primarily driven by mechanical factors (exercise and physical loading). It is important to note that increased muscle size does not always correlate with increased strength (reviewed in [38, 39]). It seems likely that increased net protein synthesis initially drives hypertrophy of (growing and mature) skeletal muscle and this then stimulates activation of the satellite cells that fuse with the growing myofiber: this addition of new myonuclei is required for maintenance of hypertrophy [54]. However, the primary importance of satellite cell proliferation in muscle hypertrophy is still debated [55, 56] and may depend on the growth stimulus (hormonal versus mechanical), age of the muscle (active growth compared with adult), species, and time of sampling [57].

Fate of satellite cells in atrophic myofibers (resulting from disuse, disease, denervation or cachexia)

A wide range of conditions including disuse (e.g., prolonged bed rest or space travel), starvation, disease, and aging lead to a loss of muscle mass (atrophy) and strength [39]. Muscle mass is normally maintained by a balance between protein synthesis and protein degradation and either of these aspects (or both) can be disturbed to result in a net loss of muscle protein. Exercise with muscle activity and loading stimulates the IGF-1 signaling pathway that increases protein synthesis and also inhibits protein degradation, thus leading to hypertrophy. Many factors that cause hypertrophy act through this crucial signaling pathway. Conversely, lack of stimulation, or factors that inhibit the IGF-1 pathway lead to muscle atrophy. For example, inflammatory cytokines such as tumor necrosis factor (TNF) that are elevated in cancer and other disease and also in aging appear to cross-talk and inhibit IGF-1 signaling [58]. Apart from the complexity of molecular mechanisms regulating the size of the myofiber [59], there is considerable interest in the question of what happens to satellite cells when a mature myofiber decreases in size.

This situation has been studied in denervated muscle where experiments in rodents established that denervation initially causes activation and sustained proliferation of satellite cells (for up to one month) followed by a steady decline in the number of satellite cells in long-term-denervated muscle [60]. Autoradiographic studies in mice show progressive loss of labeled nuclei adjacent to muscle fibers (presumed to be replicated satellite cells) in the 1–3 weeks after denervation: it was concluded that these proliferating (labeled) satellite cells migrated out from their original position beneath the basal lamina and did not fuse with the denervated parent myofiber [61]. The nuclear/myofiber ratio remains constant in denervated muscle (at least up to 3 weeks after denervation), indicating that activated satellite cells fail to fuse to the atrophic myofibers (discussed in [62]), supporting the proposal that mature myofibers are generally refractory to fusion. Ultrastructural studies show activated satellite cells and a transient increase in numbers at 2 months but a loss of satellite cells by 18 months after denervation of rat muscles [60, 62].

Elegant ultrastructural examination of short- and long-term-denervated muscles in rodents by the group of Bruce Carlson in the USA [63] and others, as well as in human muscle biopsy samples [64], shows tiny degenerative myotubes/myofibers with minimal cytoplasm and few myofibrils: some of these dwarf myotubes are located beneath the basal lamina whereas others are within the interstitial ECM. Myofiber atrophy is conspicuous by 2 months after denervation and beyond this time there is excessive deposition of fibrous interstitial connective tissue and multiple layers of basal lamina surround the satellite cells [62]. Strong evidence that there is no inherent problem with the myogenic potential of the satellite cells, but that the abortive myogenesis is due to adverse events related to the ECM environment and excessive fibrosis in the denervated muscle is provided by the excellent capacity of the satellite cells to form fully mature myofibers in tissue culture [65]. The tiny thin myotubes in the interstitial connective tissue are presumed to have been formed by satellite cells that have migrated into the interstitial ECM and represent "abortive myogenesis" outside the original myofiber (rather than severely atrophic myofibers) [65], although the contribution of muscle progenitors initially located outside the myofiber is difficult to formally exclude. Interpretation of events in human muscle is complicated in situations of partial denervation where there is a mix of denervated and reinnervated myofibers, especially since nerves can modulate the muscle properties (e.g., satellite cell proliferation, expression of myogenic factors such myogenin and MyoD) by activity-independent mechanisms as well as by nerve activation [66]. The ability of satellite cells to exit the juxtasarcolemmal position beneath the basal lamina means that these cells can no longer be identified using the classic geographic criteria; the extent to which such migration accounts for the decreased number of satellite cells reported in long-term-denervated (and aged) muscle is unknown.

Aging muscle – numbers and function of satellite cells

The progressive loss of muscle mass and function with age is a major problem that has attracted much attention. There are many complex reasons for this including age-related changes in myofiber biochemistry, denervation of myofibers, and an altered ECM environment with increased fibrosis (that also affects blood vessels and innervation) [67, 68], in addition to the issues of possibly decreased satellite (stem) cell numbers, a slightly delayed myogenic response, and possibly impaired new muscle formation (reviewed in [69, 70, 71]. Problems with extracting myogenic cells from aged skeletal muscle for tissue culture studies and a delay in their myogenic response initially led to the conclusion that the number of these cells was reduced in aged muscles and they had impaired replicative capacity and myogenesis [21]. Classical counting of satellite cells in tissue sections using electron microscopy or immunostaining generally concludes that numbers decrease in aged muscles from human and other species [19]; however, Pax7 is downregulated in many apparent satellite cells in aged muscle [22] and this may apply to many other molecular markers with age. Overall there are conflicting data concerning reduced numbers of satellite cells in aged muscles [22]. It has recently been demonstrated that a subpopulation of satellite cells in aged muscles retains excellent myogenic capacity [22] and some decline in satellite cell function may be more important than actual numbers in aging muscles [70]. Proliferation of aged satellite cells is improved by culture under low oxygen conditions and there is increasing evidence that the environment of these cells in vivo plays a major role in influencing their myogenic capacity; this parallels the situation with adverse effects of fibrosis on myogenesis in dystrophic muscle (discussed above). Classical cross-transplantation experiments between old and young rats demonstrated problems with long-term functional restoration of grafted muscles in old hosts (that may mainly reflect issues of re-innervation) and the importance of the systemic host environment in the adverse outcome [72].

Recent experiments using cross-transplantation of whole muscle grafts between young and old (up to 21 months) mice have addressed the effects of aging on the very early events of regeneration (during the first week) and new muscle formation per se [71]. Overall, these studies continue to enforce the idea that excellent new muscle formation can occur in aged muscles. Such studies emphasize that the overall muscle regeneration is influenced by the nature of the injury inflicted (e.g., grafting compared with intramuscular barium chloride injection or cold injury); this may largely reflect problems with the important early events of angiogenesis and inflammation that precede myoblast activation, proliferation, and fusion. Angiogenesis and inflammation are modified by (systemic and local) factors associated with the aged host environment, combined with intrinsic changes within aging muscle cells (e.g., production/availability of angiogenic factors and chemokines)

[71]. It is now generally considered that while myogenesis can be slightly delayed in aged muscle, this is not necessarily due to an intrinsic loss of satellite cell numbers or capacity but instead is determined by systemic host factors and can be reversed by exposure to a young environment: again emphasizing the importance of the host environment in the age-related decline in muscle repair (reviewed in [70]). Elegant experiments have started to unravel the molecular events controlling activation of postnatal satellite cells and myogenesis in aged muscle and show that the balance and cross-talk between the signaling pathways for Notch and Wnt orchestrate progression of satellite cells through proliferation and differentiation [50, 70, 73]. In brief, activation of the Notch-1 receptor is necessary during early activation and proliferation of satellite cells and that upregulation of Delta-1, the ligand for the Notch receptor, is very low in satellite cells after injury of old (compared with young) muscles. Thus impaired Notch signaling seems to account for the poor myogenic response to some types of injury seen in very old muscles. Notch signaling can also be inhibited by Numb. Members of the Wnt family may antagonize Notch-mediated satellite cell proliferation and inhibition of differentiation, and thus control this process. Notch signaling maintains the activity of GSK3β but this is inhibited by Wnt to result in myoblast differentiation. High levels of Wnt in quiescent or activated satellite cells leads to a loss of myogenic capacity and conversion into a fibrogenic fate in some experimental situations. It is proposed that an unidentified serum factor is associated with the Wnt pathway and is involved in the delayed activation of satellite cells, lineage conversion into non-myogenic cells, and increased fibrosis in aged muscle.

Cell therapy: stem cells and other sources of myoblasts

The transplantation of skeletal muscle progenitor cells is used in a range of clinical situations. Myoblast transfer therapy (MTT) is a strategy for therapeutic gene replacement in human diseases such as DMD, using normal donor nuclei derived from either myoblasts or stem cells. Another use for transplanted myoblasts is to improve the outcome of heart function after ischemic damage [9] and, while the benefits do not seem to depend on fusion of myoblasts with cardiomyocytes, such cardiac therapy shows promise in clinical trials [74]. Myoblasts are also needed for tissue engineering and the construction of muscle tissue ex vivo for potential reconstruction surgery [75]. All of these applications require a good source of autologous donor myoblasts and strategies to enhance their myogenicity and transplantation efficacy. Conventional myoblasts and different non-myogenic (stem) cell sources of myoblasts are discussed below with respect to MTT for dystrophic muscle.

Myoblast transfer therapy relies on the delivery of normal muscle nuclei into the dystrophic muscle fibers by biological fusion, as routinely occurs during muscle repair. Unfortunately, rapid and extensive cell death occurs after
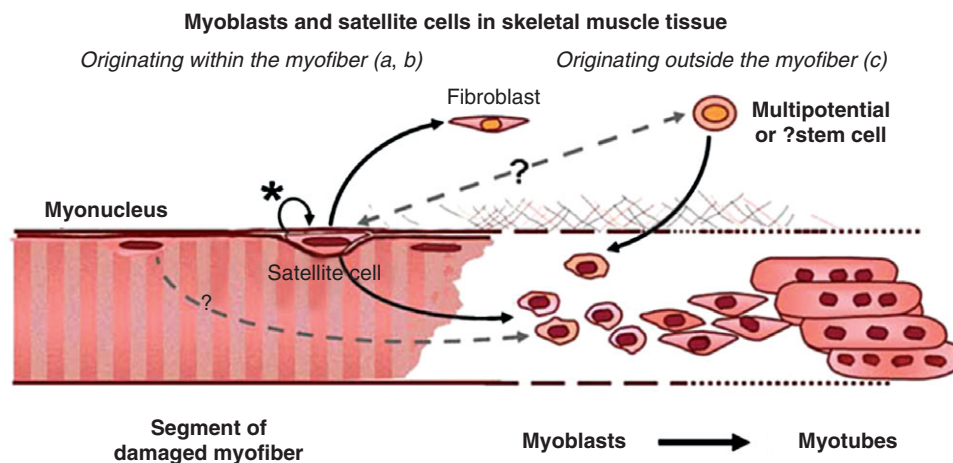
Figure 2.4. Origin and fate of myoblasts and satellite cells in mature muscle in vivo. Diagram of a segment of regenerating adult muscle tissue to indicate that myoblasts are (a) normally derived from satellite cells. The heterogeneity of satellite cells, the possibility of a stem cell subpopulation and replacement of satellite cells after (asymmetric) division (*) are topics of much discussion. The myogenic capacity of satellite cells may also be diverted into a fibroblast-like (or possibly adipogenic) fate in vivo, by alterations to the extracellular matrix (ECM) environment such as fibrosis in diseased and aged muscle. The theoretical possibility that myoblasts might be derived by sequestration of myonuclei after damage (b), is not widely endorsed for mammals but is difficult to test. Much recent attention has focused on myoblasts originating from cells lying beyond the myofiber (c), e.g., from circulating cells, mesenchymal stem cells or blood-vessel-associated cells (such as pericytes, mesangioblasts): the extent to which these may form satellite (stem) cells is unclear. The extent of potential trafficking of myogenic precursor cells between the juxtasarcolemmal satellite cell position and the interstitial connective tissue in damaged or normal tissue is unclear, although emigration of satellite cells is quite widely documented.

intramuscular injection of cultured donor myoblasts (extracted from normal donor muscles) into dystrophic mdx muscles, with about 80% of donor myoblasts dying within days. Trials with transplanted human myoblasts showed a similarly rapid loss of injected myoblasts and were disappointing [76]. Attention then turned to the possibility that there might be a stem cell subpopulation of satellite cells, more suitable for myoblast transplantation. The ideal source of stem cells (often in combination with gene correction) is autologous, i.e., from the patient themselves, to avoid problems of immune rejection. Such autologous myogenic cells would need considerable expansion of numbers in order to effectively re-populate the target muscle [77]. In addition, the ideal delivery system is through the circulation, to reach all muscles. While much research initially focused on bone-marrow-derived stem cells, many different types of stem cells have now been explored for the treatment of muscular dystrophies such as DMD. The great enthusiasm for alternative sources of muscle stem cells was fueled in part by overestimating the promise of tissue culture observations to the in vivo situation, combined with problems and limitations subsequently identified with various markers used to track putative stem cells. However, many valuable ideas have arisen from the stem cell debate, with topics of continuing interest being as follows. Is there a true stem cell subpopulation of satellite cells? Is there a cell population lying outside the myofiber that might be an ongoing source of satellite cells? What is the best source of muscle progenitors for cell therapy? Can the dream of systemic delivery of a myogenic stem cell become a therapeutic clinical reality? Some of these vital issues are discussed with respect to potential applications for cellular therapy (Figure 2.4).

Markers to track donor cells in vivo, conversion of non-myogenic cells into the myogenic lineage, and contribution of bone-marrow derived circulating cells

There was always interest in the idea that, under certain conditions, myoblasts might also be able to arise from other non-myogenic sources of precursor cells, e.g., fibroblasts, macrophages, cells derived from blood vessels such as pericytes, smooth muscle and endothelial cells, myoid cells of the thymus, in addition to circulating cells; in the 2000s there were dozens of reviews on this topic [9, 20, 21, 76, 78, 79].

It is relatively easy to cause cells from different lineages to switch into another cell type (known as plasticity) by manipulating conditions in tissue culture. However, such in vitro observations of plasticity may provide little insight into the capacity of the same cells for self-renewal, a property that is central to the stem cell concept. While such lineage conversion may be readily demonstrated in the artificial conditions of tissue culture (that may bear little resemblance to the in vivo situation), the extent to which this might normally occur in vivo, plus the precise conditions and molecular factors required for recruitment of such cells into the myogenic lineage within skeletal muscle, have barely been investigated. It is a major challenge to clarify these events in living skeletal muscle. One important aspect for conversion of cells into the myogenic lineage in vivo may be physical contact between cells, as illustrated by the need for proximity to a myogenic cell (e.g., myotube) in tissue culture [23]. In order to harness the tantalizing potential of stem cells for clinical myoblast

therapy (or other transplantation uses) due consideration should be given to: the complexity of the in vivo environment, the importance of mechanical properties that influence cell behavior in vivo, the interface between the environment and cell behavior (widely referred to as the "stem cell niche") and the ultimate definition of stem cells by the end-point of functionality. The recent recognition that the fibrotic environment in dystrophic muscle can alter the fate of muscle progenitor cells (from myogenic into fibrogenic) emphasizes the importance of an adverse environment and this needs to be considered when contemplating implanting fresh sources of myogenic precursors into dystrophic muscles (see "Fibrosis and impaired regeneration").

It was initially difficult to test the capacity of non-myogenic sources of stem cells to give rise to muscle nuclei in animals due to the lack of good markers to identify donor cells and track their fate in host animals. Some of the best markers available in the 1980s were the different forms (isoenzymes) of enzymes such as glucose-6-phosphate isomerase, a dimer that had different electrophoretic mobility on gels and could be used to distinguish between cells derived from two strains of mice. In 1983, this relatively insensitive cell marker system was used to test the possibility that bone-marrow-derived cells could give rise to myoblasts in vivo and found no evidence to support this notion [80]. Dramatic improvements in cell marker systems to specifically identify transplanted donor cells and especially to visualize them in tissue sections (a very important point) then occurred: there were two major advances. In 1991, highly specific Y-chromosome probes were developed to identify male nuclei transplanted into female hosts (in a range of species). However, the powerful tool that revolutionized the field was the sophisticated genetic engineering of cells and animals (initially mice) with reporter genes that can readily identify (transgenic) donor cells after transplantation. In 1998 a highly significant paper was published (using the transgenic reporter gene technology) that unequivocally demonstrated that bone-marrow-derived cells can indeed give rise to myonuclei in adult skeletal muscle in mice [81]. This heralded in the era of intensive stem cell research at the turn of the century.

To date the huge investment in stem cells as possible therapies for neuromuscular disorders has not converted the much-vaunted promise into reality [78]. Unfortunately, the initial potential contribution of exogenous bone-marrow-derived muscle precursor cells to new myonuclei (and the promise they offered for systemic stem cell therapy) was overestimated, due in large part to problems with expression of cell markers (used to identify the donor cells), and the phenomenon of fusion of bone marrow cells to myofibers without conversion of donor nuclei into the myogenic lineage (this applied to over 80% of bone-marrow-derived donor nuclei within myofibers) [82]. Thus myogenic conversion of bone-marrow-derived stem cells in vivo is now widely considered to be trivial and of little current interest for cell replacement

therapies. Recently, attention has moved to the use of blood-vessel-associated progenitors as an alternative source of myogenic precursors.

Relationship between satellite cells and blood-vessel-associated cells

The intriguing relationship between satellite cells and other cells within skeletal muscle tissue has attracted much attention. This is difficult to investigate because when a satellite cell moves out from the juxtasarcolemmal, classical, position beneath the basal lamina of the myofiber into the interstitial space, it cannot be readily identified. There is certainly evidence that satellite cells can emigrate, but how frequently might this occur? Conversely, how often might the same cells or another incognito myogenic progenitor originating beyond the myofiber migrate into the classical satellite cell position? The dynamics of such potential trafficking in vivo are hard to measure. These issues are central to a putative functional relationship between satellite cells and the blood-vessel-associated cells (mesangioblasts, pericytes, CD133+/AC133+) that have stimulated much recent interest as a promising alternative source of myoblasts for cell therapy.

A significant relationship between myogenic and vascular precursor cells is suggested by the close proximity of satellite cells to endothelial cells of capillaries in postnatal skeletal muscle [11, 83]. In addition, a close proximity of blood vessels and (extrasynaptic) myonuclei (up to 81% in rodent soleus) is emphasized in normal muscle and this is disturbed in denervated muscle [84]. The proximity of these myogenic nuclei to capillaries, combined with the ability of (stem) cell precursors to give rise to both endothelial and myogenic cells under various conditions in tissue culture and in vivo after cell transplantation [79, 85] presents interesting possibilities. Whether these vascular-related myogenic precursors are distinct from (or can give rise to) satellite cells is unclear. Whether the common precursor is a true stem cell is also unclear. Furthermore, the relationship between these vascular precursor (stem) cells, pericytes [86], and mesangioblasts (associated with blood vessels) requires clarification, as does the relationship to DC133+(AC133+) cell populations derived from both skeletal muscle and blood [76, 85]. These vascular-related myogenic cells have attracted much recent interest in cell transplantation experiments to potentially provide healthy donor myonuclei to correct the gene defect in dystrophic mice and dogs. The striking claims of success have attracted controversy [87] but also offer hope for an alternative source of myoblasts that might be delivered through the circulation [88].

Clinical trials using mesangioblasts in boys with DMD have been initiated in Italy, although the scientific basis for this continues to be discussed. Some of the issues that require clarification with respect to blood-vessel-related progenitors as a source of myoblasts to treat DMD are: the best source of the cells (muscle or blood); heterologous cells (with immune issues) or autologous cells (requiring gene correction);

systemic delivery (ideal); amount of muscle formed from donor myonuclei; functional improvement of muscle; longevity of donor nuclei (repeat treatment?); the formation of donor satellite cells (for replenishment of cells in vivo); and the possibility of cancers from bona fide stem cells. If repeated treatments are indeed essential for sustained benefits then blood-derived autologous cells as a source of donor myogenic (stem) cells are preferable, due to issues with repeated biopsies of muscles of DMD boys.

Concluding remarks

The satellite cell has returned to reign as the main source of myogenic precursor cells (myoblasts) in adult muscle and a wealth of new information on myogenic precursors has emerged recently as indicated below.

- Much is now known at the cellular and molecular/gene level about the origins, and factors controlling the development, of myogenic and satellite cells during embryogenesis in various muscles. However, little is known about the clinical consequences of the different sources and patterns of gene expression involved in the formation of the trunk, limb, and head muscles.
- Powerful new molecular and genetics tools have revolutionized the understanding of satellite cells, provided information on the numbers of such cells in diseased and aged muscles, and their capacity to be activated and form new muscle in response to different clinical situations (regeneration, growth and hypertrophy, atrophy, denervation, and aging).
- Factors in adult muscle that control activation of the normally quiescent satellite cells (and subsequent myogenesis) have been elucidated and include molecules associated with the sarcolemma, the crucial importance of the extracellular matrix and interactions with a host of growth factors and their receptors, plus the role of systemic factors.
- The impact of the environment and especially of fibrosis in vivo for altering the fate of myogenic precursor cells has become more widely recognized.
- Whether there is a true stem cell subpopulation of satellite cells to replenish these vital myogenic precursor cells throughout life remains a hot topic.
- Information is emerging on the relationship of satellite cells to other precursors in the interstitial tissue and the possibility of movement of such progenitor cells into and out of the satellite cell compartment.
- The transfer of myogenic (stem) cells for treatment of muscular dystrophy, cardiac damage, and also tissue engineering has continued to attract attention, although the problem of the rapid and massive death of injected myoblasts has not yet been resolved satisfactorily. Intense interest since 1998 has focused on the potential contribution of non-myogenic stem cells to the myogenic

lineage with applications to therapeutic cell therapy. Unfortunately disappointing results were obtained with circulating bone-marrow-derived stem cells for systemic delivery of myoblasts. Finally, great progress has been made concerning the possibility that precursor (stem) cells derived from blood vessels might be suitable for clinical applications.

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