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REVIEWS

Muscle Regeneration in Amphibians and Mammals: Passing the Torch

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Skeletal muscle in both amphibians and mammals possesses a high regenerative capacity. In amphibians, a muscle can regenerate in two distinct ways: as a tissue component of an entire regenerating limb (epimorphic regeneration) or as an isolated entity (tissue regeneration). In the absence of epimorphic regenerative ability, mammals can regenerate muscles only by the tissue mode. This review focuses principally on the regeneration of entire muscles and covers what is known and what remains to be elucidated about fundamental mechanisms underlying muscle regeneration at this level. *Developmental Dynamics 226:167–181, 2003.* © 2003 Wiley-Liss, Inc.

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INTRODUCTION

Although skeletal muscle constitutes only an isolated but large component of the body, its regeneration encompasses many of the fundamental problems encountered in the broader field of regeneration. Muscle regeneration, although first described as early as the mid-1800s, has had a fascinating history (Field, 1960) in which the very existence of the phenomenon has often been denied. Several generations of medical practitioners, including many still in practice, were taught from standard textbooks in histology, pathology, and surgery that muscle does not regenerate. Such a belief has certainly impacted certain areas of medical practice and drug development. In fact, during the period of the 1950s and early 1960s, when many of the presently used local anesthetics were being tested clinically, what now appears to be obvious evidence of myotoxicity and regeneration was interpreted as temporary denervation effects, because mammalian muscle regeneration was not considered to be possible.

From the purely biological point of view, the broader issue of the possibility of reversing the differentiated state is embodied in the longstanding debate about dedifferentiation in muscle regeneration. The symposium volume edited by Mauro et al. (1970) captures very well the flavor of the discussions when the debate was at its peak. With the recent intense interest in stem cells in the adult, the issue of cellular origins of regenerating muscle is again being widely discussed. Questions concerning the relationship between processes occurring during the embryogenesis and regeneration of muscle are still being asked, even though many of the early issues have been settled. Finally, factors controlling both external form (morphogenesis) and internal architecture have received far less attention then they deserve.

This review is written from the perspective of one who entered the field of muscle regeneration early in its development and who has seen it mature. The current revolution in the technologies that can be used in the study of developmental phenomena is now beginning to make its mark in the field of muscle regeneration, making this an appropriate time to summarize the recent history of this field and to help to lay the groundwork for the next generation of research. Breadth, rather than depth of coverage, is the emphasis, and because this is a perspectives article, it is being written from a more personal point of view than would be a standard review.

BACKGROUND

This article will focus on two major systems of skeletal muscle regenera-

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Fig. 1. Regenerating forelimb of an adult newt 25 days after amputation. A well-developed regeneration blastema is present (top). The proximal region (bottom half) shows portions of the original bones and muscle fibers. Hematoxylin and eosin stain.

tion: that occurring within a regenerating amphibian limb and isolated muscle regeneration in mammals. To provide a starting point, the basic elements of each system will be described.

Muscle Regeneration in the Amputated Amphibian Limb

In postamputational limb regeneration, called epimorphic regeneration by Morgan (1901), the regeneration of muscles is intimately connected with regeneration of the limb as a whole (for general reviews of the events of limb regeneration, see Carlson, 1974a; Wallace, 1981; Tsonis, 1996). Within a day after amputation, the wound surface becomes epithelialized and the underlying tissues enter a still poorly understood phase that is traditionally called phagocytosis and demolition. This stage is a prelude to the period of tissue dedifferentiation, during which the tissues underlying the amputation surface largely lose their mature differentiated characteristics and a population of mesenchymal cells begins to accumulate in that area. Dedifferentiation is followed by the formation of a regeneration blastema (Fig. 1), which has many characteristics in common with an embryonic limb bud. A new limb emerges from the regeneration blastema, following a morphologic course that is remarkably similar to that which occurs during normal embryonic development. By the time this phase of morphogenesis is complete, all elements of the skeleton and musculature that were present in the original amputated limb have been faithfully replaced. A final phase of limb regeneration, especially in larger animals, is a period of growth, which will bring the regenerated limb to normal size and an external appearance that is usually identical to that of the original limb. Although very little attention has been paid to the differentiation of muscle fibers within the regenerating amphibian limb, the morphologic evidence to date suggests that it does not differ substantially from ontogenetic differentiation.

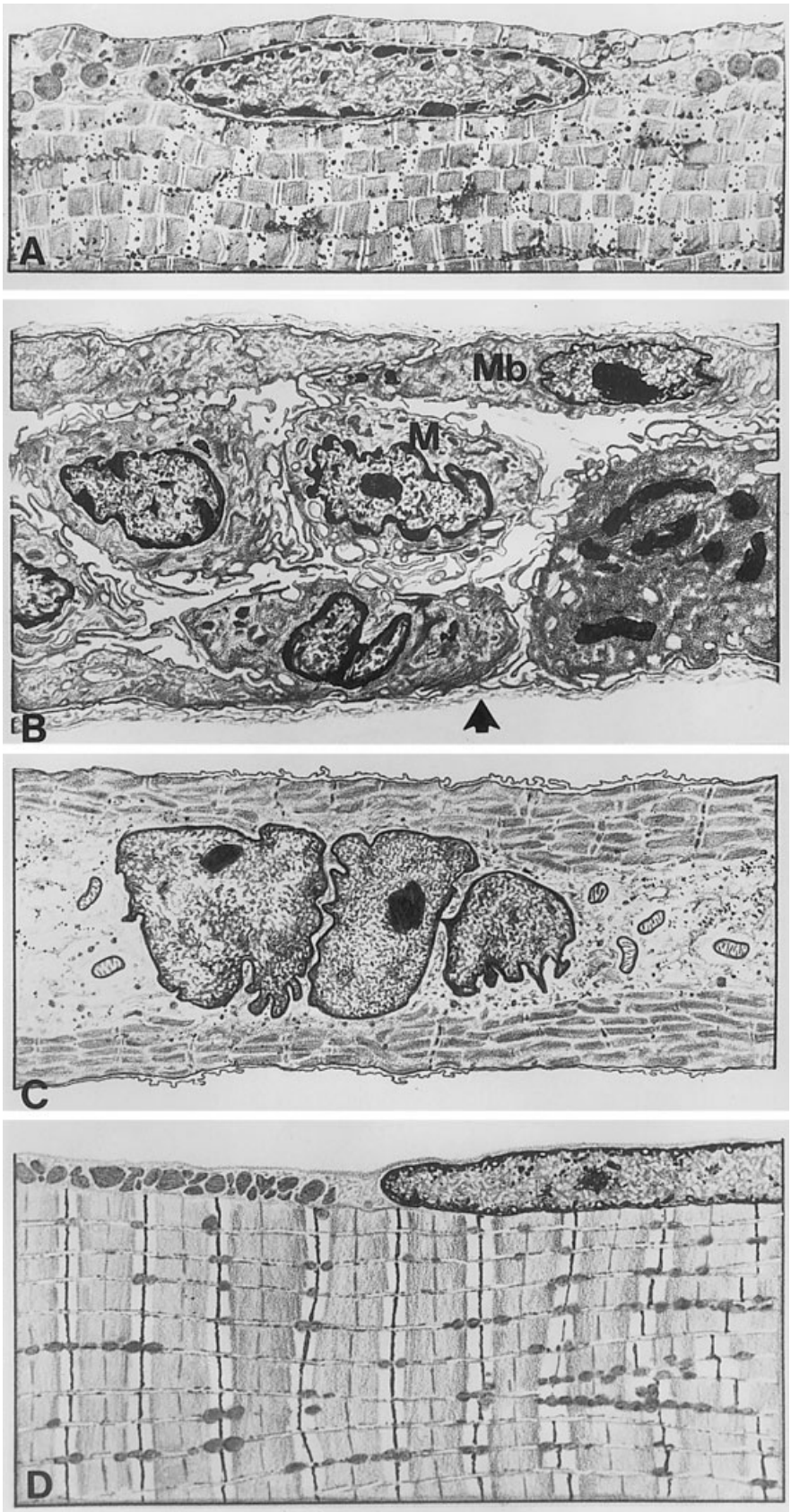
An Overview of Mammalian Muscle Regeneration

Mammalian muscle regeneration has been studied principally at the cellular and tissue level (see reviews by Carlson, 1973; Grounds, 1991, 1999; Chambers and McDermott, 1996). The regenerative process is usually initiated by some form of damage to the muscle fibers, whether through direct mechanical trauma, ischemia, thermal insults, or toxic chemicals. Characteristically there are two phases of degeneration of damaged muscle fibers. The first is an early intrinsic phase, often initiated by membrane damage, in which the activity of calcium-activated proteases causes disruption of the sarcomeric units, sometimes within minutes of the insult (Fig. 2). This stage is followed by a phase of invasion of the damaged muscle fiber by multitudes of macrophages and their phagocytosis of the damaged muscle fiber. Concurrent with phagocytic removal of the damaged muscle fiber is activation of the satellite cells associated with that muscle fiber. All of this activity typically occurs beneath the persisting basal lamina of the original muscle fiber. Proliferation of satellite cells is followed by their fusion into multinucleated myotubes and the maturation of these myotubes into muscle fibers. In mammals, one characteristic difference between regenerated muscle fibers and the original fibers is the persistence of central nuclei in regenerated fibers. This finding serves as a good marker for the presence of regeneration. The initiation of muscle fiber regeneration in mammals is dependent upon the proximity of microvasculature, and the completion of differentiation depends upon either the maintenance of innervation or the reinnervation of the regenerating muscle fiber. A fully regenerated mammalian muscle fiber returns to almost normal contractile function and biochemical characteristics.

Muscle Regeneration by the Tissue and Epimorphic Modes

There are two modes by which a muscle can be regenerated—even for the same muscle in the case of amphibians (Carlson 1970a, 1979). One is the epimorphic mode, in which the muscle regenerates as a component of a complex regenerating structure, namely the entire regenerating limb. This option is, of course, only open to those species in which limb regeneration is possible. Key to epimorphic regeneration is the formation of a regeneration blastema, with the associated tissue interactions and control mechanisms inherent in the process of blastema formation and development.

Early work (Carlson, 1970b) demonstrated that a hindlimb muscle in the axolotl could also regenerate after mincing by the tissue mode of regeneration, i.e., the process of muscle regeneration that commonly takes place in mammals. The regeneration of an entire muscle by the tissue mode does not involve the formation of a regeneration blastema, but rather involves the direct formation of muscle fibers from cellular elements remaining after tissue damage. A muscle formed by the tissue mode of regeneration typically has the general shape of the original muscle, but in contrast to a



muscle that has regenerated epimorphically, it is not an exact replica of the original.

Characteristics of each of the two modes of muscle regeneration are presented in Table 1. Specific experimental details underlying the elements of the table are presented throughout this review.

MUSCLE REGENERATION IN AMPHIBIANS

Epimorphic Regeneration

Dedifferentiation and the source of new muscle cells.

In 1938, Thornton published a detailed histologic description of the reactions of the damaged muscle fibers after limb amputation in larval *Amblystoma* (Thornton, 1938). From his studies, he concluded that during the phase of dedifferentiation, myonuclei, surrounded by a thin rim of cytoplasm, break off from the damaged muscle fibers and migrate distally to join the regeneration blastema. He left open the question of whether these cells could also form cartilage or connective tissue within the regenerated limb. In later studies, involving both light and electron microscopy, Hay (1959, 1962) and Lentz (1969) made similar observations. These studies were greeted by a torrent of criticism by other cell biologists, who believed that myonuclei in differentiated muscle fibers were incapable of re-entering the mitotic cycle.

Other proposed sources for new muscle in the regenerating limb were blood-borne cells (Hellmich, 1930), reserve cells (Weiss, 1939, p.

Fig. 2. Stages in the regeneration of an ischemic mammalian muscle fiber. **A:** Muscle fiber in a state of ischemic necrosis. The contractile proteins have separated into individual sarcomeric units, and the nucleus shows early signs of necrotic death. **B:** The peak of the cell-mediated degenerative phase. Beneath the persisting basal lamina (arrow) macrophages (M) are ingesting sarcoplasmic debris, while at the same time, activated myoblasts (M6) line up beneath the basal lamina. **C:** A regenerating myotube, with a centrally positioned nuclear chain and peripheral cross-striated myofibrils. **D:** A mature regenerated muscle fiber.

TABLE 1. Characteristics of Muscle Regenerating by the Tissue and Epimorphic Modes

Characteristic	Tissue Regeneration	Epimorphic Regeneration
Initial stimulus	Breakdown of muscle fibers	Anything that stimulates limb regeneration
Source of myoblasts	Satellite cells probably the major source; can other sources be definitively ruled out?	Dedifferentiation of muscle? Nonmuscle cells (?)
Removal of damaged cytoplasm	Phagocytosis plays a prominent role	Phagocytosis is less prominent
Regeneration blastema	Absent	Present
Relationship of regenerating muscle cells to basement membrane	Most regeneration occurs within the confines of old basement membranes	Most regeneration occurs in the absence of old basement membranes
Time course	Fast	Slow
Relation to nerves	Early differentiation and morphogenesis do not require nerves; final functional differentiation and maintenance require motor nerves	Nerves (any type) required for blastema formation; exception, aneurogenic limbs; morphogenesis is independent of nerves
Relationship between amount of damaged and regenerating muscle	Fairly direct between minimum and maximum thresholds	Amount of muscle in regenerates is independent of damaged muscle in stump
Gradients	Related to patterns of restoration of blood supply-often centripetal	Pronounced proximodistal gradient of decreasing maturity; a lesser pre- to postaxial gradient
Development of function	Development of contractile properties roughly recapitulates the ontogenetic pattern	Not investigated
Morphology of regenerate	Usually imperfect	Perfect
Amount of connective tissue	Above normal	Normal
Morphology of mature muscle fibers	Central nuclei commonly persist	Normal at the histological level
Morphology of development	Unlike that in the embryo, above the cellular level	Very close recapitulation of ontogenetic development
Morphogenetic control	Gross morphogenesis and internal architecture can be accounted for on the basis of physical factors	Morphogenetic controls seem similar to those operating in the embryo
Role of function in morphogenesis	Functional environment improves the quality of the regenerate	Function not needed for normal morphogenesis
Positional memory	Present, but not expressed	Present and expressed
Interactions between regenerative processes	Suppressed by epimorphic regeneration	Dominant over tissue regeneration

[a]Data from Carlson (1979).

468), and even epidermis (Godlewski, 1928; Rose, 1948). At this level, another controversial issue concerned stability of lineage. Could a cell originating from dedifferentiated muscle redifferentiate into a chondrocyte, for instance, or conversely, could a cartilage-derived cell redifferentiate into muscle? Although these questions were the subject of a great amount of speculation for several decades, new approaches had to be devised before there was a chance of obtaining a definitive answer.

Over many years, Brockes and colleagues (Kintner and Brockes, 1984; Lo et al., 1993; Kumar et al., 2000) have used a variety of techniques, including monoclonal antibodies and retroviral and dye markers to demonstrate that cultured newt myotubes can break up into mononucleated cells when implanted into limb regeneration blastemas. They have also shown that, in vitro, nuclei of multinucleated myotubes of newts are capable of reentering the S phase under different circumstances, such as inactivating

retinoblastoma protein by phosphorylation in response to high concentrations of serum (Tanaka et al., 1997). The breaking up of myotubes into mononucleated cells and the re-entry of individual nuclei into the cell cycle are dissociable events under different controls (Velloso et al., 2000). Working with mouse C2C12 myotubes in culture, Odelberg et al. (2000) found that ectopic expression of Msx-1 resulted in cleavage of myotubes into smaller myotubes or individual mononucleated cells. In the regenerating newt limb, Msx-1 is expressed in the early regeneration blastema and its expression could be related to the dedifferentiative process (Simon et al., 1995). In an extension of their earlier work, McGann et al. (2001) prepared protein extracts from regenerating newt limbs and exposed both newt (A1 cells) and mouse (C2C12 cells) myotubes to the extract. Approximately 18% of the murine and 25% of the newt myotubes re-entered the cell cycle. This experiment suggests that some proteinaceous element in the early regenerating limb can stimulate the dedifferentiation of muscle fibers. In the case of the mouse, it would appear that the myotubes have retained the intracellular pathways that allow dedifferentiation and that these pathways can respond to an appropriate signal. The specific nature of the signal remains unknown.

Echeverri et al. (2001) conducted an intensive in vivo study on muscle fiber dedifferentiation in the tails of larval axolotls after microinjecting individual muscle fibers with both nuclear and cytoplasmic fluorescent dextran dyes. Under direct observation, they found that the breaking up of muscle fibers into mononucleated fragments, indeed, does occur but only under specific conditions. A muscle fiber not damaged by amputation remains stable, as does a slightly injured muscle fiber associated with minimal tissue damage. A muscle fiber cut through its middle degenerates, rather than fragments. A slightly clipped muscle fiber at the plane of amputation or near an area of severe tissue damage does undergo the process of dedifferentiation (fragmentation into mononucleated cells) that was inferred from the earlier morphologic studies.

Echeverri et al. (2001) calculated that mononucleated cells derived from muscle fibers make a significant contribution to the regeneration blastema. A remaining question concerns the ability of such cells to redifferentiate into cells normally characteristic of other lineages, such as cartilage, connective tissue, or other nonmuscle cell types. Echeverri et al. (2001) refer to preliminary experiments suggesting that such lineage diversification can occur. In their in vitro experiments, Odelberg et al. (2000) reported that clonal populations of myotube-derived mononucleated cells can redifferentiate into cells expressing adipogenic, chondrogenic, myogenic, and osteogenic markers.

Research conducted during the past decade seriously challenges the dogma of the impossibility of reversal of the differentiated state of the skeletal muscle fiber. The pattern that appears to be emerging is that "dedifferentiation" of a muscle fiber in the classic sense only occurs under certain specific circumstances. Some degree of direct damage to the muscle fiber itself is important but that alone appears to be insufficient, because in the absence of significant associated tissue damage, i.e., enough to elicit an overall epimorphic regenerative response, dedifferentiation does not occur. What there is about that microenvironment remains unclear, but the common association of Msx-1 expression with an environment favorable to epimorphic regeneration provides a good starting point for further research. It is noteworthy that when the same muscle that could undergo dedifferentiation after amputation is damaged in isolation, the histologic appearance of dedifferentiation is never seen.

Much of the muscle dedifferentiation controversy occurred either before or just after the discovery of the satellite cell, an undistinguished looking mononuclear cell located between a muscle fiber and its surrounding basal lamina (Mauro, 1961). For several years, there was a question about whether or not satellite cells, as described by Mauro, exist in urodele amphibians. Although classic satellite cells have been described in 200-mm-long adult axolotls (Carlson and Rogers, 1976), newts and other related species possess what have been called postsatellite cells (Cherkasova, 1982; Cameron et al., 1986). These cells, which are often seen in locations similar to those of satellite cells, are completely surrounded by a basal lamina, so that there is a complete double basal lamina between them and the associated muscle fiber. Whether or not this configuration of satellite-type cell has any relationship to the phenomenon of muscle fiber dedifferentiation remains to be investigated. It should be noted, however, that the presence of basal lamina material between a satellite cell and muscle fiber is not unique to amphibians. In rodent muscle, this is a common finding in both old age (Snow, 1977b) and after long-term denervation (Dedkov et al., 2001).

Morphogenesis of epimorphically regenerating muscle.

From a purely descriptive standpoint, the sequence of events of muscle morphogenesis, starting with the formation of common flexor and extensor masses, and the attainment of final morphology of the regenerated muscles, is virtually identical to that which occurs in the embryonic limb (Grim and Carlson, 1974). The major difference is the much larger size of a regenerating limb, especially in species such as sexually mature axolotls. Recognizing the large difference in cross-sectional area of an embryonic and mature limb is important, because any hypothesis of overall control of limb or muscle morphogenesis in which distance is important must take into account regeneration, as well as embryogenesis.

The control of muscle morphogenesis within the regenerating (and also embryonic) limb is still not understood, other than that it occurs as an integrated component of the entire regenerating limb. In an effort to determine whether the morphology of the stump musculature plays any role in controlling the morphogene-

sis of the muscles within a regenerating limb, Carlson (1970b) surgically removed essentially all of the upper arm musculature of axolotls and amputated the limbs through the region of removed musculature. Grossly, the limbs regenerated normally, and within the regenerates, both the pattern of the musculature and amount of muscle appeared normal. Yet in the stump, where the vast majority of the musculature had been removed, no new muscle was found. In a different approach to morphogenesis, Polezhaev (1937) removed the internal tissues from limb stumps of axolotls, minced them, and then replaced the minced tissue into a cuff made by the skin of the limb stump. Despite this level of disruption, the resulting limb regenerates contained a morphologically normal skeleton and musculature. These experiments show that morphogenesis of the epimorphically regenerating musculature is independent of the form of the musculature of the stump.

It is difficult to account for the essentially normal amount of muscle fibers that appeared within the regenerates after muscle removal. This experiment (Carlson, 1970b) has sometimes been misinterpreted and criticized as an attempt to deal with the origin of the muscle cells, in a manner similar to the older experiments involving removal of bone from the limb stumps (Fritsch, 1911; Weiss, 1925), but because a handful of muscle fibers were left in the stump, it was not possible to interpret the results in that manner. Nevertheless, it is interesting that so many muscle fibers arose from a stump that contained fewer than 10 muscle fibers. One possible explanation is that cells derived from the remaining muscle fibers proliferated tremendously within the blastema. Another is that myogenic cells from more proximal regions of the limb girdle migrated into the blastema. A third is that other cell types in the blastema did compensate for a deficiency of myogenic cells. The possibility that other cells within the blastema can compensate for the lack of normal precursors of a particular cell type is ripe for future investigation.

The role of ongoing function in muscle morphogenesis was investigated in *Amblystoma* larvae by amputating limbs and then keeping the animals continuously anesthetized for the entire period of regeneration (Carlson, 1972a). Overall muscle morphogenesis was not adversely affected by continuous inactivity.

Other than the above experiments, very little attention has been paid to factors controlling the morphogenesis of muscle within the regenerating limb. As is the case with the embryo, it has been very difficult to dissociate the development of the musculature from factors that control development of the limb as a whole.

Tissue Regeneration

Early studies showed that limb muscles in amphibian species that both do and do not regenerate limbs are capable of regenerating muscle as an isolated tissue by the tissue mode. In both frogs (Carlson, 1968) and the axolotl (Carlson, 1970b), the regeneration of a minced muscle occurred according to a histologic process that did not differ significantly from the regeneration of a mammalian minced muscle (see below). In the regenerating puboischiotibialis muscle of the mature axolotl, blastema-like cells were never seen, and myotubes and striated muscle fibers were seen earlier than muscle fibers formed from blastemas of hindlimbs amputated through the level of the same muscle.

Interactions between Tissue and Epimorphic Regeneration

In the case of a muscle that is capable of participating in both tissue and epimorphic regeneration, it is logical to ask what would happen if the same muscle were afforded the opportunity to regenerate by either mode. This experiment was done by mincing an axolotl limb muscle and then amputating the leg through the level of the mince. The question was whether the muscle at the amputation surface would regenerate by the tissue or the epimorphic mode. It became quite clear that, under the influence of the epimorphic field, the distal minced muscle was swept into the field of dedifferentiation and that epimorphic regeneration took precedence over tissue regeneration (Fig. 3; Carlson, 1979). The influence of the epimorphic field extended approximately 1–2 mm proximal to the amputation surface. Dinsmore (1974) reported similar results when he transplanted distal limb blastemas over minced proximal limb muscles in the enucleated amphibian orbit. When epimorphic regeneration of a limb is prevented, the tissue regeneration of muscle extends to the amputation surface. Such interactions between tissue and epimorphic regeneration also seem to hold for cartilage formation, but because at the cellular level it is difficult to distinguish the two processes in cartilage, the distinction is less clear.

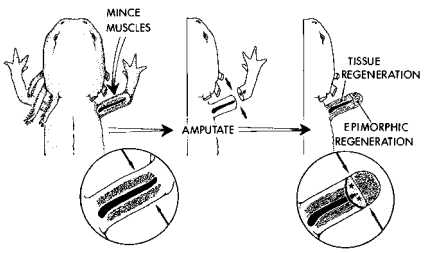


Fig. 3. Interactions between the tissue and epimorphic regeneration of muscles of the upper arm. Mincing the flexor and extensor muscles sets off a tissue regenerative process. After amputation through the level of the regenerating muscles (center), a regeneration blastema arises and sweeps the distal muscle that had begun to regenerate by the tissue mode into an epimorphic regenerative process. The stars (lower right) show the extent of the influence of the epimorphic field proximal to the amputation surface (from Carlson, 1979).

Positional Memory in Amphibian Limb Muscle

A variety of experiments by several investigators, in which stump tissues were rotated or positionally displaced, resulted in the formation of complex multiple regenerates. Collectively, these experiments suggest that certain tissues of the limb, specifically muscle and dermis, retain a memory of their original position within the cross-section of the limb (Carlson, 1983). When tissues with different positional qualities are juxta-

posed, the regenerating limb cannot integrate the disparate sources of morphogenetic information, and abnormal limb regeneration results.

The nature of positional memory remains completely unknown, but some experiments conducted on axolotl forelimb muscles shed light on some of its properties (Carlson, 1974b, 1975). If one exchanges the intact or minced flexor and extensor musculature of the forelimb and then amputates the limb, over 80% of the regenerates are multiple. Memory of positional differences is embedded principally, if not exclusively, along the anteroposterior axis of the limb (in a line through the flexor and extensor musculature). Muscle trauma without positional displacement does not elicit an epimorphic response. Mincing in situ is followed by the regeneration of the muscles alone, and exchanging minced flexor and extensor muscles without amputation is also followed by pure muscle regeneration without the formation of any supernumerary structures. But if cross-transplanted flexor and extensor muscles are allowed to regenerate and the limb is then amputated through the cross-transplanted minced muscle regenerates, multiple regenerates form. This finding shows that the positional memory inherent in muscle persists, but is not expressed, through the course of tissue regeneration and that it remains effective in a subsequent epimorphic regenerative response. In a similar experiment, extensor and flexor forelimb muscles were minced and cross-transplanted, and 5 days later the limbs were amputated through the level of the mince. In this experiment, over 90% of the limb regenerates were multiple. This finding showed that anatomic integrity of the musculature is not necessary for the preservation and expression of positional memory. One option is that positional memory is present in the connective tissue of the muscle, rather than the myocytes themselves. Other than these old attempts to map out positional memory in the amphibian limb, we still have no idea of the nature of positional memory. This discovery remains a task for the future.

MUSCLE REGENERATION IN MAMMALS

The basic histology of mammalian skeletal muscle regeneration was well described by German morphologists and pathologists in the late 1800s (reviewed by Field, 1960), but early in the 20th century, writings from the English school of pathology suggested that the regenerative capacity of mammalian muscle is limited or absent. Despite some clear-cut reports in English (Clark, 1946; Godman, 1957; Allbrook, 1962), the notion that mammalian skeletal muscle is endowed with a poor regenerative capacity flourished and persisted for decades in the literature. Largely through the discovery of the satellite cell by Mauro (1961) and the demonstrations, pioneered by Studitsky in the Soviet Union (Studitsky and Striganova, 1951; Studitsky, 1959), that entire muscles can regenerate under certain circumstances, attention was redirected toward muscle regeneration in the late 1960s.

Cellular Sources of Regenerating Mammalian Muscle

Mauro's (1961) discovery of the satellite cell in frog muscle ushered in a new era of debate concerning the cellular source of regenerating muscle. With a clear alternative to cellular dedifferentiation, the debate became polarized and intense, with most of the supporters of dedifferentiation being those who studied amphibian limb regeneration and the satellite cell supporters being those who were focused on mammalian muscle (Mauro et al., 1970). In retrospect, as often happens in science, both sides of the debate appear to have been correct within the scope of their positions. Autoradiographic studies in which either myonuclei or satellite cells of rat muscle were selectively labeled clearly showed that satellite cells contribute nuclei to regenerating myotubes (Snow, 1977a). With that demonstration, many proponents of the satellite cell side declared victory, and attention was turned to other topics. Although the satellite cell was definitely ruled in as a source of regenerating muscle, other possible sources were not rigorously ruled out.

Recently, several laboratories have demonstrated the presence of muscle-derived stem cells that have the capacity to give rise to progeny in the myogenic, hematopoietic, chondrogenic, osteogenic, and adipogenic pathways (Bosch et al., 2000; Asakura et al., 2001; Zammit and Beauchamp, 2001; Grounds et al., 2002; Jankowski et al., 2002; McKinney-Freeman et al., 2002). In addition, it has been demonstrated that bone marrow-derived cells can contribute to regenerating muscle (Gussoni et al., 1999). The lack of satellite cells in PAX-7 -/- mutants along with the presence of muscle-derived stem cells in the same animals (Seale et al., 2000) suggests that these cells are either members of separate lineages or at least representative of different steps in the differentiation of a single lineage. The seeming ability of satellite cells to give rise to adipocytes, as well as osteogenic cells (Asakura et al., 2001; Csete et al., 2001; Taylor-Jones et al., 2002), further blurs the issue of cell lineages in muscle-derived cells. The recent spate of reports, such as those cited above, concerning types of cells that can give rise to muscle provides ample evidence that much more research is needed to determine the cellular source(s) of regenerating muscle in mammals.

Whole Muscle Regeneration Models

Minced muscle regeneration.

The first whole muscle regeneration model was the minced muscle model. This regeneration model had a fascinating history. In the Soviet Union, the era of Lysenkoism was at its peak during the 1940s (Anonymous, 1949; Medvedev, 1969). A bizarre subtheme that arose under the influence of the Lysenko doctrine was the "New Cell Theory" of Olga Lepeshinskaya (1945, 1951, 1952). According to this doctrine, cells in the embryo or in regenerating systems do not have to come from preexisting cells, but rather arise from a proteinaceous "living substance." Studitsky performed an experiment that was designed to test the validity

of that theory. He took skeletal muscles from birds and rats, minced them into small fragments and replaced the fragments into the bed of the removed muscle. In this presatellite cell era, he reasoned that, if new muscle arose from the mince, the regenerated muscle must have arisen from living substance. That experiment did result in the formation of a new muscle (Studitsky, 1952), and this experiment was widely used throughout the Soviet orbit as proof of the validity of Lepeshinskaya's new cell theory (Studitsky, 1953; Hašek and Hašková, 1953, p. 105).

Regardless of the unusual theoretical origin of the minced muscle model, it proved to be a powerful regeneration model that was extensively investigated and followed-up in other experiments by Studitsky's laboratory during the 1950s and 1960s (Studitsky, 1959; Studitsky and Ignatieva, 1961; Zhenevaksya, 1974). Carlson (1968; 1970c, 1972b) repeated the basic minced muscle experiment in rats and frogs and provided a detailed histologic description of the overall regenerative process.

If the triceps surae complex (gastrocnemius, plantaris, and soleus muscles) of a young rat is minced into 1-mm^3 fragments and the fragments are replaced into the limb from which they were removed, the majority of the fragments fall into a state of ischemic necrosis. Later investigation showed that no viable myogenic cells are found within the ischemic area after 4 hr (Phillips et al., 1987). An unresolved issue is whether the myogenic cells in the center of a mince die or whether they are able to migrate away from the ischemic area toward the better-oxygenated periphery.

A thin rim of cells survives around the periphery of the muscle, probably through diffusion of oxygen and nutrients. As blood vessels grow into the mince, they bring with them macrophages that remove cytoplasm from the damaged muscle fibers. At the same time and place, satellite cells become activated. Over the course of 1-2 weeks, removal of ischemic muscle and regeneration of new muscle fibers follow a centripetal course toward the center of the original mince. The new muscle fibers, which regenerate within the basal laminae of the original minced muscle fibers, are initially chaotically organized, but starting within a week, they become oriented parallel to the long axis of the overall regenerate. The regenerate becomes innervated through regeneration of axons of the original motor nerves, and the regenerate develops a low degree of contractile function (Carlson and Gutmann, 1972). What muscle is present functions well, but mature minced muscle regenerates are considerably smaller than the original muscles, and they are typically bound to the surrounding tissues by connective tissue adhesions. Although minced muscle regenerates develop appropriate proximal and distal tendon connections with the severed tendon stumps of the host, a typical minced muscle regenerate is a generic model of a muscle, rather than having the shape of the original muscle. In general, the minced muscle model is a good one for studying early stages of muscle regeneration, but it is not satisfactory for studying mature regenerated muscle.

The minced muscle model of regeneration has also been used in mice, originally in attempts to rule in or out the neurogenic vs. the myogenic theories of muscular dystrophy (e.g., Salafsky, 1971) and later to take advantage of the Y-chromosome marker in mice to distinguish between host and donor cells (Grounds et al., 1991). Because of a lesser production of scar tissue in mice, minced muscle regenerates in mice are often relatively more successful in mice than in rats. In contemporary science, the availability of genetically modified mice would offer certain advantages over rats as model animals involving muscle regeneration if transplants from one animal to another are indicated. For regeneration within the same animal, other regeneration models, such as injection of toxic substances (see below), are easier, but only in a mince can one be certain that all original muscle fibers are destroyed.

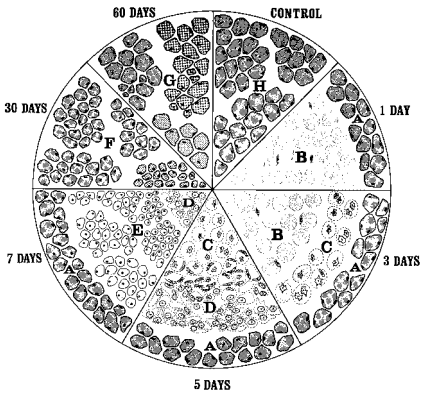


Fig. 4. Scheme illustrating the progression of regeneration in a transplanted rat muscle. **A:** Peripheral muscle fibers that survive intact through diffusion. **B:** Muscle fibers in a state of ischemic necrosis. **C:** Ischemic muscle fibers undergoing macrophage-mediated degeneration. **D:** Early regenerating myoblasts and myotubes. **E:** Maturing myotubes. **F:** Immature cross-striated muscle fibers. **G:** Mature regenerated muscle fibers. **H:** Control muscle fibers.

Free muscle grafts.

Because minced muscle regenerates were unsatisfactory for studying contractile properties of regenerating muscle, another whole muscle regeneration model was developed. This model consisted of removing entire limb muscles and grafting them back into their own bed or into the bed of another muscle (Carlson and Gutmann, 1975a,b; Studitsky, 1988). Because it is also fundamentally an ischemia model, free muscle grafts develop much like minced muscle regenerates, with the exception that, because the muscle fibers have not been mechanically damaged, a thin rim of muscle fibers survives intact at the periphery of the graft (Carlson, 1982; Fig. 4). This strategy also reduces considerably the amount of connective tissue adhesions, so that the regenerated muscles can be readily removed for the in vitro testing of contractile properties. Although most free grafts have been done in the rat, larger muscles of both experimental animals, such as rabbits, cats, monkeys (Mufti et al., 1977; Guelinckx et al., 1988; Markley et al., 1989), and humans (Freilinger et al., 1981) have been freely grafted. In larger muscles, up to 8 weeks may elapse before ingrowing blood ves-

sels reach the innermost parts of the graft. In monkeys, the center of a large muscle graft fills in with a dense rod of collagenous connective tissue, but in the cat and in large rat muscles, regenerated muscle fibers are found throughout the entire cross-section of the grafted muscle.

The final functional mass of a free muscle graft depends largely on the success of reinnervation (Carlson, 1996). If the motor nerves are allowed to regenerate spontaneously into the graft from stumps, a typical whole muscle graft in a rat regenerates to only approximately one-third to half of its original maximum tetanic force, but if the motor nerve to the muscle is left intact, the graft regenerates to its original mass and close to 90% of its original tetanic force (Carlson et al., 1981).

Anesthetic-induced muscle regeneration.

Skeletal muscles are remarkably sensitive to the toxic effects of most local anesthetics, and if such anesthetics are injected directly into a muscle, most or all of the muscle fibers in the area degenerate and subsequently regenerate (Benoit and Belt, 1970; Dolwick et al., 1977; Foster and Carlson, 1980). Several of the local anesthetics, such as bupivacaine, do not damage the motor innervation or disrupt the blood supply. Therefore, the use of this model results in a large field of synchronously regenerating muscle fibers that are well innervated.

Cell and Tissue Interactions in Regenerating Muscle

Basal lamina and extracellular matrix.

The regeneration of skeletal muscle involves a wide variety of interactions between the myocytes themselves and their surroundings. A first level of interaction is between the basal lamina and the degenerating and regenerating muscle cells beneath it. The basal lamina surrounding a muscle fiber is remarkably complex, especially at the neuromuscular junction (Sanes, 1994). After damage, the basal lamina serves as a selective cellular filter, keeping satellite cells within, fibroblasts out, and selectively allowing macrophages to penetrate. The basal lamina also serves as a mechanical scaffolding for muscle fiber regeneration, which usually takes place within existing basal laminae unless they are torn or otherwise disrupted. At the neuromuscular junction, information inherent within the basal lamina can direct the differentiation of the postsynaptic apparatus on the regenerating muscle fiber even in the absence of innervation (Burden et al., 1979; Hansen-Smith, 1986). Both the basal lamina and the extracellular matrix, in general, represent a rich source of growth factors, which have been shown to be very important in the activation of satellite cells and their further development (rev. Grounds, 1991, 1999). The original basal laminae provide the initial orientation for the regenerating muscle fibers until this is partially superseded by more powerful mechanical factors acting on the regenerate.

The microvasculature.

A local microvasculature is critical for the progression of regeneration. In ischemia-based models of muscle regeneration, the initiation and progression of the regenerative response depend on the ingrowth of blood vessels into ischemic regions (Hansen-Smith et al., 1980; Burton et al., 1987). In addition to supplying oxygen and nutrients, the ingrowing blood vessels also bring macrophages to the area of muscle damage.

Damaged muscle contains several populations of macrophages, both resident and nonresident (McLennan, 1993, 1996). Macrophages are very important in the early stages of muscle regeneration, and they function in a variety of ways. In addition to the phagocytosis of cellular debris resulting from muscle damage, they produce a great variety of growth factors that enhance satellite cell proliferation and delay differentiation (Merly et al., 1999), as well as stimulating the spread of the microvasculature that supports regeneration. Much too little research has been done on the role of macrophages in the early phases of muscle regeneration.

Innervation.

Although early mammalian muscle regeneration can proceed well in the absence of nerves, innervation is necessary for the structural and functional maturation of regenerating muscle (Zhenevskaya, 1974). In the absence of innervation, regenerating muscle fibers atrophy or undergo apoptosis, depending on the species. The process of reinnervation of regenerating muscle depends heavily on the model of muscle damage. In the case of damage induced by local anesthetics, such as bupivacaine, the innervation remains morphologically intact (Jirmanová and Thesleff, 1972; Fere et al., 1989), and muscle fiber differentiation occurs very rapidly and according to the original pattern. If the motor nerve to a regenerating muscle has been damaged, functional reinnervation does occur (Zhenevskaya, 1974; Carlson et al., 1979; Bader, 1981). However, overall reinnervation of the regenerating muscle is incomplete if regenerating axons have to make their way to the muscle fibers outside their normal endoneurial channels. Regenerating axons preferentially seek the original junctional region on the basal lamina (Sanes et al., 1978), but they readily form new neuromuscular junctions at ectopic sites as well (Bader, 1980; Womble, 1986). The most effective innervation occurs when the regenerating axons are able to grow through preexisting endoneurial channels (Carlson et al., 1981). In the nerve-intact model of muscle transplantation, there is no diminution of motor unit numbers (Cederna et al., 2001).

Not only do nerves promote the functional maturation of a regenerating muscle fiber, they also determine the specific fiber types. This finding is demonstrated by cross-transplantation studies, in which the fast extensor digitorum longus (EDL) muscle in the rat is grafted in place of the slow soleus muscle and vice versa (Gutmann and Carlson, 1975; Snoj-Cvetko et al., 1996a). In this model, regenerating slow and fast muscles are innervated by axons of

the opposite type of nerve. The regenerating muscles change their functional type, and the degree of fiber type conversion is greater than that reported in similar cross-innervation experiments, in which the muscles remain intact, but are innervated by nerves of the opposite type (Close, 1969). Although the time to peak contraction and half-relaxation time return to normal in soleus to EDL grafts, in EDL to soleus grafts, the contraction times never undergo full conversion to those of the normal soleus. Yet in the rat, regenerated EDL (fast) muscles grafted in place of the slow soleus muscle develop the same repertoire of fast myosins as the normal soleus muscle (Snoj-Cvetko et al., 1996b), whereas soleus into EDL grafts develop similar, but not identical heavy chain profiles to that of the normal EDL (Eržen et al., 1999). There is still an incomplete understanding of how, in cross-transplanted muscles, the conversion of myosin isoforms is translated into whole muscle contractile properties.

Damaged muscle spindles regenerate as long as the capsule is not greatly damaged mechanically, such as in mincing (Milburn, 1976; Rogers and Carlson, 1981; Soukup, 1988). However, as evidenced by the abnormal pattern of differentiation of the nuclear bag and chain fibers, the reinnervation of regenerating spindles is probably atypical (Rogers, 1982; Jirmanová and Soukup, 2001).

Connective tissue.

Very little attention has been paid to the connective tissue in regenerating muscle. For medical applications of muscle regeneration, fibrosis or excessive growth of connective tissue is a concern (Huard et al., 2002), but just as important, the connective tissue is the medium through which the mechanical forces on normal or regenerating muscle fibers are applied (Young et al., 2000). As with tenotomy models, the lack of tension on regenerating muscle results in abnormal regeneration (Carlson, 1972a,b).

In addition to the mechanical role of the collagenous component of connective tissue, other elements of connective tissue are also very important. The role of the basal laminae as scaffolding for early muscle fiber regeneration, as well as its informational role in reinnervation, has already been covered earlier in this review. Many of the muscular diseases that are characterized by muscle fiber damage and regeneration are due to genetic conditions that result in the absence of a molecule that is part of the link between the intracellular proteins of a muscle fiber and its connective tissue microenvironment (O'Brien and Kunkel, 2001; Blake et al., 2002).

Morphogenesis of Regenerating Mammalian Muscle

The factors leading to the morphogenesis of individual muscles are very poorly understood under any circumstance, whether in embryogenesis or during regeneration. Investigations of the minced muscle model provided an opportunity to establish some elements that underlie the morphogenesis of mammalian muscles regenerating by the tissue mode (Carlson, 1972a,b). Two elements of muscle structure are important in morphogenesis: external form and internal architecture. Because in a mince both have to be re-established, this model provides the working material for experimental studies.

The originally implanted mince is totally disorganized and has no self-standing form. In the rat within 4–5 days, the proximal and distal tendon stumps make firm connections with the mince and, through these, they transmit tension to the regenerating mince. Before that time, the regenerating myotubes are oriented according to the orientation of the basal laminae in the pieces within the mince. A few days after tendon connections become established, the myotubes at the periphery of the mince become aligned with lines of tension, and ultimately most of the muscle fibers become oriented parallel to one another. The final shape of a regenerated minced muscle is that of a generic muscle, with tendon connections on either end, a more muscular proximal belly tapering to a narrow distal end, merging with the Achilles tendon in the case of the gastrocnemius muscle. The internal architecture, as well, is never an exact replica of the original muscle.

If minced muscle fragments are implanted into a site lacking directed mechanical tension, internal reorganization does not occur and the regenerating muscle fibers remain oriented in a three-dimensional matrix, like that of the originally implanted fragments. If, however, mechanical tension is applied to this same system, parallel orientation of the regenerating muscle fibers does occur. The gross form of a minced muscle regenerate can be duplicated in the absence of any regenerating muscle fibers and appears to be due to both tension and lateral mechanical pressures on the regenerating tissue. This finding was determined by implanting pieces of the surgical sponge material Gelfoam in place of the gastrocnemius muscle (Carlson, 1972b). These reorganized to form shapes identical to those of minced muscle regenerates, including having connections with both proximal and distal tendon stumps, but they contained no muscle fibers.

In regenerating whole muscles, the original internal structure tends to be preserved throughout regeneration. This finding was demonstrated most clearly in experiments involving transplantation of the tongue musculature in place of a limb muscle, the EDL (Carlson, unpublished observations). In this case, the muscle fibers still retained the three-dimensional orthogonal relationship that they had within the tongue instead of adapting to the roughly parallel pattern of a limb muscle.

Aging and Muscle Regeneration

The loss of muscle mass, often called sarcopenia, is one of the defining characteristics of old age. One of the yet unresolved questions is whether the ill-defined mechanism(s) that account for sarcopenia also influence muscle regeneration

in old age (rev. Carlson, 1995; Grounds, 1998).

As a rule of thumb, skeletal muscle regeneration is less successful in old than in young individuals. This finding is associated with several factors, such as a reduced number and proliferative potential of satellite cells (Schultz and Lipton, 1982; Gibson and Schultz, 1983); retarded replication of myoblasts (McGeachie and Grounds, 1995); telomere shortening in satellite cells (Renault et al., 2000); reduced innervation, resulting in a decrease of motor units (Larsson, 1982; Larsson and Ansved, 1995); increased interstitial connective tissue (Marshall and Goldspink, 1989); and changes in systemic, as well as local concentrations of various growth factors (Barton-Davis et al., 1998; Yablonka-Reuveni et al., 1999; Chakravarthy et al., 2000; Grounds, 2002). Generally speaking, other than slight delays, aging does not greatly affect the early stages of muscle regeneration; rather, the most prominent age-related deficits become apparent later in the regenerative process.

One of the major questions concerning the influence of aging on muscle regeneration is whether the reduction in regenerative capacity is intrinsic to the muscle or whether it is a function of the environment in which the muscle is regenerating. This question has been approached through a cross-age muscle transplant model, in which muscles from old rats were transplanted into young adult hosts and vice versa (Carlson and Faulkner, 1989; Carlson et al., 2001). Same-age grafts of the EDL muscle show a two- to threefold greater recovery of maximum tetanic force in grafts in young vs. old rats. Old muscles transplanted into young hosts regenerate as well as young-into-young grafts, whereas young muscles placed into old hosts regenerate no better than old muscle autografts. This type of experiment shows that, (1) in vivo, there is no intrinsic age-related limitation in the regenerative capacity of a muscle, and (2) the success of a muscle graft is a function of the environment in which it is placed. A similar host effect was shown when muscles were transplanted between strains of mice that exhibit good and poor regenerative capacity (Mitchell et al., 1992). Age-related deficiencies in reinnervation were hypothesized to be an important factor in the poor regeneration of muscle grafts in old rats. To test this, muscles in young and old rats were injured by injections of the myotoxic anesthetic bupivacaine (90% reduction in maximum force within 2 days), which does not destroy the intramuscular nerves (Carlson and Faulkner, 1996). In this experiment, the success of regeneration relative to contralateral control muscles did not differ between young and old rats, but the absolute return in function was considerably greater in the young rats. If mechanical nerve injury accompanied the bupivacaine injection, regeneration in the old rats was relatively much poorer than that in young rats (Carlson and Faulkner, 1998). In extremely old rats near the end of their normal life span, EDL muscles transplanted into young hosts recovered maximum force over double that of control muscles in the old donors (Carlson et al., 2001). This finding shows the degree of environmental limitation of the old body on muscle structure and function.

Although it is not yet possible to quantify the exact nature of the environmental factors that lead to generally poorer regeneration in old animals, innervation is certainly one major factor. The humoral environment is also likely to play an important role, especially in view of recent experimentation of the effects of IGF-1 on restoration of muscle function in older animals (Barton-Davis et al., 1998; Chakravarthy et al., 2000). Experiments on parabiotic young–old rats (Carlson, unpublished observations) suggested humoral effects on the success of regeneration.

A different manifestation of regeneration is seen in the muscles of very old as well as long-term denervated muscle. As an animal approaches the end of its normal lifespan, a type of homeostatic decompensation appears to take place. This phase is manifested by the activation of myogenic regulatory factors, such as MyoD and myogenin (Kostrominova et al., 2000), the reappearance of other isoforms, such as the elongation factor eEF1A-1 as opposed to the muscle-specific eEF1A-2 (Carlson et al., 2002), and the appearance of regenerating myotubes (Dedkov et al., 2001).

PERSPECTIVES

In the past 40 years, a great deal has been learned about skeletal muscle regeneration, but as in every other field of science, each new bit of knowledge opens up many other questions. One critical area concerns the source of regenerating muscle. If we have learned anything over the past half century, the main message should be not to frame the question too narrowly. Another important observation that relates to many scientific controversies is that, if careful scientists argue on opposite sides of an issue, it is likely that there is a certain amount of truth on both sides. The dedifferentiation/satellite cell controversy also shows that too intense concentration on a narrow issue can blind one to other possibilities (in this case, stem cells). Similarly, the possible spectrum of differentiative capacities of myogenic cells, regardless of their origin, has been discussed for years but is being looked at in the new light of discoveries in the past 5 years.

One of the most important insights in mammalian regeneration is the significance of environmental factors as determinants of the success of regeneration. Although examples relating to muscle were given in this review, this principle has been applied with tremendous impact in studies of regeneration in the central nervous system (e.g., Aguayo et al., 1991; Schwab, 1998). In almost any study of regeneration, especially in mammals, distinguishing between the limits of intrinsic capacity and the effects of environmental suppression or permission is crucial.

Of great importance in the study of muscle regeneration is the issue of scale and complexity. Although the muscle fiber is the fundamental unit of a muscle, what is true for an isolated or individual muscle fiber is not necessarily true for an entire muscle. Muscles are very large structures, especially in humans, and even

though a muscle fiber may be perfectly capable of regenerating in isolation, this capacity may be totally suppressed in a situation of prolonged ischemia or aborted in the absence of innervation. Many general principles have been learned from laboratory studies on rodents, but for reasons of size alone, what works well on rodents may be totally unsuccessful in a human application. Another promising area for the future is the role of regulatory molecules, such as myostatin, in determining the final mass of regenerating muscle (Kirk et al., 2000). Manipulation of the expression of such molecules during the regenerative process could be used as an adjunct measure in attempts to increase the functional mass of muscle, especially in situations where the initial mass of regenerating muscle is compromised.

Of great relevance to mammalian muscle regeneration is the relationship between the number and condition of satellite cells and the ability of a mass of muscle to regenerate. Although numbers of satellite cells, their proliferative potential, and telomere length decreases with age, these changes may not necessarily impact the ability of the muscle to regenerate in vivo. Most organ systems in the body are endowed with considerable biological reserve, and laboratory studies, at least, suggest that even though the absolute capacity of a muscle to regenerate might be less in old age or under certain conditions, there still may be sufficient reserve capacity to allow regeneration to proceed well. Certain pathologic conditions may be exceptions to this process. The deterioration of muscles in children with Duchenne muscular dystrophy has often been attributed to satellite cell exhaustion due to frequent episodes of muscle fiber degeneration and repair, but in this case, it also remains to be determined whether or not the disease process has directly affected the proliferative capacity of the satellite cells.

The availability of genetically modified mice is revolutionizing many aspects of biology, and muscle regeneration will also be a beneficiary of the new technologies. Not only are genetic dissections of complex processes becoming possible, but the ability to combine genetic modification with lineage markers, such as green fluorescent protein, could clarify some of the issues that had formerly seemed intractable.

Many questions remain concerning the epimorphic regeneration of muscles (and other tissues, as well). Probably least is known about factors that control the overall morphogenesis of muscle in both epimorphic regeneration and in normal development. Other questions also remain. One of the most important is what stimulates the initiation of an epimorphic process and what mechanisms are released that allow an epimorphic process to dominate over a tissue regenerative process. This phrasing is in contrast to the traditional explanation that mammalian limbs do not regenerate because healing processes and tissue regeneration inhibit epimorphic regeneration. Another largely unexplored question in epimorphic regeneration is whether in the absence or a severe deficiency of a particular tissue type, cell types that usually do not contribute to the formation of that tissue in the regenerate are recruited to produce the missing tissue. This phrasing of the question would suggest a quite different mechanism from one in which blastema cells differentiate according to their position and not their origin. The nature vs. nurture type questions asked in neural crest biology closely mirror the above, but in that field, they have been asked in a much more penetrating manner. We know virtually nothing about the nature of positional memory, as it applies to muscle regeneration or the regenerating limb as a whole. The knowledge that it is preserved but not expressed in tissue regeneration could provide an entrée to future experimental approaches.

Probably the greatest difference in the approach to the study of regeneration, especially limb regeneration, over the past half century has been the change in viewing the lack of regeneration as a function of a single variable to the recognition that any biological process, even some of the most simple, is a function of a myriad of interacting networks of gene expression and environmental factors and that several different pathways can sometimes lead to the same result.

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