Gene expression in wild-type and MyoD-null satellite cells: regulation of activation, proliferation, and myogenesis

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Abstract

Regeneration is the process of renewal or repair of damaged cells and tissue. In skeletal muscle, regeneration is accomplished by satellite cells, which are rare, mononucleate, mitotically quiescent myogenic precursor cells normally present in undamaged muscle tissue. When stimulated by injury, overuse, or disease, satellite cells will become activated to proliferate and form a pool of replacement myoblasts which will differentiate to replace necrotic muscle fibers. These cells may also have the quality of self-renewal associated with stem cells. Due mainly to technical difficulties caused by their rarity, difficulty of isolation, and lack of identifying markers, satellite cells have not been as well studied as other myogenic cells. Here I present work in which I establish a reliable means of isolating and culturing mouse satellite cells resident on single explanted myofibers; a molecular marker for satellite cells which also yields information about their mechanism of activation, and a method of multiplex single-cell RT-PCR which allows simultaneous detection of six genes from a single satellite cell. Using these techniques, I have determined the temporal coexpression pattern of the four myogenic regulatory factors (MRFs) in single activated satellite cells over the first four days of a regeneration response *in vitro*. I have also assayed satellite cell cDNA pools for expression of genes important in regulating myogenesis, cell cycling, and cell fate decisions in other myogenic lineages. Finally, I have performed these analyses on MyoD-null satellite cells, which are differentiation-deficient *in vivo*, and present possible mechanisms for this based on gene expression; this analysis also suggested a potential marker for activated satellite cells which will return to the reserve satellite cell population and may act as myogenic stem cells.

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Chapter 1

Introduction

Skeletal muscle satellite cells and muscle regeneration

Regeneration is the process by which tissues or organs replace themselves when cells become senescent, worn, or damaged. It may be continuous or sporadic, and replacement of old cells with new may take place on the order of days or years. Often, the processes of growth and renewal are carried out constitutively by the same cells which will, in the case of damage or other stimuli requiring a large-scale and rapid response, be induced to mount a more extreme regeneration response. Determined cells capable of participating in these activities fall into several types. In some tissues, such as liver, terminal differentiation does not preclude further proliferation, and cells which will divide very slowly when maintenance is all that is required are also capable of rapid and extensive proliferation in the case of damage, necrosis, or partial ablation. In others, such as skin, marrow and gut ectoderm, withdrawal from the cell cycle is a required component of terminal differentiation. These tissues maintain nondifferentiating, proliferative 'stem' cells which will supply the population with replacement cells as existing cells age and are discarded, and will accelerate this process when stimulated by outside factors such as injury or infection which require more rapid replacement.

The mechanisms involved in the maintenance of normal tissue and the repair of damaged tissue, even when carried out by the same cells, are often divergent. A repair and regeneration response usually requires stimulation with a constellation of growth factors, cytokines, and other signaling molecules to be activated; these signals may emanate from the damaged cells themselves or from other cell types which are attracted to the wound site. When properly stimulated, stem cells which were not proliferating will become activated to do so, and in tissues where stem cells are already proliferating they will accelerate their division and, in some cases, change the type of cell produced.

This thesis will report on muscle satellite cells, which are normally quiescent stem cells resident in mature skeletal muscle. When muscle tissue is damaged by trauma or disease, satellite cells become activated to proliferate and form a pool of replacement myoblasts, which will contribute to repair and regeneration of the damaged tissue. *Many*

aspects of satellite cell biology were unclear when I began this work, due in large part to technical difficulties in their isolation and purification. Here I will report advances which permitted analysis of gene expression in single satellite cells over the timecourse of a regeneration response in culture. The results of this analysis yielded a series of molecular 'snapshots' of gene expression, which taken together form a framework molecular characterization of these cells which suggests possible mechanisms for key processes such as activation, proliferation, differentiation, and renewal.

Brief review of skeletal myogenesis in the embryo

In studies of avian embryos, it was determined that skeletal muscles of the trunk and limbs derive from cells of the paraxial mesoderm which lie on either side of the neural tube (reviewed in Watler and Christ, 1992). As embryogenesis proceeds, these cells assemble into regular, sequential epithelial spheres (somites) which form and mature in a rostral-to-caudal progression (reviewed in Christ and Ordahl, 1995). Cells of the epithelial somites are initially unspecified as to their eventual fate, but under the influence of a variety of signals emanating from the axial structures (notochord and neural tube) as well as the overlying ectoderm and lateral mesoderm, they become subdivided. The dermomyotome, which forms in the dorsolateral half, remains epithelial and will eventually give rise to skin and muscle, and the sclerotome, which forms in the ventromedial half, becomes mesenchymal and will eventually contribute to bone and cartilage. Cells of the dermomyotome then delaminate ventrally to form the myotome, which will form muscle, and the remaining structure is the dermotome, which will form the dermis (reviewed in Tajbakhsh and Cossu, 1997; Lassar and Munsterberg, 1996).

Myogenic patterning in the somite is dependent on both the signaling molecules available locally and a cell's ability to respond. Thus, somitogenesis and myogenic specification may be perturbed by altering either the local signaling environment or the cell positioned to receive it. Experiments done in avian embryos demonstrate that Sonic hedgehog, Wnts, noggin, BMP4, and FGF5 all influence cell fate in the developing

somite (reviewed in Tajbakhsh and Cossu, 1997). Additionally, the fate of somitic cells can be altered by moving the somite into a new position relative to the sources of these signals (Christ *et al.*, 1992). This plasticity is progressively lost as development proceeds (reviewed in Christ and Ordahl, 1995)

The myotome itself is a positionally determined structure, and is compartmentalized by signals from surrounding tissues. Based on somite rotation and transplantation experiments done in chick-quail chimeras, it was determined that the myotome contains two distinct populations of myogenic precursor cells, and that while cell fate specification is initially plastic it later becomes fixed in most cells (Ordahl and LeDouarin, 1992). Myogenic precursor cells of the medial somite will differentiate within the somite and form the differentiated myotome. These myofibers are mononucleate and are formed as cells delaminate from the rostral edge of the dorsomedial lip of the dermomyotome and traverse the somite longitudinally; they are thought to contribute to future epaxial (intervertebral and paraspinal) muscles (reviewed in Christ and Ordahl, 1995). The myogenic precursors resident in the lateral half of the somite, however, will not differentiate but will instead delaminate from the lateral edge of the dermomyotome and migrate ventrally and laterally. When these cells reach their destination, they will then differentiate into hypaxial muscles (muscles of the limbs and body wall) (Ordahl and LeDouarin, 1992).

These two populations of myogenic cells in the somite also differ in their expression of muscle-specific genes. The muscle-specific basic-helix-loop-helix transcription factors MyoD, myf5, myogenin, and MRF4 are considered to be "master regulatory genes" for myogenesis, in part because forced expression of any one in many nonmuscle cell types will serve to upregulate expression of all four and initiate the entire myogenic program (reviewed in Weintraub, 1993). These factors, in heterodimers formed with ubiquitous bHLH proteins, bind to a common consensus sequence (CANNTG, or E-box) and are required for transcription of many muscle-specific genes

(reviewed in Molkentin and Olson, 1996; Yun and Wold, 1996). While these proteins are very similar in structure, bind to the same core concensus sequence, and have similar effects in cell culture experiments, *in vivo* their functions appear to be distinct and only partially redundant.

Myf5 is the first myogenic regulatory factor (MRF) detected in the mouse embryo; it is expressed in myogenic precursor cells of the medial somite which will form the differentiated myotome and epaxial muscles of the trunk (Ott *et al.*, 1991). These cells then begin to express myogenin as they terminally differentiate (Sassoon *et al.*, 1989) and later express MRF4 and MyoD (reviewed in Ontell *et al.*, 1995). The cells of the lateral myotome, which will form hypaxial musculature, however, first express MyoD (Smith *et al.*, 1994). These differences in expression pattern between the two myogenic compartments in the somites led to speculation that either myf5 or MyoD is required for myogenic specification in general, and that myf5 is specifically required for presumptive epaxial myogenic precursor cells, while MyoD is specifically required by the presumptive hypaxial population. This was supported by evidence that deletion of either MyoD or myf5 led to upregulation and expansion of expression of the other in developing somites (Braun *et al.*, 1994; Rudnicki *et al.*, 1992). Presumably this is due to compensation by one population of myoblasts (epaxial in $MyoD^{-/-}$, hypaxial in $myf5^{-/-}$) for the other by expanding and repopulating the cellular compartment of the failed population. In addition, animals homozygous for deletion of both genes fail to specify myoblasts (Rudnicki *et al.*, 1993). Thus myf5 and MyoD are considered to be lineage-specific determination factors during somitic myogenesis.

Mice lacking the product of the *myogenin* gene, which is expressed by cells which have already expressed either MyoD or myf5 or both, successfully specified myoblasts which proliferated and migrated as they should, but which are unable to differentiate *in vivo* (Nabeshima *et al.*, 1993; Hasty *et al.*, 1993). This result, along with association of myogenin expression in differentiating myoblasts *in vivo* and upregulation

of myogenin in tissue cultures upon differentiation, led to the classification of myogenin as a differentiation factor. Based on deletion studies, MRF4 does not appear to be strictly required for myogenic differentiation in the embryo (Patapoutian *et al.*, 1995; Zhang *et al.*, 1995). However, based on its expression patterns during myogenesis *in vivo* and *in vitro* and on its impact in combination with MyoD, it is also calssed with myogenin as a differentiation factor.

Satellite cells are responsible for skeletal muscle regeneration

While the epaxial, hypaxial, and limb muscle lineages have been extremely well-studied and characterized, and the fourth myogenic lineage (that of the head and neck, which forms from unsegmented paraxial mesoderm rostral of the first somite (Couly *et al.*, 1992) and will not be further discussed here) has been the subject of some embryological and molecular studies, there exists a potential fifth myogenic lineage about which a great deal less is known. Satellite cells are mitotically quiescent, mononucleate myogenic precursor cells normally present in adult muscle. They were first identified by electron microscopy as mononucleate cells associated with the periphery of *Xenopus* muscles (hence the name satellite cells) (Mauro, 1961). Prior to this time, the role of mononucleate myoblasts in regeneration had been recognized, however their origin and character were unknown (reviewed in Bischoff, 1994). It has since been determined that satellite cells are activated by local muscle damage due to disease, acute trauma, or possibly overuse, proliferate extensively to form a pool of replacement myoblasts which differentiate to replace the damaged myofibers, and may have a 'stem cell'-like ability to generate both differentiating and reserve satellite cell progeny (reviewed in Bischoff, 1994).

Major questions in the satellite cell field are their embryonic origin and lineage history, including how they are related to myocytes which form the regular musculature and whether satellites comprise a distinct lineage or are simply a remnant of postnatal myogenesis; the mechanism(s) by which they are committed to the satellite lineage and

become quiescent rather than differentiating during development; how they become activated by local tissue damage to mount a regeneration response; how the satellite cell myogenic program resembles or differs from the better-known embryonic programs; how a response, once initiated, is maintained and amplified until the damaged tissue has been replaced; what goes wrong when satellite cells are unable to fulfill their function; and how the reserve population of satellite cells which will re-enter the quiescent state is determined. In this brief review I will address these questions as they stood when I began, and discuss advances made by myself and others towards their resolution.

What is the embryonic origin of satellite cells?

Opinions on whether or not satellite cells are considered to comprise a separate embryonic lineage tend to differ based on the system in which the work is being done (avian or mammalian) as well as the working definition of what constitutes a satellite cell. Based in part on differential resistance to certain phorbol esters and differential expression of acetylcholine-handling proteins and in part on the identification of myofiber-associated mononucleate cells during histological analysis, some workers believe that satellite cells arise as a distinct fetal lineage at about day 17 of embryonic development in the mouse (reviewed in Bischoff, 1994). Using this definition of satellite cells, these cells proliferate extensively during late fetal and postnatal development and are responsible for postnatal muscle growth during secondary myogenesis. By approximately three weeks after birth in the mouse, this proliferation appears to slow considerably, and the first satellite cells with heterochromatic nuclei (a characteristic of quiescent satellite cells in the adult) appear (reviewed in Bischoff, 1994). This view of satellite cells as a separate lineage is widely-held by researchers using avian embryos, based upon differential expression of certain structural proteins (Hartley *et al.*, 1992; Feldman and Stockdale, 1992).

Based purely on a functional definition, however, satellite cells are defined as quiescent myogenic precursor cells present in the adult which become activated *in*

response to muscle damage and carry out the myogenic regeneration program. Since no direct lineal relationship has been established between proliferating cells during secondary myogenesis and satellite cells of the adult, and since it is unclear if these cells are homogeneous in their ability to contribute to the later satellite population, many other workers in the field remain undecided concerning the conclusion that satellite cells are a separable embryonic lineage. It has been suggested (Schultz and McCormick, 1994) that satellite cells may arise as descendants of secondary myoblasts which, due to contact inhibition, depletion of growth factors, and possibly some intrinsic program fail to differentiate and enter a satellite state instead. The resolution of the status of satellite cells as a lineage, and the point at which they diverge from other myogenic cell types, may not be resolved until embryonic myogenic precursor cells of different ages are identified and labeled, and the resulting adult animals examined for satellite progeny of the marked cells.

Very recently results have been published indicating that cells fitting the description of satellite cells reside in the bone marrow and can be induced to take part in muscle regeneration (Ferrari *et al.*, 1988), apparently accessing damaged muscle tissue through the circulation. This raises the questions of the embryonic origin of these cells, their myogenic commitment status, their relationship or identity with satellite cells resident in quiescent muscle, and their function (if any) in normal muscle regeneration. Are these cells related to (or do they conceivably give rise to) satellite cells resident in undamaged muscle, and are they a functional component of the regeneration machinery or merely a fluke of development? Compared to regeneration mediated by conventional resident satellite cells, the myogenesis of these marrow-derived cells is both significantly delayed and quantitatively much less robust. This suggests that, if they do possess a function in normal myogenesis, it is probably secondary to resident satelite cells. It could be that these cells constitute the replacement population of satellite cells which will reside in muscle which has been newly-formed using the entire population of satellite cells that

had present before the damage was incurred. However, this appears unlikely given that the majority of nuclei derived from the marrow population were located in differentiated fibers rather than at their periphery. Another possibility is that these cells are continually being produced in the marrow and released into the circulation at low levels, and that they constitute a slow but steady source of new satellite cells. Again, there is evidence that this may may not be the case based on the equivalent number of clonable satellite cells present in whole muscle after successive transplantations (Mong, 1988). A less interesting hypothesis is that these cells are determined myoblasts from the somite which, due to mismigration in the embryo, were transported to an enviroment (the stroma) in which they could survive but were not stimulated to divide or differentiate, and that in the course of an inflamatory response after muscle damage they were transported to a region where they could and did receive and respond to signals to differentiate. It will be interesting to follow this story and, when more data become available, re-evaluate the possible role of these apparently nonmuscle cells in muscle regeneration.

How do quiescent satellite cells become activated?

When this work was begun, many studies of the mitogenic properties of various growth factors for satellite cells had been done in myoblast cell lines or in mass cultures of primary (usually neonatal) myoblasts. While many factors such as fibroblast growth factors (FGFs), epidermal growth factors (EGFs), insulin-like growth factors (IGFs), and transforming growth-factor β (TGFβ) could affect proliferation in activated satellite cells, no specific factor had been found which had the ability to activate quiescent satellite cells and cause them to proliferate. This could be accomplished by a saline extract of crushed muscle, however the active factor had not been purified to homogeneity (Bischoff, 1986). In recent years, due to work which will be presented here on the expression of the c-met receptor tyrosine kinase in satellite cells and converging work examining the c-met ligand hepatocyte growth factor/scatter factor (HGF/SF), it has been determined that HGF/SF is present in crushed muscle extract and capable of inducing

proliferation in quiescent satellite cells *in vitro* and *in vivo* (Allen *et al.*, 1995; Tatsumi *et al.*, 1998), and that quiescent satellite cells express c-met and are therefore capable of responding to the activation signal (Cornelison and Wold, 1997). This strongly suggests that HGF/SF, transduced by c-met, mediates activation of quiescent satellite cells.

How similar is satellite myogenesis to embryonic myogenesis?

Once satellite cells become activated they, like embryonic myogenic precursor cells, begin to express myogenic regulatory factors (MRFs). Analysis of MRF expression has been done using various methods such as *in situ* hybridization (Grounds *et al.*, 1992), RT-PCR of mass cultures (Smith *et al.*, 1994), and immunohistochemistry of satellite cells on isolated muscle fibers (Yablonka-Reuveni and Rivera, 1994). These had established roughly similar timecourses for MRF expression in activated satellite cells, basically concluding that quiescent satellite cells express no MRFs, while MyoD expression begins in the first 24 hours after activation and is followed in the next 24 hours by myf5, myogenin, and MRF4. These were important results and led to speculation that MyoD, because it is expressed first, might be uniquely required for satellite myogenesis. However, techniques such as *in situ* hybridization and immunohistochemistry, while they delivered single-cell resolution, could only be used to assay one or two genes at a time; and mass culture RT-PCR, while it allowed for the assay of many genes simultaneously, gave no measurement of heterogeneity within the cellular population. RNA collected from these cultures was also potentially contaminated by other cell types, which are endemic in satellite cell cultures.

At the time I began this work, no reliable molecular marker existed for mouse satellite cells, and they were identified by expression of MRFs once they had become activated and entered myogenesis. This had major disadvantages due to the necessity of waiting until after regeneration was well underway before satellite cells could be identified, and because it was plausible (and I later demonstrated) that at any given time, some satellite cells will not express any of the MRFs. To overcome this technical

difficulty, I adapted the specialized technique of single fiber explant culture, in which myofibers are enzymatically isolated from adult muscle and cultured along with their resident satellite cells, to be used on multiple muscles of the mouse; previously it had been thought possible only with a very short foot muscle of the rat. The advantages of this technique are that it allows identification of satellite cells based on their morphology, and therefore does not rely on muscle-specific gene expression; because of the way satellite cells decorate the fibers it is also possible to observe many more satellite cells much more readily than would be possible even in thin serial sections. Using satellite cell fiber culture in conjunction with multiplex single-cell PCR, which permits analysis of mRNA expression for several genes to be obtained from the same cell, I examined the satellite cell myogenic program and determined the progression of MRF coexpression states it involves. Satellite cells were seen to have a unique program of MRF expression and progression, distinct from that of either of the embryonic lineages. This work is presented in Chapter 2.

How are growth and differentiation balanced?

Once an activation response has been successfully initiated, it must be maintained and modulated to provide balanced and continued proliferation and differentiation. This must be the result of the coordinated effects of positive and negative myogenic regulatory factors, growth factor signaling, cell cycle regulation, and cell fate choices. While a great deal of work has been done on the MRFs and growth factors/receptors, as mentioned earlier, no large-scale screen of genes which may regulate these processes had been done, especially in a population in which all cells analyzed were certifiably satellite cells. I therefore analyzed pools of satellite cell cDNA over the first four days in culture to determine which of several genes considered relevant to myogenic progression in other cell types are expressed in satellite cells. I found that, again, satellite cells possess a unique program of gene expression unlike those seen in embryonic or cultured myoblasts. With this data, it should be possible to inquire further into the mechanisms

governing promotion and balance of proliferation and differentiation in satellite cells. This work is presented in Chapter 3.

What happens when satellite cells don't work?

Satellite cells are of clinical relevance because of their involvement in muscle repair, as well as their apparent acquired inability to successfully regenerate muscle during the terminal phases of neuromuscular wasting disease such as Duchenne's muscular dystrophy and during senile muscle atrophy. Thus, factors leading to incompetence of satellite cells in regeneration, and possible accompanying changes or blocks in their myogenic program, are of great clinical as well as biological interest. Is satellite cell failure due to intrinsic defects in the cell population (such as a possible loss of competence after many activations or cell divisions), or to a lack in an essential external mediator of the satellite cell response, or both?

A naturally-occurring mouse model for muscular dystrophy is the mdx mouse, which was found to have mutations in the dystrophin gene (Ryder-Cook *et al.*, 1988). However, unlike human patients, these mice show intense degeneration balanced by rapid regeneration from 3 weeks to 6 months of age, and thereafter are phenotypically normal (reviewed in Anderson *et al.*, 1991). Therefore the mdx mouse, while it is a good model for muscle regeneration, is a poor one for the study of satellite cell deficiencies because the murine satellite cells successfully maintain a constant and vigorous regeneration response, sparing the affected mice both many symptoms experienced by human patients and the terminal acute phase of the disease. Mouse mutants which *have* been shown to have severe regenerative disorders lack MyoD (Megeney *et al.*, 1996) and FGF6 (Floss *et al.*, 1997); severely decreased expression of MyoD in FGF6-null mice suggests that both genes may be acting in the same pathway. Both lines have been characterized as having apparently normal phenotypes during development and postnatal life save for an inability to regenerate muscle due to satellite

cells which become activated and proliferate, but rarely terminally differentiate, thus linking satellite cell dysfunction to both extrinsic and intrinsic factors.

When I assayed gene expression in single and pooled MyoD-null satellite cells as had previously been done for wild-type, the results suggested that the differention-deficient phenotype observed in MyoD-null mice may be due to failure to express the differentiation-promoting factor MRF4 and severely reduced expression of m-cadherin, a homotypic adhesion molecule which has been implicated in myoblast fusion and differentiation (Zeschnigk *et al.*, 1995). This work is presented in Chapter 4.

Another model of satellite cell insufficiency is the muscle hypotrophy associated with aging in both mouse and humans. Presumably, these satellite cells have *not* experienced gene deletions leading to a regeneration defect and were once competent to mount a full regeneration response. What, then, causes their failure to fully recapitulate regeneration as it would be in a younger mouse? Possible clues to this come from an elegant study in which satellite cell populations from young or old mice were stimulated with crushed muscle extract (the best satellite cell mitogen known at the time) which was also derived from either young or old mice. It was found that, while extract from old muscles was weakly mitogenic for young satellite cells and was not at all mitogenic for old satellite cells, the extract made from young muscles promoted robust cell division in both young and old satellite cells, although young cells were still more responsive. The same results could be obtained by coculture of young and old satellite cells with differentiated myotubes from each source (Mezzogiorno *et al.*, 1993). These results imply that both extrinsic and intrinsic factors affect satellite cell function in senile muscle.

What factor(s) mediate a return to quiescence?

Finally, it is believed that a critical part of the satellite cell response is the specification either before or during regeneration of a subset of satellite cells which will contribute not to the nascent terminally differentiated muscle but to the satellite cells which will inhabit it. Data supporting this hypothesis are the observations that the

number of resident satellite cells in a given muscle is not reduced even following repeated cycles of activation, proliferation and differentiation (Mong, 1988) and that cells meeting the criteria for satellite 'stem' cells can be isolated from clones derived from single satellite cells in that these cells, when cloned and expanded, will also yield some stem-like cells (Baroffio *et al.*, 1996; Morgan *et al.*, 1994). Determination of which satellite cell progeny will retain or acquire "stemness" has been suggested to be stochastic and based on loss of growth factor receptors during slightly assymmetric divisions (Angello and Hauschka, 1996), but a more programmed mechanism remains a possibility. For these reasons, knowledge of which of the known molecular mediators of cellular asymmetry (such as genes of the Notch system) are expressed within the satellite cell population may be a key step in answering this question.

Analysis of satellite cells from MyoD-null animals for a wide panel of genes yielded what may be an important clue about the return to quiescence after a regeneration response has ended. It had been suggested that MyoD-null satellite cells, unable to complete a regeneration response, would instead return to quiescence *en masse* (Megeney *et al.*, 1996). Consistent with this hypothesis, I observed MyoD-null satellite cells apparently returning to a position beneath the basal lamina; cDNA from cell pools enriched for these cells expressed Msx-1, a gene which is usually detected only in very recently quiescent satellite cells. Msx-1 is expressed by committed but nondifferentiating myoblasts in the embryo (Wang and Sassoon, 1995) and is thought to repress differentiation through its activity as a transcriptional inhibitor (Catron *et al.*, 1996). Therefore, upregulation in activated satellite cells could conceivably be a molecular marker for cells that will renew the satellite pool.

References

Allen, R. E., Sheehan, S. M., Taylor, R. G., Kendall, T. L. and Rice, G. M. (1995). Hepatocyte growth factor activates quiescent skeletal muscle satellite cells in vitro. *J. Cell. Phys.* **165**, 307-312.

Anderson, J. E., Liu, L. and Kardami, E. (1991). Distinctive patterns of basic fibroblast growth factor (bFGF) distribution in degenerating and regenerating areas of dystrophic (mdx) striated muscles. *Dev. Biol.* **147**, 96-109.

Angello, J. C. and Hauschka, S. D. (1996). Skeletal muscle satellite cells: timelapse videomicroscopic evidence that renewal is stochastic. *BAM* **6**, 491-502.

Baroffio, A., Hamann, M., Bernheim, L., Bochaton-Piallat, M.-L., Gabbiani, G. and Bader, C. R. (1996). Identification of self-renewing myoblasts in the progeny of single human muscle satellite cells. *Differentiation* **60**, 47-57.

Bischoff, R. (1994) *In* "Myology" (A. G. Engel andC. Franzini-Armstrong, Eds.), pp. 97-118. McGraw-Hill, New York.

Bischoff, R. (1986). A satellite cell mitogen from crushed adult muscle. *Dev. Biol.* **115**, 140-147.

Braun, T., Bober, E., Rudnicki, M. A., Jaenisch, R. and Arnold, H.-H. (1994). MyoD expression marks the onset of skeletal myogenesis in *Myf-5* mutant mice. *Development* **120**, 3083-3092.

Catron, K. M., Wang, H., Hu, G., Shen, M. M. and Abate-Shen, C. (1996). Comparison of MSX-1 and MSX-2 suggests a molecular basis for functional redundancy. *Mech. Dev.* **55**, 185-199.

Christ, B., Brand-Saberi, B., Grim, M. and Wilting, J. (1992). Local signalling in dermomyotomal cell type specification. *Anat. Embryol.* **196**, 505-510.

Christ, B. and Ordahl, C. P. (1995). Early stages of chick somite development. *Anat. Embryol.* **191**, 381-396.

Cornelison, D. D. W. and Wold, B. J. (1997). Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. *Dev. Biol.* **191**, 270-283.

Couly, G. F., Coltey, P. M. and LeDouarin, N. M. (1992). The developmental fate of the cephalic mesoderm in quail-chick chimeras. *Development* **114**, 1-15.

Feldman, J. L. and Stockdale, F. E. (1992). Temporal appearance of satellite cells during myogenesis. *Dev. Biol.* **153**, 217-226.

Ferrari, G., Cusella-DeAngelis, G., Coletta, M., Paolucci, E., Stornaiuolo, A., Cossu, G. and Malvilio, F. (1988). Muscle regeneration by bone marrow-derived myogenic progenitors. *Science* **279**, 1528-1530.

Floss, T., Arnold, H.-H. and Braun, T. (1997). A role for FGF-6 in skeletal muscle regeneration. *Genes Dev.* **11**, 2040-2051.

Grounds, M. D., Garrett, K. L., Lai, M. C., Wright, W. E. and Beilharz, M. W. (1992). Identification of skeletal muscle precursor cells *in vivo* by use of MyoD1 and myogenin probes. *Cell Tiss. Res.* **267**, 99-104.

Hartley, R. S., Bandman, E. and Yablonka-Reuveni, Z. (1992). Skeletal muscle satellite cells appear during late chicken embryogenesis. *Dev. Biol.* **153**, 206-216.

Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J. M., Olson, E. N. and Klein, W. H. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the *myogenin* gene. *Nature* **364**, 501-506.

Lassar, A. B. and Munsterberg, A. E. (1996). The role of positive and negative signls in somite patterning. *Curr. Op. Neurobiol.* **6**, 57-63.

Mauro, A. (1961). Satellite cells of skeletal muscle fibers. *J. Biophys. Biochem. Cytol.* **9**, 493-495.

Megeney, L. A., Kablar, B., Garrett, K., Anderson, J. E. and Rudnicki, M. A. (1996). MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes Dev.* **10**, 1173-1183.

Mezzogiorno, A., Coletta, M., Zani, B. M., Cossu, G. and Molinaro, M. (1993). Paracrine stimulation of senescent satellite cell proliferation by factors released by muscle or myotubes from young mice. *Mech. Aging Dev.* **70**, 35-44.

Molkentin, J. D. and Olson, E. N. (1996). Defining the regulatory networks for muscle development. *Curr. Op. Gen. Dev.* **6**, 445-453.

Mong, F. S. F. (1988). Satellite cells in the regenerated and regrafted skeletal muscles of rats. *Experientia* **44**, 601-603.

Morgan, J. E., Beauchamp, J. R., Pagel, C. N., Peckham, M., Ataliotis, P., Jat, P. S., Noble, M. D., Farmer, K. and Partridge, T. A. (1994). Myogenic cell lines derived from transgenic mice carrying a thermolabile T antigen: a model system for the derivation of tissue-specific and mutation-specific cell lines. *Dev. Biol.* **162**, 468-498.

Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Li, S., Nonaka, I. and Nabeshima, Y.-i. (1993). *Myogenin* gene disruption results in perinatal lethality because of severe muscle defect. *Nature* **364**, 532-535.

Ontell, M., Ontell, M. P. and Buckingham, M. (1995). Muscle-specific gene expression during myogenesis in the mouse. *Micr. Res. Tech.* **30**, 354-365.

Ordahl, C. P. and LeDouarin, N. M. (1992). Two myogenic lineages within the developing somite. *Development* **114**, 339-353.

Ott, M.-O., Bober, E., Lyons, G., Arnold, H. and Buckingham, M. (1991). Early expression of the myogenic regulatory gene, *myf-5*, in precursor cells of skeletal muscle in the mouse embryo. *Development* **111**, 1097-1107.

Patapoutian, A., Yoon, J. K., Miner, J. H., Wang, S., Stark, K. and Wold, B. (1995). Disruption of the mouse MRF4 gene identifies multiple waves of myogenesis in the myotome. *Development* **121**, 3347-3358.

Rudnicki, M. A., Braun, T., Hinuma, S. and Jaenisch, R. (1992). Inactivation of *MyoD* in mice leads to up-regulation of the myogenic HLH gene *Myf-5* and results in apparently normal muscle development. *Cell* **71**, 383-390.

Rudnicki, M. A., Schnegelsberg, P. N. J., Stead, R. H., Braun, T., Arnold, H.-H. and Jaenisch, R. (1993). MyoD or myf-5 is required for the formation of skeletal muscle. *Cell* **75**, 1351-1359.

Ryder-Cook, A. S., Sicinski, P., Thomas, K., Davies, K. E., Worton, R. G., Barnard, E. A., Darlison, M. G. and Barnard, P. J. (1988). Localization of the mdx mutation within the mouse *dystrophin* gene. *EMBO J.* **7**, 3017-3021.

Sassoon, D., Lyons, G., Wright, W. E., Lin, V., Lassar, A., Weintraub, H. and Buckingham, M. (1989). Expression of two myogenic regulatory factors myogenin and MyoD1 during mouse embryogenesis. *Nature* **341**, 303-307.

Schultz, E. and McCormick, K. M. (1994). Skeletal muscle satellite cells. *Rev. Physiol. Biochem. Pharmacol.* **123**, 213-257.

Smith, T. H., Kachinsky, A. M. and Miller, J. B. (1994). Somite subdomains, muscle cell origins, and the four muscle regulatory factor proteins. *JCB* **127**, 95-105.

Smith, C. K. II, Janney, M. J. and Allen, R. E. (1994). Temporal expression of myogenic regulatory genes during activation, proliferation, and differentiation of rat skeletal muscle satellite cells. *J. Cell. Phys.* **159**, 379-385.

Tajbakhsh, S. and Cossu, G. (1997). Establishing myogenic identity during somitogenesis. *Curr. Op. Gen. Dev.* **7**, 634-641.

Tatsumi, R., Anderson, J. E., Nevoret, C. J., Halevy, O. and Allen, R. E. (1998). HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. *Dev. Biol.* **194**, 114-128.

Wang, Y. and Sassoon, D. (1995). Ectoderm-mesenchyme and mesenchyme-mesenchyme interactions regulate Msx-1 expression and cellular differentiation in the murine limb bud. *Dev. Biol.* **168**, 374-382.

Watler, F. and Christ, B. (1992). The basic embryology of skeletal muscle formation in vertebrates: the avian model. *Sem. Dev. Biol.* **3**, 217-227.

Weintraub, H. (1993). The MyoD family and myogenesis: redundancy, networks, and thresholds. *Cell* **75**, 1241-1244.

Yablonka-Reuveni, Z. and Rivera, A. J. (1994). Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibers. *Dev. Biol.* **164**, 588-603.

Yun, K. and Wold, B. (1996). Skeletal muscle determination and differentiation: story of a core regulatory network. *Curr. Op. Cell Biol.* **8**, 877-889.

Zhang, W., Behringer, R. and Olson, E. N. (1995). Inactivation of the myogenic bHLH gene MRF4 results in up-regulation of myogenin and rib anomalies. *Genes Dev.* **9**, 1388-1399.

Chapter 2

Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells

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Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells

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Abstract

Repair and regeneration of adult skeletal muscle are mediated by satellite cells. In healthy muscle these rare mononucleate muscle precursor cells are mitotically quiescent. Upon muscle injury or degeneration, members of this self-renewing pool are activated to proliferate and then differentiate. Here we analyzed in single satellite cells the expression of a set of regulatory genes that are candidates for causal roles in satellite cell activation, maturation and differentiation. Individual cells were identified as satellite cells and selected for analysis based on their physical association with single explanted myofibers or their position beneath the basal lamina in unperturbed muscle tissue. Using a multiplex single cell RT-PCR assay we simultaneously monitored expression of all four MyoD family regulators of muscle determination and differentiation (MRFs) together with two candidate markers of satellite cell identity, c-met and m-cadherin. By making these measurements on large numbers of individual cells during the timecourse of satellite cell activation, we were able to define which expression states (possible combinations of the six genes) were represented and to specify how the representation of each state changed with time. Activated satellite cells began to express either MyoD or myf5 first among the MRFs; most cells then expressed both myf-5 and MyoD simultaneously; myogenin came on later in cells expressing both MyoD and myf5; and many cells ultimately expressed all four MRFs simultaneously. The results for fiber-associated satellite cells from either predominantly fast or slow muscles were indistinguishable from each other. The c-met receptor tyrosine kinase was also monitored because it is a candidate for mediating activation of quiescent satellite cells (Allen *et al.*, 1995), and because it might also be a candidate molecular marker for satellite cells. A significant difficulty in studying mouse satellite cells has been the absence of molecular markers that could identify them in the quiescent state before expression of MRFs or desmin and distinguish them from fibroblasts. We show here that c-met is expressed at both RNA and protein levels by all myofiber-associated satellite cells from the time of explant

through the course of activation, proliferation and differentiation. c-met was not detected in muscle derived fibroblasts or in other mononucleate cells from healthy muscle explants. When compared directly with m-cadherin, which has previously been suggested as a marker for quiescent satellite cells, m-cadherin mRNA was detected only in a small subset of satellite cells at early times after myofiber explant. However, at late times following activation (by 96 hours in this fiber culture system), c-met and m-cadherin were uniformly co-expressed. From the individual satellite cell expression types observed, a model of the satellite cell population at rest and during the timecourse of activation was generated.

Introduction

In adult mouse skeletal muscle the majority of myonuclei are located in syncytial myotubes that were formed by myoblast fusion during fetal and postnatal development. These myonuclei are terminally postmitotic. However, a small fraction of myonuclei are in mononucleate precursor cells called muscle satellite cells which are located between the basal lamina and sarcolemma of myofibers (Mauro, 1961), reviewed in (Bischoff, 1994). In healthy adult rodent muscle, satellite cells are mitotically quiescent and do not detectably express MRFs (MyoD family muscle regulatory factors) (Grounds *et al.*, 1992). When stimulated by damage to the muscle or by explant and culture manipulations, some fraction of satellite cells are activated to re-enter the cell cycle and/or to express myogenic regulatory factors. The resulting myoblasts subsequently differentiate and fuse to form new replacement myofibers (Bischoff, 1986a) reviewed in (Bischoff, 1994).

Although all skeletal muscle regeneration in mammals is attributed to satellite cells, including recovery phases of neuromuscular wasting diseases, we presently have an incomplete picture of the molecular mechanisms involved in establishing and maintaining the quiescent state, regulating activation and subsequent muscle differentiation, and sustaining the satellite population through multiple rounds of regeneration. This is partly due to technical problems caused by satellite cell rarity within muscle tissue and associated difficulties with identifying them, especially in the quiescent state. For the mouse there are currently no reliable molecular markers that can prospectively identify resting satellite cells or activated cells that do not yet express MRFs or desmin. This work begins to address the problem by evaluating the expression of two candidate markers of satellite cell identity, m-cadherin and c-met, in sets of individual satellite cells during the course of activation and differentiation.

Expression of c-met and m-cadherin is also relevant to muscle regeneration because of their postulated functions. c-met transduces mitogenic, migratory or

morphogenetic signals in a variety of nonmuscle tissues during development and wound healing in response to its ligand HGF/scatter factor (reviewed in (Matsumoto and Nakamura, 1996)Matsumoto and Nakamura, 1996). In muscle development HGF/c-met signaling is essential for proper emigration of muscle precursor cells of the axial lineage during embryogenesis (Bladt *et al.*, 1995). And in a mass culture system, exogenous HGF accelerates cell proliferation of freshly isolated rat satellite cells, suggesting that it may be an activation factor *in vivo* (Allen *et al.*, 1995). m-cadherin is a calcium dependent homophilic cell adhesion molecule that is expressed prominently during fetal myogenesis (Rose *et al.*, 1994). As the member of the cadherin family expressed predominately during skeletal myogenesis, it has been suggested that m-cadherin plays a significant role in alignment and fusion of myoblasts to form and expand developing myotubes (Donalies *et al*, 1991; Cifuentes-Diaz *et al.*, 1995). It has also been detected in activated satellite cells during regenerative responses after muscle damage (Moore and Walsh, 1993; Irintchev *et al.*, 1994.)

Little is known about the nature and extent of cell to cell heterogeneity of gene expression in satellite cells at present, but at least two types can be hypothesized. First, some distinction is expected between satellite cells which will differentiate as myotubes and those which maintain the progenitor pool for subsequent rounds of regeneration. Second, satellite cells from muscles of different fiber types or of different embryonic sublineages may retain distinct identities. The emerging picture for skeletal muscle development during embryogenesis is that specific combinations of regulatory genes, rather than any single myogenic master regulator, are responsible for directing determination and differentiation (reviewed in Yun and Wold, 1996). Genetic analyses of knockout mice have established that the four members of the MRF family of transcription factors are individually and collectively important for muscle precursor development and for terminal differentiation (reviewed in (Olson and Klein, 1994; Yun and Wold, 1996)Olson and Klein, 1994). It is generally believed that the MRFs will

have similarly important functions in muscle regeneration, and evidence for a specific requirement for MyoD was recently reported for regeneration in dystrophic mdx mice (Megeney *et al.*, 1996). Prior studies have also shown that these regulators are not expressed detectably in unactivated satellite cells, but that all four are transcribed beginning at different times over the course of activation in mass cultures (Table 1 and references therein). However the combinatorics of MRF family expression at the single cell level remain only partly known for embryo or satellite cell populations. For the first time we could address this problem because satellite cells are physically identifiable by positional criteria in our fiber cultures and because they are experimentally accessible for single cell RNA analysis. Thus, co-expression of c-met, m-cadherin, myf-5, MyoD, myogenin and MRF4 mRNAs was measured in a large number of individual myofiber-associated satellite cells. From these data we were able to reconstruct the satellite cell population expression pattern as the sum of distinct individual expression patterns. The sets of expression types observed over time suggested a simple developmental model for MRFs during satellite cell activation and differentiation.

Materials and Methods

Fiber isolation and culture

Myofibers from adult mice (>100 days old) were isolated essentially as in (Bischoff, 1986a), with these exceptions: Fibers were isolated from multiple muscles, including the anterior tibialis, soleus, and longissimus dorsi; in our hands it was not necessary to use only short, tendonous muscles. Dissected muscles were treated with 400 U/ml collagenase type I (Worthington) in PBS at 37° for 60 minutes. Muscle masses were not triturated, but were manually rocked before individual fibers were harvested with a fire-polished Pasteur pipette, preflushed with medium to prevent sticking. Fiber cultures were grown in DMEM supplemented with 10% equine serum (Hyclone), 5% chick embryo extract (Sigma), penicillin-streptomycin (Gibco), 2.5 μg/ml amphotericin B (Sigma) at 37° in a humidified incubator at 5% CO2. 10 μM BrdU (Boehringer) was supplied continuously in the medium to monitor proliferation history. Fresh individual fibers isolated in this way were up to 1.4 cm in length, averaging 5-7 mm and having 62 +/- 8.6 myonuclei and 0.77 +/- 0.08 satellite cells per mm. To decrease possible contamination from non-satellite cell types which were carried from the isolation, after 24 and 48 hours of culture individual fibers were re-picked with a Pasteur pipette and transferred to a fresh dish of medium (by this time, most contaminant cells emigrate from the fibers, apparently due to higher affinity for the culture dish). To derive muscle fibroblast cultures, cells adhering to the dish after fibers are transferred away which appear to be fibroblasts are identified and surrounded with a cloning cylinder, then trypsinized and removed to a new dish and expanded.

Marcaine treatment Live/Dead staining

To identify and harvest individual satellite cells immediately after fibers had been dissociated, fresh fiber preparations were treated with the myotoxic anesthetic Marcaine. The fibers and their satellite cells could then be stained with Live/Dead reagents

(Molecular Probes,) which consist of calcein AM, a fluorescent vital dye activated only in the cytoplasm of living, viable cells, and an ethidium homodimer, which stains the nuclei of dead cells. Marcaine treated fibers would hypercontract, and after treatment their cytoplasm could no longer activate calcein AM and their nuclei stained with the dead-cell reagent. Satellite cells are not affected by Marcaine treatment and can thus be identified by calcein AM staining. Fresh fiber preparations were treated with Marcaine (Winthrop; .05% in PBS) for 20-30 minutes at 25°, rinsed twice in PBS, and treated with 2μM calcein AM (Molecular Probes) for 20 minutes at 25°. We note that the fibers in which Marcaine caused most extreme hypercontraction appear to be those with ends broken from the explant surgery.

Single cell multiplex RT-PCR

The design of the multiplex single cell RT-PCR protocol (Fig. 1) was derived from that of Kato et. al. (Kato *et al.*, 1997), in which a patch-clamp pipette was used to harvest a sample of cell cytoplasm. We modified this procedure with the goal of maximizing and normalizing the amount of RNA obtained from each cell. Fibers were transferred to a dish of sterile, RNAase-free PBS immediately before harvest; collection pipettes were filled with RNAase-free PBS. The orifice of the micropipette was enlarged to be only slightly smaller than the circumference of a satellite cell, and the entire cell was collected into the micropipette. Each collected cell was used individually as substrate for reverse transcription with M-MLV RT using random primers (7.5 μl per cell RT mix containing 1x (2 μl 5x) RT buffer (Boehringer), 40 mM (4 μl 100 mM) DTT, 0.5 mM dNTPs (0.5 μl of 10 mM), 10 U (0.2 μl) RNAase inhibitor (Boehringer), 200 ng Random Primers (0.4 μl of 500 ng/μl) (Boehringer), 0.4 μl DEPC water; keep mix on ice at all times. Add cells in approximately 2 μl of collection buffer; after all cells have been added to mix tubes, add 10 U (0.5 μl) M-MLV RT (Boehringer) per tube for a final volume of 10 μl; incubate at 37° for 1 hour). In some control reactions (specified in text

and figure legends) several individual cells were collected and then pooled for analysis of sensitivity and reproducibility. The entire cDNA reaction was then added to a PCR reaction containing an outside primer pair for each gene of interest (for 1.0 cell input, 50 µl PCR reaction containing 1x reaction buffer (Qiagen), 200 nmol dNTPs, 200 µmol each outside primer, 2.5 U Taq polymerase (Qiagen); 35 cycles PCR at 60° annealing.) In reactions where more than four genes were monitored from a single cell, the cDNA reaction was first divided in half, and each half was then used in a 1/2 scale PCR reaction with primers corresponding to a subset of the genes to be assayed. All outside primer sets were designed to cross at least one intron, so that any products derived from unprocessed hnRNA or from genomic DNA could be distinguished from messenger RNA templates; this design feature is critical when using whole cells containing nuclei as the substrate. After the first round of PCR, the reaction was diluted 1:1000 into separate secondary PCR reactions, each of which contained a single set of primers positioned internal to the first set for one of the genes being tested. The second PCR reaction was then executed under the same protocol as the first. Products were analyzed on a 2% agarose gel; representative bands were sequenced to confirm identity.

Primer pairs for GAPDH, c-met, and m-cadherin were written by eye; primers for MyoD, myogenin, myf-5 and MRF4 were selected using Lasergene (DNAStar, Madison WI.) All primer sets were screened using Lasergene for possible interference with each other, and then tested empirically alone and in combination with all primers that were to be used with in a given multiplex set. Outside primers used in the first PCR were:

GAPDH: 5' GTG GCA AAG TGG AGA TTG TTG CC 3' forward, 5' GAT GAT GAC CCG TTT GGC TCC 3'reverse;

c-met: 5' GAA TGT CGT CCT ACA CGG CC 3' forward, 5' CAC TAC ACA GTC AGG ACA CTG C 3' reverse;

m-cadherin: 5' CCA CAA ACG CCT CCC CTA CCC ACT T 3' forward, 5' TCG TCG ATG CTG AAG AAC TCA GGG C 3' reverse;

MyoD: 5' GCC CGC GCT CCA ACT GCT CTG AT3' forward, 5' CCT ACG GTG GTG CGC CCT CTG C 3' reverse;

myogenin: 5'GGG CCC CTG GAA GAA AAG 3' forward, 5 AGG AGG CGC TGT GGG AGT 3' reverse;

myf-5: 5' TGC CAT CCG CTA CAT TGA GAG 3' forward, 5' CCG GGG TAG CAG GCT GTG AGT TG 3' reverse;

MRF4: 5' CTG CGC GAA AGG AGG AGA CTA AAG 3' forward, 5' ATG GAA GAA AGG CGC TGA AGA CTG 3' reverse.

Primers used for the second PCR's were:

GAPDH 5' GTG GCA AAG TGG AGA TTG TTG CC 3' forward, 5' GAT GAT GAC CCG TTT GGC TCC 3'reverse;

c-met: 5' GAA GGT ATC CGA ATT CAA GAC CGG 3' forward, 5' GAA CAT GCA GTG GAC CTC AGA CTG 3' reverse;

m-cadherin: 5' ACA GCA GCT AGG CAG TGT CAT C 3' forward, 5' AAC CTG AGG GCT GCA TTG TCT GTC 3' reverse;

MyoD: 5' CCC CGG CGG CAG AAT GGC TAC G 3' forward, 5' GGT CTG GGT TCC CTG TTC TGT GT 3' reverse;

myogenin: 5' CCG TGG GCA TGT AAG GTG TG 3' forward, 5' TAG GCG CTC AAT GTA CTG GAT GG 3' reverse;

myf-5: 5' GAG GGA ACA GGT GGA GAA CTA TTA 3' forward, 5' CGC TGG TCG CTG GAG AG 3' reverse;

MRF4: 5' TGC GGA GTG CCA TCA GC 3' forward, 5' CTC CTC CTT CCT TAG CAG TTA TCA 3' reverse

To determine the fidelity of the single-cell RT-PCR reaction when challenged with increasing numbers of primer sets or decreasing amounts of input cDNA or both, singly-harvested satellite cells were pooled prior to cDNA synthesis, reverse-transcribed, and single-cell equivalent aliquots of the resulting homogeneous pool of cDNA were

analyzed. When all six primer sets were used together, the reactions tended to fail regardless of the amount of input cDNA, but under the conditions used in this work highly consistent positive results were obtained using between 0.25 and 0.125 of a cell equivalent of input cDNA.

Technical considerations for single-cell multiplex RT-PCR

This technique has been optimized for sensitivity in order to enable detection of non-abundant transcripts from multiple regulatory genes. A significant trade-off is that the procedure intentionally operates outside the linear range of PCR and is therefore nonquantitative. A series of theoretical and technical considerations argue that reliable and meaningful quantitation (absolute RNA levels or even relative amounts of different transcripts) will not be possible by this approach and will require different techniques. It is also useful to recognize that different primer sets are differentially efficient so that empirical tests on positive samples are required for each new primer set. Moreover, primer compatibility for new multiplex combinations must also be tested on a case-by-case basis. Concordance tests such as those in Figure 6 and in Kato et al. (Kato *et al. 1997*) provide a means to evaluate robustness of a given set of measurements.

We believe that different cell types may require significantly different sample collection techniques. Specifically, we note that attempts to collect cytoplasm from mature myofibers has so far been unsuccessful because of difficulty in recovering sample from the highly structured "cytosol." Also, the variation we used here in which whole satellite cells are collected may not be possible when harvesting cells from whole embryos or from more intact tissues, and in these cases the use of cytosol-specific collection (Kato *et al*, 1997) may be preferable.

Protein blotting and immune reagent characterization

Western blotting was performed on protein extracts from pooled satellite cells derived from our fiber cultures. The anti mouse c-met polyclonal rabbit serum (Santa Cruz, cat# sc-162) specifically detected a single band of appropriate size for c-met (Fig 2). As anticipated, preincubation of the antibody with the immunizing peptide eliminated this band.

Immunohistochemistry

Cultured myofibers were fixed in fresh 4% paraformaldehyde for 20' at 25°, washed 3x with PBS, permeablized in 1% NP-40 5' at 20°, washed 3x with PBS, blocked in 10% normal goat serum, incubated overnight at 4° with primary antibodies, washed 3x with PBS, incubated 1 hour at 25° with secondary antibodies, washed 3x with PBS and mounted in Vectashield (Vector.) Fibers were photographed on a Nikon Optiphot-2 with UFX camera attachment or imaged using a Bio-Rad 600 confocal microscope with false color added in Photoshop (Adobe.)

10 μm cryotome sections of unmanipulated quadriceps muscle were double-stained for c-met and laminin sequentially by fixing, blocking and incubating with rabbit anti-met antibody as above, followed by incubation with monovalent goat anti-rabbit Fab-FITC conjugate at (Jackson) at 1:50 for 2 hours. Sections were washed in PBS and incubated with rabbit anti-laminin (Sigma) for 4 hours followed by anti-rabbit TRITC and mounted in Vectashield. The sections were then photographed or imaged as above.

Primary antibodies and dilutions used were: rabbit anti-m-met (Santa Cruz) at 1:50; F5D (mouse anti-myogenin, Wright et al., described in Cusella-DeAngelis *et al.*, 1992) at 1:5; NCL-MyoD1 (mouse, Novocastra Labs) at 1:10; mouse anti-BrdU (Boehringer Mannheim) at 1:10; rabbit anti-laminin (Sigma) at 1:250. Secondary antibodies were raised in donkey (Jackson Immunochemicals) and, except for blocking Fab used for section double-staining, used at 1:100.

Results

Expression of c-met and m-cadherin mRNAs by fiber-associated satellite cells

In the first part of this study we used isolated myofiber cultures to test individual satellite cells for the expression of m-cadherin and c-met genes. We began with these genes because each is a candidate "molecular marker" for myosatellite cells at rest or in the early stages of activation and because each is of functional interest in muscle regeneration. To serve as a satellite cell marker, expression should encompass all satellite cells and should exclude other mononucleate cells in muscle tissue such as muscle derived fibroblasts. The prime candidate thus far for a resting satellite cell marker has been the homophilic adhesion molecule m-cadherin, which has been detected by in situ hybridization (Moore and Walsh, 1993) and immunostaining (Cifuentes-Diaz *et al.*, 1995; Irintchev *et al.*, 1994) in tissue sections taken from regenerating mouse skeletal muscle. However, RNA expression was not detected in unstimulated satellite cells or in satellite cells stimulated by denervation rather than trauma injury, and the data regarding protein expression in quiescent satellite cells is conflicting. A prior study had shown that transcripts for the c-met receptor tyrosine kinase can be detected by RT-PCR in RNA from pooled cultured rat satellite cells (Allen *et al.*, 1995), indicating that it is expressed in some satellite cells. Measurements of that type could not, however, reveal what fraction of cells are responsible for the positive signal observed.

To evaluate these candidate markers an independent criterion for satellite cell identity was required. In the experiments that follow, a cell was defined as a satellite cell and picked for assay based on its anatomic association with an isolated myofiber. First utilized by Bekoff and Betz (Bekoff and Betz, 1977), the isolated fiber technique was further refined by Bischoff who focused on the rat flexor digitorum brevis muscle and used it to show that satellite cells identified initially by association with the parental myofiber proliferate in culture and are subsequently myogenic (Bischoff, 1986a). The

modification used here was designed for source material from diverse muscles, which enabled us to compare satellite cells from muscles that are mainly fast or slow in fiber type or from muscles of the two major developmental lineages, the axial and appendicular groups (Materials and Methods).

An initial question was whether either c-met or m-cadherin is expressed in all or only some satellite cells at the earliest times after fibers are explanted from healthy muscle tissue. The mRNA assay used was multiplex single cell RT-PCR modified from (Kato *et al.*, 1997); see Figure 1 and Materials and Methods). In this experiment, three genes were monitored for each cell: GAPDH, m-cadherin and c-met. GAPDH is commonly employed as a standard because of its ubiquitous expression, and was used here to show that a cell had been harvested successfully and that the reverse transcription and PCR reactions had proceeded properly. Satellite cells associated with freshly harvested myofibers were visualized for collection by treating the preparation with the myotoxic anesthetic Marcaine (Winthrop) and staining with the live-cell specific fluorescent dye calcein AM; at later timepoints morphological criteria with respect to the associated myofiber were used to define satellite cells for harvest. Satellite cells that migrated away from their fiber of origin in the culture were not picked, because their identity as satellites by anatomical criteria was uncertain.

The result of this analysis for c-met was striking: 100% of GAPDH positive cell samples also scored positive for processed c-met mRNA at all timepoints (Fig 3 A). In contrast, only a small fraction (<20%) of satellite cells scored positive for m-cadherin at the early time point. As expected, we never observed a satellite cell that scored negative for GAPDH, but positive for any other gene assayed. We next asked how the expression of c-met and m-cadherin mRNAs changed over a 96 hour timecourse in which fiber-associated satellite cells are activated to divide, express MRF family regulators, and differentiate (see below). The fraction of cells expressing m-cadherin increased gradually throughout the timecourse and was 100% at 96 hours. It is important to note that when

we examined satellite cell pools rather than groups of individual cells, all timepoints scored positive for both markers, even though the majority of individual cells did not express m-cadherin at early timepoints. A final important result was that GAPDH-positive 10T1/2 fibroblasts (data not shown) and muscle fibroblasts derived from muscle fiber cultures were entirely negative for m-cadherin and c-met (Fig 3C).

The experiments above and prior studies of rodent satellite cells have all used appendicular muscles, which develop from the c-met dependent migratory cells of the lateral somite (Ordahl and LeDouarin, 1992), as the source of satellite cells. During development the axial muscles represent a distinct population of myoblasts, and are not dependent on the c-met/HGF function (Bladt *et al.*, 1995; Daston *et al.*, 1996). This raised the question of whether c-met expression is general for all satellite cells or is restricted to the limb lineage. We therefore repeated the experiment using myofibers prepared from axial muscles of the deep back, and found consistent positive mRNA expression in satellite cells from axial muscle fibers. We also determined that satellite cells derived from predominantly fast or predominantly slow fiber types express c-met and m-cadherin similarly (see below). From these data we conclude that m-cadherin expression marks a small subset of satellite cells at early times following explant or activation but that c-met expression appears to include all satellite cells in fiber cultures.

Satellite cells express c-met protein in fiber culture

The finding that c-met mRNA is expressed by all satellite cells raised the question of c-met protein expression, which would be required for a biological function during muscle regeneration. We therefore asked whether c-met protein is expressed by fiber-associated satellite cells and how that expression is related to expression of MRF family proteins and to the proliferation status of the cell. Myofiber isolation activates fiber-associated satellite cells to express MRF family regulators, divide and differentiate over a period of several days in culture. Prior studies have shown fiber-associated satellite cells begin to enter S-phase after approximately 36 hours in culture (Bischoff, 1986a;

Yablonka-Reuveni and Rivera, 1994). When cultured in the continuous presence of BrdU, fixed at 12-hour intervals, and costained with antibodies directed against mouse c-met (Santa Cruz; see Materials and Methods for characterization) and incorporated BrdU (Boehringer), satellite cells in these fiber cultures behaved similarly, entering S-phase of the cell cycle approximately 36 hours after isolation. When costained with the antibody to mouse c-met satellite cells were positive for c-met immunoreactivity both before BrdU incorporation was detected and after, indicating that c-met expression continues even after the regeneration program has been activated and cell proliferation has begun (Fig 4A).

Prior studies have shown that MRF proteins are not expressed in quiescent satellite cells, but accumulate in activated satellite cells (Grounds *et al.*, 1992; Smith *et al.*, 1994; Yablonka-Reuveni and Rivera, 1994; summarized in Table 1). When satellite cells from fiber cultures were costained with immune reagents for c-met and either myogenin (Wright et al, described in Cusella-DeAngelis *et al.*, 1992) or MyoD (Novocastra Labs), c-met expression was observed on all cells that expressed MyoD at or before 12 hours in culture (Fig 4B) and myogenin by approximately 72 hours in culture (Fig 4C); no MyoD- or myogenin-positive mononucleate cells were ever seen that were not also positive for c-met protein. This result is important because it suggests that all cells in the culture that are activated satellite cells by the criterion of MRF expression also express c-met. Myogenin protein expression was first detected after the time at which most satellite cells had begun to divide, but it was detected in both BrdU-positive and BrdU-negative cells in experiments where BrdU had been provided continuously (data not shown). Thus a subset of satellite cells begin to express myogenin before they divide or, perhaps, without ever dividing. Myogenin expression was also observed in the nuclei of cells in the act of cytokinesis, so its expression is clearly not restricted to satellite cells that have already exited the cycle. c-met immunoreactivity was maintained when satellite cells fused with each other to form nascent myotubes on the surface of the cultured myotubes or on the dish (data not shown).

Satellite cells express c-met protein in intact muscle

To determine whether the c-met expression observed in culture is a very rapid consequence of activation or is also a property of presumably quiescent satellite cells in intact muscle, sections of undisturbed leg muscle were examined for c-met immunoreactivity. c-met positive cell outlines were detected at the edges of muscle fibers in transverse sections, and were shown to reside beneath the basal laminae by costaining with anti-laminin (Fig 5A). Costaining with DAPI showed that these outlines contain single nuclei, an observation which rules out the possibility that the c-met immunostaining belonged to small blood vessels rather than cells (Fig 5B). Unactivated satellite cells do not express MyoD or myogenin (Grounds *et al.*, 1992); when costained with antibodies to these proteins no c-met positive cells in muscle sections were seen to express either MRF. Thus by anatomical criteria and by MRF status, c-met antigen was detected on quiescent satellite cells in intact muscle, as well as on satellite cells associated with myofibers immediately after isolation and during the course of fiber culture.

Because c-met RNA and protein were not detectable in muscle derived fibroblasts or other mononucleate cells in our preparations, we think that c-met will prove to be a useful molecular marker for satellite cells within the context of myofiber cultures derived from healthy adult muscles. However, the uses of any molecular marker must be carefully matched with the context in which the putative marker was characterized. Additional studies in other muscle contexts will be needed to extend the range of application beyond those tested here. For example, while no cells other than satellite cells stained strongly for c-met protein in transverse sections through the midsection of healthy muscles, it remains possible that there will be other muscle associated cell types that express c-met. In particular, Schwann cells are neuroglial cells present at neuromuscular synapses, and it has been reported that rat Schwann cells are responsive to HGF (Krasnoselsky *et al.*, 1994) so it is likely that they express the c-met. receptor. We also do not know how the population of c-met positive cells changes in damaged muscle

where other cell types may become prominent, in addition to satellite cells (see Discussion).

Six-gene multiplex single cell RT-PCR: rationale and feasibility

As summarized in Table 1, it has been reported that all four MRF family regulators are expressed at the RNA level in mass cultures of activated satellite cells (Smith *et al.*, 1994). At the individual cell level, immunostaining studies with reagents directed against MyoD and myogenin showed positive cells on isolated myofibers and *in vivo* (Grounds *et al.*, 1992; Yablonka-Reuveni and Rivera, 1994). Because of the number of genes in the family and the absence so far of powerful immune reagents for MRF4 and myf5 proteins, the patterns of four-fold co-expression are not known for either embryo or adult muscle systems. However, our ideas about the mechanisms of MRF action as deduced from genetic and biochemical assays are strongly attached to and bounded by our knowledge of which members of the family are co-expressed at the cellular level. We therefore expanded the multiplex single cell RT-PCR to characterize co-expression of all four MRFs together with m-cadherin and c-met.

The determination of six genes for each cell is a higher level of multiplexing than has been used previously with this protocol and so raises the important question of how robust these measurements are. Using this method, we have found that the question of fidelity and sensitivity must be investigated for each combination of genes to be used together, presumably because of sequence specific interactions among primers. To evaluate fidelity for this gene set, we collected individual satellite cells from the 96 hour time point and pooled the cells together. The purpose of pooling was to create a single homogeneous master sample from which identical aliquots could be drawn and tested with the expectation that each sample should deliver the same answer. We then used this pool to make a series of identical measurements on samples containing 0.5 to 0.125 cell equivalents of starting material. We chose the 96 hour time point because we knew from our own data and from studies of satellite cell mass cultures that all four MRFs are

expressed in at least some cells at this time. The assay scored positive in all samples for each of the six genes from 0.5 to 0.25 cell equivalents per reaction (Fig. 6). However, at 0.125 cell equivalents per reaction, individual gene determinations from different samples began dropping out, indicating that at this level there would be false negative results, especially for mRNAs which are not expressed by all cells that made up the pool. We also note that if the same set of genes was used with all six primers grouped together (rather than split into group A and group B, as shown), then reactions began to fail even at the one cell equivalent input level. Further control experiments suggest that this failure is tied to the overall concentration of primers per reaction in the first round PCR as well as overall sequence complexity of the primer mix. We also noted that for some primer sets that cross short introns, PCR bands corresponding to genomic DNA or to unprocessed hnRNA were sometimes detected. This indicates that the assay is sensitive to templates present in the range of one to a few copies per cell. False positives from nuclear DNA were easily distinguished from true positives by primer design as noted above, while other possible false positives from molecular or cellular contamination were controlled by taking control samples of fiber culture media at the beginning and end of each experiment (see Materials and Methods). Any experiment in which a media sample scored as positive was eliminated in its entirety. A PCR product band was harvested and sequenced for each gene and was shown to be the expected sequence, so we are confident that the bands shown represent specific amplification from the intended target. We conclude that for this six gene set, the assay has high fidelity and sensitivity down to at least 0.25 cell equivalents of input material, and all measurements reported here were performed at 0.5 cell per reaction (half of each cell devoted to primer group A and half to primer group B).

Combinatorial expression of MRF regulators during satellite cell activation

Single cell data for co-expression of c-met, m-cadherin, myf-5, MyoD, myogenin, and MRF4 are summarized in Fig. 7. All cells shown were positive for c-met, and no cells were ever found that were positive for any of the other five genes, but negative for c-met. At time-zero following fiber isolation, no cells expressed detectable myogenin or MRF4, and only a few were positive for m-cadherin, myf-5 or MyoD. We do not know whether the infrequent MyoD or myf5 positive cells at time zero represent a small subset of cells that were activated *in vivo* before myofiber explant or whether these are the very first MRF positive cells produced in response to activation at the time of explant and fiber preparation. By the 24 hour time point, many cells were singly positive for either MyoD or myf5 (32%), and many others (35%) were positive for both. The double positive MyoD/myf5 cells might have begun as co-expressors or they may represent a second expression state that follows expression of just one of the pair, as is presently thought to occur in the embryonic lineages (reviewed in Cossu *et al.*, 1996a). At zero and 24 hour timepoints there appeared to be no correlation between MRF expression status and m-cadherin, although the fraction of m-cadherin positive cells increased with time. At 48 hours we first observed cells that are myogenin positive, and note that these were always also positive for both MyoD and myf5 as well as m-cadherin. At this time MRF4 expression was detected for the first time and it was always expressed in cells that were also positive for myogenin; with only a few rare exceptions, MRF4 expression was restricted to cells scoring positive for all four MRFs. Because MRF4 RNA is expressed at relatively high levels is differentiated muscle, the observation that it is absent from all satellite cells at early timepoints argues further that the MRF mRNA present in our samples was due to harvested satellite cells and not to contamination from the adjacent fibers. The later timepoints saw further increase in the proportion of cells that were m-cadherin positive (100% by 96 hours). The only apparent correlation between MRF expression and m-cadherin expression was noted at 48 hours where all cells expressing myogenin were positive for m-cadherin and only half in the non-

myogenin compartments were m-cadherin positive. The timing of individual MRF gene expression summed over the population at each time point here agrees well with that reported previously (Table 1.) Minor differences were detection of myf-5 and myogenin slightly earlier than by RT-PCR of rat mass cultures (Smith *et al.*, 1994) and persistence of myogenin expression longer than observed by antibody staining in rat fiber cultures (Yablonka-Reuveni and Rivera, 1994). Both could easily be explained by expected differences in assay sensitivities.

Discussion

Molecular markers of satellite cell identity

Molecular measurements currently used to identify mouse satellite cells, including DNA synthesis when the adjacent muscle is damaged or removed into culture (Bischoff, 1986b), onset of MRF expression after activation (Grounds *et al.*, 1992) or myogenic differentiation in culture (Rando and Blau, 1994) cannot be used to identify satellite cells prior to activation and they also generally preclude further study of the cells. The observations reported here suggest that expression of the c-met receptor tyrosine kinase can serve as an effective molecular marker for quiescent or activated satellite cells in fiber culture, in preparations of mononucleate muscle derived cells, and probably in sections of intact healthy muscle when coupled with associated anatomical criteria. Molecular markers in systems such as developing neural crest and the immune system have been critical for much progress, and the proposed use of c-met as a satellite cell molecular marker presents the prospect of a similarly important contribution to the study of adult muscle precursor cells and muscle regeneration. However, care must be taken in the application of c-met for marker purposes because molecular markers are necessarily context dependent. For any new or varied context such as damaged muscle from whole animals, the marker will have to be validated by appropriate independent assays akin to the positional definition of satellite identity used in this study. In particular, we do not presently know if any c-met positive cells other than activated satellite cells will be present during a regeneration response *in vivo* when other diverse cell types such as immune cells invade the healing tissue. Some of these cells might express c-met although there is no evidence for this at present. We also note that because c-met is a cell surface protein, it offers the future possibility of nondestructive identification and cell sorting, but the immune reagent used in this study is directed against an epitope located on the cytosolic portion of the receptor.

Functional implications of c-met expression patterns

The observation that the c-met receptor protein is expressed *in vivo* by quiescent satellite cells and in culture by dividing and differentiating satellite cells has interesting functional implications. The first of these concerns the initial activation of satellite cells. A number of defined growth factors, including PDGF, IGF-2, EGF, and FGF have been assayed for their effect on satellite cell activation and proliferation in culture systems. While these factors can enhance satellite cell proliferation once it has been initiated, Bischoff observed that only FGF appears capable of affecting initial activation but it is uncertain whether FGF factors are prominently available at the earliest times following damage *in vivo* (Bischoff, 1986b; Bischoff, 1990), and in other systems exogenous FGF was not found to elicit a response from quiescent satellite cells (Johnson and Allen, 1993) or affect regeneration *in vivo* (Mitchell *et al.*, 1996). Saline extracts of crushed muscle (CME) were found to be able to initiate a response, but the CME active factor has not yet been purified to homogeneity (Bischoff, 1986b). HGF/SF is a candidate for the activating factor in CME because it shares several biochemical and biological properties with the mitogenic activity in CME (Chen *et al.*, 1994) including the ability to stimulate cultured rat satellite cells to divide precociously (Allen *et al.*, 1995). If HGF/SF is an activating factor *in vivo*, a key requirement is that its receptor be expressed on quiescent satellite cells in undisturbed muscle. Our observations show that this requirement is fulfilled for c-met. The proposed initiator role for the HGF/c-met signaling system in muscle regeneration is notably similar to HGF/c-met functions in several other tissues where it is also believed to stimulate proliferative/regenerative programs following injury (reviewed in Matsumoto and Nakamura, 1996). Finally, the continued expression of the c-met receptor after activation suggests that it may also mediate one or more functions later in the regeneration response such as cell migration or morphogenesis as well as proliferation.

m-cadherin expression in satellite cells

We directly compared the expression of c-met with m-cadherin and found that m-cadherin positive cells comprised only a small fraction of muscle satellite cells at the zero-time point following fiber isolation, a time at which all satellite cells express c-met. From this result we conclude that it is very likely that m-cadherin mRNA is expressed by a subset of quiescent satellite cells in intact muscle, although we could not make the direct confirming measurements in intact muscle using this technique nor was double immunostaining possible using the current reagents. Our data support prior observations that m-cadherin is expressed on quiescent satellite cells (Irintchev *et al.*, 1994) but also emphasize that less than 20% of quiescent satellite cells are detectably m-cadherin positive at the earliest time, when all cells are c-met positive. The fact that m-cadherin positive cells comprise a minor subset of an already rare cell population may account for other reports in which m-cadherin expression could not be detected in intact muscle (Moore and Walsh, 1993; Cifuentes-Diaz *et al.*, 1995). At later times following activation in culture, an increasingly large fraction of satellite cells expressed m-cadherin, and this is consistent with studies reporting robust m-cadherin expression during some *in vivo* regeneration paradigms (Moore and Walsh, 1993; Irintchev *et al*, 1994; Cifuentes-Diaz *et al*, 1995).

The significance of the c-met/m-cadherin double positive cells at the zero timepoint compared to their more prevalent c-met positive/m-cadherin negative counterparts is uncertain. It is tempting to speculate that the early m-cadherin positive cells represent a functionally distinct subclass, perhaps satellite cells programmed to differentiate quickly upon stimulation. This notion holds some attraction because m-cadherin mediates adhesion with other m-cadherin expressing cells which could be useful for assembling clusters of early differentiating cells into the earliest myofiber framework.

MRF expression type progression

When single cell expression typing for the four MRF regulators was pooled at each timepoint to recreate the entire cell population, the picture was very similar to prior

studies of satellite cell mass cultures (Table 1). Our MRF expression results also agreed well with single MRF factor determinations made previously for satellite cells from fiber cultures that were similar to, though not identical with, ours. As in all culture preparations, it is important to recognize that the stimulus and course of satellite cell activation may be different from that in the intact animal, though the general course of MRF expression following injury is similar (Grounds *et al.*, 1992.) The multiplex single cell measurements also gave new insights not possible in prior studies of either satellite cells or embryo myoblasts and myocytes. For example, it has never been certain whether all four MRFs are ever co-expressed in individual cells or whether their joint presence within a tissue instead reflected multiple two or three member combinations. Data from our 48 and 96 hour timepoints clearly showed that simultaneous expression of all four MRFs is a preferred expression state concurrent with differentiation in this system. Cells positive for myf-5/MyoD/myogenin were also prominent at these times, and it is not clear whether all cells in our system will at some time express the full MRF set.

From the data in Figure 7, we propose a model for the use of MRF regulators in the progression of fiber-associated satellite cells from their initial MRF negative quiescent state through activation and into differentiation (Figure 8). The model is based on the frequency of individual expression states as a function of time following activation. The resulting progression is quite simple because, with respect to the MRFs, only six of the possible sixteen expression states were observed at significant frequencies at any timepoint (two other expression states, myogenin only and myogenin/MRF4, were represented by just five and two of 201 cells, respectively, and their significance therefore remains uncertain). In this model, cells enter the MRF positive compartment by expressing either MyoD or myf5 alone. These cells are prominent by 24 hours, but became rare by 48 hours. It appears that these cells become positive for both MyoD and myf5 rather quickly, and it is possible that some cells enter the MRF positive state by simultaneously expressing both determination class MRFs from the outset, as indicated.

The entry of cells into the MRF positive pool by either MyoD-first or myf5-first pathways is reminiscent of determination class MRF expression in the developing embryo. In the embryo, different inductive signals are thought to initiate the expression of either myf5 (in the early dorsomedial lineage) or MyoD (in a later ventrolateral lineage) in two distinct sublineages of the myotome (Cossu *et al.*, 1996a)(Cossu *et al.*, 1996a, reviewed in Cossu *et al.*, 1996b; Yun and Wold, 1996). It will be interesting in future studies to explore the signaling pathways active in satellite cells to see if there is more than one and to examine possible relationships to MyoD- or myf5-mediated initiation. In the fiber-associated satellite cells, the myf5/MyoD double positive state appears to be required for later myogenin expression which is absent at 24 hours but prominent by 48 hours. This differs from the earliest myotomal lineage in the embryo, where cells of the myf5 initiated dorsomedial domain apparently remain MyoD negative while they begin to express myogenin and to differentiate (Smith *et al.*, 1994; Yoon, Yu and Wold, unpublished). This myf5/myogenin positive, MyoD negative expression state common in the embryo was never observed in activated satellite cells. MRF4 expression also becomes prominent at 48 hours in satellite cells, and it was detected exclusively in cells that also express myogenin. The vast majority of MRF4 positive cells (95%) expressed all four MRFs. We do not know the experimental or biological significance of two rare expression types, myogenin alone and myogenin/MRF4, but a simple possibility indicated in the model is that they arise by the downregulation of determination class MRFs. We cannot, however, rule out the less interesting possibility that they are simply low frequency false negatives for both MyoD and myf5. Finally, it is noteworthy that even at late timepoints, there are a few cells that are c-met positive and negative for all four MRFs. It is uncertain if such cells expressed MRFs at any earlier time, but whatever their expression history, they may represent cells that possess muscle progenitor status at the time of the assay.

By coupling the use of c-met as a marker of satellite identity with the use of multiplex RT-PCR, as initially demonstrated here for the four MRFs and m-cadherin, it should be possible to characterize coordinate expression states for other genes involved in controlling and executing activation, proliferation, differentiation and, perhaps, the quality of stem-cell like renewal.

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References

Allen, R. E., Sheehan, S. M., Taylor, R. G., Kendall, T. L., and Rice, G. M. (1995). Hepatocyte growth factor activates quiescent skeletal muscle satellite cells *in vitro*. *J. Cell. Phys.* **165,** 307-312.

Bekoff, A., and Betz, W. (1977). Properties of isolated adult rat muscle fibers maintained in tissue culture. *J. Physiol.* **271,** 537-547.

Bischoff, R. (1986a). Proliferation of muscle satellite cells on intact myofibers in culture. *Dev. Biol.* **115,** 129-139.

Bischoff, R. (1986b). A satellite cell mitogen from crushed adult muscle. *Dev. Biol.* **115,** 140-147.

Bischoff, R. (1990). Interaction between satellite cells and skeletal muscle fibers. *Development* **109,** 943-952.

Bischoff, R. (1994). . *In* "Myology" (A. G. Engel and C. Franzini-Armstrong, Eds.), pp. 97-118. McGraw-Hill, New York.

Bladt, F., Riethmacher, D., Isenmann, S., Aguzzi, A., and Birchmeier, C. (1995). Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature* **376,** 768-771.

Chen, G., Birnbaum, R. S., Yablonka-Reuveni, Z., and Quinn, L. S. (1994). Separation of mouse crushed muscle extract into distinct mitogenic activities by heparin affinity chromatography. *J. Cell Phys.* **157,** 563-572.

Cifuentes-Diaz, C., Nicolet, M., Alameddine, H., Goudou, D., Dehaupas, M., Rieger, F., and Mege, R. M. (1995). M-cadherin localization in developing adult and regenerating mouse skeletal muscle: possible involvement in secondary myogenesis. *Mech. Devel.* **50,** 85-97.

Cossu, G., Kelly, R., Tajbakhsh, S., Di Donna, S., Vivarelli, E., and Buckingham, M. (1996a). Activation of different myogenic pathways: myf-5 is

induced by the neural tube and MyoD by the dorsal ectoderm in mouse paraxial mesoderm. *Development* **122,** 429-437.

Cossu, G., Tajbakhsh, S., and Buckingham, M. (1996b). How is myogenesis initiated in the embryo? *Trends Genet.* **12,** 218-223.

Cusella-DeAngelis, M. G., Lyons, G., Sonnino, C., DeAngelis, L., Vivarelli, E., Farmer, K., Wright, W. E., Molinaro, M., Bouche, M., Buckingham, M., and Cossu, G. (1992). MyoD, myogenin independent differentaition of primordial myoblasts in mouse somites. *J. Cell Biol.* **116,** 1243-1255.

Daston, G., Lamar, E., Olivier, M., and Goulding, M. (1996). Pax-3 is necessary for migration but not differentiation of limb muscle precursors in the mouse. *Development* **122,** 1017-1027.

Donalies, M., Cramer, M., Ringwald, M., and Starzinski-Powitz, A. (1991). Expression of M-cadherin, a member of the cadherin multigene family, correlates with differentiation of skeletal muscle cells. *PNAS USA* **88,** 8024-8028.

Grounds, M. D., Garrett, K. L., Lai, M. C., Wright, W. E., and Beilharz, M. W. (1992). Identification of skeletal muscle precursor cells in vivo by use of MyoD1 and myogenin probes. *Cell Tiss. Res.* **267,** 99-104.

Irintchev, A., Zeschnigk, M., Starzinski-Powitz, A., and Wernig, A. (1994). Expression pattern of m-cadherin in normal, denervated, and regenerating mouse muscles. *Dev. Dynam.* **199,** 326-337.

Johnson, S. E., and Allen, R.E. (1993). Proliferating cell nuclear antigen (PCNA) is expressed in activated rat skeletal muscle satellite cells. *J. Cell. Phys.* **154,** 39-43.

Kato, K., O'Dowd, D. K., Fraser, S. E., and Smith, M. A. (1997). Developmental changes in the co-expression of Krox20 and SEK1 in single cells from chick hindbrain. *Dev. Biol.* **191,** 259-269.

Krasnoselsky, A., Massay, M. J., Defrances, M. C., Michalopoulos, G., Zarnegar, R., and Ratner, N. (1994). Hepatocyte growth factor is a mitogen for Schwann cells and is present in neurofibromas. *J. Neurosci.* **14,** 7284-7290.

Matsumoto, K., and Nakamura, T. (1996). Emerging multipotent aspects of hepatocyte growth factor. *J. Biochem.* **119,** 591-600.

Mauro, A. (1961). Satellite cells of skeletal muscle fibers. *J. Biophys. Biochem. Cytol.* **9,** 493-495.

Megeney, L. A., Kablar, B., Garrett, K., Anderson, J. E., and Rudnicki, M. A. (1996). MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes Devel.* **10,** 1173-1183.

Mitchell, C. A., McGeachie, J. K., and Grounds, M. D. (1995). The exogenous administration of basic fibroblast growth factor to regenerating skeletal muscle in mice does not enhance the process of regeneration. *Grow. Factor.* **13,** 37-55.

Moore, R., and Walsh, F. S. (1993). The cell adhesion molecule M-cadherin is specifically expressed in developing and regenerating, but not denervated skeletal muscle. *Development* **117,** 1409-1420.

Olson, E. N., and Klein, W. H. (1994). bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. *Genes Devel.* **8,** 1-8.

Ordahl, C. P., and LeDouarin, N. M. (1992). Two myogenic lineages within the developing somite. *Development* **114,** 339-353.

Rando, T. A., and Blau, H. M. (1994). Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. *J. Cell Biol.* **125,** 1275-1287.

Rose, O., Rohwedel, J., Reinhardt, S., Bachmann, M., Cramer, M., Rotter, M., Wobus, A., and Starzinski-Powitz, A. (1994). Expression of m-

cadherin protein in myogenic cells during prenatal mouse development and differentiation of embryonic stem cells in culture. *Dev. Dynam.* **201,** 245-259.

Smith, T. H., Kachinsky, A. M., and Miller, J. B. (1994). Somite subdomains, muscle cell origins, and the four muscle regulatory factor proteins. *J. Cell Biol.* **127,** 95-105.

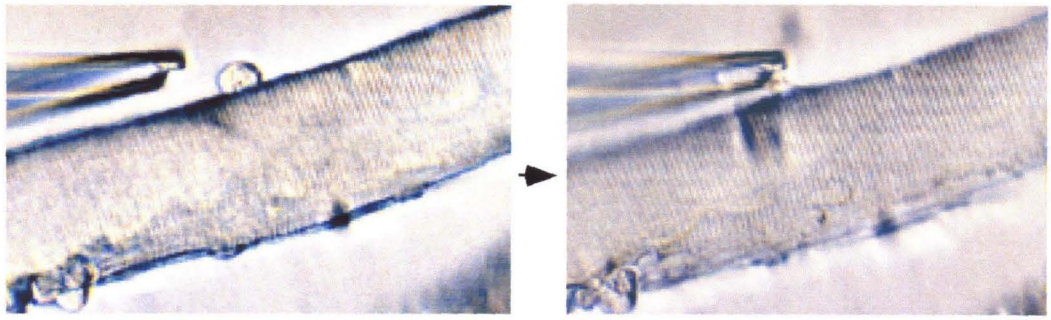
Smith, C. K., Janney, M. J., and Allen, R. E. (1994). Temporal expression of myogenic regulatory genes during activation, proliferation, and differentiation of rat skeletal muscle satellite cells. *J. Cell. Phys.* **159,** 379-385.

Yablonka-Reuveni, Z., and Rivera, A. J. (1994). Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibers. *Dev. Biol.***164,** 588-603.

Yun, K., and Wold, B. (1996). Skeletal muscle determination and differentiation: story of a core regulatory network and its context. *Curr. Op. Cell Biol.***8,** 877-889.

Figure 1: Schematic depiction of single-cell RT-PCR as used for this work. The top panels show phase images of a satellite cell (typical in appearance for cells from 24 to 96 hours) before and during harvest.

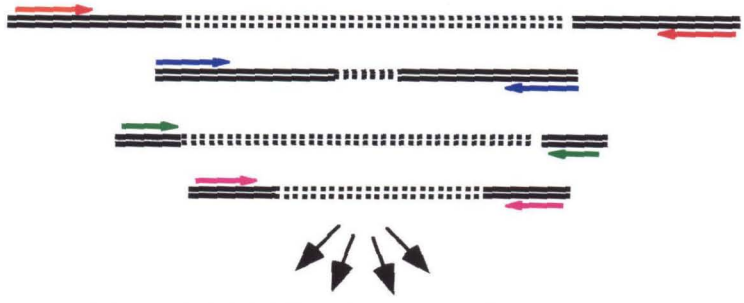
Multiplex single-cell RT-PCR



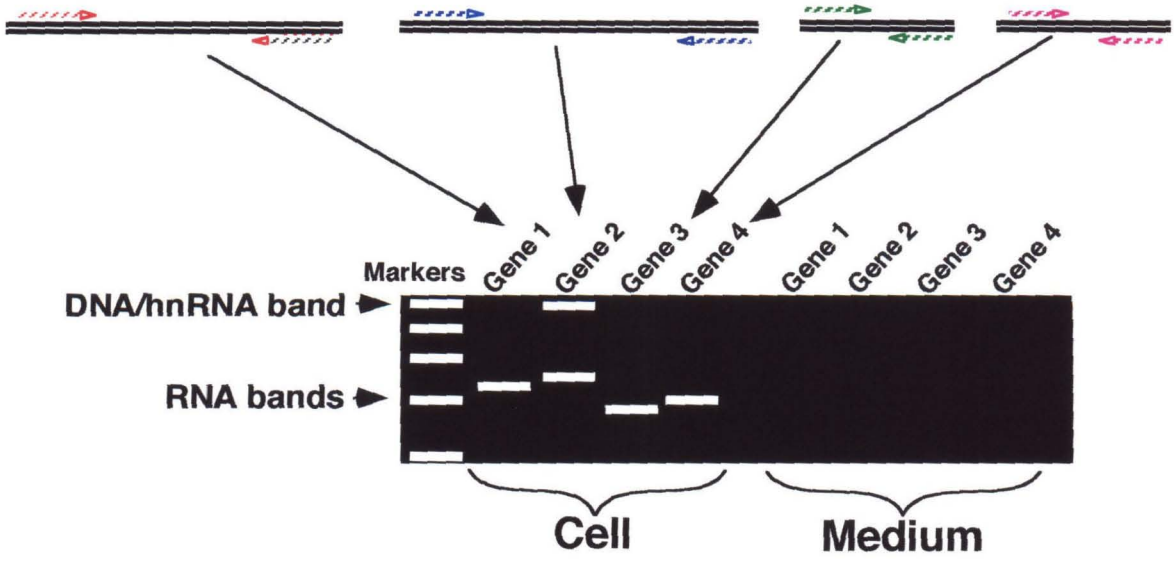
Collect satellite cells or medium control with micropipette

Random primed cDNA synthesis

1st PCR: intron-spanning outside primers for 4 genes



2nd PCR: Use 1/1000 of 1st PCR as template for 4 separate reactions using one nested inside primer set/rxn



Gel electrophoresis

Figure 2: Western blot of proteins from satellite cells expanded for 2 weeks in mass culture, with all myofibers removed. A single band of a molecular weight appropriate for the β-chain of c-met is detected, and was competed away by preincubating the antibody with excess peptide antigen.

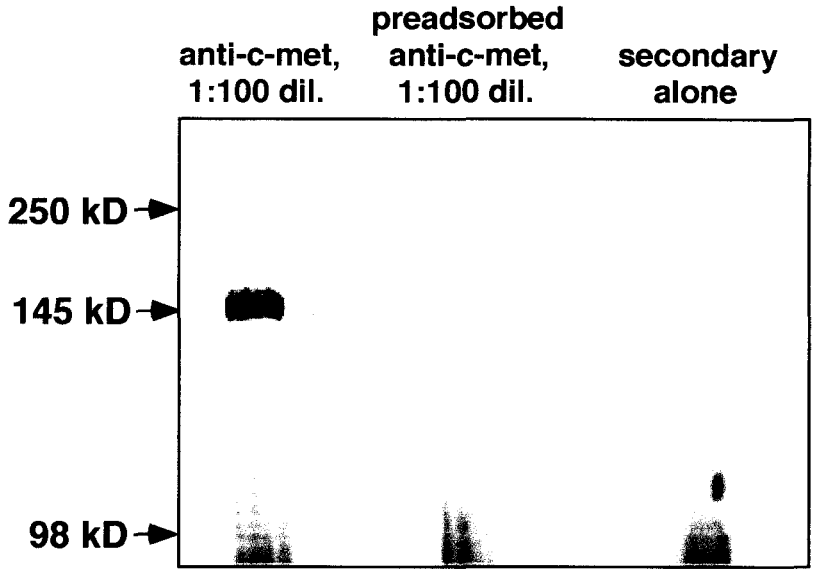
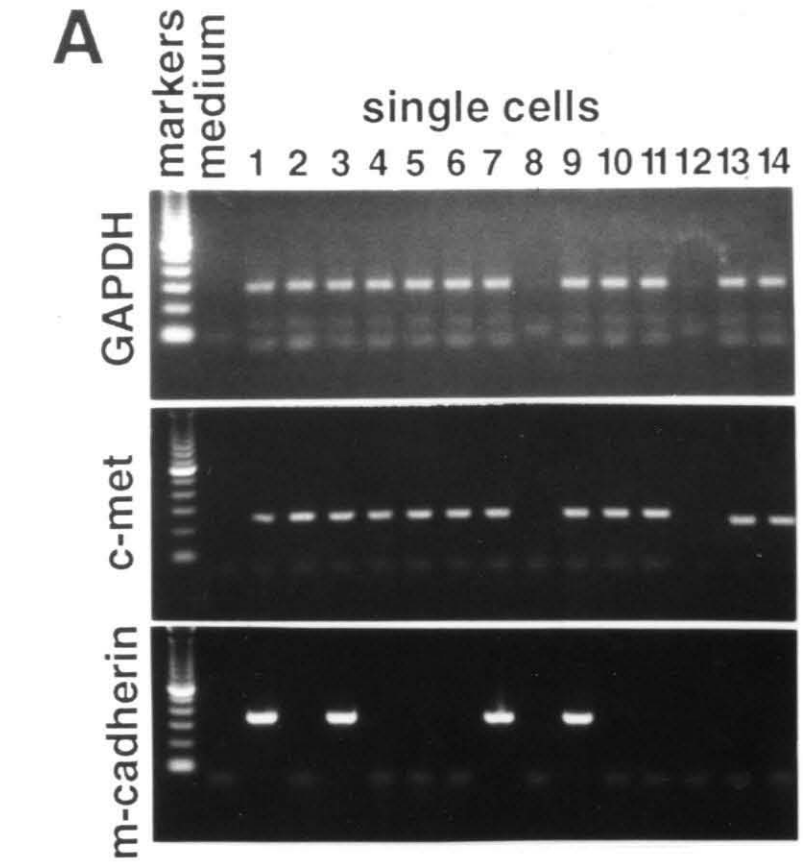

anti-c-met, 1:100 dil.
preadsorbed anti-c-met, 1:100 dil.
secondary alone
250 kD
145 kD
98 kD

Figure 3: A) Sample single-cell RT-PCR gel from 48 hour in culture samples showing GAPDH (expected product 290 bp), c-met (expected product 249 bp), and m-cadherin (expected product 316 bp); marker=100 bp ladder (Gibco). B) Line graph illustrating the population shift from c-met^{+} m-cadherin^{-} (dashed line) to c-met^{+} m-cadherin^{+} (solid line) over the first 96 hours in culture of satellite cells on isolated myofibers.


A
markers
medium
single cells
1 2 3 4 5 6 7 8 9 10 11 12 13 14
GAPDH
c-met
m-cadherin

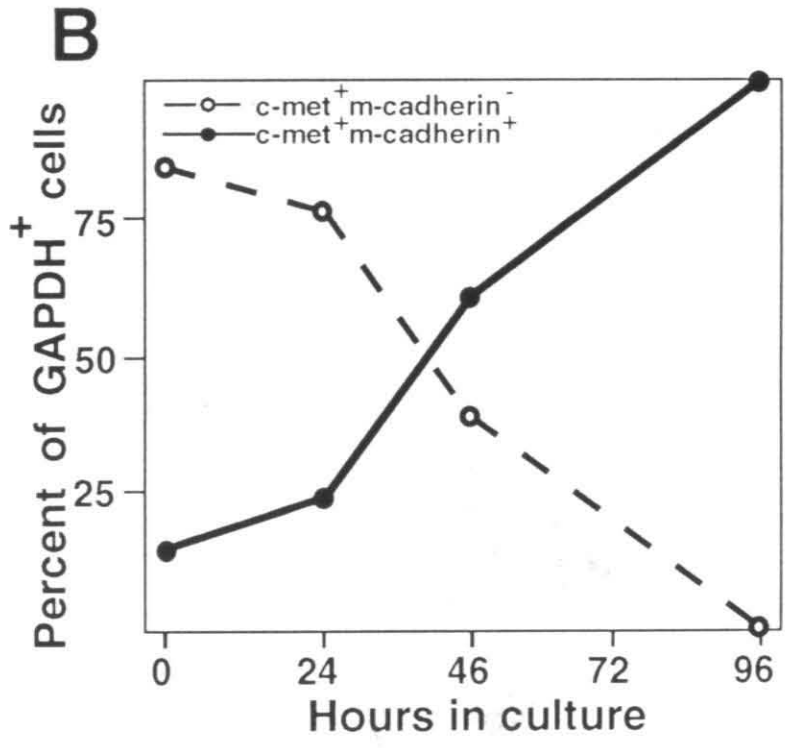

B
c-met+m-cadherin-
c-met+m-cadherin+
Percent of GAPDH+ cells
75
50
25
0
24
46
72
96
Hours in culture

Figure 3 C) Single-cell RT-PCR of muscle-derived fibroblasts harvested from the surface of a tissue culture dish. All GAPDH positive cells were negative for both c-met and m-cadherin.

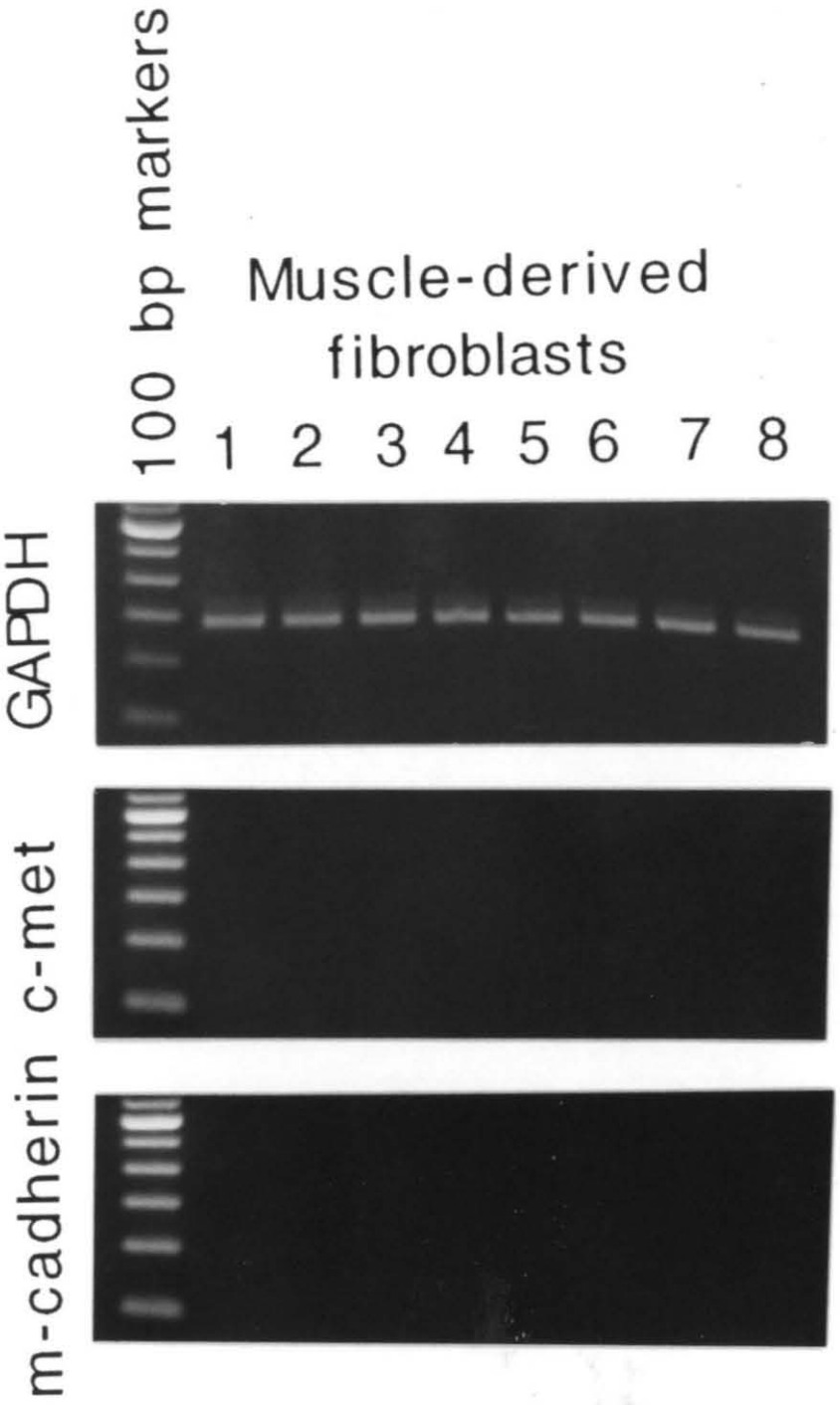

100 bp markers
Muscle-derived fibroblasts
1 2 3 4 5 6 7 8
GAPDH
c-met
m-cadherin

Figure 4: A) Myofiber stained and photographed after 48 hours in culture shown in phase and with nuclei visualized with DAPI; composite confocal image showing BrdU incorporation in nuclei of c-met positive cells. (400x) B) Myofiber stained and photographed after 96 hours in culture shown in phase and with nuclei visualized with DAPI; composite confocal image showing expression of MyoD in nuclei of c-met positive cells. (400x) C) Myofiber stained and photographed after 96 hours in culture shown in phase and with nuclei visualized with DAPI; composite confocal image showing expression of myogenin in the nucleus of a c-met positive cell. (400x)

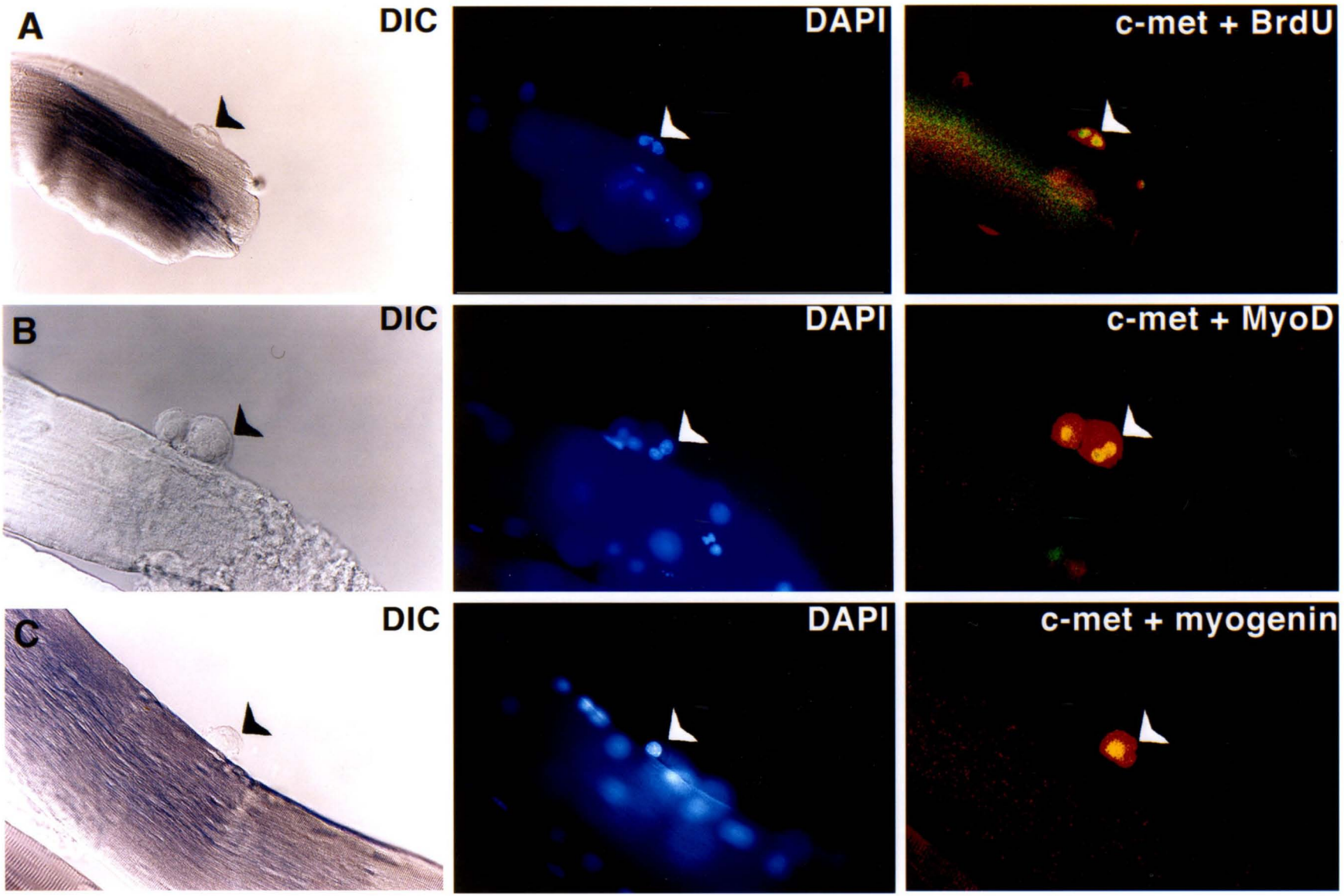

A
DIC
DAPI
c-met + BrdU
B
DIC
DAPI
c-met + MyoD
C
DIC
DAPI
c-met + myogenin

Figure 5: A) Confocal images of c-met and c-met + laminin in an unmanipulated muscle section showing localization of c-met expression (green) under the basal lamina (red) of a myofiber. (1600x) B) Composite photograph of a muscle section similar to B) showing a c-met positive (red) satellite cell and its nucleus (blue).(400x)

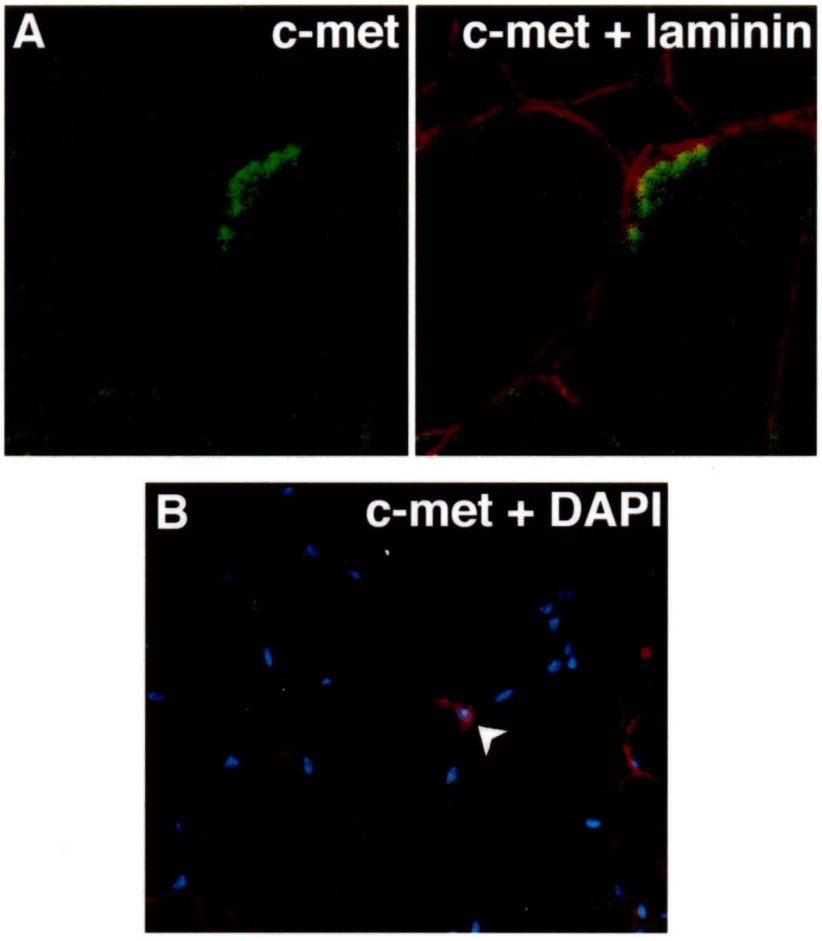

A
c-met
c-met + laminin
B
c-met + DAPI

Figure 6:Analysis of fidelity of multiplex single-cell RT-PCR reactions when challenged with decreasing input cDNA per reaction. A set of individual satellite cells after 96 hours in culture were harvested, pooled and reverse-transcribed; the resulting homogeneous cDNA was used in varying cell equivalents in first PCR reactions containing primers for either c-met + m-cadherin (Mix A), MyoD + myogenin + myf-5 + MRF4 (Mix B), or all 6 primer sets (Mix C, data not shown). Second reactions were run as usual and the products analyzed. Consistent positive results were obtained using from 0.25 to 0.125 of a cell equivalent of cDNA as input. It should also be noted that, even every cell put in the pool had been harvested successfully (the usual yield is about 80%), only about half would be expected to express myogenin or MRF4, thereby increasing the effective dilution of the mRNAs for those genes.

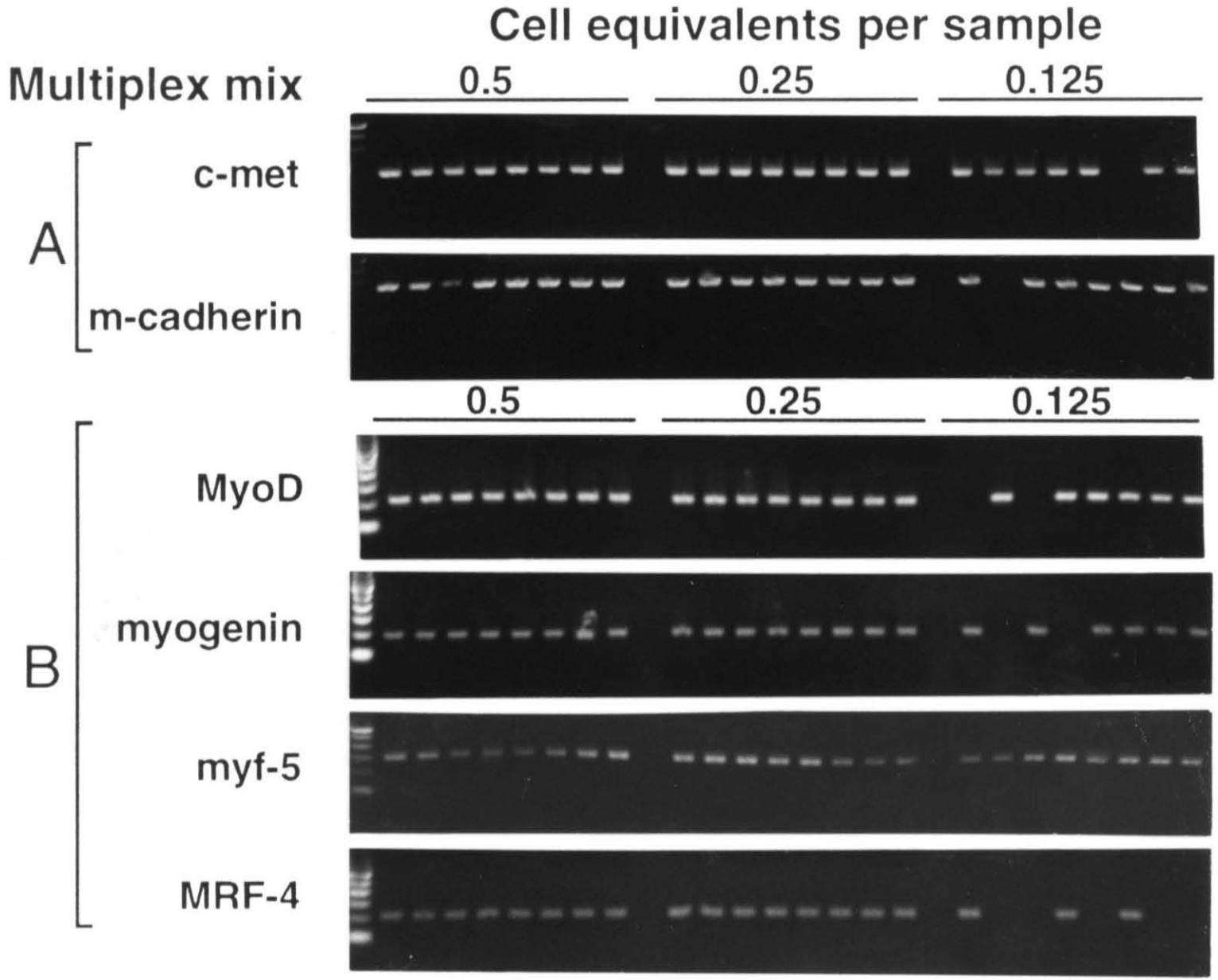
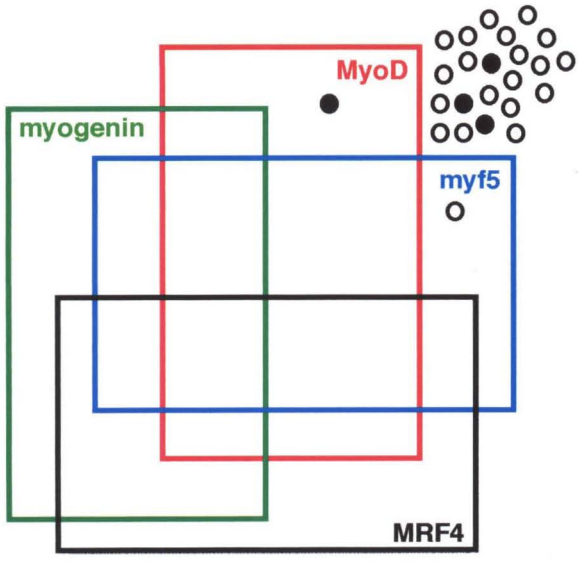
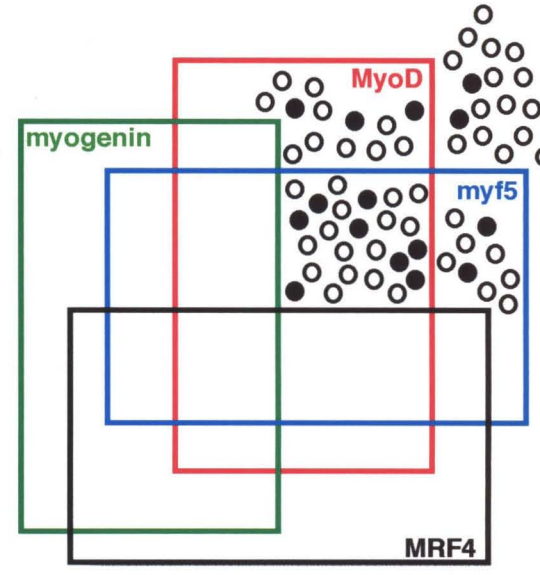

Cell equivalents per sample
Multiplex mix
0.5
0.25
0.125
A
c-met
m-cadherin
0.5
0.25
0.125
B
MyoD
myogenin
myf-5
MRF-4

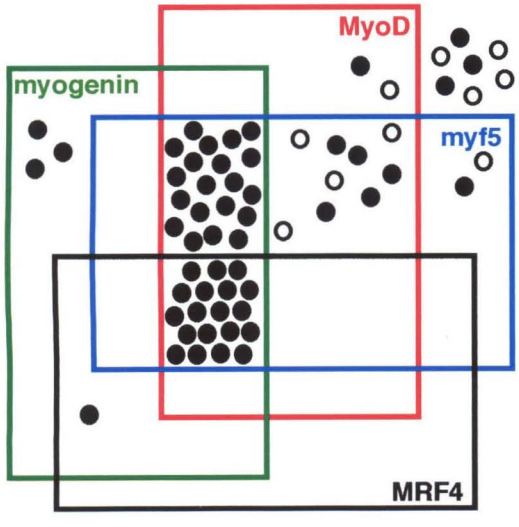
Figure 7: Representational summary of the combined gene expression status for c-met, m-cadherin, MyoD, myogenin, myf-5, and MRF4 among single satellite cells at different timepoints. Open circles indicate m-cadherin negative cells, filled circles indicate m-cadherin positive cells. The location of a cell within a colored rectangle indicates that the cell expressed that mRNA; cells in compartments formed by the overlap of two or more rectangles coexpressed those mRNAs.



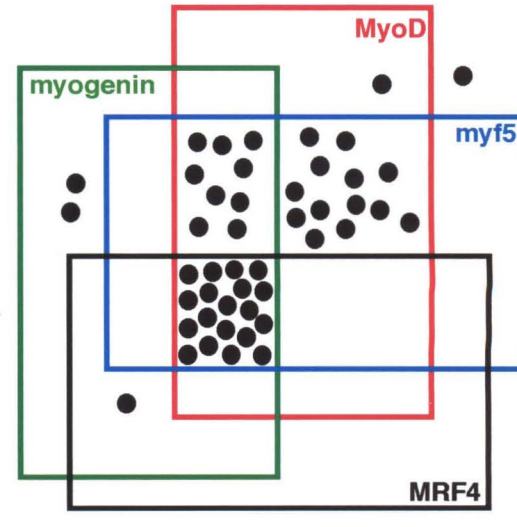
0 hours



24 hours



48 hours



96 hours

○ = c-met^{+}m-cadherin^{-}

● = c-met^{+}m-cadherin^{+}

Figure 8: Model of MRF coexpression status in satellite cells during the course of a regeneration response in fiber culture.

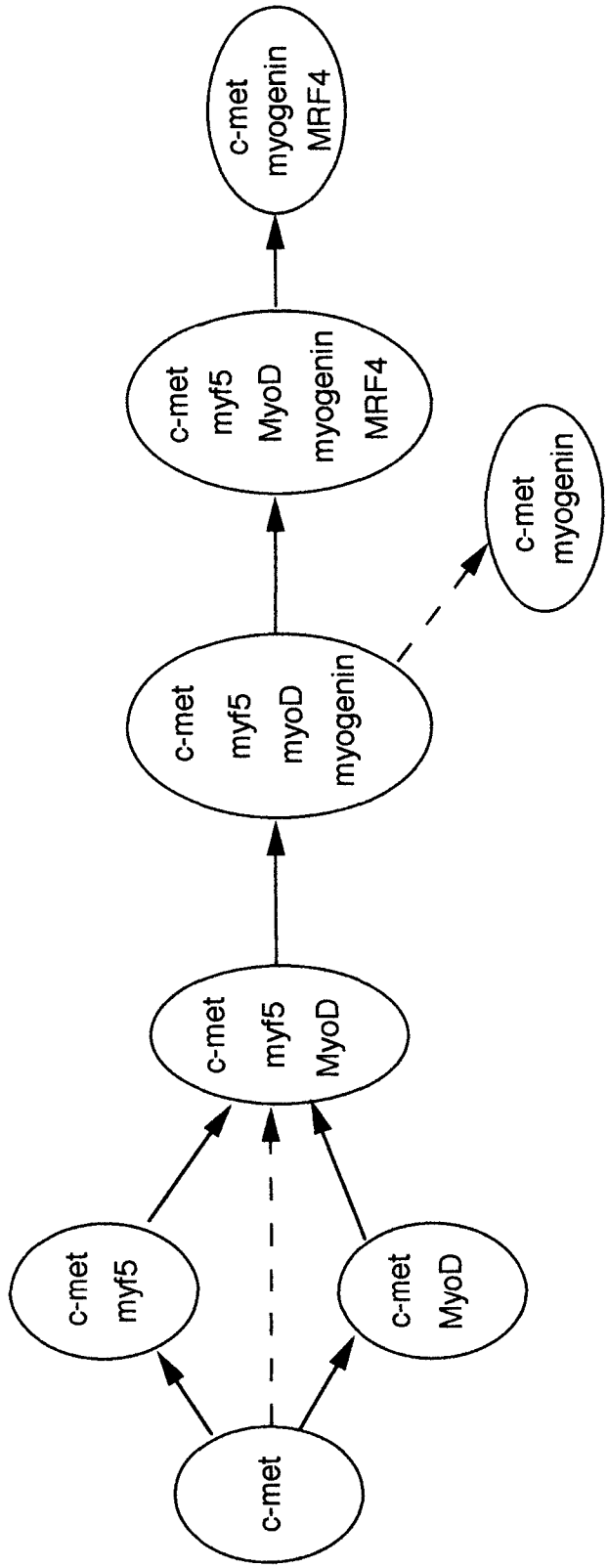

c-met
c-met myf5
c-met MyoD
c-met myf5 MyoD
c-met myf5 myoD myogenin
c-met myogenin
c-met myf5 MyoD myogenin MRF4
c-met myogenin MRF4

Table 1: Summary of prior studies of MRF expression in activated satellite cells. For this table only those studies in which satellite cells were either isolated as a highly enriched population or identified individually by morphological means were included, since MRF gene expression in other preparations cannot be unequivocally assigned to satellite cells.

Reference	Satellite cell source	Assay	MyoD					myogenin					myf5					MRF4				
			0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96
Maley *et al.*, 1994	Primary MPC mass culture, adult SJL/J or Balb/c mice	DIG *in situ*	-	+/-	+	+		-	+/-	+	+											
		Immunohist.	-	-	+	+		-	-	+	+											
Smith *et al.*, 1994	Primary MPC mass culture,	RT-PCR	-	+	+	+	+	-	-	+/-	+	+	-	-	+	+	+	-	-	+	+	+
Yablonka-Reuveni & Rivera, 1994	Cultured adult rat fibers	Immunohist.	-	+	+	+	-	-	-	+	+	+										
This study	Cultured adult mouse fibers	Immunohist.	-	+	+	+	+	-	-	+	+	+										
		SIngle cell RT-PCR	+/-	+	+	+	+	-	-	+	+	+	+/-	+	+	+	+	-	-	+	+	+

Chapter 3

Survey of gene expression in mouse skeletal muscle satellite cells and comparison to two myogenic cell lines

Abstract

While muscle satellite cells play a vital role in skeletal muscle repair after disease or injury, many basic questions regarding their biology remain unanswered due to technical difficulties. In many studies, immortalized cell lines are analyzed for clues to satellite cell processes; it is also often assumed that the much better-studied programs of embryonic myogenesis must be applicable to the satellite cell myogenic program. Exploiting the techniques of satellite cell isolation by culture of single myofiber explants and single-cell RT-PCR, I have assayed populations of satellite cells over the first four days of a regeneration response in culture (during which the cells will become activated, proliferate, and begin to differentiate into mature myofibers) for five groups of genes of general interest in myogenesis: positive and negative regulators of myogenesis, positive and negative regulators of the cell cycle, growth factors and their receptors, local signaling molecules, and markers of myogenesis in the embryo. The expression patterns detected revealed that many genes whose action is well-characterized in the embryo and widely studied in tissue culture may play a different role, or no role at all, in satellite myogenesis. This suggests that satellite cells posess a unique myogenic program, and should be considered as distinct from embryonic myoblasts and cultured cell lines. Next, the application of multiplex single-cell PCR to the genes and gene families detected in this study will serve to further define the biochemistry of satellite cell myogenesis.

Introduction

Skeletal muscle satellite cells are mitotically quiescent, mononucleate myogenic precursor cells resident in adult skeletal muscle. They are physiologically and clinically important due to their central role in regeneration of muscle tissue which has been damaged by injury or disease, yet very little is known about their biology compared to other myogenic cell lineages such as those of the developing embryo. This is largely due to their rarity, the difficulty of isolation and identification, and the difficulty of obtaining pure samples of satellite cell material in sufficient quantity for conventional biochemical and molecular analyses. For these reasons, many of the investigations into satellite cell biology have been performed on immortalized cell lines derived from presumptive satellite cells which maintain some satellite characteristics.

The technique of patch-clamp harvesting of single satellite cells resident on cultured muscle fibers is an efficient and effective method of collecting RNA from satellite cells for RT-PCR analysis (Cornelison and Wold, 1997) and provides a method of studying gene expression in satellite cells over the timecourse of an activation response. This technique avoids the drawbacks of potential contamination from nonsatellite cells present in muscle cultures or from the established muscle itself, and does not require large amounts of material for reliable analysis. This technique allowed assay of groups of satellite cells for genes whose expression pattern at various points in their myogenic program might clarify aspects of satellite cell biology, as well as establishing similarities or differences to other myogenic programs such as those of the embryonic muscle lineages.

Pools of cytoplasm collected at 0, 24, 48 and 96 hours after fiber harvest were reverse-transcribed then analyzed for the expression of a number of genes of potential importance in the satellite cell myogenic program. These included positive and negative regulators of myogenesis, positive and negative regulators of the cell cycle, signaling and cell fate determination molecules, and other genes mostly associated with somitic

myogenesis. cDNA samples from two myogenic cell lines commonly used as models for the satellite cell system, C2C12 (Yaffe and Saxel, 1977), made from thigh muscle of a 60-day old male C3H mouse and growth-transformed using a carcinogen, and MM14 (Hauschka, 1981), made from thigh muscle of a 60-day old male Balb/C mouse and the result of a spontaneous immortalization in culture, were also analyzed under conditions promoting either growth or differentiation for comparison with each other and with primary satellite cells.

The picture of satellite cells that emerges is of a dynamic population with the capacity to participate in a wide variety of intracellular and extracellular interactions, and possessing a pattern of gene expression that is quite different in several aspects from either embryonic myogenic precursor cells or model myogenic cell lines. These results mandate a rethinking of some current models of satellite cell biology, and will be further refined when combinatorial single-cell expression studies of the genes determined to be relevant in satellite cells are done.

Materials & Methods

Muscle fibers from adult mice were isolated and cultured and their satellite cells harvested with a patch-clamp pipette as described previously (Cornelison and Wold, 1997), with the exception that cells were pooled after harvest into a scaled-up reverse transcription reaction prior to transcription into cDNA; the pool cDNA was then analyzed by PCR and agarose gel electrophoresis for expression of genes of interest (Figure 1). Primers used are listed in Table 1. All primer sets were tested on known positive cDNAs for amplification of appropriate bands. All outside primer sets were designed to span at least one intron, where such information was available, and when no genomic structure information was available primers were tested on genomic DNA for non-amplification of bands equivalent to those amplified from a cDNA template.

Total RNA from C2C12 cells was obtained from cells in growth medium (DMEM supplemented with 20% FBS) or after three days in differentiation medium (DMEM supplemented with 2% horse serum) using RNeasy columns as directed by the manufacturer (Qiagen). Total RNA from MM14 cells in growth medium (DMEM + 15% horse serum + 2 ng/ml bFGF) or differentiation medium (DMEM + 2.5% horse serum + 1 μM insulin) was collected similarly. mRNA was reverse-transcribed into cDNA using the same protocol used for satellite cells, and cDNA from 0.5 ng of total RNA (2 pg, or approximately 50 cell equivalents, of cDNA) was used as template for each PCR reaction.

First and second PCRs were run as described previously, using only one outside primer set per first reaction, and the corresponding inside primer set for the second reaction, to avoid possible interactions between primer sets. Products were analyzed on a 2% agarose gel. Multiple (n>5) independently collected satellite cDNA pools were analyzed for each gene and timepoint; conditions scoring positive two or more times were counted as positive for the purposes of data analysis. Based on previous dilution studies (Cornelison and Wold, 1997), I estimate that cell pools in which 20% or more of the cells

harvested are expressing a given gene will score positive for expression of that gene; some genes may be detected at lower frequencies as well.

Results & Discussion

Positive regulators of myogenesis: MRFs and MEFs

The myogenic regulatory factors, or MRFs, are basic-helix-loop-helix transcription factors which together specify myogenic determination and differentiation (reviewed in Yun and Wold, 1996). While they have been extensively studied during execution of embryonic myogenesis, their expression patterns and function during satellite cell myogenesis have been less well studied and understood (reviewed in Cornelison and Wold, 1997). The work presented in Chapter 2 suggests a possible model for MRF progression during the transition from quiescence through proliferation and differentiation, and the work in Chapter 4 will supply further clues as to the possible function during satellite myogenesis of one member of the family, MyoD.

The MEF2 (Myocyte Enhancer Factor 2) family of DNA-binding proteins are also thought to have a positive effect on myogenic progression, although unlike the MRFs they are not expressed exclusively in muscle (reviewed in Molkentin and Olson, 1996). The four family members present in humans and mice, designated MEF2a-d, and the single factor present in Drosophila, DMEF2, all share a conserved MADS-box followed by a MEF2 domain, which together regulate dimerization and DNA binding, followed by divergent transactivation domains (reviewed in Molkentin *et al.*, 1995). They may bind to their cognate DNA sequences as either homo- or heterodimers, and are important for the activity of several muscle-specific promoters, such as that of myogenin (Cheng *et al.*, 1992; Yee and Rigby, 1993; Edmondson *et al.*, 1994) and MRF4 (Naidu *et al.*, 1995) as well as downstream muscle genes such as desmin and myosin (Kaushal *et al.*, 1994). It also appears that MEF2 dimers can, through protein-protein interactions, physically associate with MRF-E protein heterodimers when *either* complex is bound to DNA and synergistically transactivate gene transcription (Molkentin *et al.*, 1995). Deletion of the single *Drosophila* MEF2 gene results in myoblasts which form in the proper numbers and regions but which fail to differentiate into functional, multinucleate myofibers

(Ranganayakulu *et al.*, 1995). Results from tissue culture experiments as well as expression pattern analysis suggest that the MEF2 family members act to cooperatively and synergistically increase the efficiency of MRF-mediated gene activation, including MRF autoregulation (reviewed in Yun and Wold, 1996)(Molkentin and Olson, 1996) .

MEF2C is considered to be the major MEF2 family member contributing to myogenesis; in the adult mouse its expression is limited to skeletal muscle, spleen and brain (Martin *et al.*, 1993) . During embryogenesis, MEF2C is the first MEF to be detected by *in situ* hybridization. In addition to early cardiac expression, MEF2C transcript is present in myotomes at e8.5, approximately 18 hours behind the onset of *myf-5* expression, and progresses caudally as somites mature. Although it had been suggested from tissue culture experiments that MEF2 activity is required to activate transcription of *myogenin* (Cheng *et al.*, 1992; Yee and Rigby, 1993), expression of MEF2C lags behind myogenin by approximately six hours, and it is now thought to act to reinforce rather than to induce MRF expression. In caudal somites, MEF2C distribution is fairly uniform, while in more mature rostral somites transcripts are enriched between or at the edges of myotomes. In the limb buds, MEF2C transcripts are detectable at e11.5, concurrently with myogenin and MyoD, although with a different spatial distribution with regard to region of condensing muscle and, later, muscle fibers. (Edmondson *et al.*, 1994). MEF2C-null mice die during embryogenesis of a cardiac malformation (Lin *et al.*, 1997).

Expression of MEF2B mRNA in myotomes is slightly delayed relative to MEF2C, and is first detected at e9.0. Its expression pattern closely resembles that of MEF2C, and in later myogenesis MEF2B is preferentially localized at the ends of nascent myofibers. MEF2B-null mice are phenotypically normal, suggesting redundant functions shared with other family members (Molkentin *et al.*, 1996).

MEF2A transcripts are detectable in the myotome beginning at e9.5, at which time they are also present at lower levels throughout the lateral mesoderm and in migrating

neural crest cells. While it initially lags behind MEF2C expression, by e10.5 MEF2A expression extends to somites more caudal than those expressing MEF2C. Its expression pattern within the somite is very similar to that of myogenin (Edmondson *et al.*, 1994).

MEF2D is widely expressed in many embryonic and adult tissues, but has a splice variant which is preferentially expressed in skeletal and cardiac muscle (Martin *et al.*, 1994). Embryonic expression of MEF2D (using a probe which did not distinguish between the muscle-specific isoform and the more broadly expressed one) is similar to that of MEF2A, appearing at the same time and in a pattern resembling myogenin expression in the myotome; however MEF2D is more broadly expressed in several cell types, with the strongest expression in cardiac tissue and myotomes (Edmondson *et al.*, 1994).

The data for this section are summarized in Table 2. When assayed by RT-PCR, C2C12 cells express MEF2A, MEF2C, MEF2D (general) and MEF2D (muscle-specific) under both growth and differentiation conditions. A previous study found that by Western blot, C2C12 cells in growth medium express MEF2B and MEF2D, while cells in differentiation medium express all four (Molkentin *et al.*, 1996). MEF2A has also been immunolocalized to MyoD$^+$, myogenin$^+$ differentiating C2C12 cells (Yablonka-Reuveni and Rivera, 1997).

MM14 cells do not detectably express any of the MEF2 family members when in growth medium, and upregulate expression of MEF2A and both isoforms of MEF2D in differentiation medium. The lack of MEF2C expression is a notable difference from both the embryonic expression pattern and the C2C12 cell line.

Fiber-associated primary satellite cells express the general isoform of MEF2D at all times assayed, while MEF2A, MEF2C, and MEF2D muscle-specific transcripts are absent from freshly isolated cells but upregulated in activated satellite cells. MEF2A is more strongly/widely expressed, based on the high frequency with which it is detected among pools, while MEF2C expression is much weaker/rarer. While a minimum

number of pools scored positive for MEF2C at 24 and 96 hours, there is probably not a significant difference in expression and/or distribution between 24 and 96 hours, in spite of the lack of a positive cumulative score at 48 hours. This is supported by reports in which MEF2A protein was detected in differentiating C2C12 cells which had already upregulated MyoD and myogenin, but before the expression of muscle structural proteins (Yablonka-Reuveni and Rivera, 1997).

Negative regulators of myogenesis: Id family and m-twist

Id family HLH proteins

The four known members of the Id family, Id1-4 (Benezra *et al.*, 1990; Sun *et al.*, 1991; Christy *et al.*, 1991; Riechmann *et al.*, 1994), contain helix-loop-helix motifs which mediate heterodimerization with the MRFs or their pairing partners E12 and E47, but do not contain a DNA-binding basic region, rendering Id-containing dimers transcriptionally inactive (Benezra *et al.*, 1990; Sun *et al.*, 1991). Sequestration of differentiation-promoting transcription factors in inactive complexes by Ids is thought to be a mechanism for maintaining cells in a growing, undifferentiated state in spite of the presence of differentiation factors; a similar function has been suggested for the single *Drosophila* homologue, *extramacrochaetae* (*emc*) (Ellis *et al.*, 1990; Garrell and Modolell, 1990). High levels of Id are expressed in proliferating myoblasts (Benezra *et al.*, 1990), as well as in areas of active cell proliferation in the embryo, and forced expression of Id family members can delay or repress the emergence of a differentiated phenotype in cultured cells, even under low-serum conditions (Jen *et al.*, 1992). *In vivo*, overexpression of Id1 can compensate for a deleterious phenotype caused by overexpression of myogenin in mice mutant for both alleles (Gundersen *et al.*, 1995).

The expression patterns of Id-1, Id-2 and Id-3 during murine embryogenesis are mostly overlapping, but that of Id4 is unique. Id1 expression begins before gastrulation and is detected at very high levels in almost all cells of the embryo; as development

progresses expression is lost in a time- and tissue-dependent manner, declining first in precardiac mesoderm and CNS while it is still being expressed in some (but not all) areas of rapid proliferation and morphogenesis such as limb, visceral arches, and migrating neural crest. In somites, Id1 is expressed in the rostral half of the sclerotome (possibly due to the presence of migrating neural crest cells) and the dermotome. Unlike various cell lines studied, myotomal cells expressing myf5 or myogenin apparently do not express Id1 (Wang *et al.*, 1992; Evans and O'Brien, 1993). Id2 and Id3 expression are nearly identical to Id1 (Ellmeier and Weith, 1995; Jen *et al.*, 1996). Id4 expression is reportedly limited to neuronal tissues and some epithelial tissues (Jen *et al.*, 1996).

Although the actions of the Id family proteins have also been well-characterized in hematopoietic cells, where they have a similar function in repression of differentiation, studies of binding affinities of Id family members with E proteins (known as class A, or ubiquitous bHLH factors), MRFs, and hematopoietic bHLH factors (both of which belong to class B, cell type-specific bHLH factors) suggests that at least some part of their role in myogenesis may be distinct from their role in hematopoiesis. In a quantitative yeast two-hybrid assay, Id1, Id2, and Id3 were all found to bind with high affinity to E proteins, but a broader range of interaction strengths was seen for class B factors. Id2:myf5 and Id2:MyoD are high-affinity interactions, Id1:myf5 and Id1:MyoD are approximately fourfold weaker, while all other interactions are very low-affinity (Langlands *et al.*, 1997).

The data for this section are summarized in Table 2. C2C12 cells assayed by RT-PCR express transcripts for all four Id family members under both growth and differentiation conditions, while MM14 cells express no Ids in growth medium and upregulate Ids 1, 2 and 4 when cultured in differentiation medium.

The analysis of satellite cell expression of Id family genes in this paper represents the first recorded case in which myogenic cells which are not immortalized cell lines have been shown to express Ids. Based on the percentages of satellite cells also expressing

MRFs, this study suggests that Ids must also be coexpressed with MRF family members, at least in later stages of the response. Satellite cells do not express any of the Ids when freshly harvested and presumably still quiescent or newly activated, implying that Id regulation does not play a role in maintenance of quiescence. If the mechanism of Id inhibition is considered to be sequestration of myogenic bHLH factors, the lack of Id expression is not surprising since no MRFs are expressed at this time point. By 24 hours after activation, satellite cells express Ids 1, 2, and 3; this expression is maintained for the duration of the timecourse. Expression of Id4 commences by 48 hours and is also maintained at 96 hours post-activation. These data indicate that Id may be playing a role, probably in a sub-population of cells represented in the pools, in maintenance of the undifferentiated and proliferative state in the presence of MRFs.

M-twist

M-twist is the mammalian homologue of the *Drosophila* bHLH transcription factor twist, which is required for mesoderm-specific gene expression and, later, muscle formation (reviewed in Baylies and Bate, 1996). M-twist acts as a negative regulator of MRF-directed myogenesis by multiple mechanisms: 1) by sequestering MRFs and/or E proteins in a mechanism similar to that of the Ids, 2) by interacting with the basic domain of MyoD and possibly other MRFs in a way that prevents DNA binding, and 3) by actively inhibiting MEF transactivation activity (Hebrok *et al.*, 1997; Spicer *et al.*, 1996; Hamamori *et al.*, 1997).

During embryogenesis, the expression pattern of m-twist mRNA is similar to Ids 1-3. Transcripts are first detected in the anterior mesoderm at e7.0, in the somites, head mesenchyme, and somatic lateral plate by e8.0, and later in the branchial arches and their derivatives and areas of chondrogenesis. The somitic expression of m-twist originates in the dermomyotome, and is later restricted to the dermotome and the sclerotome and absent from the myotome (Fuchtbauer, 1995). A more recent study of m-twist protein distribution found a similar pattern of expression, but reduced spatially and temporally

(Gitelman, 1997); this may be due to post-translational modifications, as the author suggests, or to limitations of the antibody reagent. Twist-null mice die at e11.5 of cranial defects; they also have aberrancies in the somites and limb buds but no apparent defects in myogenic specification or progression (Chen and Behringer, 1995).

The data for this section are summarized in Table 2. C2C12 cells in this study expressed twist under both growth and differentiation conditions. Previous work in our lab found that twist mRNA is not detected by Northern blot in C2C12 cells under either growth or differentiation conditions (K. Yun, pers. comm.); however the highly sensitive and non-quantitative nature of the RT-PCR protocol employed here can lead to detection of trace amounts of message with the same apparent strength as robust signals. MM14 cells did not score positive for twist mRNA in either state; if m-twist is required for negative regulation of MRF activity, this may contribute the extreme tendency towards differentiation in this cell line.

Expression of m-twist in satellite cells is undetectable at 0 hours, present at 24 and 48 hours, and once more undetectable at 96 hours in culture. Given the assumption that twist is acting to prevent differentiation in cells expressing MyoD and myf5, this expression pattern seemed to fit very nicely- twist is not expressed in the absence of MRF expression, is upregulated when MyoD and myf5 are expressed but most cells are still proliferating, and is downregulated when differentiation begins. To test this hypothesis, single satellite cells were assayed as before for a six-gene expression set, this time replacing the primer set for m-cadherin with those detecting m-twist. The predicted result would be expression of m-twist in cells in the $MyoD^+$, $myf5^+$, or $MyoD^+myf5^+$ state but not in cells which had yet to express these genes or which had also begun to express myogenin or MRF4. Contrary to this, no obvious correlation was found between expression of m-twist and the complement of MRFs being coexpressed in the same cell (Figure 1). Clearly, the role of twist in myogenesis is not simple inhibition of MRFs; it may be required for other reasons, or not required at all, as the phenotype of twist-null

mice suggests. This result illustrates the power of single-cell analysis for understanding both the potential for and the effects of intracellular protein-protein interactions *in vivo*.

Growth factors and growth factor receptors

Hepatocyte growth factor/scatter factor and c-met

Hepatocyte growth factor is a widely-expressed cellular growth factor exhibiting mitogenic, motogenic, morphogenic, and survival effects on diverse cell types. It was cloned independently as a mitogen for primary hepatocytes, a fibroblast-derived factor which "scatters" tightly growing epithelial cell colonies, a factor inhibiting the growth of certain carcinomas, and an epithelial morphogen which induces branching tubulogenesis in endothelial cells. HGF is a mitogen for many cell types and is involved *in vivo* in hematopoiesis, chondrogenesis, and angiogenesis. It is one of the most powerful motogens known for diverse cell types including renal, hepatic, and mammary epithelial cells (for which it also functions as a morphogen), keratinocytes, thyroid cells, and vascular endothelial cells, as well as myogenic precursor cells. It is a potent mediator of cell survival in primary cultured neurons and PC12 cells, and is thought to act as a neurotrophic factor (reviewed in Matsumoto and Nakamura, 1996).

With its pleiotropic mitogenic, motogenic, and morphogenic properties, HGF is believed to be important in processes requiring coordinate cell growth, migration, and differentiation such as embryogenesis and repair of tissues and organs. This idea is borne out by the widespread expression of HGF in diverse embryonic tissues as well as in regenerating cell populations in organs such as liver, kidney and lung; most often cell types expressing HGF are mesenchymal (reviewed in Anastasi *et al.*, 1997). Null mutations of HGF result in embryonic death due to placental and hepatic defects (Schmidt *et al.*, 1995).

All of the activities of HGF are transduced by a single receptor, the c-met protooncogene. c-met is a disulfide-linked heterodimer with a small external α-chain and

a membrane-spanning β-chain with tyrosine kinase activity on its cytoplasmic domain (Bottaro *et al.*, 1991). HGF binding to c-met induces receptor autophosphorylation, which allows docking of various signal transduction proteins (Ponzetto *et al.*, 1994). c-met is most often seen expressed in epithelial cells adjacent to HGF-expressing mesenchymal cells; HGF and c-met are therefore thought to act in paracrine signaling during epithelial-mesenchymal interactions.

The null mutation of c-met (Bladt *et al.*, 1995) first gave a clue as to the role of HGF/c-met signaling in myogenesis: the null embryos had a muscle phenotype in which myogenic precursor cells otherwise destined to emigrate to the developing limb buds remained in the somite, a phenocopy of the Pax-3/*Splotch* mutant muscle defects. It was later determined that Pax-3 can directly activate c-met transcription in presumptive limb MPCs (Epstein *et al.*, 1996), nicely explaining the similarity in phenotype. Also, exogenously applied HGF could induce Pax-3 expressing myoblasts from the interlimb level of chick embryos to migrate into the lateral plate mesoderm (Brand-Saberi *et al.*, 1996). Regarding the pleiotropic effects of HGF/c-met signaling in different tissues, it is of interest to note that, while deletion of c-met produced myriad early defects resulting in death, deletion of only the Grb2-binding domain of c-met did not produce placental or liver defects, but did cause a striking reduction in limb muscle and a deficit in secondary myogenesis (Maina *et al.*, 1996).

The involvement of the HGF/c-met signaling pathway in satellite cells was first suspected in our lab due to the above results from embryonic myogenesis, and in other labs due to the emerging resemblance of HGF to the elusive satellite cell activating factor present in crushed muscle extract (discussed in Cornelison and Wold, 1997). It has since been shown (Cornelison and Wold, 1997; see Chapter 2) that c-met is expressed by all satellite cells at all stages of quiescence and activation, and that HGF is present in undamaged muscle tissue and capable of activating quiescent satellite cells (Tatsumi *et al.*, 1998).

The data for this section are summarized in Table 3. In this study, c-met and HGF are expressed by C2C12 cells under both growth and differentiation conditions, in accordance with results showing robust expression of c-met and HGF in growing C2C12s, and lower levels in differentiating cells, acting in an autocrine stimulatory loop (Anastasi *et al.*, 1997). This is abnormal for c-met/HGF signaling, which usually operates in a paracrine fashion, but is not unique (reviewed in Anastasi *et al.*, 1997). In contrast, MM14s express c-met, but do notappear to self-stimulate with HGF. This lack may be a component of their highly differentiation-prone phenotype, and their dependence on high levels of bFGF stimulation to induce cell cycling and prevent terminal differentiation; however, addition of HGF to the medium in amounts sufficient to stimulate satellite cells *in vitro* was not able to significantly repress the differentiation phenotype in the absence of bFGF (data not shown).

Primary satellite cells were also found to express HGF in an autocrine fashion approximately 36 hours after activation, inviting the theory that after initial stimulation with HGF released from locally-damaged muscle tissue, satellite cells begin to express HGF both to maintain their own proliferation in an autocrine fashion and possibly also to activate the regeneration program in satellite cells which move into the area later in the response. Expression of HGF following activation has also been noted in primary rat satellite cells (R. Allen, pers. comm.).

Fibroblast growth factors and receptors

The fibroblast growth factors, thirteen of which have now been cloned, are a family of peptide growth factors which are widely expressed during embryogenesis and in adult cell types, and mediate many processes such as gastrulation, mesoderm induction, limb outgrowth and patterning, angiogenesis, cardiac myogenesis, wound repair, stimulation of neurite outgrowth and neuronal survival, induction of ear, bone, skin, hair, limb, muscle, neural tube, tooth, and trachea, and suppression of myogenesis (reviewed in Kudla *et al.*, 1995; Pizette *et al.*, 1996). The first two isolated, *FGF1*

(acidic FGF) and FGF2 (basic FGF), are the best-characterized and most-studied. Expression studies of the various FGFs shows that, while the family as a whole is quite broadly expressed, each member has a discrete expression pattern. As possible mediators of growth and differentiation during myogenesis, it has been shown that FGF1, FGF2, FGF4 (Niswander and Martin, 1992), FGF5 (Haub and Goldfarb, 1991), FGF6 (Pizette *et al.*, 1996), FGF7 (Mason *et al.*, 1994), and FGF8 (Crossley and Martin, 1995) are expressed at some point in the developing myotomes, limb muscles, and other sites of myogenesis. Of special interest is FGF6, whose expression in the embryo and adult appears to be restricted to skeletal muscle cells and which, when deleted from the germline, leads to failure of muscle regeneration (Floss *et al.*, 1997). *In vitro*, expression of most FGFs was diminished when myoblasts differentiated and all of these factors have also been shown to delay or suppress differentiation in cultured myoblasts when expressed under a constitutive promoter (reviewed in deLapeyriere *et al.*, 1993; Pizette *et al.*, 1996). This suppression is mediated, at least in the case of FGF2, by activation of protein kinase C phosphorylation of myogenin (and possibly other MRFs) on the basic domain, abolishing DNA binding (Li *et al.*, 1992).

FGF signals are transduced by four (to date) receptor tyrosine kinases, FGFR1-4, which share sequence and structural homology. They are activated by ligand binding in cooperation with heparin sulfate proteoglycans, which promotes receptor dimerization and autophosphorylation of catalytic residues of the cytoplasmic tyrosine kinase domain (reviewed in Wilkie *et al.*, 1995). The FGFRs each have preferred FGFs which they will bind with high affinity, as well as differing expression patterns during embryonic and adult life. During embryogenesis, the only FGFR expressed in myotomes is FGFR4 (Stark *et al.*, 1991); later it is coexpressed in limb premuscle masses and differentiating muscles along with FGFR1 (Peters *et al.*, 1992). FGFR4 and FGFR1 are also the only FGF receptors known to be expressed in myoblast cell lines and satellite cells to date. In C2 and some other myoblast cell lines, expression of FGFR1 is strongly correlated with

the proliferative state, and has been suggested to be not only required to maintain that state but obligatorily lost upon differentiation (Itoh *et al.*, 1996). FGFR4, on the other hand, seems to have a biphasic expression pattern, being expressed in proliferating cells but also in differentiated cells; as opposed to FGFR1, whose expression is stimulated by FGF2 and FGF6, expression of FGFR4 is decreased on growth factor stimulation (Pizette *et al.*, 1996). FGFR1 can bind FGF1, FGF2, and FGF4; it binds FGF6 with low affinity. FGFR4 can bind FGF1 but not FGF2, and binds FGF6 with high affinity (Partanen *et al.*, 1991; Pizette *et al.*, 1996). Ligand:receptor association data for other FGFs are not available.

In accordance with previous studies, I find that C2C12 cells express FGF1 and FGF2; they also express, at levels detectable by this protocol, FGFs 5, 7, and 10. They express FGFR1 and FGFR4, also agreeing with published data, but in addition I detect FGFR2 and 3 as well. MM14 cells expressed FGF1 but not FGF2, which is not surprising given their absolute dependence on exogenously supplied FGF2 to maintain the proliferative state, which it has been shown can be replaced by transfection of a FGF2 expression construct. MM14 cells also expressed FGF6 and FGF7 in the differentiated state. The only FGF receptor detected in MM14s is FGFR1, which is also in accordance with published data (Olwin *et al.*, 1994).

Primary satellite cells express both FGF1 and FGF2 upon activation, but unlike myotomal cells, express no other FGFs. This finding should not be interpreted as a lack of a requirement for other FGFs; indeed, the requirement for at least one FGF (FGF6) in satellite myogenesis has been well established. The implication, instead, is that satellite cells receive FGF stimulation both in an autocrine fashion (FGF1 and FGF2) and in a paracrine fashion. This pattern fits well with both what is known of FGF signaling during embryogenesis and also with current knowledge of the local environment satellite cells would experience during regeneration *in vivo*.

Expression of FGFR4 was detected at 0 and 96 hours, but not at 24 or 48; this may reflect the biphasic nature of FGFR4 expression reported previously, or may be characteristic of a particular state of the population or a subset which is only present during quiescence and after the first cell division. Expression of FGFR1, while it was not detected at 0 hours, commenced before 24 hours post-activation and was maintained through the remainder of the timecourse.

Taken together, the data suggest a very early (pre-MyoD expression) role for FGF6 signal transduced by FGFR4, which is replaced during the first 24 hours by FGF2 and FGF1 acting through FGFR1. When FGFR4 is re-expressed at 96 hours, it may be that all cells, having passed some point, upregulate FGFR4 expression; alternatively, it may be expressed only in a subpopulation of satellite cells such as those which will continue to proliferate instead of differentiating.

Insulin-like growth factors and receptors

Insulin-like growth factors I and II are highly related to each other and to insulin. They have been implicated in many anabolic pathways in skeletal muscle including protein synthesis, nucleic acid synthesis, and glucose uptake. IGF I expression is low during embryogenesis in most tissues. Myogenic cells in the somite show only weak hybridization for IGF I message, however they stain more strongly for IGF I peptide and express IGFR1, implying that they have taken up IGF from the surrounding connective tissue, which actively secretes IGFs (reviewed in Engert *et al.*, 1996)). IGF mRNA and peptide have been detected in regenerating muscle, where it presumably acts as a trophic factor to recruit satellite cells to the wound site (Edwall *et al.*, 1989; Jennische *et al.*, 1987).

IGF I and IGF II can both bind to the two known IGF receptors, IGFR1 and IGFR2. IGF1 is a heterotetrameric transmembrane receptor structurally and biochemically similar to the insulin receptor. Ligand binding results in activation of both Ras-dependent MAP kinase and PI3 kinase (reviewed in (Montarras *et al.*, *1996*).

IGFR2, also known as mannose-6-phosphate receptor, is a single-chain transmembrane protein which is involved in transport of lysosomal enyzymes; it modulates IGF activity by internalizing molecules of growth factor and removing them from the cellular environment (reviewed in Stewart and Rotwein, 1996). In the muscle system, IGFR1 is considered to be the mediator of IGF signaling.

Null mutations of IGF I result in mice with a 60% decrease in size and concomitant reduction in skeletal muscle, while null mutations of IGF II have no muscle phenotype, possibly due to complementation by IGF I. IGF I has been suggested to be an anti-apoptotic factor, and IGF II will rescue apoptotic muscle cells in mdx mice (reviewed in Engert *et al.*, 1996).

The impetus for studying their effects on satellite cells comes not from their embryonic expression, where they are required for preimplantation development but are not specifically associated with myogenesis, but from their role in myogenesis of muscle cell lines *in vitro*. While they are referred to as growth factors due to their mitogenic effects on many cell types, they have been known for some time to stimulate differentiation in myogenic cell lines. C2C12 myoblasts express low levels of IGF II; when they are moved into differentiation medium expression of IGF II is strongly upregulated. This autocrine stimulation is essential for successful differentiation (Florini *et al.*, 1991). Expression of IGF II and MyoD appear to be mutually dependent: blocking either one with an antisense construct results in the levels of both transcripts being drastically reduced. Although in both cases the amount of myf5 transcript is greatly increased, the cells do not differentiate. The basal expression of IGF II in myoblasts is also required for maintenance of MyoD expression (Montarras *et al.*, 1996). Later experiments showed that IGF II antisense has no apparent effect on C2C12 cells in growth medium but promotes apoptotic death within 12-16 hours of the switch to differentiation medium, implying a role as a survival factor in the switch from proliferation to differentiation. Cells could be rescued by addition of insulin, IGF I, or

IGF II, and would then go through one round of cell division, then terminally differentiate (Stewart and Rotwein, 1996).

The dual effects of IGF on myoblasts were further elucidated by studies showing that IGF's mitogenic effects and pro-differentiation effects are sequential and separable. IGF-I stimulation initially increases levels of D-type cyclins, which return to normal levels within 48 hours, while it has the opposite effect on MyoD and myogenin, transiently downregulating their expression. Cells so treated will proliferate longer than untreated cells, but will then enter an accelerated and enhanced state of myogenic differentiation such that the end result is more and larger myotubes (Engert *et al.*, 1996). This finding was expanded upon with biochemical studies of IGFR1 in which it was found that inhibiting the Raf/Ras/MAP kinase pathway inhibited IGF-induced proliferation but led to greatly enhanced myogenesis, while inhibition of PI3 kinase activity inhibited IGF's pro-differentiation effects (Coolican *et al.*, 1997).

The data for this section are summarized in Table 3. By RT-PCR, C2C12 cells express IGF I and IGF II as has previously been reported, as do MM14s for which no data has been published. C2C12 cells express both receptors under both growth and differentiation conditions, while MM14 cells express neither receptor under growth conditions but both under differentiation conditions.

Primary satellite cells express IGFR1 and IGFR2 but do not detectably express either IGF I or IGF II. In this respect they resemble myotomal myoblasts more than they do either cell line, and do not appear to have a need for autocrine IGF stimulation in order to make the transition from proliferation to differentiation. Given the extracellular environment which surrounds satellite cells during wound repair, it is likely that a non-satellite cell source of IGFs is available and active during a regeneration response. This assumption is supported by the finding that IGF I peptide can both be localized in satellite cells *in situ* after injury (Jennische *et al.*, 1987), and IGF I mRNA is in the tissue (Edwall *et al.*, 1989), but does not explain why IGF I was not detected by RT-PCR when some

satellite cells in the previous reference were positive for IGF I message by S35 *in situ*. hybridization. I would suggest that, as in the embryo, autocrine IGF expression in satellite cells is low-level and possibly only in a fraction of the population; in support of this, when immunostaining and autoradiography are compared in both references, there are apparently many more immunoreactive satellite cells than there are satellite cells with silver grains.

Platelet-derived growth factors and receptors

Platelet-derived growth factors, or PDGFs, were first discovered as components of platelet α-granules with mitogenic activity for arterial smooth muscle cells and fibroblasts; they have since been shown to be produced by a variety of normal and transformed cell types. Active factors are dimers of any of the three possible combinations of two different but related subunits, PDGF-A and PDGF-B. These three isoforms differ in their functional properties as well as their secretability: PDGF-AA and PBGF-AB are readily secreted from producing cells, while the PDGF-BB variant mostly remains in the cell where it is produced. Genes whose expression is induced by PDGF stimulation include matrix and cytoskeletal proteins, growth-promoting and growth-inhibitory factors, and transcription factors such as c-fos, c-myc, c-jun, and c-myb (reviewed in Heldin and Westermark, 1990).

Two receptors for PDGFs, with different ligand specificities, have been identified: PDGFαR binds PDGF-AA, PDGF-AB, and PDGF-BB with high affinity, while PDGFβR binds PDGF-BB with high affinity, PDGF-AB with low affinity, and PDGF-AA not at all. PDGF receptors have tyrosine kinase activity which is activated by ligand binding and receptor dimerization and *trans*-phosphorylation. Known intracellular targets for PDGF receptor phosphorlyation are PLC-γ, PI3 kinase, and Raf1 (reviewed in Heldin and Westermark, 1990).

The influence of PDGF on myogenesis is less definite than that of, for example, the fibroblast growth factors, and has been less well studied. In C2C12 myoblasts,

PDGF stimulation increases the index of ^{3}H-thymidine labeling, possibly by reducing the time required to cycle (Yablonka-Reuveni *et al.*, 1990). This effect was seen most strongly with PDGF-BB, less so with PDGF-AB, and not at all with PDGF-AA, which was interpreted to mean that C2C12 cells express high levels of PDGFαR and low levels of PDGFβR subunits. Further studies (Yablonka-Reuveni and Rivera, 1997) indicated that this effect is quite mild, and is strongly affected by other factors such as culture density which can abrogate the mitogenic effect.

The data for this section are sumarized in Table 3. C2C12 cells in this study express both PDGFαR and PDGFβR, under both growth and differentiation conditions. C2C12 cells also expressed both PDGF-A and PDGF-B under both growth and differentiation conditions. Consistent with previous analyses, MM14 cells do not express either PDGF receptor; however, they do express both PDGF-A and PDGF-B under growth conditions, downregulating PDGF-B but not PDGF-A when switched into differentiation medium.

Stimulation by PDGFs appears to be a late and/or minor component of satellite cell progression, as expression of both receptors is not detected until late in the response (96 hours in culture); positive pools were detected at frequencies sufficient for a positive score but much rarer than the norm for many genes. Autocrine expression of either factor was never detected at any timepoint.

Positive regulators of the cell cycle: cyclins and cyclin-dependent kinases

Cells are driven through the cell cycle in response to mitogen stimulation by cyclins and their catalytic partners, the cyclin-dependent kinases (cdks), a family of Ser/Thr kinases which are dependent on cyclin binding for activity. These proteins are especially important in the context of the growth vs. differentiation decision which must take place before cells pass the G1/S boundary, also known as START or, in mammalian cells, the restriction point (reviewed in Hunter and Pines, 1994; Sherr, 1993). The

cyclin-cdk complexes expressed during the G1 phase are the D-type cyclins, D1, D2, and D3, which will usually form complexes with the cyclin-dependent kinase cdk4. Expression of the D-type cyclins is strongly growth factor-dependent, and both the RNA and protein have a very short half-life, leading to the suggestion that they may act as growth factor sensors (reviewed in Hunter and Pines, 1994). Most cells express a combination of cyclin D3 and either cyclin D1 or cyclin D2, in a lineage-specific manner (reviewed in Sherr, 1993). The mechanism of cyclin D-cdk promotion of the cell cycle is thought to involve phosphorylation of the retinoblastoma protein (Rb), and possibly the related proteins p107 and p130. Rb then binds to and inhibits transcription factors such as E2F, which are required for initiation of DNA synthesis (reviewed in Hunter and Pines, 1994).

In the context of skeletal myogenesis, in which cell proliferation and differentiation are mutually exclusive programs, expression of cyclin D1 and later D2 and D3 was shown to promote continued proliferation even in the absence of mitogens, and to inhibit MyoD-induced expression of the differentiation marker muscle creatine kinase (MCK) by phosphorylating MyoD (Rao *et al.*, 1994; Guo and Walsh, 1997). When C2C12 cells are placed in low-mitogen differentiation medium, the levels of cyclin D1 mRNA and protein drop precipitously, however cyclin D3 expression transiently decreases then returns to the same or higher level of expression, suggesting it may have some functions in differentiating cells distinct from cell cycle promotion (Rao and Kohtz, 1995). cdk2 expression is downregulated during myogenic differentiation, while cdk4 expression levels do not change (Walsh and Perlman, 1997).

Cyclin E, and more rarely cyclin A, are thought to act after the D cyclins at the G1/S boundary itself and to be important in initiation of replication (reviewed in Hunter and Pines, 1994). They also phosphorylate Rb, p107, and p130, which until recently were the only known substrates for cyclin E-cdk2. Unlike the D-type cyclins, ectopic expression of cyclin E alone cannot divert cells from myogenesis to proliferation, but

coexpression with its active partner cdk2 can do so (Guo and Walsh, 1997). It is also interesting to note that while expression of a dominant-negative form of cdk4 (the preferred cdk of D-type cyclins) cannot arrest cycling cells, expression of a dominant-negative cdk2 (which pairs with cyclin E) will arrest cells in G1 (Heuvel and Harlow, 1993). A very intriguing result was recently published in which it was discovered that Id2 is also a substrate for cyclin E-cdk2 complexes, and that its phosphorylation occurs at the G1/S boundary and renders it incapable of binding to MyoD (and possibly E proteins as well) (Hara *et al.*, 1997), leading to cell cycle arrest in G1, thus linking the cell cycle and myogenesis. This followed studies which concluded that Id proteins are somehow required for G1 progression, along with E proteins, in a mutually dose-dependent manner (Peverali *et al.*, 1994), however the mechanisms involved are still not clear.

The data for this section are summarized in Table 4. In this study, the C2C12 cell line expresses all three D-type cyclins, as well as cdk2, cdk4, and cdk 5 when in high-mitogen medium; when switched to low-mitogen differentiation medium expression of cyclin D1 and D3 are still detected, as are all three of the cdks. Expression of cyclin E, which is detected under growth conditions, disappears under differentiation conditions. In MM14s, cyclin D3 and cyclin D1 but not cyclin D2 are expressed under growth conditions, consistent with the finding that most cell types will prefer to express cyclin D3 and either D1 or D2 and that, for myoblasts, cyclin D1 seems to be the most potent.

Satellite cells, like MM14s, seem to preferentially express cyclins D1 and D3 once cell proliferation has been initiated; however, they appear to be forming complexes with a less-often used pairing partner, cdk4, as cdk2 and cdk5 were not detected in this study. Cyclin E, which normally regulates passage from G1 into S, is not expressed by satellite cells. Presumably, cyclin A-cdk4 is acting in place of cyclin-E-cdk4 in this function, a substitution which is unusual but not unheard-of (Resnitzky *et al.*, 1995) and for which there is at least some evidence in C2C12 cells (Guo *et al.*, 1995). It may also explain why attempts to immunolocalize Cyclin E to C2C12s and satellite cells have failed; such

failures were usually attributed to the assumed transience of cyclin E expression (i.e. Yablonka-Reuveni and Rivera, 1997)

Negative regulators of the cell cycle: cyclin-dependent kinase inhibitors

The cell cycle promoting activities of cyclin-cdk complexes are negatively regulated by stoichiometric interactions with two families of cyclin-dependent kinase inhibitors, or CDIs. CDIs can act as tumor suppressors by mediating G1 arrest and/or apoptosis, and as such are frequently deleted or inactivated in cell lines and primary tumors. They also act to arrest cells in response to mitogen deprivation, contact inhibition, stimulation by anti-proliferation factors, and differentiation signals (reviewed in (Walsh and Perlman, 1997)Walsh and Perlman, 1997).

The p16/INK4 (for inhibitor of cdk4) family of CDIs includes p15, p16, p18, and p19; they share structural homology such as four conserved ankyrin repeat motifs, and all appear to act to induce cell cycle arrest in G1 by binding to cdk4 or cdk6 at the expense of D cyclins and do not bind to other cdks or any cyclins (Hall *et al.*, 1995). At least for p16 and p18, INK4 action is only effective in cells in which Rb is present in its hypophosphorylated, growth-suppressive form (reviewed in Sherr and Roberts, 1995). p16/INK4a is expressed at very low levels or not at all during embryogenesis and early postnatal development, and during adulthood is only expressed in spleen, lung and liver (Zindy *et al.*, 1997). Expression of p16 can counteract cyclin D1 inhibition of MyoD-induced differentiation, linking cell cycle regulation and myogenesis (Rao *et al.*, 1994; Rao and Kohtz, 1995; Skapek *et al.*, 1995). p16-null mice have abnormal hematopoiesis and are susceptible to sarcomas and lymphomas (reviewed in Yan *et al.*, 1997). p15/INK4b is also not detected prenatally, and is later expressed primarily in testis and lung (Zindy *et al.*, 1997). It is induced upon differentiation or upon stimulation with TGF-β (Hannon and Beach, 1994). p18/INK4c is expressed during mid to late embryogenesis, and in adult tissues including kidney, lung, heart, and skeletal muscle

(Zindy *et al.*, 1997). It is expressed ubiquitously in proliferating cultured cells and in normal mouse tissues (reviewed in Sherr and Roberts, 1995), and is upregulated in differentiating myoblasts (reviewed in Walsh and Perlman, 1997). It may have a function in maintaining the differentiated state (Zabludoff *et al.*, 1998). p19/INK4d (not to be confused with $p19^{ARF}$, a protein generated from an alternate open reading frame within the p16INK4a locus), is expressed from early embryogenesis and is widely expressed in many adult tissues (Zindy *et al.*, 1997). Its expression is downregulated in differentiating myocytes (reviewed in Walsh and Perlman, 1997). Its specific functions apart from those shared by the entire family remain largely unclear.

The p21/Cip/Kip family of CDIs includes p21, p27, and p57; they also function to arrest cells in G1 but do so by binding to cyclins or cyclin-cdk complexes, not to uncomplexed cdks (Hall *et al.*, 1995). p21, also called Cip1 and Waf1, is induced in a p53-dependent manner in response to DNA damage, and through a p53-independent, MAP kinase-mediated pathway in response to mitogen deprivation or differentiation cues (Liu *et al.*, 1996). It is expressed in differentiating cells and has been implicated in cellular senescence and protection from apoptosis in differentiating myocytes (Wang and Walsh, 1996). p21 is strongly upregulated during myogenic differentiation in the embryo, as well as in cultured myoblasts upon serum deprivation, and remains high even if the cells are restimulated with mitogens, implying it has a role in maintenance of differentiation-induced withdrawal from the cell cycle (reviewed in Walsh and Perlman, 1997). p21 also contains a domain which directly binds to the proliferating cell nuclear antigen (PCNA) and directly inhibits entry of the cell into S-phase (Watanabe *et al.*, 1998). p21 has been implicated in integration of Raf signaling to cause either proliferation or cell cycle arrest (Woods *et al.*, 1997; Sewing *et al.*, 1997) and BRCA1-induced cell cycle arrest (Somasundaram *et al.*, 1997). p21-null mice have no developmental defects and are not abnormally susceptible to tumors (reviewed in Yan *et al.*, 1997). p27, also called Kip1, is expressed in quiescent cells, downregulated upon

re-entry into the cell cycle, and upregulated in differentiating cells (reviewed in Grana and Reddy, 1995). It is induced by stimulation with TGF-β, by serum deprivation, and by contact inhibition (reviewed in Katayose *et al.*, 1997).. During embryogenesis, it is expressed in myotomes and is thought to have a transient role during the transition from proliferation to differentiation during myogenesis (Zabludoff *et al.*, 1998). Overexpression of p27 in cultured myogenic cells greatly enhanced MyoD-induced myogenesis (Zabludoff *et al.*, 1998). p27-null mice are larger than wild-type, presumably due to failure of cells to exit the cell cycle properly (reviewed in Yan *et al.*, 1997). In human cancer cells, overexpression of p27 promotes apoptosis (Katayose *et al.*, 1997), but the relevance of this result to untransformed cells expressing normal levels of p27 has yet to be determined. p57/Kip2 has homology to p21 and p27 but also has unique domains. It is expressed during mid-embryogenesis in newly differentiated neurons, myotomes of somites and cardiac muscle; at later times it is expressed in a variety of postmitotic tissues (Yan *et al.*, 1997). Null mutations of p57, unlike those of p15, p16, p18, and p21, exhibit embryonic malformations which in highly penetrant forms are lethal at birth (Yan *et al.*, 1997). Expression of p57 is associated with terminally differentiated, nonproliferating cells and appears to have an antiapoptotic effect (Yan *et al.*, 1997).

It should be noted that most studies of these proteins have been performed in transfected tissue culture lines, in which they are expressed at superphysiological levels, or in virally transformed or tumor cells, in which they are often inactivated or deleted. In the course of the cell cycle in normal cells, they are tightly regulated in response to various checkpoints but do allow cell cycling even when actively expressed, unless the cell fails a checkpoint assessment or is receiving external signals to cease proliferating and/or to differentiate.

The data for this section are summarized in Table 4. In C2C12 cells assayed here, p18, p19, p21, and p27 are expressed by cells in growth medium; all CDI's

assayed for were expressed in differentiating cells. MM14s expressed p19 and p21 in growth medium, and p18, p21, and p27 in differentiation medium. Given that p18 is suspected to act as a maintenance factor for terminal differentiation, p21 is present in cycling myoblasts and upregulated in differentiating myocytes, and p27 is upregulated during mitogen withdrawal and the switch from proliferation to differentiation, the only puzzling factor is the lack of expression of p57, which is usually strongly associated with terminal myogenic differentiation.

Satellite cells expressed p15 at all times surveyed, and upregulated p18, p21, p27, and p57 over the course of differentiation. The significance of the p15 expression is unclear; p15 has been implicated in TGFβ-induced cell cycle arrest, which also induces myogenic differentiation in cultured myogenic cell lines (Hannon and Beach, 1994), however why it should be expressed so ubiquitously is not apparent. The lack of expression of p27 at very early timepoints is distinctive when compared to other types of quiescent cells. Either activation had progressed sufficiently by the time the cells were harvested that p27 levels had already been downregulated, which is possible but unlikely as proliferating cells still express basal levels of p27, or p27 is not a factor in maintaining quiescence in satellite cells.

Cell signaling and cell fate determination molecules

Many of the genetic and molecular pathways involved in cell-cell signaling leading to the adoption of different fates by equivalent neighbor cells have been best studied in *Drosophila*. Their mammalian homologs, often appearing in families in which the function of a single *Drosophila* gene has been distributed and elaborated, are now being studied to elucidate parallel signalling pathways in mammalian embryos. While many of these genes have been best studied in the developing nervous system of both flies and mammals, they often have functions during somitogenesis and/or myogenesis. The mechanisms by which satellite cells separate into populations of proliferating,

differentiating, and possibly 'pre-quiescent' cells, are unknown. Possibly one or more cell-cell signalling pathway is involved, either among satellite cells or between satellite cells and some other cell type. Therefore, I investigated the expression patterns of several of these gene families over the timecourse of satellite cell activation.

Mammalian Notch, Serrate, and Delta homologs

Drosophila Notch, Serrate, and Delta proteins are related transmembrane proteins which act together to restrict cell fate in neighboring cells via cell-cell signaling and feedback loops. A well-characterized system requiring these genes is the specification of neuroblasts from precursor populations (equivalence groups) all of which are equally competent to become neuroblasts. In each group, the single cell which will become the neuroblast will express Delta, a signal which is received by Notch expressed on neighboring cells. Ligand-binding by Notch results in cleavage of the intracellular domain, which then becomes localized to the nucleus and acts through several other gene products to repress transcription of differentiation-promoting genes. This intracellular signaling cascade then feeds back to reinforce the secondary cell fate decision in the Notch-expressing cell in a process known as lateral inhibition (reviewed in (Dunwoodie *et al.*, 1997). Many other cell types use signaling to Notch via Delta or Serrate expression on neighboring cells to specify alternative cell fates in equivalent cells; in these tissues as well as in the neural precursors described previously, loss-of-function mutations for either ligand or receptor result in the expansion of one cell type at the expense of another.

The four mammalian Notch homologs cloned so far, Notch 1, Notch 2 (previously known as Motch A and B, respectively), Notch 3, and Notch 4, have not only structural homology but also apparently similar functions to their *Drosophila* counterpart: constitutively active forms will inhibit neurogenesis in *Xenopus*, as well as inhibiting myogenesis in *Xenopus* and cultured mouse myoblasts (reviewed in Dunwoodie *et al.*, 1997). This homology also extends to the two mammalian Serrate

genes cloned thus far (Jagged-1 and 2) and the two mammalian Delta homologs, Dll-1 and Dll-3. These genes have been shown to be important in neurogenesis, which will not be discussed here, and in somitogenesis. Dll-1 and Dll-3 are expressed in two stripes in the posterior and anterior of nascent somites, respectively, in regions where Notch activity is required to establish somite boundaries. Notch 1-null mutants have disorganized and irregular somite boundaries, supporting this interpretation (reviewed in Dunwoodie *et al.*, 1997). In adult tissues, only Notch 1 is detected in skeletal muscle by Northern blot (Lardelli and Lendahl, 1993); during embryonic and adult development, Notch 3 and Notch 4 are neuronally- and endothelially-restricted, respectively (Lardelli *et al.*, 1994; Uyttendaele *et al.*, 1996).

Within the framework of myogenesis, Notch signaling has been implicated in repression of the myogenic phenotype in *Xenopus* embryos and in cultured myoblasts, and appears to act by interfering with activation of target genes by MyoD, based on studies of a constitutively active form of Notch (Kopan *et al.*, 1994). Similar results were obtained by coculture of Notch-expressing C2C12 cells with Jagged-1 (rat Serrate-1) expressing fibroblasts: even under differentiation conditions, the C2C12 cells failed to differentiate (Lindsell *et al.*, 1995).

The data for this section are summarized in Table 5. In this study, C2C12 cells detectably expressed Notch 1, 2, and 3, under both growth and differentiation conditions. MM14 cells, however, only expressed Notch 1, which is the most prominently-expressed family member in embryonic and adult myoblasts. C2C12 cells expressed Jagged-2 and Dll-1 but not Dll-3 in both conditions, as did MM14 cells.

Satellite cells expressed Notch 1 and Notch 2, over the entire course of the differentiation response. If interpreted according to the current theory of Notch function, this would imply that at least a fraction of satellite cells at all times in a regeneration response are capable of receiving anti-differentiation signals. As satellite cell pools at all timepoints are also positive for Dll-1 (but, interestingly, never express Dll-3) there may

be a mixture of 'sending' and 'receiving' cells. Alternatively, both ligand and receptor may be coexpressed by the same population of cells at some or all timepoints, requiring cell-cell signalling-induced feedback loops to modulate expression of both genes and subsequently direct alternative cell fate decisions. Jagged-2, which is also expressed at all times post-activation, may also play a role in addition to or in concert with Delta in activation of Notch proteins.

Mammalian Fringe homologs

Drosophila Fringe is a signaling protein which has homology to bacterial glycosyltransferases. It acts to establish wing margin formation at Notch-ligand boundaries, possibly by altering glycosylation of other proteins on the surfaces of or secreted by neighboring cells. Three mammalian homologs have been cloned, Radical fringe, Lunatic fringe, and Manic fringe. Their expression also defines boundaries between Notch ligands (i.e. between areas of Dll-1 expression and areas of Jagged-1 expression). During somitogenesis, Lunatic fringe is highly expressed in two stripes defining the anterior and posterior boundaries of each somite as it forms, as well as in the myotome and proliferating and/or undifferentiated neural progenitor cells. Conversely, Radical fringe and Manic fringe are expressed in differentiated cells, often physically juxtaposed with less-differentiated Lunatic fringe-expressing cells. In addition to somitogenesis, these patterns appear in the neural ventricular and marginal zones, the thymic cortex and medulla, and a variety of other tissues; in all cases expression coincides with a Notch-ligand boundary. It is proposed that the mammalian fringe proteins are differentially required to activate Notch signalling in such border domains (Cohen *et al.*, 1997).

The data for this section are summarized in Table 5. Consistent with the somitic expression pattern, Lunatic fringe (which is associated with proliferating or predifferentiated cells) was detected in this study in C2C12 cells under growth conditions, while it was not detected under differentiation conditions. Manic fringe,

which is associated with terminally differentiated cells, was expressed in the reciprocal pattern. Radical fringe, which is also associated with differentiated cells, was detected under both conditions. Data do not exist regarding the relative strength and abundance of Radical fringe vs. Manic fringe; it may be that Radical fringe is more widely expressed or less strongly associated with the differentiated state, or that this highly sensitive RT-PCR assay detects expression from the small percentage of differentiating cells unavoidably present even under growth conditions.

Satellite cells express Radical fringe within 24 hours of activation but not when quiescent or freshly isolated; this is accordance with the association of Radivcal fringe with the more differentiaed state in other cell types. Manic fringe, which is also associated with differentiation, was never detected in satellite cells. Strangely, Lunatic fringe, which is instead associated with predifferentiated cells, is not detected in freshly harvested satellite cells or at24 or 48 hours after activation, but only at the 96 hour timepoint. The meaning of this is unclear; one possible explanation would be that by this time in culture, expression of Lunatic fringe is required to modulate Notch signaling in order to maintain cells in proliferation due to widespread expression of myogenin and MRF4.

Mammalian Numb homologs

Drosophila numb is a gene which has only recently been cloned and characterized; it encodes a membrane-associated protein which becomes asymmetrically distributed prior to cell division, such that one daughter cell receives the Numb protein and one does not. This differential distribution leads to alternative cell fates, mediating intrinsically the same sort of decisions mediated extrinsically by Notch-Delta signaling (Rhyu *et al.*, 1994). Indeed, during *Drosophila* myogenesis, in which numb is required to segregate cell fate in the terminal division of myogenic precursor cells, numb acts to antagonize Notch-mediated transcriptional repression (Gomez and Bate, 1997; Carmena *et al.*, 1998). This is very similar to *Drosophila* neurogenesis, in which selection of alternate

cell fates during the two asymmetric divisions of a sensory organ precursor cell are mediated by Numb distribution in a Notch-dependent manner (Guo *et al.*, 1996; Spana and Doe, 1996).

Two mouse homologs of Numb have been cloned, Numb and Numblike. While numb function has been better studied, both can complement *Drosophila numb* mutant phenotypes and, when overexpressed, can induce phenotypes typical of *numb* overexpression (Verdi *et al.*, 1996; Zhong *et al.*, 1997). The expression and function of numb in mammalian neurogenesis appear to be equivalent to the *Drosophila* counterpart, however numblike has a more restricted expression pattern and is not asymmetrically localized on dividing cells, implying that it may have unique functions.

Mammalian numb is broadly expressed in the developing embryo, and is expressed in adult tissues including kidney, gut, lung, heart, brain, spleen, and liver, but skeletal muscle expression is extremely weak or not detected (Verdi *et al.*, 1996; Zhong *et al.*, 1997). Numblike is only expressed in areas of the embryonic and adult nervous system, and there its expression is restricted to a subset of numb-expressing cells which are more differentiated (Zhong *et al.*, 1997).

The data for this section are summarized in Table 5. RT-PCR shows that both C2C12 cells and MM14 cells express Numb under both growth and differentiation conditions, while both cell lines only express numblike when differentiated. Satellite cells as well express Numb at all times, while expression of numblike is upregulated concurrently with differentiation in the population. Numb protein expression is also detectable in some but not all satellite cells at different timepoints (data not shown); its expression appears to be uniform across the cell surface rather than positionally restricted, as seen in other mammalian cell types assayed with this immune reagent (Verdi *et al.*, 1996). Little is known about the biological function of numblike, especially outside the context of neurogenesis, but numblike does appear to be associated with more-differentiated cells (Zhong *et al.*, 1997). These factors may conceivably have a role

in specifying, via interactions with expressed Notches or other factors, the proliferation program versus differentiation, or may have a role in maintenance of the satellite 'stem cell' population, or both.

Markers of embryonic myogenesis

Pax-3

Pax genes, which are homologs of *Drosophila paired*, encode transcription factors which individually play roles in determination and morphogenesis in a number of different tissues. One of these family members, Pax-3, is expressed in MRF-negative myoblasts during embryonic myogenesis. While myotomal cells downregulate Pax-3 expression just prior to or immediately following activation of the first MRF to be expressed, cells resident in the ventrolateral portion of the dermomyotome, which will delaminate and migrate laterally to populate the limb buds, maintain Pax-3 expression until they reach their destination and upregulate MRFs (Williams and Ordahl, 1994). In the absence of Pax-3 activity these cells fail to migrate and instead differentiate in the somite (Daston *et al.*, 1996). One function of Pax-3 in these cells was elucidated with the findings that the activity of the c-met receptor tyrosine kinase is required for this migration (Bladt *et al.*, 1995), and that Pax-3 activity is necessary and sufficient for c-met expression in these cells (Epstein *et al.*, 1996). Another possible function which has been suggested more recently is that Pax-3, in parallel with myf5, is upstream of MyoD in the genetic hierarchy (Tajbakhsh *et al.*, 1997). This result was based on the analysis of embryos carrying null mutation of both Pax-3 and myf5, and the observation that these embryos, unlike either of the singly-homozygous mutants, almost entirely lack body muscles and fail to express MyoD.

The data for this section are summarized in Table 5. Given that Pax-3 is essential for c-met expression in the myogenic precursors of the limbs, which are in many ways very similar to satellite cells in that they are committed but predifferentiated, proliferative,

migratory, MRF-negative myogenic precursor cells, and that it is important in initiating expression of MyoD, whose expression appears to be required for satellite myogenesis (Megeney *et al.*, 1996), Pax-3 expression was predicted in satellite cells. However, neither C2C12 cells, MM14 cells, or satellite cells ever detectably expressed Pax-3 in this study. The Pax-3-specific primers, like all primers used here, amplified an appropriate band from cDNA samples known to be positive (in this case, e10.5 embryonic cDNA.)

What are possible explanations for this counterintuitive failure to express Pax-3? In the case of c-met, the *cis*-regulatory sequences are sufficiently complex as to allow c-met expression in a large array of widely different cell types. It would arguably be *more* reasonable for satellite cells, which exist in a very different environment than embryonic myogenic precursor cells, where they are probably exposed to a different array of stimuli and serve a very different function, to have different factors which will promote c-met expression as well. The observations here and elsewhere that c-met expression, while it occurs initially when satellite cells are in an MRF-negative state, persists well into a regeneration response over many days of active MRF expression also support this argument. The question of a requirement for Pax-3 or myf-5 to activate MyoD cannot directly be studied in satellite cells at this time, as such doubly-homozygous embryos die *in utero* and satellite myogenesis is a phenomenon of the adult animal. However, given that expression of the different MRFs in satellite cells occurs with different timing and sequence than in either type of somitic myoblast (Cornelison and Wold, 1997), and that the functions of MyoD and myf5 differ at least in that there appear to be nonredundant requirements for MyoD expression during satellite myogenesis (Megeney *et al.*, 1996; Chapter 4, this work), the requirement for Pax-3 expression may also be different in these cells.

Msx-1

The Msx genes, a small family of genes resembling the *Drosophila muscle segment homeobox* (*msh*) gene are an ancient family of homeobox-containing genes,

present in species ranging from coelenterates to mammals with an extremely high degree of homology in the homeobox domain, although quite divergent elsewhere. This similarity and their tendency to be expressed early in the differentiation of diverse tissues suggests a fundamental role in development which has been evolutionarily conserved. In the mouse, two members of this family, Msx-1 and Msx-2, have been cloned and characterized; a third, Msx-3, was cloned only recently and is not well-studied. Msx-1 and 2 are widely expressed in mostly overlapping patterns during embryogenesis, usually in regions of epithelial-mesenchymal interactions, including the primitive streak, lateral mesoderm, dorsal ectoderm, neural plate, dorsal neural tube and brain, cranial neural crest cells, branchial arches, facial processes, tooth germs, eye, ear, nose, vibrissae, heart, pericardium, limb bud, and tail. Surprisingly, given this widespread expression, null mutants of Msx-1 die perinatally of defects in derivatives of the first branchial arch; no other defects are noted (reviewed in Davidson, 1995).

Functionally, Msx-1 appears to act as a transcriptional repressor, however this function is not dependent on DNA binding by the homeodomain and appears instead to involve direct interaction with the basal transcriptional apparatus (Catron *et al.*, 1996). This effect was strongly heightened in transfected C2C12 cells, where other factors present in sites of endogenous expression (such as limb bud) might be positively influencing Msx-1 activity. Forced expression of Msx-1 in cultured myoblasts led to inhibition of differentiation, including repression of MyoD expression, and acquisition of a transformed phenotype; Msx-2 did not have this effect (reviewed in Davidson, 1995).

In the limb bud, Msx-1 is expressed in migrating myogenic precursor cells and is dependent on signals from the limb ectoderm as well as cell-cell interactions for maintenance of expression. Signals which have been shown to induce Msx-1 expression in the limb bud in the absence of ectoderm include BMP-2, BMP-4, FGF2, and FGF4 (reviewed in Davidson, 1995). Msx-1 is also associated with tissue and organ regeneration in urodeles, chick, and mouse, in that its expression is reactivated in

regenerating newt blastemas (Crews *et al.*, 1995) and successfully (but not unsuccessfully) regenerating chick limbs (Kostakopoulou *et al.*, 1996) and is required for mouse digit tip regeneration (Reginelli *et al.*, 1995).

The data for this section are summarized in Table 5. In this study, Msx-1 expression is detected in both C2C12 and MM14 cells under both growth-promoting and differentiation-promoting conditions. Satellite cells, which due to their similarities to limb myoblasts might be proposed to express Msx-1, do indeed do so but only at extremely early timepoints (<30 minutes post-fiber isolation.) The implication is that quiescent satellite cells express Msx-1 but quickly downregulate its expression once activated. In limb myoblasts which downregulate Msx-1 when dissociated, extinction of expression occurs within 30 minutes (D. Sassoon, pers. comm.) Thus, in the best case, expression of Msx-1 may prove to be a molecular marker for quiescent satellite cells, or satellite cells destined to re-enter quiescence after a regeneration response.

Conclusions

Cricket, not baseball

Many of the genes and gene families whose expression, based on the current knowledge of myogenesis in the embryo and on studies of myogenic cell lines, was predicted to be a component of satellite myogenesis were indeed detected in satellite cells undergoing a regeneration response in culture. However, other genes considered critical for myogenesis in the embryo are not expressed by satellite cells, and even genes likely to be filling similar or identical functions in embryonic and satellite myogenesis exhibit subtle differences in their expression. Clearly, the program of satellite myogenesis under the specific culture conditions used is unique and distinct from those of the developing embryo and of myogenic cell lines.

In order to further define the interactions within and among the genes surveyed, analysis at the single-cell level should be very informative. Combinations to be assayed should include the MRFs and the Ids, to determine the degree of correspondence of expression in general, and the degree of preference, if any, for specific combinations, and the MRFs and MEFs. Single-cell analysis of the suite of cell cycle regulatory factors, in combination with each other and with other families such as MRFs and growth factor receptors, may also yield information concerning the coregulation of proliferation and differentiation. Combinatorial analysis of components of the Notch signalling pathway should also prove interesting, especially between satellite cells in contact with each other (probable siblings).

In addition, this technique should be applicable to not only fiber-associated satellite cells, but also myogenic cells in the developing embryo via cell sorting or patch-clamp harvest from living sections. When the same combinatorial measurements can be made at the single-cell level for all primary myogenic cells, general and specific programs of myogenesis can be formulated and compared.

References

Anastasi, S., Giordano, S., Sthandier, O., Gambarotta, G., Maione, R., Comoglio, P., and Amati, P. (1997). A natural hepatocyte growth factor/scatter factor autocrine loop in myoblast cells and the effect of the constitutive met kinase activation in myogenic differentiation. *J. Cell Biol.* **137,** 1057-1068.

Baylies, M. K., and Bate, M. (1996). twist: a myogenic switch in Drosophila. *Science* **272,** 1481-1484.

Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L., and Weintraub, H. (1990). The protein Id: A negative regulator of helix-loop-helix DNA binding proteins. *Cell* **61,** 49-59.

Bladt, F., Riethmacher, D., Isenmann, S., Aguzzi, A., and Birchmeier, C. (1995). Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature* **376,** 768-771.

Bottaro, D. P., Rubin, J. S., Faletto, D. L., Chan, A. M. L., Kmiecik, T. E., Woude, G. F. V., and Aaronson, S. A. (1991). Identification of hepatocyte growth factor receptor as the met proto-oncogene product. *Science* **251,** 802-804.

Brand-Saberi, B., Muller, T. S., Wilting, J., Christ, B., and Birchmeier, C. (1996). Scatter factor/hepatocyte growth factor (SF/HGF) induces emigration of myogenic cells at interlimb level *in vivo*. *Dev. Biol.***179,** 303-308.

Carmena, A., Murugasu-Oei, B., Menon, D., Jimenez, F., and Chia, W. (1998). *inscuteable* and *numb* mediate asymmetric muscle progenitor cell divisions during Drosophila myogenesis. *Genes Dev.* **12,** 304-315.

Catron, K. M., Wang, H., Hu, G., Shen, M. M., and Abate-Shen, C. (1996). Comparison of MSX-1 and MSX-2 suggests a molecular basis for functional redundancy. *Mech. Dev.* **55,** 185-199.

Chen, Z.-F., and Behringer, R. R. (1995). *twist* is required in head mesenchyme for cranial neural tube morphogenesis. *Genes Dev.* **9,** 689-699.

Cheng, T.-C., Hanley, T. A., Mudd, J., Merlie, J. P., and Olson, E. N. (1992). Mapping of *myogenin* transcription during embryogenesis using transgenes linked to the *myogenin* control region. *J. Cell Biol.* **119,** 1649-1656.

Christy, B. A., Sanders, L. K., Lau, L. F., Copeland, N. G., Jenkins, N. A., and Nathans, D. (1991). An Id-related helix-loop-helix protein encoded by a growth fator-inducible gene. *PNAS USA* **88,** 1815-1819.

Cohen, B., Bashirullah, A., Dagnino, L., Campbell, C., Fisher, W. W., Leow, C. C., Whiting, E., Ryan, D., Zinyk, D., Boulianne, G., Hui, C.-c., Gallie, B., Phillips, R. A., Lipshitz, H. D., and Egan, S. E. (1997). Fringe boundaries coincide with Notch-dependent patterning centres in mammals and alter Notch-dependent development in *Drosophila*. *Nature Genetics* **16,** 283-288.

Coolican, S. A., Samuel, D. S., Ewton, D. Z., McWade, F. J., and Florini, J. R. (1997). The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. *JBC* **272,** 6653-6662.

Cornelison, D. D. W., and Wold, B. J. (1997). Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. *Dev. Biol.* **191,** 270-283.

Crews, L., Gates, P. B., Brown, R., Joliot, A., Foley, C., Brockes, J. P., and Gann, A. A. F. (1995). Expression and activity of the new*t Msx*-1 gene in relation to limb regeneration. *Proc. Royal Soc. Lon, Series B* **259,** 161-171.

Daston, G., Lamar, E., Olivier, M., and Goulding, M. (1996). Pax-3 is necessary for migration but not differentiation of limb muscle precursors in the mouse. *Development* **122,** 1017-1027.

Davidson, D. (1995). The function and evolution of Msx genes: pointers and paradoxes. *Trends Gen.* **11,** 405-411.

deLapeyriere, O., Ollendorff, V., Planche, J., Ott, M.-O., Pizette, S., Coulier, F., and Birnbaum, D. (1993). Expression of the Fgf6 gene is restricted to developing skeletal muscle in the mouse embryo. *Development* **118,** 601-611.

Dunwoodie, S. L., Henrique, D., Harrison, S. M., and Beddington, R. S. P. (1997). Mouse Dll3: a novel divergent Delta gene which may complemet the function of other Delta homologues during early pattern formation in the mouse embryo. *Development* **124,** 3065-3076.

Edmondson, D. G., Lyons, G. E., Martn, J. F., and Olson, E. N. (1994). Mef2 gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis. *Development* **120,** 1251-1263.

Edwall, D., Schalling, M., Jennische, E., and Norstedt, G. (1989). Induction of insulin-like growth factor I messenger ribonucleic acid during regeneration of rat skeletal muscle. *Endocrinology* **124,** 820-825.

Ellis, H. M., Spann, D. R., and Posakony, J. W. (1990). *extramacrochaetae*, a negative regulator of sensory organ development in *Drosophila*, defines a new class of helix-loop-helix proteins. *Cell* **61,** 27-38.

Ellmeier, W., and Weith, A. (1995). Expression of the helix-loop-helix gene Id3 during murine embryonic development. *Dev. Dynam.* **203,** 163-173.

Engert, J. C., Berglund, E. B., and Rosenthal, N. (1996). Proliferation precedes differentiation in IGF-I-stimulated myogenesis. *JCB* **135,** 431-440.

Epstein, J. A., Shapiro, D. N., Cheng, J., Lam, P. P., and Maas, R. L. (1996). Pax3 modulates expression of the c-met receptor during limb muscle development. *PNAS USA* **93,** 4213-4218.

Evans, S. M., and O'Brien, T. X. (1993). Expression of the helix-loop-helix factor Id during mouse embryonic development. *Dev. Biol.* **159,** 485-499.

Florini, J. R., Magri, K. A., Ewton, D. Z., James, P. L., Grindstaff, K., and Rotwein, P. S. (1991). "Spontaneous" differentiation of skeletal myoblasts is dependent upon autocrine secretion of insulin-like growth factor-II. *JBC***266,** 15917-15923.

Floss, T., Arnold, H.-H., and Braun, T. (1997). A role for FGF-6 in skeletal muscle regeneration. *Genes Dev.***11,** 2040-2051.

Fuchtbauer, E.-M. (1995). Expression of *M-Twist* during postimplantation development of the mouse. *Dev. Dynam.* **204,** 316-322.

Garrell, J., and Modolell, J. (1990). T*he Drosophila extramacrochaetae* locus, an antagonist of proneural genes that, like these genes, encodes a helix-loop-helix protein. *Cell* **61,** 39-48.

Gitelman, I. (1997). Twist protein in mouse embryogenesis. *Dev. Biol.* **189,** 205-214.

Gomez, M. R., and Bate, M. (1997). Segregation of myogenic lineages in *Drosophila* requires Numb. *Development* **124,** 4857-4866.

Grana, X., and Reddy, E. P. (1995). Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). *Oncogene* **11,** 211-219.

Gundersen, K., Rabben, I., Klocke, B. J., and Merlie, J. P. (1995). Overexpression of *myogenin* in muscles of transgenic mice: interaction with Id-1, negative crossregulation of myogenic factors, and induction of extrasynaptic acetylcholine receptor expression. *Mol. Cell. Biol.* **15,** 7127-7134.

Guo, K., and Walsh, K. (1997). Inhibition of myogenesis by multiple cyclin-cdk complexes. *JBC***272,** 791-797.

Guo, K., Wang, J., Andres, V., Smith, R. C., and Walsh, K. (1995). MyoD-induced expression of p21 inhibits cyclin-dependent kinase activity upon myocyte terminal differentiation. *Mol. Cell Biol.* **15,** 3823-3829.

Guo, M., Jan, L. Y., and Jan, Y. N. (1996). Control of daughter cell fates during asymmetric division: interaction of numb and Notch. *Neuron* **17,** 27-41.

Hall, M., Bates, S., and Peters, G. (1995). Evidence for different modes of action of cyclin-dependent kinase inhibitors: p15 and p16 bind to kinases, p21 and p27 bind to cyclins. *Oncogene* **11,** 1581-1588.

Hamamori, Y., Wu, H.-Y., Sartorelli, V., and Kedes, L. (1997). The basic domain of myogenic basic-helix-loop-helix (bHLH) proteins is the novel target for direct inhibition by another bHLH protien, twist. *Mol. Cell. Biol.* **17,** 6563-6573.

Hannon, G. J., and Beach, D. (1994). $p15^{INK4b}$ is a potential effector of TGFβ-induced cell cycle arrest. *Nature* **371,** 257-261.

Hara, E., Hall, M., and Peters, G. (1997). Cdk2-dependent phosphorylation of Id2 modulates activity of E2A-related transcription factors. *EMBO J.* **16,** 332-342.

Haub, O., and Goldfarb, M. (1991). Expression of the fibroblast growth factor-5 gene in the mouse embryo. *Development* **112,** 397-406.

Hauschka, S. (1981). Myogenic differentiation in permanent clonal mouse myoblast cell lines: Regulation by macromolecular growth factors in the culture medium. *Dev. Biol.* **86,** 19-.

Hebrok, M., Fuchtbauer, A., and Fuchtbauer, E.-M. (1997). Repression of muscle-specific gene activation by the murine twist protein. *Exp. Cell Res.* **232.**

Heldin, C.-H., and Westermark, B. (1990). Platelet-derived growth factor: mechanism of action and possible *in vivo* function. *Cell Regul.* **1,** 555-566.

Heuvel, S. V. D., and Harlow, E. (1993). Distinct roles for cyclin-dependent kinases in cell cycle control. *Science* **262,** 2050-2054.

Hunter, T., and Pines, J. (1994). Cyclins and cancer II: cyclin D and cdk inhibitors come of age. *Cell* **79,** 573-582.

Itoh, N., Mima, T., and Mikawa, T. (1996). Loss of fibroblast growth factor receptors is necessary for terminal differentiation of embryonic limb muscle. *Development* **122,** 291-300.

Jen, Y., Manova, K., and Benezra, R. (1996). Expression patterns of Id1, Id2, and Id3 are highly related but distinct from that of Id4 during mouse embryogenesis. *Dev. Dynam.* **207,** 235-252.

Jen, Y., Weintraub, H., and Benezra, R. (1992). Overexpression of Id protein inhibits the muscle differentiation program: *in vivo* association of Id with E2A proteins. *Genes Dev.* **6,** 1466-1479.

Jennische, E., Skottner, A., and Hansson, H.-A. (1987). Satellite cells express the trophic factor IGF-I in regenerating skeletal muscle. *Acta Physiol. Scand.* **129,** 9-15.

Katayose, Y., Kim, M., Pakkar, A. N. S., Li, Z., Cowan, K. H., and Seth, P. (1997). Promoting apoptosis: a novel activity associated with the cyclin-dependent kinase inhibitor p27. *Cancer Res.* **57,** 5441-5445.

Kaushal, S., Schneider, J. W., Nadal-Ginard, B., and Mahdavi, V. (1994). Activation of the myogenic lineage by MEF2A, a factor that induces and cooperates with MyoD. *Science* **266,** 1236-1247.

Kopan, R., Nye, J. S., and Weintraub, H. (1994). The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. *Development* **120,** 2385-2396.

Kostakopoulou, K., Vogel, A., Brickell, P., and Tickle, C. (1996). 'Regeneration' of wing bud stumps of chick embryos and reactivation of Msx-1 and Shh expression in response to FGF-4 and ridge signals. *Mech. Dev.* **55,** 119-131.

Kudla, A. J., John, M. L., Bowen-Pope, D. F., Rainish, B., and Olwin, B. B. (1995). A requirement for fibroblast growth factor in regulation of skeletal muscle growth and differentiation cannot be replaced by activation of platelet-derived growth factor signaling pathways. *Mol. Cell. Biol.* **15,** 3238-3246.

Langlands, K., Yin, X., Anand, G., and Prochownik, E. V. (1997). Differential interactions of Id proteins with basic-helix-loop-helix transcription factors. *JBC* **272,** 19785-19793.

Lardelli, M., Dahlstrand, J., and Lendahl, U. (1994). The novel Notch homologue Notch 3 lacks specific epidermal growth factor-repeats and is expressed in proliferating neuroepithelium. *Mech. Dev.* **46,** 123-136.

Lardelli, M., and Lendahl, U. (1993). Motch A and Motch B- two mouse Notch homoogues coexpressed in a wide variety of tissues. *Exp. Cell Res.* **204,** 364-372.

Li, L., Zhou, J., James, G., Heller-Harrison, R., Czech, M. P., and Olson, E. N. (1992). FGF inactivates myogenic helix-loop-helix proteins through phosphorylation of a conserved protein kinase C site in their DNA-binding domains. *Cell* **71,** 1181-1194.

Lin, Q., Schwarz, J., Bucana, C., and Olson, E. N. (1997). Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. *Science* **276,** 1404-1407.

Lindsell, C. E., Shawber, C. J., Boulter, J., and Weinmaster, G. (1995). Jagged: a mammalian ligand that activates Notch1. *Cell* **80,** 909-917.

Liu, Y., Martindale, J. L., Gorospe, M., and Holbrook, N. J. (1996). Regulation of $p21^{Waf1/Cip1}$ expression through mitogen-activated protein kinase signaling pathway. *Cancer Res.* **56,** 31-35.

Maina, F., Casagranda, F., Audero, E., Simeone, A., Comoglio, P. M., Klein, R., and Ponzetto, C. (1996). Uncoupling of Grb2 from the met receptor *in vivo* releals complex roles in muscle development. *Cell* **87,** 531-542.

Martin, J. F., Miano, J. M., Hustad, C. M., Copeland, N. G., Jenkins, N. A., and Olson, E. N. (1994). A Mef2 gene that generates a muscle-specific isoform via alternative mRNA splicing. *Mol. Cell. Biol.* **14,** 1647-1656.

Martin, J. F., Schwartz, J. J., and Olson, E. N. (1993). Myocyte enhancer factor (MEF) 2C: a tissue-restricted member of the MEF-2 family of transcription factors. *PNAS USA* **90,** 5282-5286.

Mason, I. (1994). The ins and outs of fibroblast growth factors. *Cell* **78,** 547-552.

Mason, I. J., Fuller-Pace, F., Smith, R., and Dickson, C. (1994). FGF-7 (keratinocyte growth factor) expression during mouse development suggests roles in myogenesis, forebrain regionalisation and epithelial-mesenchymal interactions. *Mech. Dev.* **45,** 15-30.

Matsumoto, K., and Nakamura, T. (1996). Emerging multipotent aspects of hepatocyte growth factor. *J. Biochem.* **119,** 591-600.

Megeney, L. A., Kablar, B., Garrett, K., Anderson, J. E., and Rudnicki, M. A. (1996). MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes Dev.* **10,** 1173-1183.

Molkentin, J. D., Black, B. L., Martin, J. F., and Olson, E. N. (1995). Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. *Cell* **83,** 1125-1136.

Molkentin, J. D., Firulli, A. B., Black, B. L., Martin, J. F., Hustad, C. M., Copeland, N., Jenkins, N., Lyons, G., and Olson, E. N. (1996). MEF2B is a potent transactivator expressed in early myogenic lineages. *Mol.and Cell. Biol.* **16,** 3814-3824.

Molkentin, J. D., and Olson, E. N. (1996). Combinatorial control of muscle development by basic helix-loop-helix and MADS-box transcription factors. *PNAS USA* **93,** 9366-9373.

Montarras, D., Aurade, F., Johnson, T., Ilan, J., Gros, F., and Pinset, C. (1996). Autonomous differentiation in the mouse myogenic cell line, C2, involves a mutual positive control between insulin-like growth factor II and MyoD, operating as early as the myoblast stage. *J. Cell Sci.* **109,** 551-560.

Naidu, P. S., C.Ludolph, D., To, R. Q., Hinterberger, T. J., and Konieczny, S. F. (1995). Myogenin and MEF2 function synergistically to activate the MRF4 promoter during myogenesis. *Mol. Cell. Biol.* **15,** 2707-2718.

Niswander, L., and Martin, G. R. (1992). Fgf-4 expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development* **114,** 755-768.

Olwin, B. B., Arthur, K., Hannon, K., Hein, P., McFall, A., Riley, B., Szebenyi, G., Zhou, Z., Zuber, M. E., Rapreager, A. C., Fallon, J. F., and Kudla, A. J. (1994). Role of FGFs in skeletal muscle and limb development. *Mol. Reprod. Dev.* **39,** 90-101.

Partanen, J., Makela, T. P., Eerola, E., Korhonen, J., Hirvonen, H., Claesson-Welsh, L., and Alitalo, K. (1991). FGFR-4, a novel acidic fibroblast growth factor receptor with a distinct expression pattern. *EMBO J.* **0,** 1347-1354.

Peters, K. G., Werner, S., Chen, G., and Williams, L. T. (1992). Two FGF receptor genes are differentially expressed in epithelial and mesenchymal tissues during limb formation and organogenesis in the mouse. *Development* **114,** 233-243.

Peverali, F. A., Ramqvist, T., Saffrich, R., Pepperkok, R., Barone, M. V., and Philipson, L. (1994). Regulation of G1 progression by E2A and Id helix-loop-helix proteins. *EMBO J.* **13,** 4291-4301.

Pizette, S., Coulier, F., Birnbaum, D., and deLapeyriere, O. (1996). FGF6 modulates the expression of fibroblast growth factor receptors and myogenic genes in muscle cells. *Exp. Cell Res.* **224,** 143-151.

Ponzetto, C., Bardelli, A., Zhen, Z., Maina, F., Zonca, P. D., and Giordano, S. (1994). A multifunctional docking site mediates signalling and transformation bythe hepatocyte growth factor/scatter factor receptor family. *Cell* **77,** 261-271.

Ranganayakulu, G., Zhao, B., Dokidis, A., Molkentin, J. D., Olson, E. N., and Shulz, R. A. (1995). A series of mutations in the D-MEF2 transcription factor reveal multiple functions in larval and adult myogenesis in Drosophila. *Dev. Biol.* **171,** 169-181.

Rao, S. S., Chu, C., and Kohtz, D. S. (1994). Ectopic expression of cyclin D1 prevents activation of gene transcription by myogenic basic-helix-loop-helix regulators. *Mol. Cell. Biol.* **14,** 5259-5267.

Rao, S. S., and Kohtz, D. S. (1995). Positive and negative regulation of D-type cyclin expression in skeletal myoblasts bybasic fibroblast growth factor and transforming growth factor b. *JBC* **270,** 4093-4100.

Reginelli, A. D., Wang, Y.-Q., Sassoon, D., and Muneoka, K. (1995). Digit tip regeneration correlates with regions of *Msx1 (Hox 7)* expression in fetal and newborn mice. *Development* **121,** 1065-1076.

Resnitzky, D., Hengst, L., and Reed, S. I. (1995). Cyclin A-associated kinase activity is rate limiting for entrance into S phase and is negatively regulated in G1 by $p27^{Kip1}$. *Mol. Cell. Biol.* **15,** 4347-4352.

Riechmann, V., Cruchten, I. V., and Sablitzky, F. (1994). The expression pattern of Id4, a novel dominant negative helix-loop-helix protein, is distinct from Id1, Id2, and Id3. *Nucleic Acids Res.* **22,** 749-755.

Schmidt, C., Bladt, F., Goedecke, S., Brinkmann, V., Zschiesche, W., Sharpe, M., Gherardi, E., and Birchmeier, C. (1995). Scatter factor/hepatocyte growth factor is essential for liver development. *Nature* **373,** 699-702.

Sewing, A., Wiseman, B., LLoyd, A. C., and Land, H. (1997). High-intensity Raf signal causes cell cycle arrest mediated by $p21^{Cip1}$. *Mol. Cell. Biol.* **17,** 5588-5597.

Sherr, C. J. (1993). Mammalian G1 cyclins. *Cell* **73,** 1059-1065.

Sherr, C. J., and Roberts, J. M. (1995). Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev.* **9,** 1149-2263.

Skapek, S. X., Rhee, J., Spicer, D. B., and Lassar, A. B. (1995). Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent kinase. *Science* **267,** 1022-1024.

Somasundaram, K., Zhang, H., Zeng, Y.-X., Houvras, Y., Peng, Y., Zhang, H., Wu, G. S., Licht, J. D., Weber, B. L., and El-Deiry, W. S. (1997). Arrest of the cell cycle by the tumour-suppressor BRCA1 requires the CDK-inhibitor $p21^{Waf1/Cip1}$. *Nature* **389,** 187-190.

Spana, E. P., and Doe, C. Q. (1996). Numb antagonizes Notch signaling to specify sibling neuron cell fates. *Neuron* **17,** 21-26.

Spicer, D. B., Rhee, J., Cheung, W. L., and Lassar, A. B. (1996). Inhibition of myogenic bHLH and MEF2 transcription factors by the bHLH protein Twist. *Science* **272,** 1476-1480.

Stark, K. L., McMahon, J. A., and McMahon, A. P. (1991). FGFR-4, a new mwmber of the fibroblast growth factor receptor family, expressed in the definitive endoderm and skeletal muscle lineages of the mouse. *Development* **113,** 641-651.

Stewart, C. E. H., and Rotwein, P. (1996). Insulin-like growth factor-II is an autocrine survival factor for differentiating myoblasts. *JBC* **271,** 11330-11338.

Sun, X.-H., Copeland, N. G., Jenkins, N. A., and Baltimore, D. (1991). Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix-loop-helix proteins. *Mol. Cell. Biol.***11,** 5603-5611.

Tajbakhsh, S., Rocancourt, D., Cossu, G., and Buckingham, M. (1997). Redefining the genetic heirarchies controlling skeletal myogenesis: Pax-3 and myf-5 act upstream af MyoD. *Cell* **89,** 127-138.

Tatsumi, R., Anderson, J. E., Nevoret, C. J., Halevy, O., and Allen, R. E. (1998). HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. *Dev. Biol.* **194,** 114-128.

Uyttendaele, H., Marazzi, G., Wu, G., Yan, Q., Sassoon, D., and Kitajewski, J. (1996). Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene. *Development* **122,** 2251-2259.

Verdi, J. M., Schmandt, R., Bashirullah, A., Jacob, S., Salvino, R., Craig, C. G., Amgen EST Program, Lipshitz, H. D., and McGlade, C. J. (1996). Mammalian NUMB is an evolutionarily conserved signaling adaptor protein that specifies cell fate. *Curr. Biol.* **6,** 1134-1145.

Walsh, K., and Perlman, H. (1997). Cell cycle exit upon myogenic differentiation. *Curr. Op. Gen. Dev.* **7,** 597-602.

Wang, J., and Walsh, K. (1996). Resistance to apoptosis conferred by cdk inhibitors during myocyte differentiation. *Science* **273,** 359-361.

Wang, Y., Benezra, R., and Sassoon, D. A. (1992). Id expression during mouse development: a role in morphogenesis. *Dev. Dynam.* **194,** 222-230.

Watanabe, H., Pan, Z.-Q., Schreiber-Agus, N., DePinho, R. A., Hurwitz, J., and Xiong, Y. (1998). Suppression of cell transformation by the cyclin-dependent kinase inhibitor $p57^{KIP2}$ requires binding to proliferating cell nuclear antigen. *PNAS USA* **95,** 1392-1397.

Wilkie, A. O. M., Morriss-Kay, G. M., Jones, E. Y., and Heath, J. K. (1995). Functions of fibroblast growth factors and their receptors. *Curr. Biol.* **5,** 500-507.

Williams, B. A., and Ordahl, C. P. (1994). Pax-3 expression in segmental mesoderm marks early stages in myogenic cell specification. *Development* **120,** 785-796.

Woods, D., Parry, D., Cherwinski, H., Bosch, E., Lees, E., and McMahon, M. (1997). Raf-induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by $p21^{Cip1}$. *Mol. Cell. Biol.* **17,** 5598-5611.

Yablonka-Reuveni, Z., Balesteri, T. M., and Bowen-Pope, D. F. (1990). Regulation of proliferation and differentiation of myoblasts derived from adult skeletal muscle by specific isoforms of PDGF. *J. Cell Biol.* **111,** 1623-1629.

Yablonka-Reuveni, Z., and Rivera, A. J. (1997). Influence of PDGF-BB on proliferation and transition through the MyoD-myogenin-MEF2A expression program during myogenesis in mouse C2 myoblasts. *Growth Factors* **15,** 1-continues.

Yaffe, D., and Saxel, O. (1977). Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature* **270,** 725-727.

Yan, Y., Frisen, J., Lee, M.-H., Massague, J., and Barbacid, M. (1997). Ablation of the CDK inhibitor $p57^{Kip2}$ results in increased apoptosis and delayed differentiation during mouse development. *Genes Dev.* **11,** 973-983.

Yee, S.-P., and Rigby, P. W. J. (1993). The regulation of *myogenin* gene expression during embryonic development in the mouse. *Genes Dev.* **7,** 1277-1289.

Yun, K., and Wold, B. (1996). Skeletal muscle determination and differentiation: story of a core regulatory network and its context. *Curr. Op. Cell Biol.* **8,** 877-889.

Zabludoff, S. D., Csete, M., Wagner, R., Yu, X., and Wold, B. J. (1998). $p27^{Kip1}$ is expressed transiently in developing myotomes and enhances myogenesis. *Cell Growth Diff.* **9,** 1-11.

Zhong, W., Feder, J. N., Jiang, M.-M., Jan, L. Y., and Jan, Y. N. (1996). Asymmetric localization of a mammalian numb homolog during mouse cortical neurogenesis. *Neuron* **17,** 43-53.

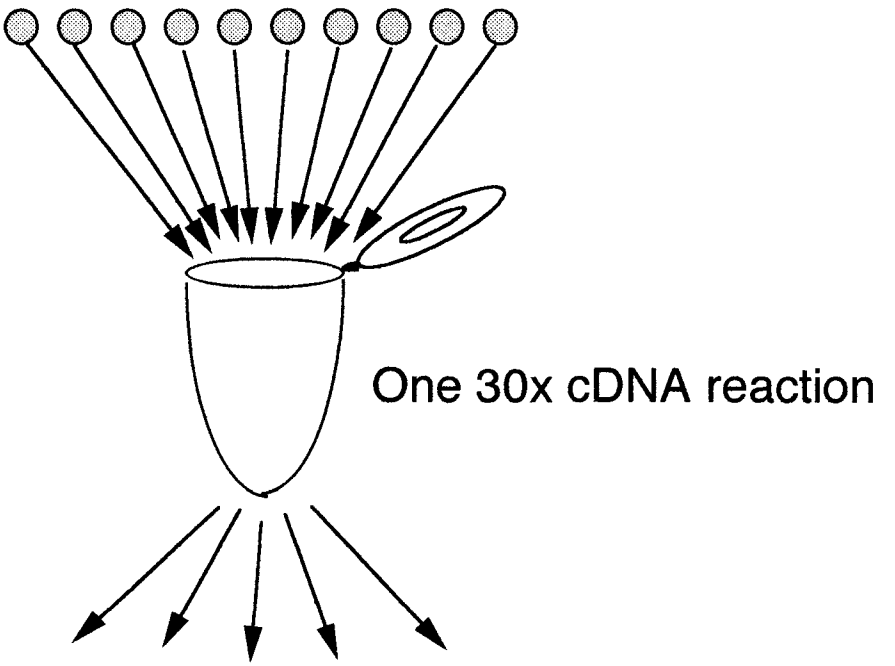
Zhong, W., Jiang, M.-M., Weinmaster, G., Jan, L. Y., and Jan, Y. N. (1997). Differential expression of mammalian numb, numblike and Notch 1 suggests distinct roles during mouse cortical neurogenesis. *Development* **124,** 1887-1897.

Zindy, F., Quelle, D. E., Roussel, M. F., and Sherr, C. J. (1997). Expression of the $p16^{INK4a}$ tumor suppressor versus other INK4 family members during mouse development and aging. *Oncogene* **15,** 203-211.

Figure 1: Cartoon depicting the single-cell harvest, RNA pooling, reverse-transcription and RT-PCR analysis used in this study.

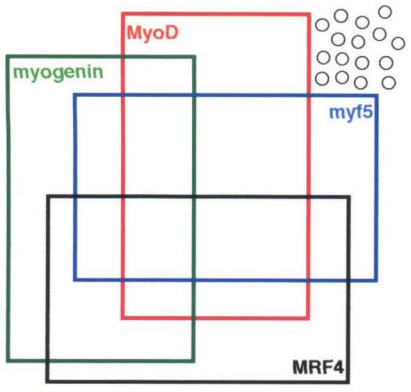
Pool collection for gene survey

30 individually-harvested satellite cells

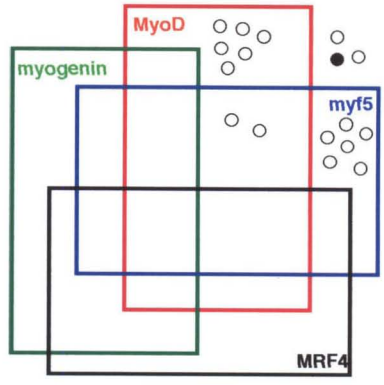


PCR single-cell equivalents for 30 genes of interest

Figure 2: Representational depiction of coexpression states of single satellite cells for c-met, m-twist, MyoD, mty5, myogenin, and MRF4 from 12 to 48 hours of fiber culture.



0 hours



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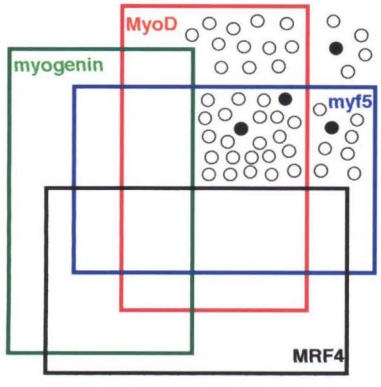

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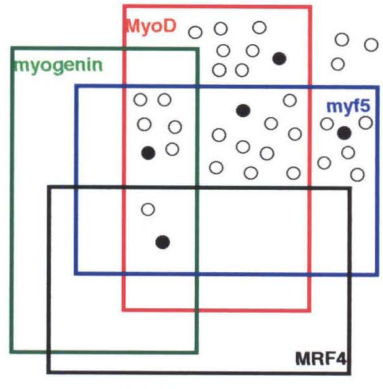
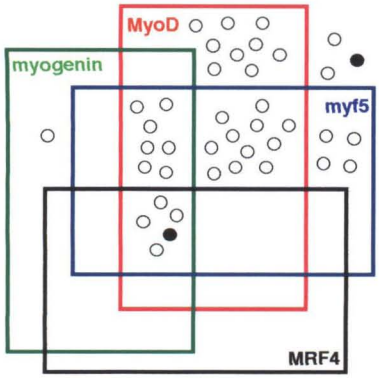
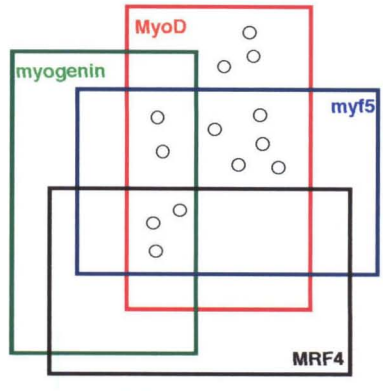


36 hours



48 hours



72 hours

○ =c-met^{+} twist^{-} ● =c-met^{+} twist^{+}

Table 1: Primer sets used in this study. All primers are written based on mouse cDNA sequence from GenBank using Lasergene (DNAstar). All outside primer sets were written to cross at least one intron to distinguish cDNA template from genomic DNA.

Table 1 PCR primer sets used in gene survey

Gene	Forward primers	Reverse primers	Product size
myf-5	5' TGC CAT CCG CTA CAT TGA GAG 3'	5' CCG GGG TAG CAG GCT GTG AGT TG 3'	353
	5' GAG GGA ACA GGT GGA GAA CTA TTA 3'	5' CGC TGG TCG CTG GAG AG 3'	293
MyoD	5' GCC CGC GCT CCA ACT GCT CTG AT 3'	5' CCT ACG GTG GTG CGC CCT CTG C 3'	397
	5' CCC CGG CGG CAG AAT GGC TAC G 3'	5'GGT CTG GGT TCC CTG TTC TGT GT 3'	234
myogenin	5' GGG CCC CTG GAA GAA AAG 3'	5' AGG AGG CGC TGT GGG AGT T 3'	364
	5' CCG TGG GCA TGT AAG GTG TG 3'	5' TAG GCG CTC AAT GTA CTG GAT GG 3'	198
MRF4	5' CTG CGC GAA AGG AGG AGA CTA AAG 3'	5' ATG GAA GAA AGG CGC TGA AGA CTG 3'	367
	5' TGC GGA GTG CCA TCA GC 3'	5' CTC CTC CTT CCT TAG CAG TTA TCA 3'	215
MEF2A	5' CAG TCT TGG AAT GAA CAG TCG GAA ACC 3'	5' TCA CTG CAG TAA CAC AAA GAA CAA ACA T 3'	758
	5' GCC CAA AGG ATA AGC AGT TCT CAA G 3'	5' AGT ATT AGC AGG TCG GCC AAG CAC A 3'	554
MEF2B	5' CGC AAC GCC TCT TCC AGT ATG 3'	5' GAG GCG GGG GCG TCT TG 3'	429
	5' GAC CGG GTG CTG CTC AAA TAC A 3'	5' GCA GGG GCC ACG GGA TAG A 3'	221
MEF2C	5' AAC ACG GGG ACT ATG GGG AGA AAA 3'	5' TAT GGC TGG ACA CTG GGA TGG TAA 3'	469
	5' GAG TAC AAC GAG CCG CAC GAG A 3'	5' TAT GGC TGG ACA CTG GGA TGG TAA 3'	247
MEF2D	5' CCC CCA GCA GCC AGC ACT ACA 3'	5' GAC TTG GGG ACA CTG GTT CTG ACT TGA 3'	779
	5' CCC CTG GCC TCC TCC CTG TG 3'	5' CTG CTG TGG CTG TGG CTG TGG TAA 3'	485
Id-1	5' GCC CCA GAA CCG CAA AGT GA 3'	5' TGA TTA ACC CCC TCC CCA AAG TCT 3'	429
	5' GCA TGT GTT CCA GCC GAC GAT 3'	5' CCC TGG GGA ACC GAG AGC AC 3'	116
Id-2	5' CCC AGC ATC CCC CAG AAC AAG A 3'	5' CCA GGC CGG AGA ACG ACA CC 3'	453
	5' ACG CGC CTG ACC ACC CTG AAC 3'	5' TCT CCT GGT GAA ATG GCT GAT AAC AAA A 3'	219
Id-3	5' GCG CCT GCG GGA ACT GG 3'	5' CAG GGT GGG GAC AGA GTG ACG 3'	480
	5' GGT GGA AAT CCT GCA GCG TGT CAT AG 3'	5' TCG AGG CGT TGA GTT CAG GGT AAG TG 3'	329
Id-4	5' CGC CGC GGA CCC CAC TCA 3'	5' CAG CGG TCA TAA AAG AAG AAA CGA AAG A 3'	365
	5' GGA CCC CAC TCA CCG CGC TCA ACA CTG 3'	5' TTA ATT TCT GCT CTG GCC CTC CCT TCC T 3'	161
m-twist	5' CCC TCG GAC AAG CTG AGC AAG AT 3'	5' ACA AAC GAG TGT TCA GAC TTC TAT CAG 3'	497
	5' CGG CCAGGT ACA TCG ACT TCC 3'	5' TCA TCT GCC CCT CTG GGA ATT CT 3'	334

Table 1 page 2

HGF	5' TTG GCC ATG AAT TTG ACC TC 3'	5' ACA TCA GTC TCA TTC ACA GC 3'	558
	5' ACG GTA TCC ATC ACT AAG AGT GGC 3'	5' CGC ACG TTT TAA TTG CAC AAT ACT CCC 3'	450
c-met	5' GAA TGT CGT CCT ACA CGG CC 3'	5' CAC TAC ACA GTC AGG ACA CTG C 3'	370
	5' GAA GGT ATC CGA ATT CAA GAC CGG 3'	5' GAA CAT GCA GTG GAC CTC AGA CTG 3'	249
FGF-1	5' CAC CGT GGA TGG GAC AAG GGA CAG 3'	5' CAC CGG GAG GGG CAG AAA CAA GAT 3'	325
	5' GCG GAA AGT GCG GGC GAA GTG 3'	5' TCC GAG GAC CGC GCT TAC AG 3'	226
FGF-2	5' CGG CAT CAC CTC GCT TCC 3'	5' CTT CTG TCC AGG TCC CGT TTT G 3'	418
	5' CGG CTT CTT CCT GCG CAT CCA TCC 3'	5' AGC CAG CAG CCG TCC ATC TTC CTT CAT 3'	169
FGF-4	5' GCG CAG CAC TCA CCG AAC T 3'	5' CTT GCC CCT GCT GCT CAT A 3'	482
	5' AAC GCG GGC CGA CCA CAG G 3'	5' GCC CAC GTT GCA GTA GAG C 3'	254
FGF-5	5' GGC AGC CGG GGC AGA AGT AG 3'	5' CTG GGT AGG AAG TGG GTG GAG ACG 3'	521
	5' TCG TCT TCT GCC TCC TCA CCA 3'	5' CCA CGT ACC ACT CTC GGC CTG TCT TT 3'	406
FGF-6	5' CGC TGC AGG CTC TCG TCT TCT TAG 3'	5' GCC CCG CTT TAC CCG TCC TAT 3'	514
	5' TGG TGG GCA TTA AGC GAC AGC GGA GAC 3'	5' CGT AGG CGT TGT AGT TGT TTG GAA GGA G 3'	278
FGF7	5' ACG GAT CCT GCC AAC TCT GCT CTA CA 3'	5' TCT TTC TTC GTT TTC TTC CCT TTG ACA 3'	525
	5' CGG AGC AAA CGG CTA CGA GTG TG 3'	5' CTT TGC ATA GAG TTT CCC TTC CTT GTT CA 3'	281
FGF-8	5' ATC AAC GCC ATG GCA GAA GAC 3'	5' CGT GAA GGG CGG GTA GTT GAG G 3'	384
	5' CAT TGT GGA GAC CGA TAC TTT TG 3'	5' AGG CTC TGC TCG GTG GTG TGG TG 3'	309
FGF 10	5' CCG GGC TGC TGT TGC TGC TTC TT 3'	5' TGC CAC GTA CAT TTG CCT GCC ATT G 3'	492
	5' GGC CAC CAA CTG CTC TTC TTC CTC CTC 3'	5' TCC CGC TGA CCT TGC CGT TCT TCT 3'	164
FGFR-1	5' GGG ATG TGG GGC TGG AAG TGC 3'	5' CAG GCC TAC GGT TTG GTT TGG TGT 3'	454
	5' ATG GGG TGC AGC TGG TGG AGA G 3'	5' CAG GCC TAC GGT TTG GTT TGG TGT 3'	246
FGFR-2	5' CGG CGT GGC GAC TGC TCT CC 3'	5' AGG CGG CTG TGC TGC TGT TGC 3'	419
	5' CGC CGG CTG CTA CCC AAG GAA T 3'	5' TGC TGC TGC TGC TGC TGC TGT G 3'	154
FGFR-3	5' CGC GGC GCT GCT TGA GG 3'	5' GCA TCT TCG TGG GAG GCA TTT AGC 3'	347
	5' TGG TCC AGA GCA GCG AGT TGT 3'	5' GGC CAC CAG ACC TGT ACC ATC CTT 3'	175

Table 1 page 3

FGFR-4	5' AGC CCT GCC TAG CCC CAA TCC 3'	5' CAG CCG AAT GCC TCC AAT ACG A 3'	515
	5' CTG GGC GAG TAC GGG GTT GG 3'	5' GTA GGG TGC TTG CTG TGG GTA GAC 3'	218
IGF-1	5' CTG GCG CTC TGC TTG CTC ACC TTC 3'	5' TCT TGT TTC CTG CAC TTC CTC TAC TTG T 3'	346
	5' GTG TGG ACC GAG GGG CTT TTA CTT CAA 3'	5' TGG CGC TGG GCA CGG ATA GAG 3'	183
IGF-2	5' AGC CGT GGC ATC GTG GAA GAG T 3'	5' GCA TGG GGG TGG GTA AGG AGA AA 3'	497
	5' CCC CGC CAA GTC CGA GAG G 3'	5' GCG GGG TCT TTG GGT GGT AAC A 3'	243
IGF-1 R	5' GTG GGC CCG GCA TTG ACA TC 3'	5' TGC AGG CTC GCT TCC CAC AC 3'	576
	5' TTC CCC AAG CTC ACA GTC ATC ACC 3'	5' CAT TCC TTT GGG GGC TTG TTC C 3'	311
IGF-2 R	5' CCT TCG GGA CCC CAG CAC AC 3'	5' TTC TCT TGA CAA ACA GCA GCC TCT T 3'	504
	5' TGA AGG GGA ACC AGG CAT TTG AC 3'	5' TGT GGC AGG CAT ACT CGG TGA T 3'	249
PDGF α	5' CCG GGA CCT CCA GCG ACT CTT G 3'	5' GGC CGG CTC TATCTC ACC TCA CAT CT 3'	482
	5' GAC CCC ACA TCG GCC AAC TTC C 3'	5' AGG TCG CAC ATG CAC ACT CCA G 3'	214
PDGF β	5' CTG CTG GGC GCT CTT CCT TCC T 3'	5' TGG GCT TCT TTC GCA CAA TCT CAA TCT T 3'	482
	5' CGA CCA CTC CAT CCG CTC CTT TGA 3'	5' CGT CTT GCA CTC GGC GAT TAC AGC 3'	196
PDGF α R	5' CGG TAT GAA ATT CGC TGG AGG GTT ATC G 3'	5' CTT CGG CTT CTC TGG GTG TTG GCT CAT 3'	447
	5' GGA CCC CAT GCA GTT GCC TTA CGA 3'	5' GAG CAT CTT CAC AGC CAC CTT CAT TAC 3'	163
PDGF β R	5' ACC AAA GGA GGG CCC ATC TAC ATC ATC A 3'	5' CGA GCC AGG CCG AAG TCA CAG 3'	533
	5' TGG ACT ACC TGC ACC GGA ACA AAC A 3'	5' GGT AGC TGA AGC CCA CGA GGT C 3'	357
cyclin A	5' TGA GAC CCT GCA TTT GGC TGT GAA CT 3'	5' TTG TGG CGC TTT GAG GTA GGT CTG GTG A 3'	523
	5' CCC CCA GAA GTA GCA GAG TTT GTG TA 3'	5' TCC AGC AAT GAG TGA AGG CAG GTA 3'	258
cyclin D1	5' CCT GTG CTG CGA AGT GGA GA 3'	5' CTG GCA TTT TGG AGA GGA AGT GT 3'	494
	5' AAG TGC GTG CAG AAG GAG ATT GTG 3'	5' TCG GGC CGG ATA GAG TTG TCA GT 3'	269
cyclin D2	5' TGG CCG CAG TCA CCC CTC AC 3'	5' TCT CTT GCC GCC CGA ATG G 3'	446
	5' GCT CTG TGC GCT ACC GAC TTC AAG 3'	5' TTG GAT CCG GCG TTA TGC TGC TCT 3'	257
cyclin D3	5' CAG CGC TGC GAG GAG GAT G 3'	5' GTA GCA CAG AGG GCC AAA AAG GTC 3'	362
	5' CGA AAC CAC GCC CCT GAC TAT TGA GAA 3'	5' CAC GGC AGC CAG GTC CCA CTT GAG C 3'	124

Table 1 page 4

cyclin E	5′ TTG TGT CCT GGC TGA ATG TCT ATG TCC 3′	5′ CTG CTC GCT GCT CTG CCT TCT TAC T 3′	486
	5′ TGT CCT CGC TGC TTC TGC TTT GTA TCA T 3′	5′ GGC TTT CTT TGC TTG GGC TTT GTC C 3′	244
cdk-2	5′ GAA ATT CAT GGA TGC CTC TGC TCT CAC 3′	5′ GAA ATC CGC TTG TTG GGG TCA TAG T 3′	564
	5′ CTT TCT GCC ATT CTC ACC GTG TCC TTC A 3′	5′ TTT GCT AAA ATC TTG CCG AGC CCA CTT G 3′	404
cdk-4	5′ GTA CAA AGC CCG AGA TCC CCA CAG T 3′	5′ CAG CCA GCT TGA CGG TCC CAT TAC 3′	413
	5′ CTT CCC GTC AGC ACA GTT CGT GAG GT 3′	5′ CAG GCC GCT TAG AAA CTG ACG CAT TAG A 3′	240
cdk-5	5′ CAG GCT GGA TGA TGA CGA TGA GG 3′	5′ TAG CAG CGG ACG GGG ATA CCA A 3′	369
	5′ CAG CTG CAA TGG TGA CCT GGA CCC TGA GAT 3′	5′ CAG CCA ATT TCA ACT CCC CAT TCC TGT TTA 3′	155
p15	5′ ACC CCA CCC CGC CTA TTT GTC TC 3′	ACT TGC CCA GCT TGT ACG GAA CCA G 3′	555
	5′ ACC CGG CGT GAG TCG TCT GTG C 3′	5′ CGC CCT TGT CCC CGG TCT GTG G 3′	178
p16	5′ TTG GGC GGG CAC TGA ATC T 3′	5′ AGG CAT CGC GCA CAT CCA 3′	361
	5′ CGC CCA ACG CCC CGA ACT CTT 3′	5′ GTG CAC CGG GCG GGA GAA GG 3′	140
p18	5′ CTG CGA AGG ACC TGA CTC TGA A 3′	5′ GGC TCC CCC AAC CCC ATT T 3′	556
	5′ GCC GAG CCT TGG GGG AAC G 3′	5′ CCA AGT CGA AGG CGG TGT C 3′	427
p19	5′ AAG GTG CCA GCC CCA ATG TCC AAG ATG C 3′	5′ CCA AAA GGG GTG AGG AAA AAC AAA TGA G 3′	499
	5′ CCT AGC TCC TGA ATC TGA TCT CCA CCA C 3′	5′ AAC TGC TCC TCC CAC TCC CTT CTT CAA T 3′	234
p21	5′ ACC CGG GTC CTT CTT GTG TTT C 3′	5′ CGT TTT CGG CCC TGA GAT GTT C 3′	445
	5′ CCC GTG GAC AGT GAG CAG TT 3′	5′ GCA GCA GGG CAG AGG AAG TA 3′	235
p27	5′ AAA TCT CTT CGG CCC GGT CAA TCA 3′	5′ CTC CAC AGT GCC AGC GTT CG 3′	466
	5′ AGC ACT GCC GGG ATA TGG AAG AA 3′	5′ GCG CGG GGG CCT GTA GTA GAA C 3′	140
p57	5′ CCG CAG GAG CCG TCC ATC AC 3′	5′ AGC CGG CGG CCC AGA ACG 3′	415
	5′ CGA CGT GTA CCT CCG CAG CAG AAC 3′	5′ GCG GTA GAA GGC GGG CAC AGA CT 3′	271
M-Notch 1	5′ CCG CCC GTG GAT TCA TCT GTA GGT 3′	CTC AAT CTG CGG TGG GGG AAT GTC 3′	356
	5′ ACG CTG CCT CAA CGG TGG TA 3′	5′ CAG GGC ATA GAC AGC GGT AGA AAG 3′	188
M-Notch 2	5′ TGG GCG CTG CAT TGA CCT G 3′	5′ GGG GCA TAT ACA CCG GAA ACC AT 3′	271
	5′ GTG GAC GGC ATC AAT CGC TAC A 3′	5′ GGG GCA TAT ACA CCG GAA ACC AT 3′	150

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M-Notch 3	5′: GCA CCG CCC GAT TCT CCT 3′	5′ TGC AGC TCT CAC CCG TCC AG 3′	636
	5′ CAA AGC TGC CAA AGT GAC ATA GAT 3′	5′ TCC AGC CAT TGA CAC ATA CAC AG 3′	463
M-notch 4	5′ GCG CTG TGA GGG AGA TGT GGA T 3′	5′ ACC GGG GCC CTG GAG AGT C 3′	506
	5′ CAG CGG TGT GAG GTG GAG ATG G 3′	5′ GCG GTG GTG ACC CTG GCT TAG 3′	217
Dll-1	5′ CCG GGA GGT ACT GCG AGG ACA ATG 3′	5′ CAG CCG GAC GCA GAC CAC CAC A 3′	410
	5′ CCA CCT GGC TAC ACG GGC AAG AAC T 3′	5′ GCA GGA GGA CAA GCA CCA C 3′	268
Dll-3	5′ GCC CTT GCC GCC TCT TCT TCA 3′	5′ CGG GGC TTC GCA CTC GTC TG 3′	490
	5′ TCC CAG GAG GCC ACC GAG TC 3′	5′ CCA CAC GTG CTA GCA GGT TCC 3′	234
Jagged-2	5′ GCC GTG CCT TAA TGC TTT TTC TTG 3′	5′ TGG GCA GGC GCA GTA GTA GTC AC 3′	400
	5′ CCG GGC TGG AAG GGC ATC A3′	5′ AGG CAG GGG CTT GGT TCA CAG AGA 3′	287
Lunatic fringe	5′ GCG CGG CTC GAT CTG CTG TT 3′	5′ GGA TGC GCT CTG CCG TGC TC 3′	475
	5′ CCT GCA AGA TGG CTG TGG AGT ATG ACC 3′	5′ ACT TTG TGC TCG CTG ATC CGT TCT GTG 3′	202
Manic fringe	5′ CTC CAG GAT CAG GCA ACA GAC A 3′	5′ GGG GAG CCG GAT GAG AGC AGA AGT G 3′	454
	5′ TTC TGCCAC GTG GAT GAT GAC AAC TA 3′	5′ GGC AAA CCA GAA CCG CAC CAG 3′	174
Radical fringe	5′ CAG GCG GCC GCA TGA TCA ACA CCA AT 3′	5′ ACG GGC GCC CAG AAG TCC T 3′	440
	5′ GCC TGC TGC ACC TGC TTT CCA CCT TC 3′	5′ AAC CCA GCC CCA CCA GTA GCA AAC CAG A 3′	154
numb	5′ GCG GAT GGG CTC AGA GTT GTG GA 3′	5′ GGC GTG GGA TAG CAT GGG GAT TGT 3′	517
	5′ AGC GTA AAC AGA AGC GGG AGA AGG AGT G 3′	5′ CGG AGT GGG AGA GGT GGG AGA GGA T 3′	242
numblike	5′ GAG GCG GGG GAC AAG AAG AAA GAA G 3′	5′ CAG CGC TGG CGA AAG ACG AG 3′	412
	5′ CCT GGG CAC GTT TCT CCG ACA C 3′	5′ TGG CAG CTC ATT CAA CCG TAG G 3′	219
Msx-1	5′ GCC GAA AGC CCC GAG AAA CTA 3′	5′ CAG GAC CGC CAA GAG GAA AAG AG 3′	379
	5′GGA CGC CTT TCA CCA CAG C 3′	5′ TCG CGG CCA TCT TCA GC 3′	201
Pax-3	5′ GCC AGG GCC GAG TCA ACC AG 3′	5′ GAT CCG CCT CCT CCT CTT CTC CTT 3′	414
	5′ GCC TCT GCC CAA CCA TA 3′	5′ TTC CTC AAT TTT CTT CTC CA 3′	214

Table 2: Summary of detected expression of positive and negative regulators of myogenesis in myogenic cell lines and primary satellite cells.

Table 2: Positive and negative regulators of myogenesis

	C2C12	MM14	Satellite cells			
	G/D	**G/D**	**0**	**24**	**48**	**96**
MyoD	+/+	+/+	-	+	+	+
myf-5	+/+	-/-	-	+	+	+
myogenin	+/+	+/+	-	-	+	+
MRF4	+/+	-/+	-	-	+	+
MEF2A	+/+	-/+	-	+	+	+
MEF2B	- / -	-/-	-	-	-	-
MEF2C	+/+	-/-	-	+	-	+
MEF2D	+/+	-/+	+	+	+	+
MEF2D-MS	+/+	-/+	-	-	+	+
Id1	+/+	-/+	-	+	+	+
Id2	+/+	-/+	-	+	+	+
Id3	+/+	-/-	-	+	+	+
Id4	+/+	-/+	-	-	+	+
twist	+/+	-/-	-	+	+	-

Table 3: Summary of detected expression of growth factors and their receptors in myogenic cell lines and in primary satellite cells.

Table 3: Growth factors and growth factor receptors

	C2C12	MM14	Satellite cells			
	G/D	**G/D**	**0**	**24**	**48**	**96**
HGF/SF	+/+	-/-	-	-	+	+
c-met	+/+	+/+	+	+	+	+
FGF1	+/+	+/+	-	+	+	+
FGF2	+/+	-/-	-	+	+	+
FGF4	- / -	-/-	-	-	-	-
FGF5	+/+	-/-	-	-	-	-
FGF6	- / -	-/+	-	-	-	-
FGF7	+/+	-/+	-	-	-	-
FGF8	- / -	-/-	-	-	-	-
FGF10	+/+	-/-	-	-	-	-
FGFR1	+/+	+/+	-	+	+	+
FGFR2	+/+	-/-	-	-	-	-
FGFR3	+/-	-/-	-	-	-	-
FGFR4	+/+	-/-	+	-	-	+
IGF-1	+/+	-/+	-	-	-	-
IGF-2	-/+	-/+	-	-	-	-
IGF1-R	+/+	-/+	+	+	+	+
IGF2-R	+/+	-/+	-	+	+	+
PDGFα	+/+	+/+	-	-	-	-
PDGFβ	+/+	+/-	-	-	-	-
PDGFαR	+/+	-/-	-	-	-	+
PDGFβR	+/+	-/-	-	-	-	+

Table 4: Summary of detected expression of positive and negative regulators of cell cycle progression through G1 and G1/S in myogenic cell lines and in primary satellite cells.

Table 4: Positive and negative regulators of the cell cycle

	C2C12	MM14	Satellite cells			
	G/D	**G/D**	**0**	**24**	**48**	**96**
cycA	+/+	+/+	-	+	+	+
cycD1	+/+	+/-	-	+	+	+
cycD2	+/-	-/+	-	-	+	-
cycD3	+/+	+/+	-	+	+	+
cycE	+/-	-/+	-	-	-	-
cdk2	+/+	+/+	-	-	-	-
cdk4	+/+	+/+	-	+	+	+
cdk5	+/+	+/+	-	-	-	-
p15	-/+	-/-	+	+	+	+
p16	-/+	-/-	-	-	-	-
p18	+/+	-/+	-	-	+	+
p19	+/+	+/-	-	-	-	-
p21	+/+	+/+	-	-	-	+
p27	+/+	-/+	-	-	+	+
p57	-/+	-/-	-	-	+	+

Table 5: Summary of detected expression of cell-cell signaling molecules and markers of embryonic myogenesis in myogenic cell lines and in primary satellite cells.

Table 5: Cell signaling molecules and markers of embryonic myogenesis

	C2C12	MM14	Satellite cells			
	G/D	G/D	0	24	48	96
M-Notch 1	+/+	+/+	-	+	+	+
M-Notch 2	+/+	-/-	+	+	+	+
M-Notch 3	+/+	-/-	-	-	-	-
M-Notch 4	- / -	-/-	-	-	-	-
Dll-1	+/+	+/+	+	+	+	+
Dll-3	- / -	-/-	-	-	-	-
Jagged-2	+/+	+/+	+	+	+	+
Radical fringe	+/+	-/+	-	-	+	+
Lunatic fringe	+/-	-/-	-	-	-	+
Manic fringe	-/+	-/-	-	-	-	-
numb	+/+	+/+	+	+	+	+
numblike	-/+	-/+	-	-	+	+
Pax-3	- / -	-/-	-	-	-	-
Msx-1	+/+	+/+	+	-	-	-

Chapter 4

MyoD-deficient satellite cells are differentiation-defective *in vitro*, express reduced m-cadherin, and fail to express MRF4

Abstract

MyoD-null mice, while without obvious deleterious muscle phenotypes during embryogenesis, have recently been shown to have deficiencies in adult muscle regeneration. Satellite cells from homozygous null animals are unable to completely replace damaged muscle tissue, and in the mdx (dystrophin-null) background the dystrophic phenotype is greatly enhanced. It was suggested that mutant satellite cells become activated normally, but cannot properly differentiate as replacement muscle, and so many satellite cells return to quiescence at the expense of new muscle formation. Here I show that, consistent with this hypothesis, satellite cells are present in excess in experimentally undamaged MyoD$^{-/-}$ adult skeletal muscle. In fiber-explant satellite cell culture, the majority become activated normally, enter the cell cycle and upregulate expression of myogenic factors. However, their MRF expression profile is aberrant, and they do not successfully upregulate MRF4, the last MRF in the satellite program to be expressed in wild-type satellite cells. Mutant satellite cells also show a remarkable lack of m-cadherin expression when compared to wild-type. Failure to properly express these two genes could account for aspects of their phenotype *in vitro* and *in vivo*, including a general failure to fuse properly into multinucleate myotubes as well as failure to assume a differentiated morphology. Analysis of a panel of genes of potential interest in satellite cells and comparison to wild-type reveals other differences in gene expression, one of which may be relevant to the return to quiescence in mutant and wild-type satellite cells.

Introduction

Skeletal muscle is formed by myoblast fusion during fetal and neonatal development in mice. Myonuclei that have fused into muscle fibers are terminally postmitotic and therefore unable to participate in repair of damaged muscle; this function is filled by satellite cells (reviewed in (Cornelison and Wold, 1997)Cornelison and Wold, 1997). Several naturally-occurring cases are known in which satellite cells are, due to unknown mechanisms, unable to successfully regenerate damaged tissue. In terminal phases of neuromuscular wasting diseases such as Duchenne's muscular dystrophy, satellite cells which have previously been able to mediate recovery from acute periods of muscle degeneration lose their regenerative capacity, leading to severe muscle atrophy and death. In muscle hypotrophy characteristic of aging, it is also suspected that previously competent satellite cells have somehow lost at least some portion of their regenerative capacity.

The mouse model of Duchenne's muscular dystrophy, the dystrophin-null mdx mouse, shows a less severe phenotype than do human patients due to more active and persistent satellite cell activity; these mice experience intense muscle necrosis and vigorous regeneration from 21 days to 6 months of age, by which time they return to a grossly normal phenotype (Megeney *et al.*, 1996). A mouse mutant which has persistent deficiencies in muscle regeneration is the MyoD-null mouse. While they have no obvious degenerative phenotype, when bred into a dystrophin-null background, the additional lack of MyoD gene expression amplifies the mdx phenotype and leads to premature death. In addition, when acutely injured during adulthood, MyoD-null mice show deficiencies in muscle regeneration (Megeney *et al.*, 1996). MyoD-null satellite cells in crushed muscle become activated, but are unable to properly execute the regeneration program. Few replacement myofibers are formed, with the majority of satellite cells remaining mononucleate within the damaged area. I was proposed that, in the absence of MyoD gene expression, some essential step in myogenic progression was blocked,

leading to a population of activated satellite cells which, instead of terminally differentiating, returns to a quiescent state.

Here I present data indicating that in the isolated fiber culture system, MyoD-null satellite cells are present in excess over wild-type in experimentally undamaged muscle tissue; that they have aberrant morphology during proliferation and differentiation *in vitro*; and that they exhibit major differences in gene expression when compared to wild-type satellite cells. These data then suggest a possible genetic mechanism for the differentiation-defective phenotype. Finally, I identify a gene whose expression in activated satellite cells may correspond to cells which are re-entering the quiescent state.

Materials and Methods

MyoD homozygous null adult mice were obtained from Dr. M. Rudnicki and muscle fibers were prepared and cultured as described previously (Cornelison and Wold, 1997). Single satellite cells were harvested with a patch-clamp pipette and their cellular mRNA was reverse-transcribed as in (Cornelison and Wold, 1997) Single cells were assayed for the combined expression of c-met, m-cadherin, myf-5, myogenin, and MRF4; cell pools were assayed at the single cell level for expression of a panel of regulatory genes (see Chapter 3 for primer sets and methodologies). Total percentages of wild-type and MyoD-null satellite cells in each of 16 possible coexpression states were analyzed for significance by two-way correlation using Fisher's exact test; $p > 0.05$ was considered to be significant. Analysis was performed in StatView (Abacus)..

Immune staining was performed on wild-type and mutant satellite cell cultures after 7 days in growth medium (DMEM + 10% horse serum + 5% chick embryo extract + antibiotic/antimycotic) followed by 7 days in differentiation medium (DMEM + 2% horse serum.) Cells were fixed for 20 min. in ice-cold methanol/acetone at -20° and dried for 24 hours, then rehydrated in PBS and stained with anti-myosin heavy chain (MF-20, Developmental Studies Hybridoma Bank) and detected with anti-mouse HRP and DAB; alternatively, they were stained with MF-20 and anti-c-met (Santa Cruz) or MF-20 and anti-m-cadherin (Santa Cruz) and detected with donkey anti-mouse FITC and donkey anti-rabbit TRITC (Jackson.) Hoechst 33347 was added to the final wash after staining to visualize nuclei.

Results

MyoD$^{-/-}$ satellite cells are present in excess in undamaged muscle

MyoD$^{-/-}$ satellite cells resident on single explanted myofibers became activated (as determined by emergence from under the basal lamina of the host fiber and an increase in cytoplasm) within the first 24 hours after harvest similar to wild type. At harvest, satellite cells were present in excess over wild-type based on both the number of satellite cell nuclei/mm fiber and the ratio of satellite nuclei/myofiber nuclei (Figure 1.) While their morphology was basically normal at 24 hours after harvest (Figure 2 a and b), by 48 hours after harvest they were enlarged compared to wild-type and had begun to aggregate abnormally (Figure 2 c and d), and by 96 hours after harvest many mutant satellite cells were contained in very large, multicellular calluses adherent to the fibers (Figure 2 e and f.)

At 48 hours, and more strikingly at 96 hours, some MyoD-null satellite cells had become associated with depressions in the host fiber, and were in some cases partially enclosed by the fiber sarcolemma (Figure 2 g and h.) This phenotype was never observed for wild-type satellite cells, and may represent cells which have failed to execute the myogenic program and are returning to the sub-laminal position of resting satellite cells.

It was also observed that myonuclei of fibers from MyoD-null mice were shaped diferently than those of wild-type myofibers: while wild-type myonuclei, expecially when freshly harvested, are extremely elongated, myonuclei of mutant fibers were only slightly oval (Figure 3a). Nuclei of some MyoD-null satellite cells also had unusual morphology after several days in fiber culture, becoming teardrop-shaped with a distinct point on one end (data not shown). The explanations and significance of these morphological changes are unknown at present. Additionally, MyoD-null myofibers were observed to have a much greater than normal rate of fiber forking, splitting, and sprouting, a phenomenon associated with the pathologies of Duchene's and Becker's muscular dystrophies as well

regenerated muscle (Bradley, 1978) (Figure 3b). This is unexpected, as the MyoD-null mouse is not considered to have a degenerative phenotype, nor would individuals be assumed to have undergone extensive regeneration. Taken together with the excess of satellite cells observed in 'undamaged' muscle, these observations suggest that normal muscle function in MyoD-null mice is indeed impaired and may have undergone periods of injury and partial regeneration which have been undetected thus far.

MyoD$^{-/-}$ satellite cells are differentiation-defective *in vitro*

When MyoD$^{-/-}$satellite cells which had become adherent to the culture plate during fiber culture were subsequently cultured in differentiation medium and assayed for expression of the differentiated muscle marker myosin heavy chain (MHC), very few multinucleate myotubes could be found compared to wild-type, and the majority of those which did form were abnormal in appearance (i.e. stunted, having very few nuclei, or excessively poor in cytoplasm, as in Figure 4b, arrow.) The majority of MHC-positive cells were round, mononucleate cells which had not morphologically differentiated (Figure 4b, arrowhead). When compared to wild-type satellite cells under the same conditions, 17% of nuclei in MHC-positive cells are in elongated, spindle-shaped myotubes, as opposed to 95% in the wild-type. When costained with the satellite cell marker c-met (Cornelison and Wold, 1997) which is also retained on newly fused myotubes ((Tatsumi *et al.*, 1998); Cornelison and Wold, unpub. res.), 11% of MyoD-/- satellite cell nuclei are found in c-met^{+}MF-20^{+} cells, while the percentage for wild-type cells is 86% (Figure 5.)

MyoD$^{-/-}$ satellite cells underexpress m-cadherin and fail to express MRF4

When single satellite cells from mutant animals are analyzed for coexpression of c-met, m-cadherin, myf5, myogenin, and MRF4 over the first four days in culture, several departures from the wild-type program are evident (Figure 6; Table 1).

Myogenin, which is not seen in wild-type cells until 48 hours, is expressed by a small fraction of cells at 0 and 24 hours. This could reflect the presence of a population of already-activated satellite cells present prior to fiber harvest, or of *de novo* expression due to activation by harvest; however, the difference between wild-type and MyoD-null is not significant for this gene expression state (see Table 1).

At 24 hours, cells begin to accumulate in the myf5-positive compartment. 30% of the cells, comparable to 27% in wild-type, expressed no MRFs; 61% expressed myf5 alone. Wild-type cells at this timepoint were expressing MyoD (20%), myf5 (14%), or both (39%). Additionally, rarer mutant satellite cells were expressing myogenin (3%) or myogenin and myf5 (6%.) At this timepoint, reduced m-cadherin expression becomes apparent; only about 3% of cells assayed are positive for m-cadherin, compared to 24% in wild-type.

By 48 hours in culture, the expression patterns diverge from the wild-type even more. 21% of mutant cells express no MRFs, compared to 11% in wild-type. While wild-type satellite cells have mostly transited through the MyoD^{+}, myf5^{+}, and MyoD^{+}myf5^{+} states, leaving 3%, 3%, and 14% respectively in those compartments while 32% occupy the MyoD^{+}myf5^{+}myogenin^{+} state and 30% express all four, most mutant satellite cells remain in the myf5^{+} only state (56%) and some have gone on to be myf5^{+}myogenin^{+}, which may be analogous to the triple-positive state in wild-type cells. It is also evident by this timepoint that m-cadherin expression has become uncoupled from MRF expression. None of the MyoD$^{-/-}$ satellite cells assayed expressed m-cadherin at this point, while in wild-type cells m-cadherin expression was present in 84% of cells and correlated absolutely with myogenin expression. Unlike wild-type cells, mutant satellite cells did not express MRF4 at this timepoint.

After 96 hours in culture, MyoD$^{-/-}$ satellite cells still did not express MRF4. 30% (compared to 2% in wild-type) did not express any MRFs, while the remainder expressed either myf5 alone (47%) or myf5 and myogenin (20%); in the wild-type no cells

expressed myf5 alone, 28% expressed myf5 and MyoD and 20% expressed myf5, MyoD and myogenin. While more cells in this period are expressing m-cadherin, it is not universally expressed as it would be in normal cells, nor is its expression correlated with expression of myogenin.

To determine whether MRF4 is never expressed in MyoD-null satellite cells in fiber culture or if its expression is simply delayed, mutant satellite cells were assayed at six and eight days after fiber harvest. Even at these late timeepoints, no MRF4 expression was detected (data not shown).

Given the scarce m-cadherin mRNA expression, the sugested role of m-cadherin in mediating myoblast fusion and subsequent differentiation (Zeschnigk *et al.*, 1995). and the large number of mononucleate cells expressing MHC but not fusing into myotubes, it was expected that costaining for MHC and m-cadherin would show coexpression only in myotubes (deriving from a small population of fusion-competent, presumably m-cadherin expressing myoblasts). What was observed, however, was that while m-cadherin protein was present on only a small fraction of cells as would be predicted based on the RT-PCR results, very few MHC^+ myotubes expressed m-cadherin protein. Instead, m-cadherin immunoreactivity was usually found at points of contact between two MHC^+ mononucleate blast-like cells (Figure 7a) and occasionally uniformly distributed over the surface of multinucleate blast-like cells at a slightly lower level (Figure 7b).

$MyoD^{-/-}$ satellite cells differ from wild-type in the expression of myogenic, cell-cycle, and other genes

The expression of a number of other genes of interest was assayed for in pools of MyoD-null satellite cells and compared to data for wild-type cells (Table 2; for review of satellite cell gene expression see Chapter 3.) Genes whose expression appears to be down-regulated in whole or in part in the mutants include MRF4; MEF2D; Id 2, 3, and 4; twist; and p57. MRF4 and p57 are associated with terminal differentiation in muscle and

the Id factors and twist are considered to be inhibitors of MyoD-initiated myogenesis. Genes with expanded expression compared to wild-type are myogenin, p21, and p27, which are detected at earlier timepoints than in wild-type cultures, and cdk2, p19, PDGFα, which are reproducibly detected in mutant but never in wild-type satellite cells.

When a pool of MyoD-null satellite cells is enriched for cells which rest in depressions in the host fiber and are possibly ingressing, several variations from the 'normal' MyoD-null gene expression program were detected, including the expression of IGF1, GDF8, and, perhaps most interestingly, Msx-1 (Hox 7.1), a gene normally only detected in very recently activated satellite cells (see Chapter 3).

Discussion

The program of skeletal muscle regeneration can be subdivided into four essential and sequential events: initial activation by local damage; proliferation to form a population of replacement myoblasts; differentiation of those myoblasts to repair the damaged tissue; and finally the supposed respecifiction of a new population of progenitor myoblasts which will not terminally differentiate during the current regeneration response but will return to quiescence to provide material for future responses. Defects in any of the first three processes will cause an acute regeneration response to fail in part or completely; defects in the last process would be expected to affect the success of future regeneration responses.

The first two steps, activation and proliferation, are difficult to distinguish from one another because proliferation requires activation, and activation is normally assessed by initiation of proliferation, along with later expression of myogenic regulatory factors. Following initial activation, however, satellite cell proliferation can be affected *in vitro* and may be regulated *in vivo* by stimulation with various growth factors, including fibroblast growth factors (FGFs), insulin-like growth factors (IGFs) (Allen and Boxhorn, 1989; Maley *et al.*, 1995), and hepatocyte growth factor/scatter factor (Allen *et al.*, 1995). Growth factor signals lead to biochemical alterations in proteins affecting the cell cycle, contributing to continued cellular proliferation or, in some cases, cell cycle arrest and terminal differentiation. The expression of the myogenic regulatory factors (MRFs) is also affected by growth factor signaling and by cell cycle status, and once expressed will in turn regulate growth factor, growth factor receptor, and cell cycle regulator expression (for review see Chapter 3, this work). The return of a subset of activated satellite cells to the quiescent state is suggested to happen based on xxx and xxx. Howver, the mechanisms which may be involved are at this time completely unknown, as are the genes which may specify or regulate this cell fate decision.

Several different mechanisms are available for MyoD to influence cellular myogenesis: MyoD and the other MRFs are transcriptional activators, and their binding to their cognate recognition site (E-box) in the promoters of many muscle-specific genes is essential for their expression (reviewed in (Molkentin *et al.*, 1995)Molkentin *et al.*, 1995). MyoD also upregulates the expression of factors such as p21 (Halevy *et al.*, 1995; Guo *et al.*, 1995) and to promote terminal differentiation. Expression of MyoD also has indirect effects via genes and gene products which, when activated by MyoD, promote the muscle program as a whole, as evidenced by the fact that exogenous expression of MyoD alone in many cell types will result in terminal myogenesis (reviewed in (Weintraub, 1993)Weintraub, 1993). An example of still less direct action of MRFs is seen in the myf5-null mouse, which has sclerotomal (bone and cartilage) defects, probably due to loss of a required cellular interaction with myotomal (muscle) cells lacking myf5 (Braun *et al.*, 1994)

MyoD-null satellite cells do not appear to be activated at a lower frequency or at a slower rate than wild-type, based on cell number, cell morphology, time of first cell division, and timing of myf5 upregulation. Proliferation *in vitro* of MyoD-null satellite cells is reported to be threefold less than in wild-type based on population doubling time and ^{3}H-thymidine incorporation (Megeney *et al.*, 1996); this might also be caused by cell-cycle arrest in a large portion of the population. Thus, it appears that MyoD plays a role in positive regulation of the cell cycle in activated satellite cells.

Expression of multiple MRFs is required for myogenic differentiation; they act together to specify the myogenic program, and while individual MRFs appear dispensable in some myogenic cells, the absence of two or more is usually highly deleterious to myoblast specification or differentiation (Rawls *et al.*, 1995; Rudnicki *et al.*, 1993). Since both myf5 and myogenin are present in at least some MyoD-null satellite cells, yet none of them upregulate MRF4, the implication is that MyoD plays an essential and nonredundant role in expression of MRF4 in satellite cells. The absence of

MRF4 expression may be directly due to the absence of MyoD as a transcriptional activator or indirectly due to failure of as aspect of MyoD-induced satellite myogenesis which occurs before MRF4 upregulation. In favor of the direct effect, at least in non-myotomal myoblasts, are the data that embryonic limb myoblasts and wild-type satellite cells express MyoD prior to MRF4 (Ott *et al.*, 1991; Cornelison and Wold, 1997) and MyoD is also capable of inducing MRF4 (as well as the other MRFs) when transfected into permissive cells in culture (reviewed in Weintraub, 1993). Arguing against a unique requirement for MyoD in regulating MRF4 expression is the observation that during embryogenesis MyoD-null mice will express MRF4 normally, due to complementation by myf5 (Rudnicki *et al.*, 1992). It is important to note, however, that promoter-transactivator interactions required for expression in embryonic myogenic precursor cells may not be sufficient or even necessary in satellite cells (i.e., embryonic MPCs require Pax-3 to activate c-met expression, while satellite cells constitutively express c-met but never express Pax-3 (Bladt *et al.*, 1995; Epstein *et al.*, 1996).

Terminal differentiation occurs after MRF expression is established and for most myogenic populations includes fusion of myoblasts into myotubes. M-cadherin is a homotypic cell-cell adhesion molecule that may be required for this process: when m-cadherin activity is masked by blocking peptides or antisense RNA, C2C12 cells fail to fuse; they also fail to withdraw from the cell cycle or terminally differentiate (Zeschnigk *et al.*, 1995). Therefore, the extreme reduction in m-cadherin expression accompanying the fusion- and differentiation-defective phenotype of MyoD-null satellite cells may be causal. It is unlikely that MyoD is required for m-cadherin expression only as a transactivator. While there have been no detailed studies of the m-cadherin promoter/enhancer region, in both the embryo and wild-type satellite cells m-cadherin can be expressed proir to MyoD expression (Moore and Walsh, 1993; Rose *et al.*, 1994; Cornelison and Wold, 1997) and it is expressed, albeit at extremely low frequency, in satellite cells lacking MyoD (this work). It is interesting and possibly relevant that

targeted deletion of MRF4 in the mouse (which partially reduces myf5 activity as well) also causes a 75% reduction in m-cadherin mRNA expression in developing myotomes (Patapoutian *et al.*, 1995).

MyoD-null satellite cells which do express m-cadherin protein in culture have an abnormal pattern of expression: while in wild-type cells immunoreactivity is generalized over the entire cell surface, in mutant cells it is usually expressed asymmetrically in a stripe between two unfused mononucleate MHC^+ cells (Figure 7a) and at lower levels on the entire surface of mutliniucleate MHC^+ blast-like cells (Figure 7b). These expression patterns suggest that even cells capable of expressing m-cadherin are not necessarily competent to fuse (possibly fusion must take place between two of the rare m-cadherin$^+$ cells) and that failure to express m-cadherin must be only partially responsible for the fusion-deficient phenotype.

Another possible factor in the MyoD-null satellite cell phenotype may be not in the satellite cells themselves, but in their environment. Conceivably, as deletion of myf5 causes malformation of the ribs, deletion of MyoD may have an effect on muscle-associated nonmuscle tissue. During dissection of MyoD-null muscles for fiber culture, it was noticed that while the muscle fibers themselves are not fragile, they are not as strongly associated with each other as wild-type muscles, leading to fraying of muscle groups (DDWC). It was also noted hat, unlike wild-type cells which have an extremely strong affinity for the surface of dead myofibers in culture, MyoD-null satellite cells do not colonize dead fibers (DDWC). This may indicate a connective-tissue defect, which could conceivably also affect satellite cell differentiation. Results presented here which suggest that MyoD-null satellite cells are also differentiation-defective *in vitro* , which would tend to discount this possibility; however under other culture conditions MyoD-null satellite cells are reported to differentiate normally *in vitro* (Megeney *et al.*, 1996), implying that availability of secreted factors available to MyoD-null satellite cells may modulate their phenotype.

Other genes which relate to myogenesis also display more limited expression in MyoD-null satellite cells than in wild-type. MEF2D and Ids 2, 3, and 4 are detected later in the timecourse than in wild-type satellite cells, while m-twist and p57 are never detected in mutant satellite cells. These may all be results of a delayed or incomplete myogenic program, particularly in the case of p57, which is normally associated with terminal differentiation in muscle cells (Yan *et al.*, 1997). Conversely, p21and p27, which are detected at *earlier* timepoints than in wild-type satellite cells, are often correlated with cells undergoing the switch from proliferation to differentiation (Zabludoff *et al.*, 1998; Cohen *et al.*, 1997).

It is more difficult to speculate as to the cause and function for expression of genes which, although never detectably expressed in wild-type satellite cells, are widely and reproducibly detected in MyoD-null satellite cells: cdk2, p19, and PDGF-A. cdk2 is a cyclin-dependent kinase (reviewed in Chapter 3) whose absence in wild-type satellite cells is unusual; in most cells it associates with cyclin E to promote transition from G1 to S phase of the cell cycle (reviewed in (Sherr, 1993). p19 is an inhibitor of the cyclin-dependent kinases cdk4 and cdk6 (Harai *et al.*, 1995), but unlike many other CDIs its specific functions in cell cycle regulation have not been well characterized. PDGF (platelet-derived growth factor) A is one of two PDGF chains which form the active dimeric factor; unlike PDGF-BB it is not considered to have a potent effect on myogenic cells due presumably to lower levels of PDGFα receptor (Yablonka-Reuveni *et al.*, 1990). The mechanisms leading to expression of these genes in MyoD-null but not wild-type satellite cells, and their predicted function for the MyoD-deficient muscle regeneration program, are unknown at this point.

Observation of MyoD-null satellite cells in fiber culture apparently ingressing beneath the lamina of their host myofibers, as was suggested based on the MyoD-null regeneration phenotype *in vivo* (Megeney *et al.*, 1996), prompted attempts to harvest these cells explicitly and query them for differences from either the wild-type or non-

ingressing MyoD-null patterns of gene expression. The most striking difference observed (Table 2) was the expression of MSX-1 in these pools. Among myogenic cells, Msx-1 (Hox 7.1) is expressed by migratory, predifferentiated, MRF-negative embryonic limb myoblasts (Wang and Sassoon, 1995) and also by quiescent or very freshly-activated satellite cells (Chapter 3, this work). Re-expression of Msx-1 in ingressing cells reinforces the idea that they may be returning to quiescence; if such expression is found to be characteristic of cells which will form the reserve satellite population *in vivo* it would be extremely important, both clinically and biochemically.

The data presented here suggest a necessary and nonredundant role for MyoD in progression of activated satellite cells through a myogenic regeneration response. This is in contrast to embryonic myogenesis, in which MyoD can be functionally replaced by myf5, and suggests a modification of the model for satellite cell myogenesis presented in our earlier work (Cornelison and Wold, 1997). The new model (Figure 8) incorporates this new data and suggests a pathway for cells failing myogenesis to downregulate myogenin and myf5 once again and return to the original state.

References

Allen, R. E. and Boxhorn, L. K. (1989). Regulation of skeletal muscle satellite cell proliferation and differentiation by transforming growth factor-beta, insulin-like growth factor I, and fibroblast growth factor. *J. Cell. Phys.* **138**, 311-315.

Allen, R. E., Sheehan, S. M., Taylor, R. G., Kendall, T. L. and Rice, G. M. (1995). Hepatocyte growth factor activates quiescent skeletal muscle satellite cells in vitro. *JJ. Cell. Phys.* **165**, 307-312.

Bladt, F., Riethmacher, D., Isenmann, S., Aguzzi, A. and Birchmeier, C. (1995). Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature* **376**, 768-771.

Bradley, W. G. (1978) *In* "Muscle regeneration", (A. Mauro, Ed.), pp. 215-230. Raven Press, New York.

Braun, T., Bober, E., Rudnicki, M. A., Jaenisch, R. and Arnold, H.-H. (1994). MyoD expression marks the onset of skeletal myogenesis in *Myf-5* mutant mice. *Development* **120**, 3083-3092.

Cohen, B., Bashirullah, A., Dagnino, L., Campbell, C., Fisher, W. W., Leow, C. C., Whiting, E., Ryan, D., Zinyk, D., Boulianne, G., Hui, C.-c., Gallie, B., Phillips, R. A., Lipshitz, H. D. and Egan, S. E. (1997). Fringe boundaries coincide with Notch-dependent patterning centres in mammals and alter Notch-dependent development in Drosophila. *Nature Genetics* **16**, 283-288.

Cornelison, D. D. W. and Wold, B. J. (1997). Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. *Dev.Biol.* **191**, 270-283.

Epstein, J. A., Shapiro, D. N., Cheng, J., Lam, P. P. and Maas, R. L. (1996). Pax3 modulates expression of the c-met receptor during limb muscle development. *PNAS USA* **93**, 4213-4218.

Guo, K., Wang, J., Andres, V., Smith, R. C. and Walsh, K. (1995). MyoD-induced expression of p21 inhibits cyclin-dependent kinase activity upon myocyte terminal differentiation. *Mol. Cell Biol.* **15**, 3823-3829.

Halevy, O., Novitch, B. G., Spicer, D. B., Skapek, S. X., Rhee, J., Hannon, G. J., Beach, D. and Lassar, A. B. (1995). Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science* **267**, 1018-1021.

Harai, H., Roussel, M. F., Kato, J.-Y., Ashmun, R. A. and Sherr, C. J. (1995). Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. *Mol. Cell.Biol.* **15**, 2672-2681.

Maley, M. A. L., Davies, M. J. and Grounds, M. D. (1995). Extracellular matrix, growth factors, genetics: their influence on cell proliferation and myotube formation in primary cultures of adult mouse skeletal muscle. *Exp. Cell Res.* **219**, 169-179.

Megeney, L. A., Kablar, B., Garrett, K., Anderson, J. E. and Rudnicki, M. A. (1996). MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes Dev.* **10**, 1173-1183.

Molkentin, J. D., Black, B. L., Martin, J. F. and Olson, E. N. (1995). Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. *Cell* **83**, 1125-1136.

Moore, R. and Walsh, F. S. (1993). The cell adhesion molecule M-cadherin is specifically expressed in developing and regenerating, but not denervated skeletal muscle. *Development* **117**, 1409-1420.

Ott, M.-O., Bober, E., Lyons, G., Arnold, H. and Buckingham, M. (1991). Early expression of the myogenic regulatory gene, *myf-5*, in precursor cells of skeletal muscle in the mouse embryo. *Development* **111**, 1097-1107.

Patapoutian, A., Yoon, J. K., Miner, J. H., Wang, S., Stark, K. and Wold, B. (1995). Disruption of the mouse MRF4 gene identifies multiple waves of myogenesis in the myotome. *Development* **121**, 3347-3358.

Rawls, A., Morris, J. H., Rudnicki, M., Braun, T., Arnold, H.-H., Klein, W. H. and Olson, E. N. (1995). Myogenin's functions do not overlap with those of MyoD or myf5 during mouse embryogenesis. *Dev. Biol.* **172**, 37-50.

Rose, O., Rohwedel, J., Reinhardt, S., Bachmann, M., Cramer, M., Rotter, M., Wobus, A. and Starzinski-Powitz, A. (1994). Expression of M-cadherin protein in myogenic cells during prenatal mouse development and differentiation of embryonic stem cells in culture. *Dev.Dynam.* **201**, 245-259.

Rudnicki, M. A., Braun, T., Hinuma, S. and Jaenisch, R. (1992). Inactivation of *MyoD* in mice leads to up-regulation of the myogenic HLH gene *Myf-5* and results in apparently normal muscle development. *Cell* **71**, 383-390.

Rudnicki, M. A., Schnegelsberg, P. N. J., Stead, R. H., Braun, T., Arnold, H.-H. and Jaenisch, R. (1993). MyoD or myf-5 is required for the formation of skeletal muscle. *Cell* **75**, 1351-1359.

Sherr, C. J. (1993). Mammalian G1 cyclins. *Cell* **73**, 1059-1065.

Wang, Y. and Sassoon, D. (1995). Ectoderm-mesenchyme and mesenchyme-mesenchyme interactions regulate Msx-1 expression and cellular differentiation in the murine limb bud. *Dev. Biol.* **168**, 374-382.

Weintraub, H. (1993). The MyoD family and myogenesis: redundancy, networks, and thresholds. *Cell* **75**, 1241-1244.

Yablonka-Reuveni, Z., Balesteri, T. M. and Bowen-Pope, D. F. (1990). Regulation of proliferation and differentiation of myoblasts derived from adult skeletal muscle by specific isoforms of PDGF. *J. Cell Biol.* **111**, 1623-1629.

Yan, Y., Frisen, J., Lee, M.-H., Massague, J. and Barbacid, M. (1997). Ablation of the CDK inhibitor p57Kip2 results in increased apoptosis and delayed differentiation during mouse development. *Genes Dev.* **11**, 973-983.

Zabludoff, S. D., Csete, M., Wagner, R., Yu, X. and Wold, B. J. (1998). p27Kip1 is expressed transiently in developing myotomes and enhances myogenesis. *Cell Growth Diff.* **9**, 1-11.

Zeschnigk, M., Kozian, D., Kuch, C., Schmoll, M. and Starzinski-Powitz, A. (1995). Involvement of M-cadherin in terminal differentiation of skeletal muscle cells. *J. Cell Sci.* **108**, 2973-2981.

Figure 1: Histograms comparing myonuclei per mm of fiber (A), satellite nuclei per mm of fiber (B), and satellite nuclei per 100 myonuclei (C) in fiber explants from wild-type and three individual MyoD-null mice.

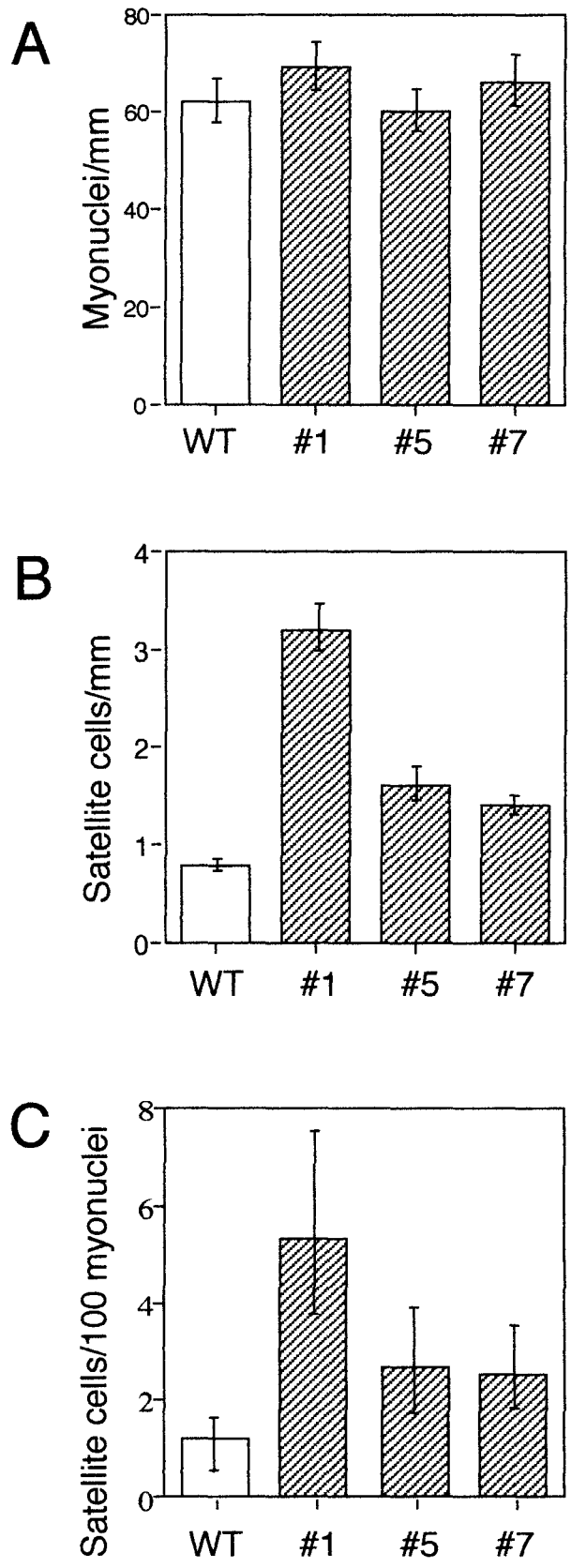
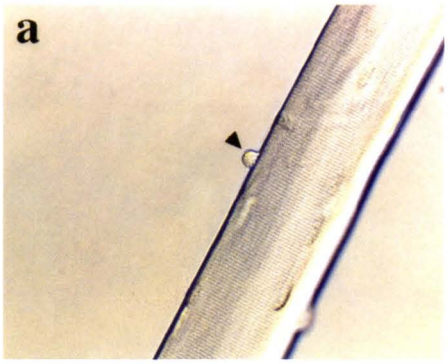
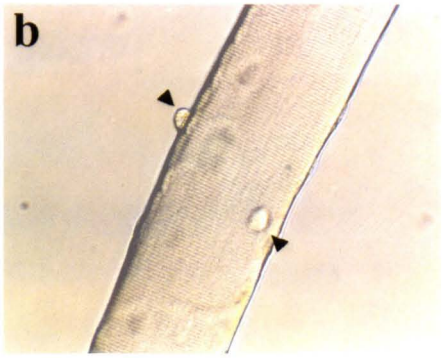
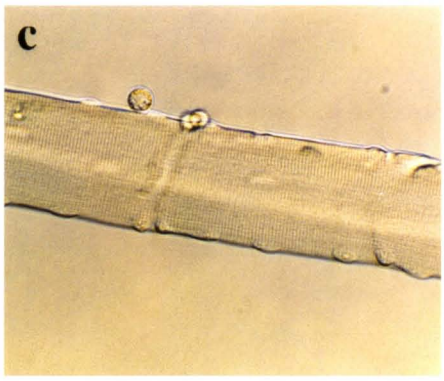
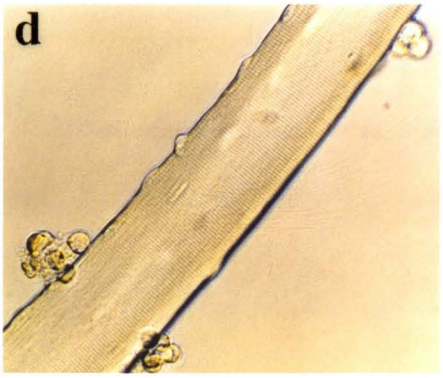

A
Myonuclei/mm
80
60
40
20
0
WT
#1
#5
#7
B
Satellite cells/mm
4
3
2
1
0
WT
#1
#5
#7
C
Satellite cells/100 myonuclei
8
6
4
2
0
WT
#1
#5
#7

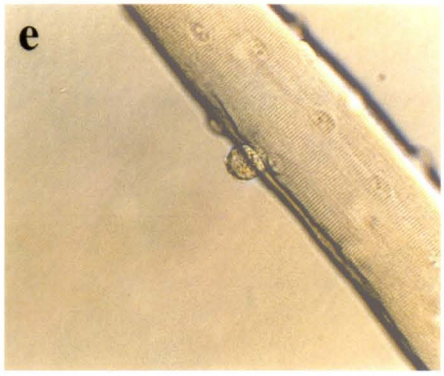
Figure 2: Wild-type (a, c, e) and MyoD-null (b, d, f) satellite cells on myofibers at 24, 48, and 96 hours in culture. Arrowheads in a and b point to small satellite cells. Note the abnormal clustering of mutant cells at 48 hours and formation of large multicellular calluses at 96 hours in culture. Arrowheads in g and h point to a MyoD-null satellite cell at 96 hours which has begun to ingress beneath the basal lamina of the host fiber, before (g) and after (h) harvest by patch-clamp.

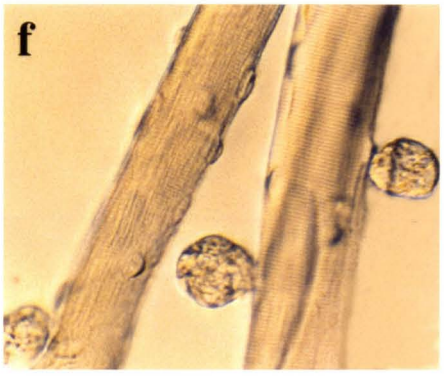

a

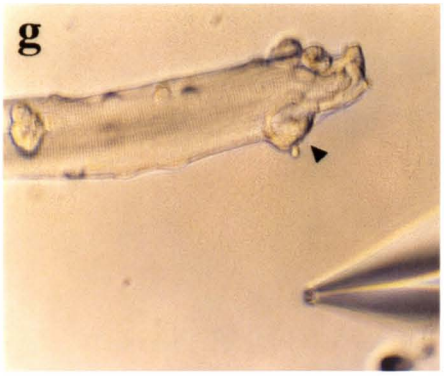

b


c


d


e


f


g

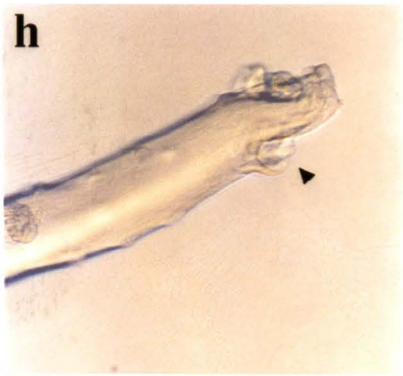
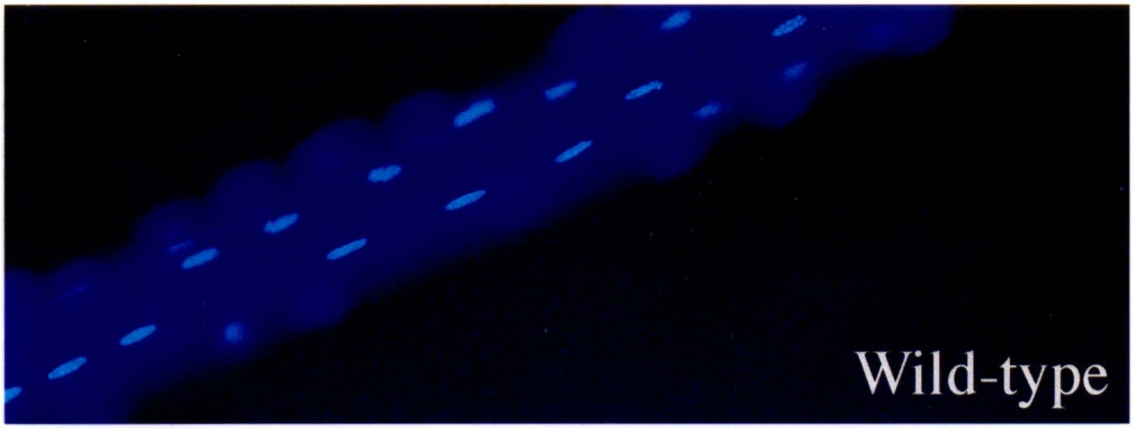
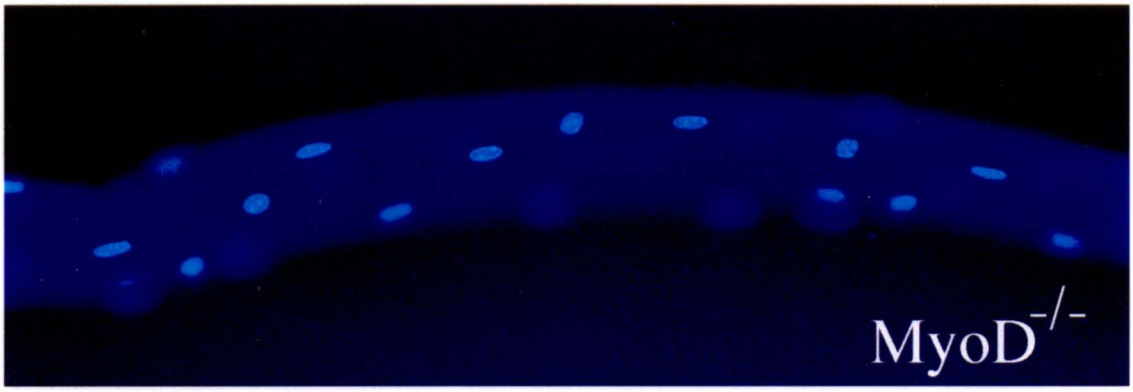

h

Figure 3: (a) Comparison of myonucleus morphology between wild-type and MyoD-null myofibers. (b) MyoD-null myofiber with a fork. MyoD-null fibers which forked and rejoined, and fibers with small sprouts, were also observed.

a





b

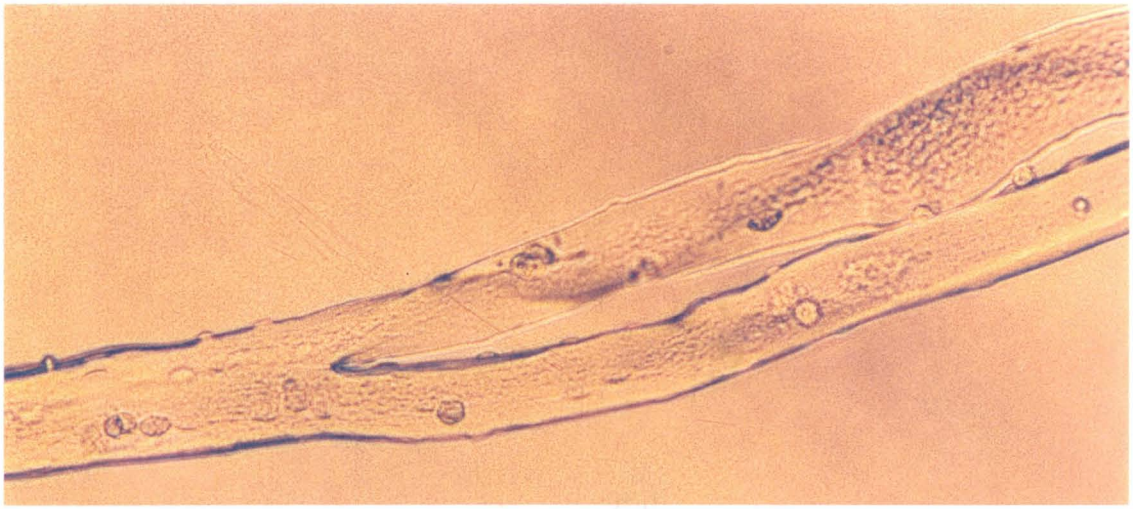
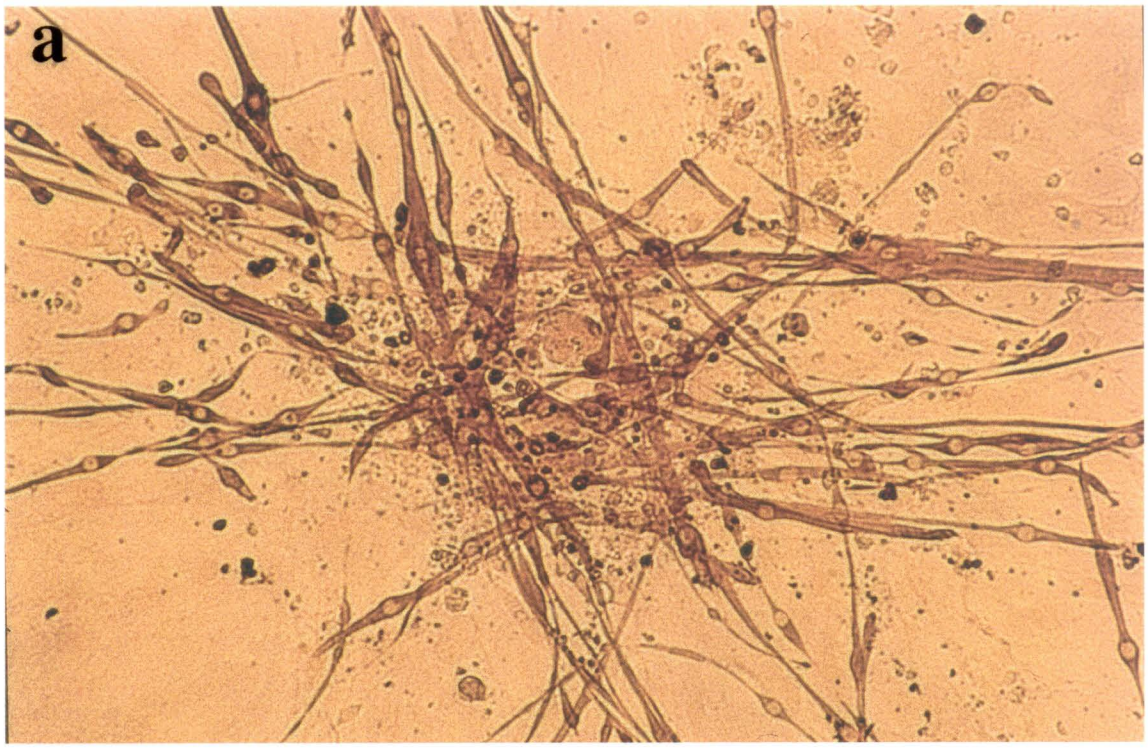


Figure 4: Immunostain for myosin heavy chain (MHC) on colonies of adherent satellite cells cultured for 7 days in growth medium and 7 days in differentiation medium. In wild-type, the majority of MHC^+ cells are multinucleate myotubes (a); in cultures of MyoD-null cells the majority of MHC^+ cells are round, mononucleate blasts (arrowhead), while those myotubes which do form are abnormally stunted and have few myonuclei (arrow)


a

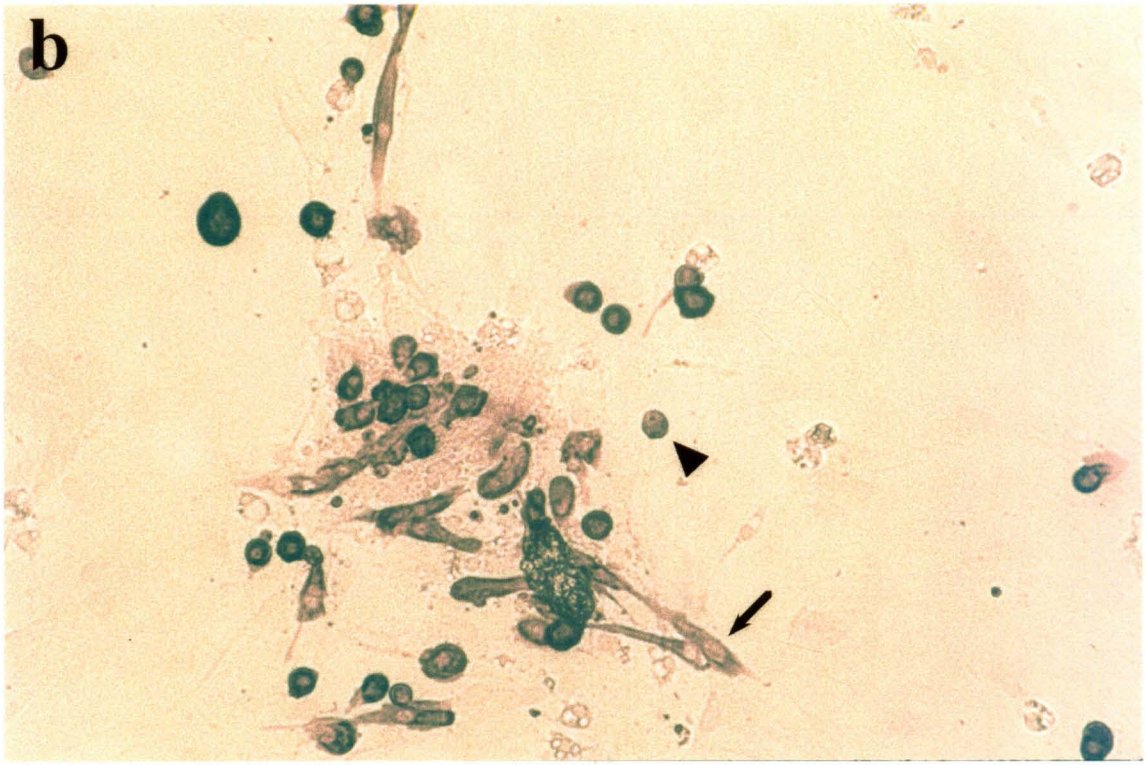

b

Figure 5: Column stack histogram comparing the percentage of c-met expressing cells which coexpress MHC (hatched area), or coexpress MHC and have a myotube morphology (shaded area) in wild-type and MyoD-null satellite cell cultures.

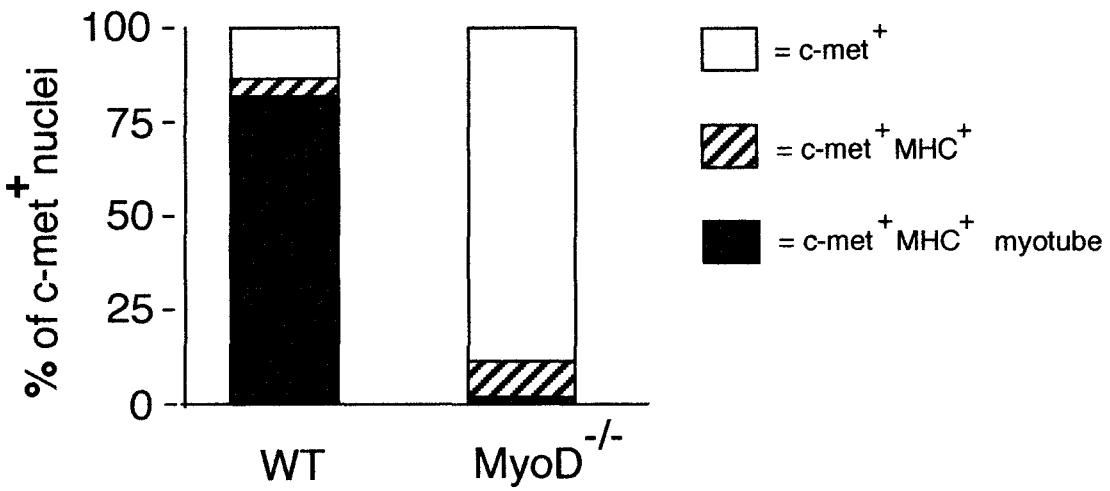
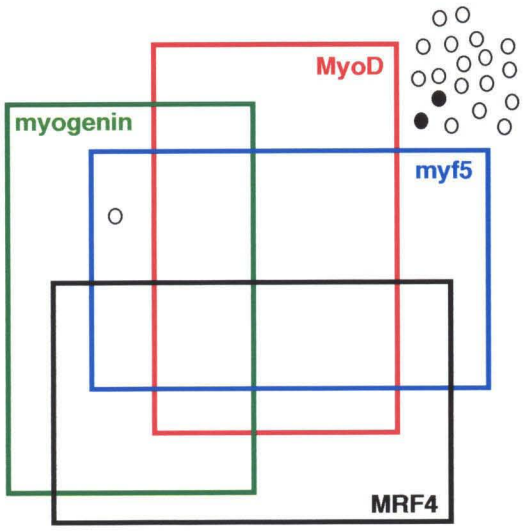
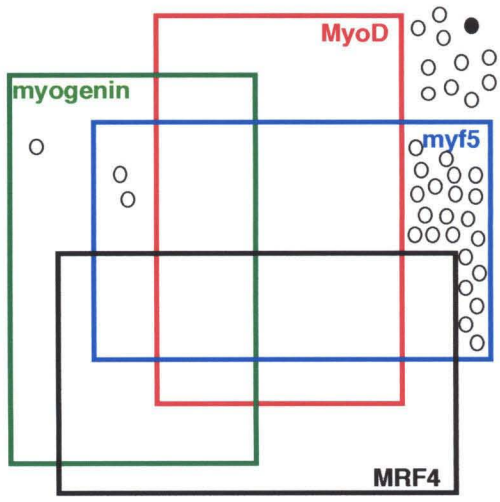

100
75
50
25
0
% of c-met+ nuclei
WT
MyoD-/-
= c-met+
= c-met+MHC+
= c-met+MHC+ myotube

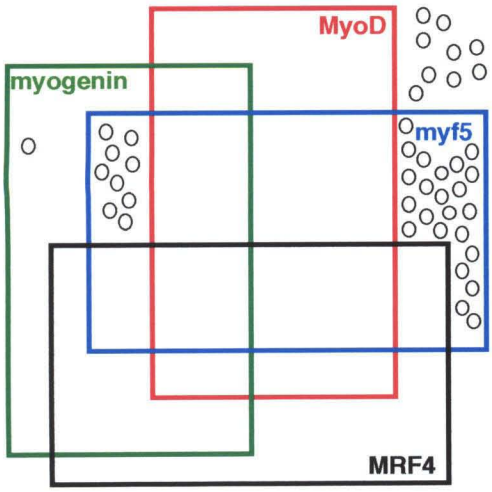
Figure 6: Representation of the combinatorial expression states of c-met, m-cadherin, MyoD, myf5, myogenin, and MRF4 in MyoD-null satellite cells during the first 4 days in fiber culture. Note precocious myogenin expression at 0 and 24 hours, absence of MRF4 expression at 48 and 96 hours, and the general decrease and failure to correlate with myogenin of m-cadherin.



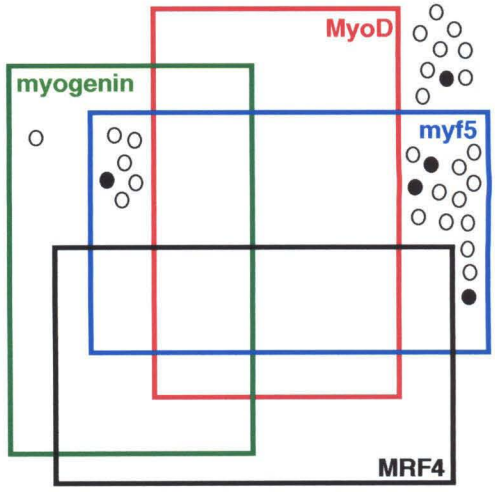
0 hours



24 hours



48 hours



96 hours

○ = c-met^{+}m-cadherin^{-}

● = c-met^{+}m-cadherin^{+}

Figure 7: Coexpression of myosin heavy chain (green) and m-cadherin (red) in adherent MyoD-null satellite cells. M-cadherin is most often observed at the interface of two mononucleate MHC^+ cells which are in contact with each other but clearly not fused (a). Occasionally expression more similar to that of wild-type cells is seen, in which m-cadherin is more uniformly distributed (b); the yellow color in the m-cadherin panel is an artifact of the TRITC filter set used and indiciates more intense fluorescence than does the red color.

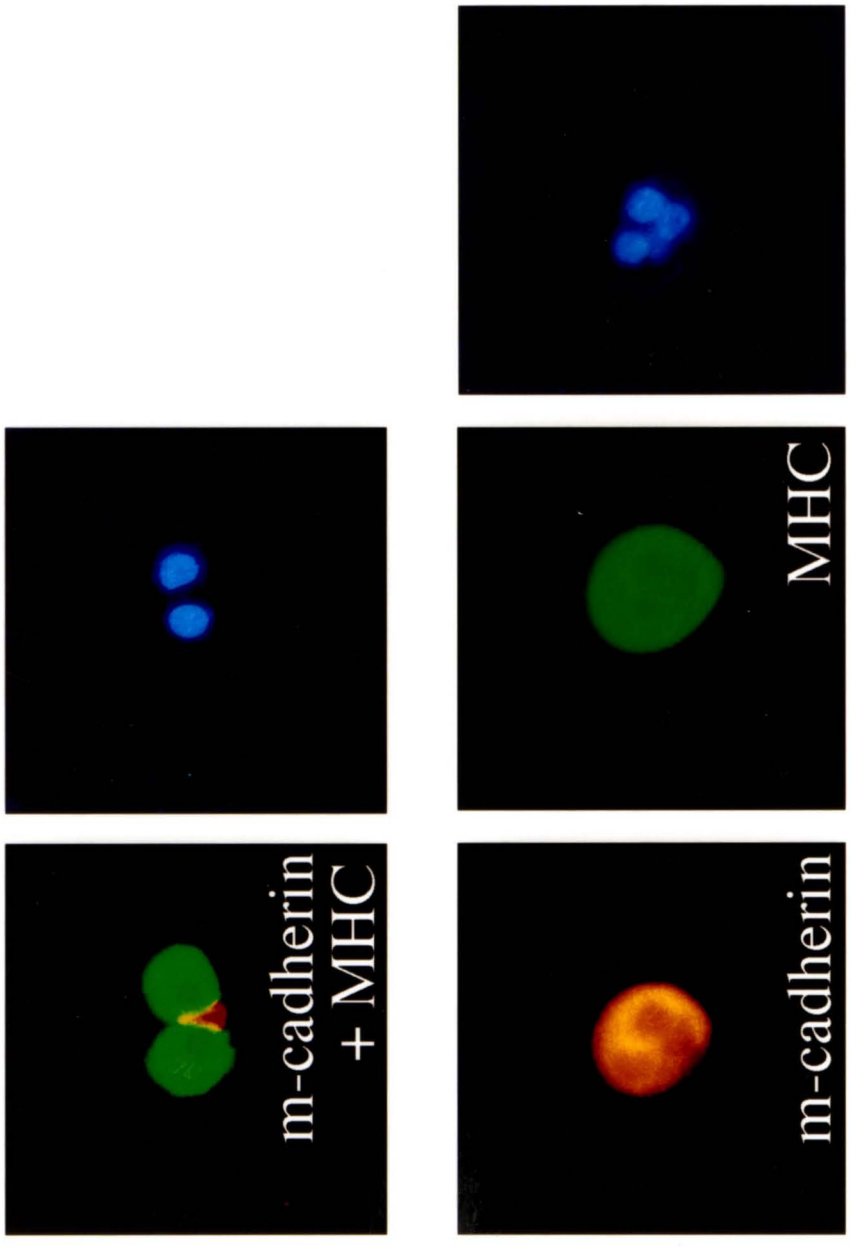

a
m-cadherin
+ MHC
b
m-cadherin
MHC

Figure 8: Revised model of MRF progression in wild-type and MyoD-null satellite cells encompassing the data from this work. Dashed lines indicate pathways which are suggested by the expression pattern but for which no direct evidence has been found.

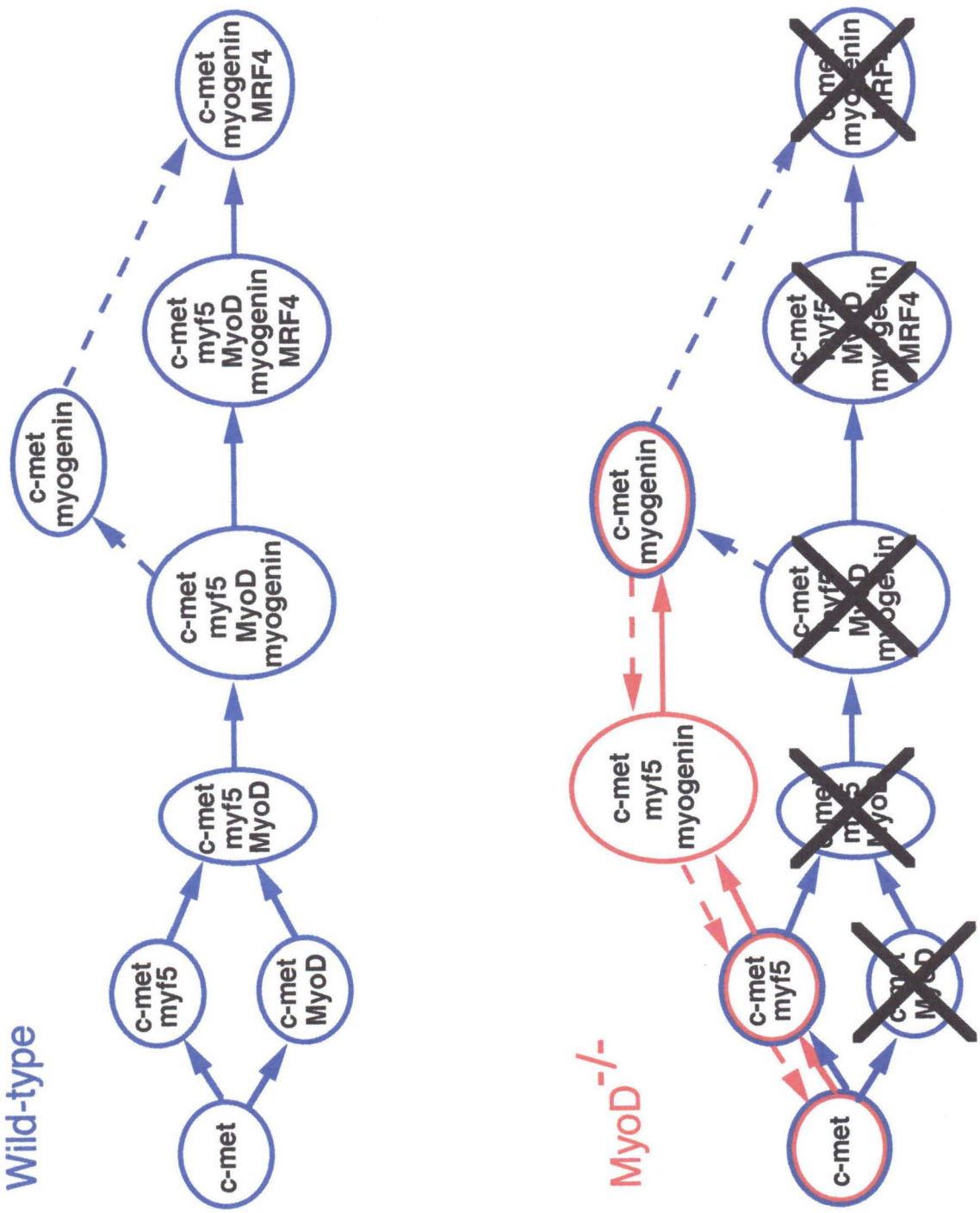

Wild-type
c-met
c-met myf5
c-met MyoD
c-met myf5 MyoD
c-met myf5 MyoD myogenin
c-met myogenin
c-met myf5 MyoD myogenin MRF4
c-met myogenin MRF4
MyoD-/-
c-met
c-met myf5
c-met myf5 myogenin
c-met myogenin
c-met myogenin MRF4

Table 1: Percent occupation of all possible coexpression states in single satellite cells from wild-type or MyoD-null fiber cultures (percentages are derived from single-cell data from (Cornelison and Wold, 1997), figure 7, and this work, figure 5). Percentages which represent statistically significant differences are in bold type.

Table 1: Quantitative analysis of expression states

				Wild-type				MyoD-null			
MyoD	myf5	myg	MRF4	0	24	48	96	0	24	48	96
–	–	–	–	92%	27%	11%	2%	95%	**30%**	21%	**30%**
+	–	–	–	4%	20%	3%	2%	0%	0%	0%	0%
–	+	–	–	4%	14%	3%	0%	0%	**61%**	**56%**	**47%**
–	–	+	–	0%	0%	5%	4%	**5%**	3%	2%	3%
–	–	–	+	0%	0%	0%	0%	0%	0%	0%	0%
+	+	–	–	0%	39%	14%	28%	0%	**0%**	**0%**	**0%**
+	–	+	–	0%	0%	0%	0%	0%	0%	0%	0%
+	–	–	+	0%	0%	0%	0%	0%	0%	0%	0%
–	+	+	–	0%	0%	0%	0%	0%	**6%**	**21%**	**20%**
–	+	–	+	0%	0%	0%	0%	0%	0%	0%	0%
–	–	+	+	0%	0%	1%	2%	0%	0%	0%	0%
+	+	+	–	0%	0%	32%	20%	0%	0%	**0%**	**0%**
+	+	–	+	0%	0%	0%	0%	0%	0%	0%	0%
+	–	+	+	0%	0%	0%	0%	0%	0%	0%	0%
–	+	+	+	0%	0%	0%	0%	0%	0%	0%	0%
+	+	+	+	0%	0%	30%	41%	0%	0%	**0%**	**0%**

Table 2: Genes whose expression is altered from wild-type to MyoD-null; the final colums (96*) represents expression observed in MyoD-null satellite cell pools enriched for cells which are apparently ingressing beneath the fiber lamina.

Table 2: Genes whose expression varies between wild-type and MyoD-null satellite cells

	Wild-type				MyoD$^{-/-}$				
	0	**24**	**48**	**96**	**0**	**24**	**48**	**96**	**96***
MyoD	-	+	+	+	-	-	-	-	-
myogenin	-	-	+	+	-	+	+	+	+
MRF4	-	-	+	+	-	-	-	-	-
MEF2D	+	+	+	+	-	-	+	+	+
MEF2D-MS	-	-	+	+	-	-	+	+	-
twist	-	+	+	-	-	-	-	-	-
cdk2	-	-	-	-	-	+	+	+	-
p19	-	-	-	-	+	+	+	+	+
p21	-	-	-	+	-	+	+	+	-
p27	-	-	+	+	+	+	+	+	+
p57	-	-	+	+	-	-	-	-	-
IGF-2	-	-	-	-	-	-	-	-	+
PDGFα	-	-	-	-	-	+	+	+	+
Msx-1	+	-	-	-	+	-	-	-	+

Chapter 5

Conclusions & future directions

The work presented in this thesis addresses many of the general questions regarding the nature and function of satellite cells as adult myoblasts presented in Chapter 1. The results clearly indicate that while satellite cells may resemble myoblasts of the embryo in some aspects, they differ in both their gene expression and gene usage, leading to alternate pathways for some common functions and at least partially explaining the mechanisms of some functions unique to satellite cells.

In the embryo, cells become committed myoblasts and proceed to differentiate in a continuous and fairly rapid process. In contrast, satellite cells are presumably committed to the myogenic lineage but do not differentiate until long after embryogenesis, and then only when the host muscle tissue is damaged. While it remains unknown how either embryonic myoblasts or satellite cells become committed to the myogenic lineage, work presented here presents a mechanism by which committed but quiescent satellite cells become activated to differentiate. The hepatocyte growth factor/scatter factor receptor c-met, which is demonstrated in Chapter 2 to be expressed by quiescent satellite cells *in vivo*, is an excellent candidate for the transducer of the initial activation signal. This argument is supported by the biochemical similarity of the c-met ligand to the major active component in crushed muscle extract (Bischoff, 1986) and the ability of exogenous HGF/SF to induce activation in rat satellite cells *in vitro* (Allen *et al.*, 1995) and *in vivo* (Tatsumi *et al.*, 1998). Additional functions of c-met in satellite cells, such as inducing cell migration (Bischoff, 1997), may also be important for robust regeneration.

During embryonic myogenesis, the myogenic regulatory factors (MRFs) play partially redundant roles in myogenic determination and differentiation. In particular, MyoD and myf5 share a function in determination of somitic myoblasts: deletion of either gene from the germline does not result in a myogenic defect due to compensation by cells expressing the other factor (Rudnicki *et al.*, 1992), but deletion of both leads to the absence of myoblasts (Rudnicki *et al.*, 1993). MRF expression is crucial for all known skeletal myogenesis, and satellite cells were known to express MRFs during

differentiation, so it was extremely likely that MRF activity also played a pivotal role in satellite cell myogenesis. However, when this work was begun the roles of specific MRFs in satellite myogenesis had yet to be defined; since adult muscles and potentially their satellite cells are heterogeneous (i.e. derived from axial vs. appendicular myoblasts, or expressing fast vs. slow myosin heavy chain isoforms) there also existed the possibility of differential gene expression within the satellite cell population. Possibilities for MRF activity in satellite cells included a mechanism similar to that of embryonic myoblasts, in which either MyoD or myf5 is directly required for initiation of myogenesis in different populations of satellite cells; one in which expression of either MyoD or myf5 was sufficient for any satellite cell; one in which either MyoD or myf5 was required for all satellite cells; or a completely different mechanism requiring, for example, myogenin expression.

In Chapter 2, it was determined that primary satellite cell in culture first expressed either MyoD or myf5, followed shortly by coexpression of both. This differed from the temporal coexpression seen in either embryonic myoblast population, and indicated that at least a mechanism for initiation of myogenesis in satellite cells which involved neither MyoD nor myf5 was unlikely. In Chapter 4, building on *in vivo* morphological studies published while this work was in progress (Megeney *et al.*, 1996), it was determined that MyoD is specifically required for robust myogenesis in satellite cells. That satellite cells unable to express MyoD also failed to express MRF4 at later points in the response and that very few MyoD-null satellite cells ever expressed m-cadherin are probably symptomatic of the failure of myogenic progression in these cells as well as being themselves causes of certain aspects of the differentiation-defective phenotype.

The broader examination of genes whose activity may affect processes such as proliferation and differentiation, and the balance between them, presented in Chapter 3 was meant to suggest sets of genes for later coexpression analysis in single cells. Many of these genes and gene families were chosen for study because they are known to

influence myogenic development in the embryo. It is important to realize, however, that the extracellular environments in which satellite cells exist before and after muscle damage are different from each other as well as from the somitic system; therefore, it is unlikely that all genes known to affect embryonic myogenesis will be expressed by satellite cells, or that genes which are expressed will necessarily serve the same function.

Within gene families, it was hoped that a preferred suite of genes would be expressed in satellite cells, thus reducing the complexity of the system. While within some families most or all members were found expressed in satellite cells (i.e. the MRFs, MEFs and Ids), in other families (i.e. Notches and Dlls) there were some family members which were never detected in satellite cells. Given the results presented in Chapter 3, several sets of genes whose coexpression patterns in single cells would be of interest suggest themselves; following are a few possible sets.

Since the MEF2 family of transcription factors are thought to act synergistically with MRFs to activate muscle-specific genes, determining the fractional representation of MEF2A, C, and D and any possible coexpression preferences with MRFs and correlation to the differentiated state may suggest specific roles for each factor in satellite myogenesis. Fractional representation and coexpression with MRFs should also be determined for Ids 1-4; if there appears to be an ordered progression of MEFs and Ids during the course of differentiation, it may also be possible to correlate sets of MRFs, MEFs, and Ids in order to form a more complete picture of the myogenesis-promoting and -inhibiting factors at work within a single satellite cell.

Similarly, the coexpression of genes in the Notch cell-cell signaling pathway (including Notches 1 and 2, Dll-1, Jagged-2 and possibly Jagged-1) with the MRFs is also of interest, especially in adjacent satellite cells after 48 hours, which are likely to be siblings. It may be possible to determine specific roles for these signaling molecules in promoting or inhibiting the differentiated state. Their coexpression with Radical and

Lunatic Fringe and numb and numblike, especially if these genes are indeed differentially expressed based on a given cell's terminal differentiation status, is also of interest.

The pathology of MyoD-null satellite cells in culture offers several opportunities for studying processes which do not occur in wild-type cells *in vitro*, such as a potential to return to a state similar to quiescence. The analysis of genes specifically expressed in apparently regressing cells may yield clues as to the mechanism of return to quiescence. The first such differentially-expressed gene, Msx-1, is expressed in quiescent satellite cells in both wild-type and MyoD-null mice, thus allowing protein and RNA reagents to be characterized in sections of intact muscle before being applied to sections of damaged muscle or to cultured fibers in order to confirm and extend the pool RT-PCR data.

In conclusion, this work presents significant technical advances in the study of satellite cells, significant new data based on these techniques, and suggests new lines of questioning which have the potential to further extend knowledge of this system.

References

Allen, R. E., Sheehan, S. M., Taylor, R. G., Kendall, T. L. and Rice, G. M. (1995). Hepatocyte growth factor activates quiescent skeletal muscle satellite cells in vitro. *J. Cell. Phys.* **165**, 307-312.

Bischoff, R. (1997). Chemotaxis of skeletal muscle satellite cells. *Dev. Dynam.* **208**, 505-515.

Bischoff, R. (1986). A satellite cell mitogen from crushed adult muscle. *Dev. Biol.* **115**, 140-147.

Megeney, L. A., Kablar, B., Garrett, K., Anderson, J. E. and Rudnicki, M. A. (1996). MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes Dev.* **10**, 1173-1183.

Rudnicki, M. A., Braun, T., Hinuma, S. and Jaenisch, R. (1992). Inactivation of *MyoD* in mice leads to up-regulation of the myogenic HLH gene *Myf-5* and results in apparently normal muscle development. *Cell* **71**, 383-390.

Rudnicki, M. A., Schnegelsberg, P. N. J., Stead, R. H., Braun, T., Arnold, H.-H. and Jaenisch, R. (1993). MyoD or myf-5 is required for the formation of skeletal muscle. *Cell* **75**, 1351-1359.

Tatsumi, R., Anderson, J. E., Nevoret, C. J., Halevy, O. and Allen, R. E. (1998). HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. *Dev. Biol.* **194**, 114-128.