1

Introduction
Skeletal muscle formation is a highly ordered process, orchestrated by a family of master regulators coordinating a series of events that culminate in the formation of mature myofibers. This family of transcription factors called the myogenic regulatory factors (MRFs) is comprised of four members, MyoD, Myf-5, Myogenin and MRF-4. MRFs have the remarkable property that when over-expressed, they can not only activate muscle-specific genes in a large number of cell types but also convert some cell types into skeletal muscle (Davis et al, 1987; Braun et al, 1989; Wright et al, 1989; Rhodes et al, 1989; reviewed by Buckingham, 2001).

A) Muscle formation and differentiation

1) Embryonic myogenesis

Myogenesis in the embryo is initiated by a population of precursor cells located in transient mesenchymal entities called somites. Signaling molecules from the surrounding tissues such as the dorsal ectoderm and the notochord specify myogenic fate in the precursor cells by the expression of the two MRFs, MyoD and Myf5. These committed precursor cells or myoblasts expand their pool, express myogenin, exit the cell cycle and differentiate followed by fusion to give rise to multinucleated myofibers. The myofibers are characterized by the expression of Myogenin and MRF4 (Figure 1) (reviewed in Tajbaksh, 2005; Buckingham, 2001).

The individual functions of the MRFs in vivo have been investigated by determining their expression during development, and by analyzing mice with mutations in these genes. Mutant mice with homozygous targeted deletions for MyoD and Myf-5 have been invaluable in establishing the function of MyoD and Myf-5 during early myogenic differentiation. Surprisingly, loss of MyoD function does not result in any gross muscle phenotype (Rudnicki et al, 1992). Instead, these mice are viable and display a developmental delay indicating a specific requirement for MyoD in a subset of muscles (hypaxial muscles). Similarly, although they die shortly after birth, Myf-5 mutant mice show no severe muscle abnormalities but a delay in the differentiation of a different set
Figure 1: Skeletal muscle differentiation
of muscles that do not overlap with the MyoD mutant mice (epaxial muscles) (reviewed in Kablar and Rudnicki, 2000). Importantly, the Myf5-MyoD double mutant is an early embryonic lethal, completely lacking determined myoblasts. These data suggest that the lineage determination of myoblasts by Myf5 and MyoD is an essential event during myogenesis in the embryo, wherein MyoD and Myf5 have largely overlapping functions. However, mice that do not express myogenin (Hasty et al, 1993; Nabeshima et al, 1993) display normal numbers of determined myoblasts but a severe deficit in differentiated fibers. MRF-4 knockout mice appear normal but possess elevated levels of myogenin as a compensatory mechanism (Olson et al, 1996).

These observations support the notion that there are differences in the temporal requirement of the MRFs and therefore specific functions for sets of MRFs in the myogenic program. While early expression of MyoD and Myf5 in the myogenic program is essential for lineage determination, Myogenin and MRF4 have later roles in myogenic differentiation (by Weintraub, 1993; Cooper et al, 1999).

2) Muscle regeneration

Skeletal muscle formation in the adult occurs in response to injury, in a repair process that recapitulates many of the events involved in embryonic muscle development (Figure 2). The myofibers constituting skeletal muscle tissue are post-mitotic and incapable of participating in the regenerative process. Therefore, the source for new cells comes from a population of mononucleated precursor cells satellite cells (SC) that are found closely associated with the muscle fiber. SC were first identified by their anatomical location, peripheral to the myofiber and juxtaposed between the basal lamina and the sarcolemma of the myofiber (Mauro, 1961; reviewed by Campion, 1984). SC are capable of giving rise to myogenically determined cells that can differentiate, as well as self-renew. Following injury, SC associated with myofibers re-enter the cell cycle, proliferate, differentiate and fuse to replace the lost myofiber. However in normal muscle, SC are mitotically quiescent and undifferentiated in that they do not express any of the known muscle
Figure 2: Skeletal muscle regeneration
regulatory factors (reviewed by Seale and Rudnicki, 2000; Zammit and Beauchamp, 2001; Partridge, 2003; Collins et al, 2005). This transition of SC from an arrested undifferentiated state to an activated, lineage determined state in response to external stimuli is a complex molecular process that is not well understood.

3) Expression of MyoD during regeneration in the adult skeletal muscle

MyoD is the first and most abundantly expressed myogenic regulatory gene (Smith et al, 1993) and in vivo, the transcript can be detected by in situ hybridization, a few hours after injury to the skeletal muscle (Grounds et al, 1992). Although adult mice lacking MyoD do not display any macroscopic degenerative phenotype under normal conditions, (Rudnicki et al, 1992), regeneration was significantly impaired in the MyoD null mice when challenged with an injury (Megeney et al, 1996). A detailed analysis of individual fiber cultures from MyoD null mice showed that the SC are differentiation defective and fail to up-regulate the terminal differentiation marker MRF4 (Sabourin et al, 1999; Cornelison et al, 2000). Thus, unlike its partially redundant role in the embryo, MyoD has an essential function during the early stages of myogenic differentiation in the adult. These studies also corroborate the inferences drawn from tissue culture studies on the importance of stage specific-induction of MyoD expression (see below).

4) MyoD: A master regulator that initiates the myogenic program

Since its discovery in 1987, MyoD has been extensively analyzed in tissue culture studies that have allowed its function as a master regulator of the skeletal muscle transcriptional hierarchy to be established.

Once expressed, MyoD can interact with cell cycle regulators, chromatin remodellers and synergize with other ubiquitous and muscle specific transcription factors to induce cell cycle withdrawal and initiate the expression of muscle specific genes characteristic of terminal muscle differentiation (Figure 3). Although it was suggested that MyoD transactivation
is checked in growing myoblasts by degradation or posttranslational modifications (Thayer et al, 1989), microarray analysis of MyoD−/− fibroblasts transfected with MyoD in high serum conditions have shown that MyoD also regulates the expression of growth phase target genes. However, MyoD is more efficient in inducing differentiation-phase target genes due to a novel cooperation between its amino and carboxy-terminal ends (Ishibashi et al, 2005).

The structure of the MyoD protein revealed that many of its functions can be attributed to a basic region, the adjacent helix-loop-helix region and a 15 amino acid stretch in the C-terminus such that mutations in these regions can affect MyoD’s ability to regulate muscle specific genes and inhibit growth. More importantly, the bHLH (basic helix-loop-helix) domain represents the DNA binding and dimerization motif through which MyoD can efficiently bind to DNA as a heterodimer with other HLH-motif containing proteins (Figure 4) (Tapscott et al, 1988; Murre and Lassar, 1989).

As a result of MyoD’s ability to interact with diverse proteins, all of which play integral roles in muscle differentiation, MyoD emerges as a versatile and potent inducer of differentiation. Therefore, several mechanisms that are known to prevent precocious myogenic differentiation are aimed towards repressing MyoD function. However, very few regulators are known to regulate MyoD at the level of expression.

The objective of this chapter is to review the importance of the timing of MyoD expression in adult skeletal muscle, and address the necessity for the identification of regulators of MyoD expression in the adult. The latter section of this chapter (Section C) examines the contribution of adhesion and specific adhesion dependent pathways that regulate MyoD expression in vitro. The significance of this mode of regulation on myogenic gene expression and cell cycle progression has also been presented.
Figure 3: Interactions of MyoD

MyoD expression and function is tightly regulated by its interactions with cell cycle regulators (cdk4, in red), chromatin remodellers (p300, HDAC1, in green), transcription factors (E47/E12, MEF2, Id1, Twist, E2a, in peach), other myogenic regulators (MyoD itself and Myogenin, in pink) and regulating the transcription of muscle specific genes and cell cycle inhibitory genes through E-boxes in their promoters (in blue). (Double arrow heads and double T's indicate interacting partners of the MyoD protein, single arrow head towards target indicate regulation by MyoD at the level of the promoter, single arrow head towards MyoD indicate modulation of MyoD expression). Transcription factors, Msx1 and l-mfa, Pax3/Pax7 and SRF (in purple) regulate MyoD expression. Myogenic activity of MyoD is potentiated by interaction with transcription factors (MEF2, E2a, E47, Myogenin, MyoD) and acetylation events (p300). MyoD function is kept repressed by transcription factors (Id1, Twist) and posttranslational modifications (phosphorylation by cdk4 and deacetylation by HDAC1). Msx1 and l-mfa have been recently shown to suppress MyoD expression while Pax3/Pax7 potentiate its expression.
<table>
<thead>
<tr>
<th>NH2</th>
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- **Affects myogenic activity, cell cycle progression, nuclear localization**
- **Regulates myogenic activity**
- **Reduction in trans-activating functions**
- **Regulates myogenic activity, cell cycle progression**
- **Induction of differentiation phase target genes**

**Figure 4: MyoD structure and function**

*Schematic of MyoD protein showing domains responsible for several functions.* The basic region and the adjacent region containing the helix-loop-helix motif is the primary region that accounts for nuclear localization, myogenic conversion activity and inhibition of cell cycle progression as revealed by site directed mutagenesis (shown as crossed bars). A 15 amino acid stretch in the carboxy-terminal region of MyoD has been shown to interact with cdk4 that is also important for regulating the myogenic activity of MyoD and cell cycle progression (Wei and Paterson, 2001). The HLH region represents the DNA binding and dimerization motif through which MyoD can bind as a heterodimer through interactions with other HLH-motif containing proteins. The NH2 and COOH-ends can cooperate to induce the expression of differentiation phase target genes (Ishibashi et al, 2005).
B) Myogenesis and the cell cycle

1) Satellite cells
   a) The Quiescent state

Most cells in the adult body are post-mitotic. The few cells that retain the capacity to proliferate during tissue replacement are the stem cells. Unlike organ systems like the vascular system or the skin that is characterized by frequent stem cell activity, SC activity is evident only in the regenerating muscle. In the normal muscle, however, SC are quiescent, dividing only rarely (Bischoff, 1990; Schultz, 1985).

Mitotic quiescence is a cellular state characterized in addition to the absence of DNA synthesis by low metabolic activity, heterochromatic nuclei and suppression of tissue-specific gene expression. Studies on the quiescent state in naive lymphocytes have shown that there are signaling molecules and transcription factors (e.g. Polycomb group of proteins) that inhibit activation and cell cycle progression (Glynne et al, 2000). These pathways also actively suppress the expression of lymphoid lineage markers, and absence of these signals leads to deregulated lymphopoiesis. Thus, quiescence is understood as an actively maintained state rather than a default state that is characterized only by the suppression of gene expression (Yusuf and Fruman, 2003). Ongoing studies in the lab have specifically addressed the question of whether there is gene induction during quiescence rather than a mere absence of positive regulators (Sachidanandan et al, 2002; Ramkumar et al, unpublished; Subramaniam et al, unpublished). These studies have found a large number of genes to be actively induced during the quiescent state. More importantly, the absence of expression of tissue-specific genes is central to the theme of quiescence.

There is a paucity of information on molecular details in SC because studying the phenomenon of quiescence in SC in vivo poses a considerable challenge: they are a rare population representing only 2-6% of nuclei in the entire muscle and are invariably activated upon isolation (reviewed in Dhawan
and Rando, 2005). In addition to transgenic mice, in vitro and ex vivo model systems have contributed substantially to our understanding of SC behaviour (Section B-c).

In uninjured muscle, SC are quiescent and divide slowly (Bischoff, 1994; Grounds and Yablonka-Reuveni, 1993). Although single cell RT-PCR revealed that none of the MRF transcripts are expressed in quiescent SC (Cornelison and Wold, 1997) the presence of Myf-5, in the quiescent SC has been controversial. βgal activity in SC of the Myf-5lacZ knock-in mouse (Beauchamp et al, 2000) suggests that the Myf-5 promoter may be active at a basal level. However, Myf-5 protein itself has not been detected.

Thus, the predominant pathways existent in the quiescent SC must involve not only the suppression of cell cycle progression, but also inhibition of muscle specific gene expression to avoid spontaneous proliferation and precocious differentiation, which would deplete the stem cell pool. Importantly, these pathways or regulators that are hypothesized to suppress cell cycle progression must be different from the irreversible cell cycle arrest associated with terminally differentiated cells.

b) Genes expressed in quiescent Satellite Cells

Studies to understand the molecular events responsible for the relationship between undifferentiated SC and their progeny led to the identification of tissue-restricted genes expressed in the SC myogenic lineage. However, none of these markers are associated exclusively with quiescence and are also expressed by activated SC. Of these, the paired box transcription factor Pax7 is of particular importance because of its similarity with Pax3, a paralogue earlier shown to regulate the developmental program of embryonic myoblasts. Using the technique of Representational Differential Analysis, Pax7 was found to be expressed in cultures of proliferating SC-derived myoblasts, but not in differentiated myotubes, and also found localized to both quiescent and proliferating SC in adult muscle in vivo (Seale
et al, 2000). At the time, the drastic reduction in the number of SC in postnatal Pax7 knockout mice was interpreted to reflect a key role for this transcription factor in SC specification. The observation that these mutant mice had apparently normal embryonic myogenesis supported that interpretation. However, recent detailed studies that examined postnatal development in Pax7 null mice suggest a role for Pax7 in survival via prevention of apoptosis and therefore the maintenance of SC rather than specification per se (Oustanina et al, 2004; Kassar-Duchossoy et al, 2005). Myostatin, a member of the TGF-β super family (McCroskery et al, 2003) is a serum factor known to induce SC quiescence and MNF-beta, a transcription factor may modulate SC arrest (Hawke et al, 2001).

Quiescent SC have also been shown to express several surface markers, of which some imply a state of preparedness for activation, but the function of others is as yet unknown. The isolation and identification of the active factor in crushed muscle extract that caused rapid activation of SC (Bischoff, 1986b) as the ECM-bound growth factor, HGF, led to the finding that quiescent SC express c-met, a receptor tyrosine kinase (Tatsumi et al, 1998). Localization of other surface markers on the quiescent SC such as CD34, the hematopoietic stem cell marker (Beauchamp et al, 2002), a novel epitope defined by the SMC2.6 antibody (Fukada et al, 2004), and M-cadherin, a muscle specific calcium dependent adhesion molecule (Irintchev et al, 1994), have aided SC identification although no signaling function has been defined for the individual markers. The first attempt to prospectively isolate a purified population of stem cells from skeletal muscle used flow cytometric analysis with multiple antigens to isolate cells defined by the signature, CD45−Sca1−Mac1−CXCR4+β1Integrin+ (CSM4B) (Sherwood et al, 2004a).

Thus, the molecular characterization of SC has aided their identification and isolation from adult skeletal muscle tissue, but this is still far from routine and additional markers that would be required to improve the specificity and ease of isolation. Although some markers appear to be essential for
persistence of SC in the adult muscle (Pax7), or contribute to G0 arrest (myostatin) or early activation events (c-met), there is a conspicuous dearth of knowledge about mechanisms responsible for inducing the state of reversible arrest and preventing the expression of the MRFs during quiescence.

c) Activation of satellite cells

Damage to the muscle fiber activates SC, causing them to exit quiescence, become determined myoblasts and rapidly proliferate. Damage may be caused by mechanical or chemical injury, excessive load bearing or genetic deficiency as in the muscular dystrophies. Multiple biochemical pathways triggered during activation including those initiated by inflammatory cytokines (listed in Figure 2) (Tidball, 2005), evolutionarily conserved growth factor pathways such as Notch, Wnt and Shh (Conboy and Rando, 2002; Husman et al, 1996; Pola et al, 2003; Polesskaya et al, 2003 and Tidball, 2005) have been implicated in the 2 major events that govern SC activation, i.e., G0-G1 transition and the expression of MyoD.

(i) G0-G1 transition

Satellite cells are considered to exist in the G0 state in uninjured adult muscle. Reversible arrest, however, has largely been characterized by studies in cultured cell lines and much of our understanding of this “out of cycle” state is derived from studies of synchronized fibroblasts (Pledger et al, 1978; Campisi and Pardee, 1984). G0 arrest differs from G1 arrest in that, although both states are characterized by a G1 DNA content and the absence of DNA synthesis, G0 cells show different patterns of gene expression and importantly, upon activation, require several hours longer to enter the S phase compared to G1 cells. This additional period is termed the G0-G1 transition.

The most extensively used model for the study of the cell cycle is the serum-starved fibroblast system. Serum stimulation of quiescent fibroblasts causes them to exit G0 and respond to growth-promoting signals by up-regulating a set of genes that allow the cell to slowly re-enter the cell cycle.
(reviewed in Pardee et al; 1989, Herschman, 1991). The expression of these genes confers on the cell the competence to enter the cell cycle and is a distinct feature of the G0-G1 transition. They include

(i) Transient induction of the growth regulatory transcription factors- c-myc, c-fos and c-jun that characterize the immediate early response (Greenberg et al, 1984)
(ii) Transcription of variant histone genes specific to G1 (DeLisle et al, 1983).
(iii) Increase in the rate of protein synthesis (Benecke et al, 1978; Ben Ze'ev et al, 1980).

In vivo in SC, exit from G0 is inferred from expression of muscle specific markers and induction of DNA synthesis after a lag period. Thus, although a detailed analysis of the hallmarks of the G0-G1 transition is lacking, activated SC are known to express the muscle regulatory factor MyoD a few hours after entry into the cell cycle following damage (Grounds et al, 1992).

Although myogenic cell lines and primary myoblast cultures have been available for several decades, these systems were exclusively used for the analysis of muscle differentiation. Only recently did synchronized myoblast systems become available for modeling reversible arrest in SC (Dhawan, 1991; Milasincic et al, 1996; Kitzman et al, 1998). This resulted in the finding that MyoD is cell cycle regulated and is maximally expressed in G1.

(ii) Synchronized myoblast cultures to model satellite cell activation

Since SC represent <5% of nuclei in muscle tissue and are activated upon isolation, detailed molecular analyses of SC behavior poses a considerable challenge. Further, activated SC may vary temporally in their response in gene expression to stimuli depending on the extent of injury or distance from the site of injury. Thus, it becomes difficult to address the control of molecular events such as MyoD activation that are associated with specific cell cycle stages.
To circumvent these problems, different synchronized model systems have been used to generate a homogenous population of cells (Figure 5). A comparison of these systems is presented below:

**Suspension Culture**

This system was the first one used to demonstrate that myogenic cells can actually be synchronized without differentiation (Dhawan, 1991; Milasincic et al, 1996). When deprived of adhesion, C2C12 myoblasts undergo a state of reversible arrest (G0) despite the presence of serum. When these G0 synchronized myoblasts are replated in the presence of serum, they exit G0, enter the cell cycle and express myogenic regulators (Figure 5).

**Advantages**

- Almost 100% cell viability
- Can study the G0 state and events during the G0-G1 transition.
- Extensively characterized for all known molecular markers (Sachidanandan et al, 2002). Although the overall rate of protein synthesis decreases during suspension, the continued presence of serum during adhesion deprivation permits a basal level of transcription and translation. Indeed, a large number of proteins and transcripts are upregulated during suspension-arrest (Dhawan, 1991; S. Subramaniam and J. Dhawan, unpublished).
- Can be used to study the role of specific adhesive interactions or of specific mitogens during reactivation
- Homogeneous population of synchronized cells, useful for molecular analysis.

**Disadvantage**

Cells are isolated in suspension and do not mimic the SC niche. Thus, the contribution of the myofiber to SC activity is not considered.
Figure 5: Synchronized model systems to study molecular events in G1 (See text for detailed description)

A) Undifferentiated cells or "reserve cells" in a C2C12 myotube culture
B) Suspension culture system
C) Deprivation of methionine and serum
D) Single fiber culture.
Combined nutrient and mitogen deprivation

G0 myoblasts can also be generated using a combination of low serum and deprivation of the essential amino acid, methionine (Kitzman et al, 1998). While C2C12 myoblasts differentiate in low serum, the simultaneous depletion of methionine slows down translation and pushes the cells into G0 that represents a much lower metabolic state than what is required for terminal differentiation (Figure 5).

Advantages
- Has been used in combination with hydroxyurea to block cells at the G1-S border and generate a high degree of synchrony
- Details of cell cycle regulation of MyoD and Myf-5 were discovered using this system
- Homogeneous population of synchronized cells, useful for molecular analysis

Disadvantages
- Not well characterized for known molecular markers
- Does not account for signals from the myofiber or mimic the SC niche
- Because of the absence of essential nutrients and serum, the viability of cells is low, perhaps due to stress response pathways, thus limiting the application of this system.

Mitogen deprivation-generation of Reserve cells

Deprivation of serum induces C2C12 myoblasts to differentiate. However, it was observed that only a fraction of the myoblasts undergo differentiation upon serum deprivation (Miller, 1990). At least 30% of the cells continued to remain mono-nucleated, and did not express any myogenic markers (Yoshida et al, 1998). Isolation and sub-culturing these "reserve cells" in growth medium caused them to re-express MyoD and progress through the cell cycle thus mimicking the activation of G0 myoblasts (Figure 5).

Advantages
• Cultures contain differentiated cells and quiescent cells and could be used to study their interactions which are largely unknown
• Can study G0 and G0-G1 events
• Distinct from the combined effects of mitogen and nutrient deprivation (see above) as the reserve cells show good viability.

Disadvantages
• Not well characterized for known markers of SC quiescence and activation
• Cannot address the contribution of adhesion to SC activity as the cells are always attached to the substratum.

**Single Fiber culture**

Primary cultures containing single fibers with their accompanying SC have been generated after isolating a single intact muscle and releasing individual fibers by enzymatic digestion. The SC associated with the myofiber are activated and proliferate into myoblasts (Figure 5).

Advantages
• Best characterized ex vivo system thus far
• Mimics the SC niche at least with respect to the association of the myofiber
• Clonal populations available for in situ analysis

Disadvantages
• Limited samples for analysis
• Isolated myofibers exhibit a denervation response which, in the absence of infiltrating immune cells, may have altered effects on the associated SC
• When more than one SC is associated with the myofiber, the activation profile between SC may vary temporally. Thus, this system is not suitable to study stage specific events at a molecular level.
2) *MyoD expression is cell cycle regulated*

Although in situ hybridization had shown that MyoD mRNA was induced in presumptive SC during regeneration, detailed analysis of activation using single cell RT-PCR was more informative (Cornelison and Wold, 1997). Examination of mRNA levels of multiple genes in SC associated with isolated single myofibers revealed that some SC enter an MRF+ve compartment by first expressing either MyoD or Myf-5, followed by rapid co-expression of the two MRFs. The mutually exclusive pattern of expression in the initial stages has been interpreted to suggest that two parallel pathways might regulate MyoD and Myf-5 induction.

More insights into the cell cycle regulation of MRF expression has been possible in culture. The first report of G0-synchronized myoblasts in culture revealed that MyoD was suppressed in G0 and induced in G1 (Dhawan, 1991; Milasincic et al, 1996). Extending this observation, detailed analysis of myoblasts synchronized by a different means (see above), mapped the profile of MyoD and Myf-5 separately, revealing that MyoD and Myf-5 undergo distinct and specific cell cycle dependent regulation (Kitzman et al, 1998). MyoD was absent in G0, peaked in G1, fell to a minimum in G1/S and gradually increased from S to M. By contrast, Myf-5 protein was high in G0 and decreased during G1. Therefore, although both MRFs are expressed in proliferating myoblasts, the two have distinct patterns of expression in the cell cycle. This raises the possibility that despite the overall synchrony into S phase, there could be inherent differences in cell cycle times of activated myoblasts. Thus, the MyoD expressing cells could be slightly out of phase with the Myf-5 expressing cells. Alternatively, there might be stochastic differences in levels of regulators which are not linked to cell cycle position, but which require a threshold level for activation of MyoD. Whatever the mechanism for this control, the result is that the peak of expression of MyoD occurs in G1 coinciding with the early activation of SC.
3) The significance of MyoD expression in G1

The G1 phase of the cell cycle represents the point of integration of several extracellular signals including growth factors, chemokines and attachment factors (reviewed by Pardee, 1989). The myogenic bHLH factors are known to activate muscle specific gene transcription only in cells cultured in low mitogen medium. Mitogens suppress myogenic activity because of a PKC-dependent (Spizz et al, 1986) and a cdk4-dependent phosphorylation of MyoD leading to its inactivation (Zhang et al, 1999). Therefore, the induction of MyoD in G1 bestows myogenic competence that is only unmasked in response to differentiation-promoting cues or a mitogen-poor environment. (Panel B of Figure 6 and Panel C of Figure 7). Activated MyoD directly regulates the expression of genes associated not only with terminal differentiation such as myogenin and muscle contractile proteins, but also those associated with irreversible cell cycle arrest, such as the CDKI, p21, Rb and cyclin D3 (Halevy, 1995; Lassar et al, 1994; Andres and Walsh, 1996) (Figure 7). Thus, MyoD expression and enhanced MyoD activity in G1 couples the myogenic program with terminal cell cycle exit.

In contrast, myogenic activity of MyoD is kept low in growth promoting conditions by multiple inhibitory mechanisms that counter MyoD activity in proliferating myoblasts, enabling progression through the cell cycle (reviewed in Wei and Paterson, 2001) (Panel B of Figure 7). Thus, modulation of the myogenic activity of MyoD enables the myoblast to decide between terminal differentiation and cell cycle progression (reviewed by Olson, 1992; Lassar et al, 1994; Puri et al, 2000; Kitzmann et al, 2001). Recent evidence suggests that MyoD may have targets even in proliferating myoblasts, but the significance of this is not well understood (Wyzykowski et al, 2002). Interestingly, the genome-wide “ChIP-on-chip” location analysis has revealed that MyoD protein occupies its target promoters even in proliferating myoblasts prior to the transcriptional activation of these genes in differentiation (Bergstrom et al, 2002). For the purposes of this review, the presence of MyoD protein in G1 confers on the myoblast the competence to differentiate in response to external signals.
Figure 6: Regulation of MyoD expression and activity in A) quiescence versus B) Terminal differentiation

A) MyoD expression (in blue) and activity (in red) in quiescent myoblast are low. Activation cues permit entry into G1. MyoD expression is upregulated but remains inactive in proliferating myoblasts. In S phase MyoD expression falls but gradually rises in G2-M. In the second round of the cell cycle, quiescence cues in G1 push the myoblast into G0 and suppress MyoD expression accompanied by the upregulation of the cdki, p27. Here cell cycle arrest is uncoupled from myogenic gene expression.

B) In G1 when MyoD expression is high, differentiation cues increase myogenic activity of MyoD. Active MyoD postively regulates p21 and myogenin, markers of irreversible arrest. In this case cell cycle exit is coupled to terminal differentiation.

(Modified from Dhawan and Rando, 2005)
Figure 7: Interactions between MyoD and the cell cycle regulators

A) Schematic of the cell cycle showing that the cell is responsive to growth regulatory effects of adhesion and growth factors only in G1 before the Restriction point (R). The G0 phase that represents the state of reversible arrest is shown.

B) Activity of MyoD is suppressed in proliferating myoblasts. Presence of growth factors and adhesion increases active cyclinD1-ckd4 complex. MyoD is inactivated by the binding of cyclinD-ckd4 complex. Association of HDAC 1 prevents acetylation of MyoD resulting in inactivation. Phosphorylation of Rb releases E2F from repression allowing transactivation of S phase genes.

C) Activity of MyoD is enhanced in a poor mitogen medium
Ratio of available active cyclinD-ckd4 complexes to MyoD decreases due to the lack of growth factors. Hypophosphorylated Rb sequesters HDAC relieving repression on MyoD and allowing acetylation by PCAF. Active MyoD enhances transcription of p21 and Rb and hypophosphorylated Rb sequesters E2F. These two activities contribute to terminal cell cycle arrest.
4) **MyoD and satellite cell self-renewal**

While the expression of MyoD is a hallmark of SC activation, absence of its expression has been associated with the return to quiescence in cells that renew the progenitor pool (Zammit et al, 2004). This population of cells would therefore be hypothesized to possess mechanisms that operate to actively suppress MyoD expression and the cell cycle (Panel A of Figure 6). In this regard, comparative analysis of gene expression in MyoD null cells with the wild type may provide useful clues to the program of quiescence. Indeed, MyoD null cells are known to express high levels of myostatin, a TGF-β superfamily member that has an anti-proliferative role and Msx1, a potent transcriptional repressor of MRF expression implicated in dedifferentiation of myotubes in vitro (Cornelison and Wold, 2000; Odelberg et al, 2000).

The previous sections have described the importance of MyoD in regeneration in adult muscle and its potent transcriptional ability depending on the signals that the cell receives during G1. Additionally, inhibition of its expression is obligatory for entry into quiescence. Thus, regulators that potentiate or inhibit MyoD expression impact upon the ability of the SC to proliferate, differentiate or quiesce.

5) **Regulators of MyoD expression**

a) **Transcriptional regulators**

Much of the evidence for the regulators of MyoD expression comes from studies in the embryo. Forced expression of Pax3 in non-muscle tissues in avian embryos (Epstein et al, 1995), and of Pax7 in Pax7-null myoblasts induces the expression of MyoD (Zammit et al, 2006). However, whether the mechanism of regulation of MyoD is direct or indirect is unknown. Each member of a group of transcription factors that was identified downstream of Pax3 including Six1, Eya2 and Dach2, when individually co-expressed with Pax3 was sufficient to activate MyoD expression in embryonic precursor cells
Pax7 belongs to the same family of paired box transcription factors as Pax3, but the possibility that Pax7 may employ an analogous network in the adult has not yet been explored. However, we did not detect the intermediate transcription factors in C2C12 myoblasts and there are no reports of these factors in SC in vivo.

The Sox family of proteins (HMG proteins related to SRY) also regulates MyoD expression. While Sox 8 was found to repress MyoD expression in cultured myoblasts (Schmidt et al., 2003), Sox 15 null myoblasts displayed reduced MyoD expression during skeletal muscle differentiation (Lee et al., 2004). However, in neither case has the regulation been shown to involve direct interaction with the MyoD promoter.

b) Signaling pathways

The MAP kinase, p38 is essential for myogenic differentiation since the pharmacological inhibition of p38MAPK activity abolishes MyoD expression in L8 myoblasts (Zester et al., 1999). Recently, activation of the p38 MAPK has been shown to be essential for the activation of SC associated with isolated single fibers (Jones et al., 2005). Inhibition of this central signaling molecule promotes cell cycle exit, prevents differentiation and appears to prevent SC activation. Interestingly, p38 signals to different substrates in mitogen-rich versus mitogen-poor media, making it a good candidate for MyoD regulation. However, while p38MAPK was shown to directly modulate the expression of myosin light chain by the induction of MEF2 the mechanism of MyoD regulation remains unaddressed.

Activation signals mediated by conserved signaling pathways such as the Notch pathway or the Wnt pathway that are known to play a critical role in embryonic myogenesis (reviewed in Tajbaksh et al., 1998) have recently been implicated in myogenic determination in the adult. Activation of the Notch signaling pathway is critical for SC activation and proliferation (Conboy and Rando, 2002). The transcriptional outcome of the Notch pathway points towards repression of the myogenic program and suggests that SC use Notch
signaling to avoid precocious differentiation. The observation that blood stem cells (CD45+) undergo myogenic specification upon exposure to specific Wnts has been used to propose that the Wnt pathway operates in adult skeletal muscle (Polesskaya et al, 2003) and results in the activation of the Wnt effector, β-catenin, which alleviates the repression mediated by a potent transcriptional inhibitor I-mfa (Inhibitor of the MyoD family a) on the MRFs (Pan et al, 2005). The possibility that this mechanism could also operate during adult muscle regeneration is yet to be addressed.

Overall, despite the extensive studies on these signaling pathways in the embryo there is scant evidence in literature that implicates specific biochemical pathways and the immediate targets of the transcriptional regulators in regulating MyoD expression in postnatal myogenesis.

6) Upstream transcriptional control regions of the myod gene

A different approach to identify the regulators that directly mediate MyoD expression has been to identify transcription factors that can bind to the MyoD promoter. Transgenic analyses of various deletion constructs of the 24-kb mouse MyoD promoter defined two MyoD enhancers in mammals, which in conjunction with a basal promoter are sufficient to recapitulate MyoD expression in developing and mature muscle. A proximal regulatory region (PRR) along with a distal regulatory region (DRR) that is located 5 kb upstream of the transcriptional start site was shown to be sufficient for high levels of skeletal muscle-specific activity (Tapscott et al, 1992). The DRR is a non-conventional enhancer in that chromosomal integration appears necessary for productive interactions with the PRR. A second enhancer located 20 Kb upstream (core enhancer) was shown to be sufficient for early MyoD activation in the embryo (Goldhamer et al, 1995).

Targeted mutagenesis of both enhancers revealed that while the activity of the core enhancer is dispensable for MyoD expression in neonatal muscle, the DRR is essential for MyoD expression in the adult (Chen et al,
2002). Surprisingly the PRR, DRR and the core enhancer lack binding sites for muscle specific regulatory factors other than the MRFs themselves (Tapscott, 1992; Goldhamer, 1995) suggesting either a combinatorial control or an epigenetic mechanism for its observed muscle-specific activity.

MRFs can bind to DNA on the consensus sequence CANNTG (E box), either as homodimers or as heterodimers with the ubiquitous E-protein family of transcription factors (reviewed in Parker et al, 2003). Although E-boxes are present in the MyoD promoter, they cannot account for the initiation of myogenic gene expression. The identification of a single Serum Response Element (SRE or CArG box) in the DRR (Minty and Kedes, 1986; L'honore et al, 2003) is consistent with the known role of SRF in regulating muscle differentiation (Section C-5). Although the DRR contains putative sites for regulators of muscle differentiation such as the Myocyte enhancer factor-2 (MEF-2) and the Forkhead family of transcription factors (FOXO), there have been no reports that demonstrate a direct binding of these factors to the DRR or their direct involvement in the regulation of MyoD expression.

In the next section I review what is known about the contribution of adhesion to the regulation of myogenic gene expression, in particular the role of known pathways in regulating expression of MyoD.

C) Role of adhesion dependent signaling pathways in regulating MyoD expression

1) Adhesion-dependent signaling

Growth factors and anchorage to the substratum are essential parameters that regulate cell cycle progression. However, the requirement for adhesion-mediated growth varies between different cell types. While tumorigenic cells and some cells of the blood lineage do not depend on adhesion for growth (Stoker et al, 1968), culture of non-transformed fibroblasts in the absence of a substratum (such as ECM or even tissue
culture plastic) was demonstrated to inhibit the production of mRNA and protein synthesis (Benecke et al, 1978). Subsequent studies showed that rather than attachment to the substrate per se, a spread cell shape supported growth (Folkman, 1978). Consistent with this notion, artificial substrates that prevent cell spreading have been shown to inhibit proliferation while those that support cell extension permit cell cycle progression (Ben Ze’ev et al, 1980; Farmer et al, 1983; Ingber and Folkman, 1989).

Much of our current understanding of the molecular basis of adhesion dependent effects on the signaling machinery is based on the “dynamic reciprocity” model that postulates information exchange between the ECM, the cytoskeleton (CSK) and the nuclear matrix. It was proposed that a physico-chemical influence of the ECM was exerted on gene expression via transmembrane receptors, by changing the interaction of polysomes with the CSK, and in an analogous manner, the interaction of chromatin with the nuclear matrix. These set of events re-configure the ECM, thereby creating a feedback loop (Bissell, 1982). More detailed observations confirmed that the interactive zones between the ECM and the cell indeed contain focal adhesion complexes (Assoian et al, 1997; Geiger et al, 2001; Assoian et al, 2001) and transmembrane glycoproteins that promote cell-cell interactions (cadherins) (Kemler, 1993), and cell-ECM interactions (integrins), to initiate intracellular signaling cascades (Assoian et al, 1997).

It is noteworthy that unlike other models that use nutrient deprivation to obtain synchrony (see above), synchronization of C2C12 myoblasts by manipulating the adhesion requirement models cell-ECM interactions, as the cells are plated at sub-confluent densities. It therefore serves as a good model for the identification of adhesion-dependent regulators of myogenic gene expression.

2) Adhesion dependent control of the G0-G1 transition

A better understanding of the cell cycle effects of adhesion came from studies with fibroblasts arrested in G0 by contact inhibition or serum
deprivation. Activation of these cells initiated the transition from G0-G1 by the up-regulation of a set of genes that was not dependent on ongoing protein synthesis. These genes could generate second messenger mRNAs that allowed subsequent progression through G1 (Nishizuka, 1986; Rozengurt, 1984). A similar molecular profile characterizes the response that has been documented in fibroblasts and myoblasts, but in response to deprivation of adhesion. Unlike epithelial cells that undergo anoikis (programmed cell death) in the absence of a substrate (Frisch and Francis, 1994), fibroblasts and myoblasts undergo a state of reversible arrest in G0 that is typically characterized by the absence of DNA synthesis, and suppression of several growth related genes (Benecke et al, 1978; Dike and Farmer, 1987; Dhawan, J. 1991; Milasincic et al, 1996; Sachidanandan et al, 2002). Restoration of adhesive contacts in these cells is sufficient to initiate the entry of quiescent cells into G1 (Assoian, 1997) and is mainly initiated by molecules that facilitate adhesion and cell spreading (Dike and Farmer, 1988).

3) Rho GTPases: key intermediaries in adhesion dependent signaling

Since it was well known that adhesion-dependent cell cycle progression was an important feature of non-transformed cells in culture, the discovery that most cancers contained oncogenic mutations in the ras genes stimulated much interest in the biological roles of ras and ras-related low molecular weight GTP-binding proteins (Bos, 1988). Of the 4 subfamilies of ras homology proteins, the Rho family of proteins that includes the Rho subfamily, Rac and cdc42, was found to be the most versatile, in that its members mediate the known effects of ras on cellular proliferation and differentiation (Hall, 1990). The Rho subfamily in turn has several members RhoA, RhoB and RhoC (Chardin et al, 1988). All Rho proteins are regulators of the cytoskeleton-while Rho proteins control stress fiber dynamics, Rac controls lamellipodia and cdc42 regulates filopodia (Figure 8) (Ridley and Hall, 1992; Fukata et al, 2003).

a) Effects of Rho on the Cytoskeleton: The first clue of the biological functions of Rho came from the disappearance of actin stress fibers in
response to a toxin C3 transferase that ADP-ribosylates Rho (Rubin, 1988). Microinjection of recombinant Rho induced the rapid reorganization of actin stress fibers in a variety of cell lines, mimicking the effects induced by serum (Paterson et al, 1990). These results indicated that Rho was involved in actin polymerization and can mediate the formation of specific actin structures even in the absence of soluble mitogens (Clark, 1998). Unlike Rho, rac and cdc42 appear to be involved in the formation of actin structures only during motility.

b) Effects of Rho on the cell cycle: The use of dominant negative mutants revealed multiple functions accomplished by Rho that point towards a dominant role in mid-G1 (Assoian, 1997). Although Rac and cdc42 can also induce genes for G1 progression, in particular cyclin D1, it was shown that Rho could inhibit the alternative Rac- and cdc42-dependent pathway to regulate the timing of expression of cyclin D1 (Welsh et al, 2001). Although Rho has been implicated in LPA-stimulated induction of G1, formal proof for the role of Rho in the G0-G1 transition comes only from recent studies. C2C12 myoblasts expressing dominant-negative Rho caused a cell cycle arrest with the up regulation of a marker of reversible arrest, p27 and the absence of markers of terminal differentiation, p21 and myogenin (Dhawan and Helfman, 2004). Thus, absence of Rho activity leads to G0.

c) Rho and cell fate: Recent studies have shown that Rho GTPase plays a crucial role in the decision adopted by mesenchymal stem cells to differentiate into adipogenic or myogenic lineage in response to IGF-1. Inhibition of p190GAP, an inhibitor of Rho activity resulted in the stem cells adopting a myogenic rather than adipogenic fate (Sordella et al, 2003). Although the mechanism of this process was proposed to not involve the established role of Rho in the actin cytoskeleton, the considerable differences in actin structures between myocytes, adipocytes or even fibroblasts still leaves open the question of the involvement of the cytoskeletal role of Rho in cell fate determination (Saltiel, 2003).

Thus, Rho GTPase represents a nodal point within the cell that integrates and conveys mechano-chemical signals from the ECM to the
Figure 8: Rho proteins are regulators of the cytoskeleton

The Rho family consists of 3 members: Rho, Rac and cdc42. Rho regulates stress fiber dynamics. Rac regulates the formation of broad projections at the end of the cell called ruffles or lamellipodia and cdc42 controls the formation filopodia that are rod like structures filled with bundles of parallel actin filaments. Lamellipodia and filopodia have been implicated in aiding motility.
internal cytoskeletal apparatus as well as to the cell cycle machinery (Figure 9). More importantly for this work, the functions of Rho in myoblasts have significant implications in early myogenic events.

4) **Rho and myogenic gene expression**

Historically, the prominent role of Rho GTPases in cytoskeletal rearrangement and regulating G1 progression raised the question of their potential implication in muscle differentiation, given that myofibers are highly specialized cytoskeletal machines. Dominant negative forms of Rho GTPase and their upstream regulators RhoGDI were shown to suppress promoter activity of myogenin, an early differentiation marker, while mutationally activated forms induced myogenin transcription (Takano et al, 1998; Lee et al, 1999). Additionally, suppression of Rho activity was accompanied by decrease in the transcript levels of MEF2, a regulator of myogenin. Thus, Rho regulates muscle differentiation through its effects on myogenin and MEF2 proteins.

Another approach to investigate Rho's role in myogenesis was to determine if Rho interacted with pathways or factors previously important in muscle differentiation. In this respect, work from two independent groups yielded two outcomes. 1) Consistent with Rho's role in myogenic differentiation, a muscle specific integrin (Menko et al, 1987), PI3 kinase (Jiang et al, 1998) and FAK (Illic et al, 1995) previously reported to be independently important for the expression of late myogenic markers, form a regulatory network that required the presence of functional Rho to mediate their muscle specific effects (Lei et al, 2000). 2) The ability of Rho to modulate the activity of a MADS box transcription factor, SRF in response to serum, previously reported to regulate the expression of MyoD (discussed in detail below) (Gauthier et al, 1996) implicated a role for Rho for the first time in early myogenesis (Carnac et al, 1998).

Taken together, several lines of evidence suggested that consistent with its role in G1, functional Rho is a prerequisite for muscle differentiation.
Rho GTPase activity is negatively regulated by GTPase activating proteins (GAPs) and GDP dissociation inhibitors (GDIs). GTP exchange factors (GEFs) positively activate Rho by stimulating the exchange of GDP-GTP. In myoblasts, the muscle specific integrin β-1D integrin potentiates Rho activity in response to adhesion. Active Rho regulates the expression of cell cycle regulators and cytoskeletal dynamics. Modulation of actin dynamics by Rho affects SRF activity. SRF target genes include muscle specific genes, cytoskeletal genes and growth regulatory genes. Listed in the schematic are the individual muscle specific genes which have CArG boxes and display SRF-dependence. Rho-stimulated acto myosin contractility is important for MyoD expression by an unknown mechanism. The downstream consequence of Rho's effects on microtubule stability and polarity have not been explored.
More importantly, Rho plays a role in the regulation of early myogenic events, in particular the expression of MyoD.

5) Role of SRF in myogenic gene expression

The crucial link that explained the ability of Rho, a membrane-associated signaling molecule, to affect nuclear events was the finding that Rho is required for serum and LPA induced activation of the transcription factor SRF, and that neither functional Rac or cdc42 were necessary (Hill and Treisman, 1995).

Although SRF was initially implicated in the activation of mitogen-stimulated genes, the identification of a sequence similar to the SRE -termed the CArG box- in many muscle-specific genes (Treisman, 1992; Boxer et al, 1989) immediately suggested an involvement in myogenesis. The demonstration that the CArG box was competent to interact with SRE binding factors led to the speculation that in addition to its function in cell proliferation, SRF may play a role in muscle differentiation (Tuil et al, 1993; Molkentin and Olson, 1996). Indeed, antibodies to SRF prevented the myoblast to myotube transition and inhibited the expression of myogenin, troponin T, MLC1/3, and skeletal alpha actin (Vandromme et al, 1992). Subsequently, investigations into the early requirement of functional SRF for myogenesis suggested a role in the regulation of MyoD expression (Gautheir et al, 1996). The presence of a non-canonical SRE element in the DRR of the MyoD promoter that was shown to bind SRF and activate reporter expression in transgenic mice further substantiated a role for SRF in MyoD expression during postnatal myogenesis (L'honore et al, 2003). Thus, these experiments clearly indicated that MyoD is regulated by the activity of two proteins Rho and SRF that are well-established regulators of the actin cytoskeleton. However, only recently has the mechanism of the Rho mediated effects on SRF been revealed in experiments on fibroblasts.
6) Mechanism of Rho regulation of SRF

Activation of Rho induces the formation of polymeric actin (F-actin) through 2 effector pathways. One pathway involves the Rho-effector kinase (ROCK) that promotes the recruitment of an actin modulating protein-cofilin (Geneste et al, 2002; Sotiropoulos, 1999), while the other involves the activation of a scaffold protein, mDia that recruits actin assembly proteins like VASP (Copeland and Treisman, 2002; Tominaga et al, 2000). Interference with the ability of Rho effector mDia to promote F-actin formation disrupted SRF activity suggesting the involvement of a factor that is modulated by actin dynamics and thereby affects SRF activity (Copeland and Treisman, 2002; Geneste et al, 2002).

These studies culminated in the identification of a co-activator protein, MAL, that is sequestered by monomeric G-actin in the cytoplasm when actin is depolymerized (Miralles et al, 2003). MAL belongs the myocardin-related family of transcription factors and is a ubiquitously expressed gene. When actin polymerizes, MAL is released, translocates to the nucleus and facilitates SRF-dependent transcription (Figure 10). Thus, alterations in actin dynamics are both necessary for the activation of SRF by extracellular signals and sufficient when enforced in the absence of signal (Sotiropoulos, 1999; Mack et al, 2001).

In retrospect, the Rho-SRF pathway appears to be a pivotal discovery in the regulation of nuclear events by mechano-chemical pathways. Being a key regulator of several growth promoting genes (Treisman, 1987), SRF's involvement explained the role of Rho not only in proliferation, but also in myogenic differentiation, and in particular the induction of MyoD. At the time when this study was initiated, SRF was the only known transcriptional effector of Rho-mediated regulation of MyoD.
Figure 10: Actin dynamics regulate SRF activity

See text for description. Pharmacological drugs Latrunculin B and Cytochalasin D also modulate SRF activity depending on their effects on actin polymerization.
7) SRF, a ubiquitous regulator of growth related genes and muscle specific genes: two inherent paradoxes

Paradox 1: A ubiquitous regulator preferentially affects muscle differentiation.

Studies in transgenic embryos showed that while expressed ubiquitously, at E11.5, SRF expression is enriched in skeletal, cardiac and smooth muscle (Croissant et al, 1996; Chang et al, 2001). Consistent with this observation, SRF-null mutants preferentially display a defect in the formation of embryonic mesoderm and die during early gastrulation (E12.5) (Arsenian et al, 1998) The presence of SREs in most muscle-specific promoters coupled with its requirement for myogenic differentiation underscores the importance of SRF as a critical upstream factor in myogenesis. Thus, the dependence of muscle specific gene expression on SRF can be correlated with muscle-restricted abundance of SRF and its ability to complex with muscle specific factors like MyoD (in skeletal muscle), Nkx2.5 (in cardiac muscle) and GATA (in smooth muscle) to affect downstream myogenic events. However, SRF activates many genes in non-muscle tissues and it is unclear why its activation of muscle-specific genes is restricted to muscle.

Paradox 2: SRF promotes expression of involved in two opposing pathways: growth progression and terminal differentiation

Generally, myogenic differentiation occurs concomitant with cell cycle withdrawal. SRF-activated genes are involved both in differentiation as well in cell cycle progression. How does SRF regulate genes involved in two apparently incompatible processes? Investigations into this interesting paradox showed that SRF target genes fall into 2 classes based on their sensitivity to Rho-Actin dynamics (Gineitis and Treisman, 2001). Growth-promoting targets like egr1 and junB are unaffected by actin polymer status and display an absolute co-dependence of SRF with another ubiquitous transcription factor called ternary complex factor (TCF, not to be confused with TCF/LEF the target of Wnt signaling). Consistent with this notion, the promoters of several immediate early genes possess SRF and ternary
complex factor sites in close conjunction with each other. Other targets such as vinculin and SRF itself are sensitive to actin depolymerising drugs and are expressed independent of ternary complex factor. Recent reports have demonstrated that phosphorylation of a cluster of amino acids in the SRF α1 coil DNA binding domain is key in enabling SRF to distinguish between its growth related targets and muscle specific targets (Iyer and Schwartz, 2006).

8) Unanswered questions

Several lines of evidence indicate that downstream of Rho, there could be multiple adhesion dependent pathways other than SRF, which regulate early myogenic gene expression. Work from the lab has shown that Rho can sustain MyoD expression through its effects on contractility and focal adhesions. Disruption of this function can abolish MyoD expression (Dhawan and Helfman, 2004) by a mechanism as yet unexplained.

In addition, Rho has multiple effects on the cytoskeleton (Figure 9) whose roles have hitherto been unexplored in early myogenic gene expression. Interestingly, while dominant negative Rho suppresses MyoD and arrests cells in the G0 phase, constitutive wild type Rho results in sustained MyoD expression with premature differentiation. Thus, it has been speculated that Rho could function as a switch that enables myoblasts (and activated SC) to decide between quiescence and differentiation and therefore has implications in self-renewal and differentiation pathways during regeneration (Dhawan and Helfman, 2004). Identification of the pathway downstream of Rho that mediates this decision would facilitate a better understanding of the mechanisms that enable cell fate choice in activated SC.