Reversibly growth arrested muscle cells: a model system for skeletal muscle satellite cells
Introduction

Growth and differentiation are mutually exclusive pathways

In muscle cells, as in many lineages, growth arrest is a prerequisite for differentiation and the two are coupled programs (Walsh and Perlman, 1997). Cultured myoblasts have served as a useful system for understanding the mechanisms by which the cessation of proliferation is linked to the onset of differentiation. Skeletal myogenesis can be induced in culture by depriving cycling myoblasts of serum: this mitogen-dependent cell cycle withdrawal is irreversible, and is followed by fusion and the activation of the differentiation program (Florini et al, 1991; Olson, 1992).

A large number of molecules and pathways that interact to bring about the differentiation of muscle cells have been identified (Lassar et al, 1994). Broadly, antagonistic interactions between regulators of the cell cycle and regulators of myogenesis are integrated to lead to a mutually exclusive relationship between proliferation and differentiation (Lassar et al, 1994). Irreversible arrest is initiated by the action of growth inhibitors such as the retinoblastoma protein (pRb), cyclin dependent kinase inhibitors such as p21 and p57, and the myogenic regulator MyoD, which inhibits cell cycle progression independent of its myogenic function (Crescenzi et al, 1990; Sorrentino et al, 1990). Mitogen activated pathways in cycling myoblasts suppress the differentiation promoting activities of the MRFs (reviewed in Puri and Sartorelli, 2000).

Reversible arrest in stem cells

Because of the coupling of arrest with differentiation, most differentiated cells are incapable of reentry into the cell cycle. Replenishment of cells in tissues that turn over rapidly such as blood and skin, and regeneration in tissues such as muscle and liver that respond to
tissue damage requires a population of cells capable of proliferation. In most tissues this ability lies within a small population of stem cells, which are either quiescent or slowly cycling. The stem cells that play a central role in muscle regeneration are the satellite cells (SC). The characteristic features of SC in muscle are their reversible growth arrest and their latent myogenic potential: as these cells do not express any of the known MRFs that are critical for muscle cell identity, but re-express the MRFs concomitant with cell cycle reentry.

**Reversible arrest in cultured myoblasts**

Cultured fibroblasts and myoblasts enter a reversible arrest upon anchorage deprivation. This property is termed anchorage dependence and is considered a criterion for normal cell growth. By contrast, the transformed counterparts of these cell types show anchorage independent growth in culture that correlates with tumorigenicity in vivo (Stoker et al, 1968). Proliferation of anchorage dependent cells requires spread cell shape, rather than adhesion per se (Folkman and Moscona, 1978). Loss of adhesion triggers growth arrest in fibroblasts (Benecke et al, 1978; Dike and Farmer, 1988) and myoblasts (Dhawan, 1991; Milasincic et al, 1996) despite the presence of mitogens, indicating that both growth factors and adhesion are necessary elements in promoting the proliferation of these cells. The cell cycle machinery such as the cyclins and the CDKs are responsive to signals from growth factor induced pathways as well as ECM induced cascades, at multiple levels (Assoian, 1997). Therefore, it is evident that the two pathways are integrated at a number of points and progression through the cell cycle is a function of the availability of both signals.

In this study I have used suspension culture of C2C12 myoblasts in methylcellulose medium (Milasincic et al, 1996) (Fig. 4) to induce reversible growth arrest.
Myoblasts (Mb)
Asynchronous growth

Adhesion deprivation

Mitogen deprivation

MyoD
Myf5
Myogenin

Suspension myoblasts (S)
Reversible arrest

MyoD
Myf5
Myogenin

Reactivated myoblasts (R)
Synchronous reentry into cell cycle

MyoD
Myf5
Myogenin

Myotubes (Mt)
Irreversible arrest
Differentiation

Fig. 4 Reversible Arrest in Myoblasts
In this chapter I present evidence that the suspension-arrested myoblasts in culture mimic SC in vivo in terms of growth state and expression of some key genes.

Results

Sub-cloning of C2C12 myoblasts to obtain stringently anchorage-dependent clones

Suspension culture of the original stock of C2C12 cells resulted in a significant proportion of the cells (~95%) entering a state of growth arrest (as assessed by BrdU incorporation) as previously observed (Milasincic et al, 1996). However, 5% of cells in this population continued to progress through the cell cycle during suspension culture. In addition, the myoblast population also contained approximately 3% Myogenin positive (differentiated) cells. Since the presence of anchorage independent or differentiated cells is a source of heterogeneity in the system, we subcloned these cells (in collaboration with S Ramkumar and J Dhawan) in order to obtain a homogenous population of myoblasts for further analysis. Of the 23 clones tested, 3 showed stringent anchorage dependence (~1% nuclei were BrdU positive after 48 hr in suspension). These subclones, designated C2C12A2, C2C12A12 and C2C12C3 were used for further studies in the lab. The work described in this study has been done using the C2C12A2 subclone and it will be described as C2C12 throughout this work.

Reversible arrest in C2C12 myoblasts

The morphology, growth arrest and gene expression of the anchorage dependent C2A2 subclone were studied in detail using fluorescent detection of actin, BrdU labeling and Northern analysis for a number of growth regulatory and differentiation dependent genes.
To assess the morphological changes that occur in myoblasts during adherent culture and suspension I determined the organization of the actin cytoskeleton. Microfilaments, the polymerized network of actin cables, are a reliable indicator of cell shape. In attached well-spread cells actin microfilaments form bundles of ‘stress fibres’, which can be revealed using a fluorescent probe, Phalloidin-Oregon Green (Fig. 5). Myoblasts in asynchronously growing adherent cultures exhibited a typical spread cell morphology, and abundant well-organized stress fibres were visible. Upon suspension of these cells in methocel, the rounded cell morphology was accompanied by a disorganized actin cytoskeleton (Fig. 5), consistent with the requirement for adhesion-plaque insertion of microfilaments in the maintenance of cell shape. Replating of suspended cells under adhesive conditions (on tissue culture plastic dishes) resulted in rapid reattachment within minutes. By two hours after replating (R2) cells achieved a well-spread morphology and developed filopodia and ruffling membranes with the formation of the cortical microfilaments by R4. However, stress fibre formation became evident only at 6-8 hr after replating (not shown) and was well established at R24.

To determine the kinetics of reversible arrest, DNA synthesis was measured during a time course of suspension and replating using BrdU incorporation. In asynchronous adherent myoblasts, approximately 35% of cells were in S phase (Fig. 6). Following culture in suspension for 48 hr, there was an almost complete withdrawal of the cells from cell cycle, as is evident from the decrease in the number of BrdU-positive nuclei to ~1%. Growth arrest is reversible and upon re-plating, cells reenter the cell cycle in a synchronous manner, with the maximum number of cells (~42%) entering S phase at around 24 hr (Fig. 6). This delay in entering S phase suggests that the cells might be growth arrested in the G0 phase of the cell cycle. Support for this hypothesis comes from FACS analysis that unambiguously shows that the reversibly arrested cells in suspension have a G1 (2n) DNA content (S. Ramkumar and J. Dhawan, unpublished)
Fig. 5

Suspension culture of myoblasts causes a disorganization of the actin stress fibres that reform upon reattachment to the substratum. Detection of actin cytoskeleton with Phalloidin-Oregon Green as an indication of cell shape. Well developed stress fibres are present in adherent myoblasts (Mb). Loss of contact with substratum (S60) results in rounding up of cells and general disruption of the actin cytoskeleton. Cells regain adhesive interactions between 2-4 hr of replating (R2-R4). By 24 hr of replating, myoblasts are attached and spread completely and also form stress fibres. Since cell density was low in these dishes, S60, R2 and R4 are composites of images of several fields.
Fig. 6
Quiescence induced by suspension culture is reversible. BrdU incorporated into DNA during a 15 min pulse was detected using an anti-BrdU antibody to estimate the frequency of cells in S phase. Asynchronous myoblasts (Mb), cells in suspension for 48 hr (S48), cells replated for 6-29 hr (R6-R29) following 48 hr in suspension. Data shown in this figure is the representative of more than 3 independent experiments.
results). Absence of DNA synthesis in cells with a G₁ complement of DNA defines G₀.

To determine the percentage of arrested cells that are capable of reentering cell cycle, cumulative DNA synthesis during replating was analysed. Cells recovered from suspension were replated and maintained in medium containing BrdU, thus labeling all cells which go through S phase until the time of harvest. The BrdU positive cells were detected by immunostaining and representative fields are shown in Fig. 7A. Following suspension culture for 48 hr (which leads to growth arrest, Fig. 6), no increase in DNA synthesis was observed at 2 and 6 hr after replating. By 22 hr after replating, >50% were labeled, reaching ~100% by 46 hr (Fig. 7B). Thus, all arrested cells in the population reenter the cell cycle and reversibility is not a property of a subset of arrested myoblasts. Moreover, the entry of the large percentage of cells (~58%) into S phase by 22 hr indicates that this entry is synchronous.

**Anchorage deprivation in C2C12 myoblasts uncouples cell cycle arrest from differentiation**

During arrest that accompanies myogenic differentiation, MyoD is induced and maintained at high levels but Myf5 is downregulated (Yoshida et al, 1998; Kitzmann et al, 1998). To determine the status of these MRFs during reversible arrest I used Northern blot analysis (Fig. 8A). Histone 2B (H2B) mRNA was used as an indicator of the growth status of myoblasts. As expected, H2B transcript levels were high in asynchronous, growing myoblasts and decreased as these cells were maintained in suspension culture for 12 hr. After suspension for 48 hr H2B mRNA levels were undetectable, consistent with the near absence of DNA synthesis in these cultures. H2B transcript levels increased, as cells reattached and reentered the cell cycle, reaching a maximum at R18. Expression of H2B detected in differentiated myotube cultures can be
All arrested cells reenter the cell cycle. (A) Immunodetection of cells in S phase. Representative fields of BrdU labeled cells (green: FITC) with DNA shown in blue (Hoechst 33342). Myoblasts (Mb) and Myotubes (Mt) were pulse labeled for 15 min with BrdU while replated myoblasts were cumulatively labeled for 2 or 50 hr (R2, R50) following 48 hr in suspension. The % S phase cells are noted below the figure. (B) Quantification of S phase in replated cells by immunostaining of cells cumulatively labeled with BrdU for 2-50 hr of reattachment (R2-R50). All cells that growth arrested during suspension reenter cell cycle upon reattachment: the majority of cells have entered S phase by 24 hours of replating.
Reversibly arrested myoblasts downregulate expression of muscle determination factors and do not activate differentiation specific markers.

(A) Northern analysis of 20 μg RNA from myoblasts in asynchronous growth (Mb), held in suspension for 12 or 48 hr (S12, S48), activated for 2-30 hr by replating after 48 hr suspension (R2-R30), and 72 hr differentiated myotube cultures (Mt). Downregulation of H2B during suspension indicates growth arrest. Asynchronously growing Mb and reactivated cells express H2B mRNA. Mt cultures also express lower amounts of H2B showing that all cells have not growth arrested/differentiated. MyoD and Myf5, both expressed in Mb are downregulated in arrested cells and induced during reactivation. In Mt, MyoD is maintained but Myf5 is downregulated. Myogenin and p21, early markers of differentiation are not expressed in arrested myoblasts but are highly induced in Mt. p21 is also induced at low levels during reactivation of arrested cells. MCK, a late marker of differentiation is not induced in growing or arrested myoblasts and is expressed only in Mt. Fibronectin is not expressed in growing or differentiated myoblasts, but is induced in arrested cells and maintained until R18. L7 has been used as a loading control.

(B) Immunodetection of Myosin heavy chain (blue, AMCA) shows that this differentiation specific protein is not expressed in growing or arrested myoblasts but is expressed abundantly in differentiated myotubes. Nuclei are counterstained with propidium iodide (red).
attributed to the large number (~10%) of undifferentiated cells that continue to cycle.

Consistent with previous reports, both MyoD and Myf5 mRNAs were expressed in cycling myoblasts and during differentiation MyoD was highly expressed in myotubes but Myf5 was suppressed (Fig. 8A). However, unlike the arrest accompanying differentiation, both MyoD and Myf5 transcripts were downregulated in reversibly arrested myoblasts. Reactivation into the cell cycle leads to an increase in the levels of these mRNAs although Myf5 is again downregulated at R30. Thus, genes for the myogenic specification factors show cell cycle dependent expression.

Since growth arrest in myoblasts is usually a prelude to differentiation, I analysed the expression pattern of known differentiation specific genes during reversible arrest. The levels of Myogenin (an MRF expressed early in differentiation) and muscle creatine kinase (MCK, a late marker of muscle differentiation) mRNAs were analysed on Northern blots (Fig. 8A). As expected, neither Myogenin nor MCK was expressed in growing myoblasts and were markedly induced in differentiated myotubes. By contrast, reversibly arrested myoblasts did not induce these mRNAs. Further, expression of myosin heavy chain (MHC), a major component of the contractile apparatus induced in differentiated myotubes, was analysed using immunodetection (Fig. 8B). As expected, myoblasts did not express this sarcomeric protein and myotubes showed abundant expression in the cytoplasm. However, myosin was not induced in a significant number of reversibly arrested myoblasts, supporting the observation that arrested myoblasts do not differentiate.

During arrest that accompanies differentiation, the CKI p21 is induced and participates in coupling the two pathways (Andrés and Walsh, 1996). p21 mRNA was undetectable in asynchronous myoblasts and was strongly induced in differentiated cells in agreement with previous studies. Importantly, p21 was not induced when myoblasts
entered quiescence in suspension. However, a marginal increase in p21 mRNA was noted as arrested myoblasts were reactivated. Thus, arrest in adhesion-deprived myoblasts appears to be independent of p21.

Based on the observations that C2C12 myoblasts reversibly arrest growth in non-adhesive conditions, suppress myogenic specification factor expression (MyoD and Myf5) and do not induce markers of differentiation (Myogenin, MCK, p21 and MHC), I conclude that suspension-induced arrest uncouples cell cycle arrest from differentiation.

**Not all genes are downregulated in reversibly arrested myoblasts**

Growth arrest in suspension has been reported to be accompanied by general suppression of gene expression (Dike and Farmer, 1988; Dhawan and Farmer, 1990; this study). However, some genes are known to be induced specifically in arrested cells. For example, in suspension-arrested fibroblasts, fibronectin mRNA is upregulated (Dike and Farmer, 1988). Northern blot analysis showed that fibronectin mRNA levels were elevated in suspended myoblasts as well and decreased as these cells entered cell cycle upon replating (Fig. 8A). Thus, although a number of genes are downregulated concomitant with growth arrest in suspended myoblasts, as illustrated in the previous section, the induction of fibronectin mRNA shows that some transcripts can be induced. This observation has implications for the DD-PCR strategy used in the following chapter.

**Cell cycle dependent expression of satellite cell markers during arrest and activation of C2C12 cells in culture**

In addition to quiescence, SC in vivo are characterized by an absence of MRF expression. Since the expression patterns of growth-associated markers and MRFs in arrested C2C12 cells resemble the pattern seen in SC (Cornelison and Wold, 1997; Cornelison et al, 2000.)
Fig. 9A
Suspension arrested myoblasts upregulate CD34, a marker implicated in the regulation of quiescence of SC in vivo. Semi-quantitative RT-PCR analysis of CD34 expression. Compared with asynchronous myoblasts (Mb), relative levels of CD34 mRNA rise in suspension-arrested cells (S12, S60) and decline during cell cycle activation upon replating (R1, R12, R30). Primers used detect a region common to both splice variants of CD34 mRNA; values represent the mean of duplicate assays for CD34 RNA normalized with respect to L7 control RNA for each sample. Similar results were obtained with 2 independent time course experiments.

Fig. 9B
PEA3, a transcription factor induced in activated SC, is upregulated during myoblast reactivation in culture. Northern analysis of 20 μg RNA from a time course of reversible arrest in cultured myoblasts. PEA3 is expressed in growing myoblasts (Mb) and in myotubes (Mt) and downregulated in arrested myoblasts (S48). There is a sharp induction in myoblasts replated in growth medium (R2G, R6G, R24G). However, a corresponding increase is not seen in myoblasts replated in fusion medium (R24F).
I analyzed the expression of other molecules known to play a role in SC during muscle regeneration.

Transcripts for CD34, a marker of hematopoietic stem cells, have recently been shown to be expressed in quiescent SC in muscle and in SC associated with single fibres. As CD34 undergoes alternative splicing within 3 hr, followed by downregulation by 24 hr of activation of SC upon single fibre isolation (Beauchamp et al., 2000), it has been proposed to play a role in the maintenance of quiescence. The levels of this transcript in cultured C2C12 myoblasts were determined by semi-quantitative RT-PCR analysis. CD34 transcripts were detected in growing C2C12 cells in agreement with earlier reports (Beauchamp et al., 2000), however there was a ~6-8 fold upregulation of the mRNA levels in the G0-arrested cells (Fig. 9A). Concomitant with cell cycle entry, CD34 transcript levels decreased, consistent with the downregulation reported in SC, but do not return to the basal levels seen in myoblasts. Thus, CD34 transcripts are regulated during the cell cycle in a manner consistent with a role for CD34 in quiescent cells.

Expression of polyoma enhancer activator protein (PEA3) a transcription factor expressed by activated satellite cells during regeneration (Taylor et al, 1997) was analysed during activation in culture. PEA3 mRNA levels were low in growing myoblasts and differentiated myotubes and were undetectable in arrested myoblasts (Fig. 9B). However, there was a significant induction by 6 hr after reactivation (G1) of the arrested cells, which increased by 24 hr (entry into S phase). A similar induction was not observed when arrested cells were replated in medium containing low serum that does not promote cell cycle progression, suggesting a correlation between cell cycle progression and PEA3 expression.
Thus, two molecules implicated in growth arrest (CD34) and activation (PEA3) of SC, are regulated in reversibly arrested myoblasts in a cell cycle dependent manner that resembles the regulation in vivo.

Discussion

Reversible arrest is one of the key features of skeletal muscle SC. Accompanying this reversible arrest are the induction or downregulation of some genes which characterize this population of cells in the muscle in vivo. Since studying this minor population directly in the adult muscle is difficult, I utilized a culture model for the SC. In this chapter, I present evidence that this cell culture system can be used as a model for SC owing to a number of similarities between the arrested cells in culture and the SC in vivo.

Non-adherent conditions lead to a growth arrest not accompanied by differentiation

Unlike epithelial cells, C2C12 myoblasts do not undergo anoikis or death upon anchorage deprivation (Assoian, 1997), but enter a state of growth arrest (Milasincic et al, 1996). Adhesion to substratum affects cell shape, an important variable that influences the growth state of cells. Adhesion-deprived myoblasts, unlike adherent cells, have a disorganized cytoskeleton that reflects the absence of matrix-membrane-cytoskeleton interactions. When adhesive conditions are restored, arrested cells regain the ‘spread’ cell shape and reenter cell cycle after a long lag phase. Flow-cytometric analysis has shown that suspended myoblasts possess a 2n DNA content, indicating arrest in G₀/ G₁ and not in G₂ (S. Ramkumar and J. Dhawan, unpublished results). From cumulative DNA synthesis analysis it is clear that the arrest is completely reversible and ~100% of the suspended cells in G₀ are capable of entering S phase upon attachment. Thus non-adherent conditions cause synchronization of myoblasts in G₀.
which is also reflected in their simultaneous reentry into cell cycle when reactivated by replating.

The expression of a number of molecules known to be important in growth and differentiation was analysed to assess the similarity of this culture model to SC in vivo. The expression pattern of MyoD in arrested myoblasts in culture (Milasincic et al, 1996; Yoshida et al, 1998; Kitzmann et al, 1998) has consistently tallied with the pattern observed in SC in vivo using different techniques (Grounds et al, 1992; Cornelison and Wold, 1997).

However, there is still debate as to the expression of Myf5. The reversible quiescence exhibited by a small population of residual "reserve" cells present in a differentiated culture of myotubes is characterized by presence of Myf5 protein (Yoshida et al, 1998). Also, during reversible arrest induced by a combination of serum and methionine deprivation, Myf5 protein is present in G₀ and is downregulated during G₁ phase as cells are reactivated (Kitzmann et al, 1998). In the reversible arrest induced by changes in the adhesive state of myoblasts described here, I detect very low levels of Myf5 mRNA. Both MyoD and Myf5 proteins are undetectable in arrested myoblasts studied by western blot (S. Ramkumar, unpublished) and by immunofluorescence (J Dhawan, unpublished). Moreover, both MyoD and Myf5 mRNAs are induced concomitantly in reattached cells, at 6 hr (G₁ phase) instead of the mutually exclusive pattern of expression noted in serum-deprived myoblasts (Kitzmann et al, 1998).

In SC also the expression of Myf5 is still debated. RT-PCR analysis of SC associated with single fibres showed that quiescent SC do not express Myf5 RNA (Cornelison and Wold, 1997; Cornelison et al, 2000). Myf5 promoter activity in quiescent SC of uninjured, adult transgenic mice detected by a lacZ 'knock-in' has been shown to be absent by Cooper et al (1999) but present by Beauchamp et al (2000).
Thus, my observations on the downregulation of both MRFs in reversible arrest are in register with several studies on expression patterns in SC during quiescence as well as activation (Grounds et al, 1992; Cooper et al, 1999; Cornelison and Wold, 1997). The issue of Myf5 protein expression in vivo may be resolved when a reliable antibody becomes available.

In differentiating populations of myoblasts, MyoD is upregulated and is instrumental in inhibiting cell cycle progression. Cell cycle arrest precedes and is independent of the subsequent differentiation. However, the presence of MyoD in dividing myoblasts is not sufficient to cause growth arrest since multiple mechanisms cooperate to keep MyoD in an inactive form (see Introduction). Cycling myoblasts display a heterogeneity with respect to the MyoD expression that is most likely a function of the cell cycle phase. When shifted to differentiation medium it is the MyoD positive cells that exit cell cycle irreversibly and enter the myogenic program (Yoshida et al, 1998). MyoD negative myoblasts do not differentiate and reversibly arrest (the reserve cells) in response to differentiation stimulating program. Therefore it appears that in the absence of mitogens, the default response of myoblasts is to irreversibly exit the cell cycle and differentiate, and only the absence of MyoD prevents this pathway from operating. In adhesion-deprived myoblasts it is not clear whether the reversible arrest is owing to an absence of MyoD or whether MyoD downregulation is just a consequence of this process.

During differentiation, the CKI p21$^{Cip1/Waf1}$ has been shown to be activated in Myogenin positive myoblasts as they exit the cell cycle and commits these cells to irreversible arrest (Andrés and Walsh, 1996). During suspension-arrest p21 mRNA is not upregulated nor is p21 protein expressed (J Dhawan, unpublished). Intriguingly, this transcript is slightly induced in myoblasts reentering cell cycle at the G1 phase (R1-R6), which is unexpected for an inhibitor of proliferation. Indeed, p21 and Myogenin
proteins are upregulated in 20% of a reactivated population with similar kinetics as the peak of S phase, but in a distinct subset from those synthesizing DNA (J. Dhawan, unpublished). This observation indicates that all cells exiting quiescence are not identical and based on the expression of p21 and Myogenin and BrdU incorporation, there are at least two distinct populations. Whether these two populations respond differently to differentiation-inducing stimuli would be of interest because of the implications for cell fate choices to be made by a single population of cells exposed to identical conditions.

**CD34 and PEA3 expression reflect a molecular resemblance of myoblasts in culture to satellite cells**

In the wake of several studies showing pluripotentiality of the muscle precursor cells, the presence of the HSC marker CD34 at the mRNA and protein levels in SC (Beauchamp et al, 2000) is particularly interesting. Downregulation of CD34 mRNA 24 hr after isolation of single fibres (a time when the fibre-associated SC are expected to be activated) implicated this molecule in the maintenance of quiescence in SC. Further, it was speculated that CD34 expression was suppressed at the G₀/ G₁ transition. A synchronized system allowed a direct test of this hypothesis. RT-PCR analysis of CD34 mRNA levels in reversibly arrested myoblasts in culture shows an induction of this mRNA in G₀ and declining levels during reactivation. This data supports the suggestion that CD34 plays a role in reversible quiescence of myoblasts (Beauchamp et al, 2000).

Activation of SC in vivo is associated with induction of the ets-domain transcription factor, PEA3. During synchronized cell cycle activation in culture, PEA3 transcripts were induced in G₁ and peaked coincident with S phase. Thus, in the expression of a marker of quiescence (CD34) and one of activation (PEA3), synchronized myoblasts in culture resemble SC in vivo.
Reversible arrest and activation of adhesion-deprived C2C12 cells in culture mirror quiescence and activation of SC in vivo

The most comprehensive survey of gene expression in SC has been the RT-PCR analysis of single SC attached to isolated myofibres in culture (Cornelison and Wold 1997, 2000), an intermediate between the cultured cell and the tissue environment in vivo. My observations regarding the absence of MyoD, Myf5, Myogenin and p21 mRNAs in the arrested myoblasts are consistent with their profile in the single-cell analysis. Moreover, the induction of CD34 during arrest and PEA3 during reactivation is also consistent with previous studies on SC (Beauchamp et al, 2000; Taylor et al, 1997). I conclude that reversible arrest in culture recapitulates many of the alterations of gene expression seen in SC in vivo.

In vivo, SC proliferation and differentiation occur over a period of 2-3 days after injury (Grounds, 1991) and the time courses of these processes overlap: some cells may be dividing when others are differentiating. Therefore, analysis of the molecular mechanisms that control any discrete stage is difficult. In addition, SC are rare, representing only ~5% of total muscle nuclei (Schultz, 1996). Thus, a culture model would mitigate the complications of working with rare asynchronous cells. An obvious drawback of culture is the absence of the complex environment of the true SC. However, a model that mimics key features of SC has the advantage that large numbers of cells can be obtained, allowing molecular analyses that would be untenable in vivo. For example, mis-expression of candidate regulators is far simpler in culture. Synchrony also permits the detection and analysis of processes not obvious in asynchronous populations.

Taken together, the reversible arrest and the expression of several important genes (MyoD, Myf5, Myogenin, p21, CD34 and PEA3) that
accompany this arrest in myoblasts suggest that synchronized myoblasts can be used to model molecular events in quiescent and activated SC.