

# Tristetraprolin and LPS-inducible CXC chemokine are rapidly induced in presumptive satellite cells in response to skeletal muscle injury

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Accepted 11 April 2002

Journal of Cell Science 115, 2701-2712 (2002) © The Company of Biologists Ltd

## Summary

Myogenic precursor cells known as satellite cells persist in adult skeletal muscle and are responsible for its ability to regenerate after injury. Quiescent satellite cells are activated by signals emanating from damaged muscle. Here we describe the rapid activation of two genes in response to muscle injury; these transcripts encode LPS-inducible CXC chemokine (LIX), a neutrophil chemoattractant, and Tristetraprolin (TTP), an RNA-binding protein implicated in the regulation of cytokine expression. Using a synchronized cell culture model we show that C2C12 myoblasts arrested in G0 exhibit some molecular attributes of satellite cells *in vivo*: suppression of MyoD and Myf5 expression during G0 and their reactivation in G1. Synchronization also revealed cell cycle dependent expression of *CD34*, *M-cadherin*, *HGF* and *PEA3*, genes implicated in satellite cell biology. To identify other genes

induced in synchronized C2C12 myoblasts we used differential display PCR and isolated LIX and TTP cDNAs. Both LIX and TTP mRNAs are short-lived, encode molecules implicated in inflammation and are transiently induced during growth activation *in vitro*. Further, LIX and TTP are rapidly induced in response to muscle damage *in vivo*. TTP expression precedes that of MyoD and is detected 30 minutes after injury. The spatial distribution of LIX and TTP transcripts in injured muscle suggests expression by satellite cells. Our studies suggest that in addition to generating new cells for repair, activated satellite cells may be a source of signaling molecules involved in tissue remodeling during regeneration.

Key words: Synchronized C2C12 myoblasts, Satellite cell, LIX, TTP

## Introduction

A central feature of stem cells and lineage-restricted progenitor cells involves the ability to arrest growth reversibly without activating differentiation (Fuchs and Segre, 2000; Weissman, 2000). By contrast, in committed precursor cells such as myoblasts, quiescence is coupled to the onset of differentiation (Andres and Walsh, 1996). Analyses of myogenic cell lines such as C2C12 (Yaffe and Saxel, 1977; Blau et al., 1983) have contributed significantly to our understanding of this process, revealing antagonistic interactions between regulators of the cell cycle and muscle gene expression (reviewed by Olson, 1992; Lassar et al., 1994). Despite expression of the muscle regulatory factors (MRFs) MyoD and Myf5, proliferating myoblasts remain undifferentiated owing to inhibition of MRF function by mitogen-induced pathways (Li et al., 1992). Thus, serum withdrawal leads to irreversible cell cycle exit, activation of muscle-specific genes and fusion into multinucleated myotubes (Andres and Walsh, 1996). However, there are instances in culture where arrest occurs without differentiation (Milasincic et al., 1996; Yoshida et al., 1998; Kitzmann et al., 1998), mimicking growth control in muscle precursor cells (MPC) *in vivo*.

Quiescent MPC, known as satellite cells (SC), persist within adult skeletal muscle tissue (Mauro, 1961) and facilitate its regeneration after damage (reviewed in Grounds, 1991; Bischoff, 1994; Seale and Rudnicki, 2000). SC lie sequestered

between the basal lamina and plasma membrane of myofibers and are induced to proliferate when the muscle is injured. Quiescent SC do not express MRFs, but activated SC express MyoD, Myf5 and Myogenin (Grounds et al., 1992; Cornelison and Wold, 1997; Cooper et al., 1999). Until recently, molecular studies on SC were impeded by the lack of unambiguous markers. Owing to their proximity to the myofiber, SC are difficult to distinguish from peripheral myonuclei and from interstitial mononucleated cells. Recently, genes such as *M-cadherin* [*M-cad*; (Irintchev et al., 1994)], *c-met* (Cornelison and Wold, 1997), *myocyte nuclear factor* (Garry et al., 1997), *Pax7* (Seale et al., 2000) and *CD34* (Beauchamp et al., 2000), have been demonstrated to be specifically expressed in SC. Of these, *Pax7* is required for the specification of SC (Seale et al., 2000), *c-met* has been implicated in SC activation (Tatsumi et al., 1998) and *CD34* has been suggested to contribute to maintenance of arrest (Beauchamp et al., 2000).

Activation of SC occurs when muscle experiences increased workload, mechanical or toxic injury or genetic defects such as dystrophin deficiency that lead to myofiber damage (reviewed by Grounds, 1991; Seale and Rudnicki, 2000). Although the proximal activating factor of SC *in vivo* has yet to be identified, hepatocyte growth factor/scatter factor (HGF/SF) is a strong candidate (Tatsumi et al., 1998; Sheehan and Allen, 1999). However, little is known of early activation events in SC.

Direct identification of molecular correlates of SC activation *in vivo* is complicated by problems of asynchrony and of dilution by the non-SC components of muscle. SC associated with isolated single myofibers (Bischoff, 1986) are more amenable to a molecular analysis of activation (Cornelison and Wold, 1997; Cornelison et al., 2000; Beauchamp et al., 2000). The first documented molecular transformation in activated SC is the appearance of a splice variant of CD34 mRNA (Beauchamp et al., 2000), the defining marker of hematopoietic progenitors (reviewed by Krause et al., 1996). Altered splicing of CD34 is detected 3 hours after the isolation of single fibers from muscle, a process that leads to SC activation. Induction of MyoD mRNA in activated SC is detected within 6 hours of crush injury (Grounds et al., 1992) or single fiber isolation (Cornelison and Wold, 1997; Beauchamp et al., 2000).

Although single fiber cultures retain the association between myofiber and SC, an important feature of the muscle environment, isolated myoblasts provide cellular homogeneity and large numbers that are advantageous for molecular analysis. Despite the loss of extrinsic interactions characteristic of the tissue milieu, cultured cells retain some important intrinsic attributes of SC. For example, representational difference analysis of proliferating versus differentiated primary muscle cultures led to the identification of *Pax7* as a gene involved in SC specification (Seale et al., 2000). However, to date few genes other than the *MRFs* have been identified that are induced during the activation of resting SC.

In this study, we report the identification of two genes that are acutely induced in response to skeletal muscle injury and are expressed in a spatial pattern consistent with activated SC. Our strategy employed culture conditions (Milasincic et al., 1996) that enabled cell cycle synchrony. Synchronized C2C12 myoblasts share with SC their core property of reversible arrest without differentiation. Using differential display PCR (DD PCR), we isolated cDNAs that could be detected during arrest and activation of synchronized cultures but not during asynchronous growth or differentiation.

We found that two of the cDNAs, LIX and TTP, are rapidly expressed in response to muscle injury *in vivo* and encode molecules implicated in cell-cell signaling. LIX, a chemokine implicated in damage-induced neutrophil chemo-attraction (Wuyts et al., 1996; Wuyts et al., 1999; Chandrasekar et al., 2001) is expressed in skeletal muscle within 6 hours of injury. TTP, an RNA-binding protein that regulates cytokine mRNA stability (Carballo et al., 1998; Carballo et al., 2000), is dramatically and transiently induced within 30 minutes of injury, before any other recorded molecular event in this tissue. In injured muscle, LIX and TTP transcripts appear to be located in mononuclear cells that abut myofibers and lie beneath the basal lamina sheath, a location reminiscent of SC. Taken together, our observations support the notion that SC could themselves be an early source of signaling molecules that play a role in the regeneration of damaged muscle.

## Materials and Methods

### Cell culture

C2C12 mouse myoblasts (Blau et al., 1983) obtained from H. Blau (Stanford University, CA) were subcloned by ring cloning. Adherent cells were cultured in growth medium (GM: DMEM with 20% FBS, Life Technologies, Inc.). For differentiation, near-confluent

cultures were incubated in differentiation medium (DM: DMEM with 2% horse serum) for 3 days. Suspension culture (Milasincic et al., 1996) was performed with modifications. Briefly, adherent cultures harvested using trypsin-EDTA were suspended in methyl cellulose (4000 centipoise, Sigma Chemical Co., final composition 1.5% Methocel in GM) at a density of  $10^5$  cells/ml. Cells were held in suspension for up to 60 hours: viability of cells even at 72 hours (assessed by propidium iodide exclusion) was >95%. Cells were harvested from methyl cellulose by dilution and centrifugation.

### DNA synthesis assay

Cells were seeded on cover slips for staining. To cumulatively label S phase cells, 10  $\mu$ M BrdU was added to the culture medium for 2-48 hours, cultures were fixed in cold 70% ethanol, DNA denatured in 2 N HCl, 0.5% Triton X-100, 0.5% Tween-20 and neutralized with NaBH<sub>4</sub> (1 mg/ml). Staining with anti-BrdU monoclonal antibody (Sigma, 1:500) was detected using a biotinylated goat anti-mouse secondary antibody (1:200) and the Vectastain ABC reaction (Vector Labs). Antibodies were diluted in blocking buffer (10% horse serum, 0.5% Tween 20). Controls excluding primary antibody or BrdU were negative. The frequency of cells in S was determined after counting three fields (~250 nuclei) per sample using a Zeiss Axioskop equipped with DIC optics.

### Flow cytometry

Cells were fixed in cold 80% ethanol, washed in PBS and incubated in PBS +1% Triton X100, 50  $\mu$ g/ml propidium iodide and RNase (100  $\mu$ g/ml final) for 30 minutes at 37°C.  $10^4$  cells/sample were analyzed on a FACStar Plus flow cytometer (Becton Dickinson) using the CellQuest software.

### Western blot analysis

Cell pellets were solubilized in 2 $\times$ SDS-PAGE sample buffer, total protein estimated (Biorad protein assay), 100  $\mu$ g samples separated by 12.5% SDS-PAGE and transferred to Hybond C (Amersham-Pharmacia). Blots were blocked in 25 mM Tris-Cl, pH 8.0, 125 mM NaCl, 0.05% Tween 20 (TBST) +5% nonfat dry milk. Antibodies were diluted in blocking buffer: MyoD polyclonal (Santa Cruz) 1:400; Myf5 polyclonal (Santa Cruz) 1:1000; actin monoclonal (Developmental Studies Hybridoma Bank) 1:500; desmin polyclonal (Sigma Chemical Co.) 1:500. Alkaline-phosphatase-conjugated secondary antibodies (anti-rabbit or anti-mouse, Bangalore Genei) were used at 1:2000. Washes were for 3 $\times$ 15 minutes in TBST. Antibody binding was detected using chemiluminescence (CDP-Star, Amersham-Pharmacia).

### Northern blot analysis

RNA was isolated from cells and tissue using Trizol (Life Technologies, Inc). 10-20  $\mu$ g samples were separated in 1% agarose gels containing 2% HCHO, transferred to Hybond N and immobilized by UV crosslinking. Probes used were histone H2B (DeLisle et al., 1983), MyoD (Davis et al., 1987), Myf5 (Braun et al., 1989), HGF (Bladt et al., 1995), c-met (Takayama et al., 1996), PEA3 (Taylor et al., 1997), muscle creatine kinase (MCK) and ribosomal protein L7 mRNA [loading control, (Cornelison and Wold, 1997)]. Probes labeled with [ $\alpha^{32}$ P]-dCTP (>3000 Ci/mmol, BRIT, India) by PCR or by random priming of purified inserts were used at > $10^6$  cpm/ml of hybridization solution (7% SDS, 0.5 M sodium phosphate pH 7.0, 1 mM EDTA). Blots were washed with 1 $\times$ SSC, 0.1% SDS and 0.1 $\times$ SSC, 0.1% SDS at 65°C; for initial screening of DD-PCR cDNAs (see below) washes were at 60°C. Hybridization was detected either by autoradiography or on a phosphor imager (Fuji); L-Process and Image Gauge programs (Fuji) were used to quantify background-subtracted signals.

### Differential display PCR analysis

RNA was isolated from growing, arrested and differentiated cultures. Residual proliferating cells in day 3 myotube cultures were eliminated by exposure to cytosine arabinoside ( $10^{-5}$  M) for a further 2 days. 0.2  $\mu$ g of RNA (DNase-treated using MessageClean, GenHunter Corp.) was used for DD RT-PCR (Liang and Pardee, 1992), with the RNAimage kit (GenHunter Corp.) and [ $\alpha^{33}$ P]-dATP according to manufacturer's instructions. Purified fragments were cloned into pBS (KS) (Stratagene). The differentially expressed fragments described in the Results are as follows. CF1 (333 bp) is the 3'UTR of Matrilin2 (Accession # U69262). CF2 (253 bp) spans the junction of the coding and 3'UTR regions of Znf216 (Accession # AF062071). 740 bp of the coding region of Znf216 was amplified from muscle RNA using primers FZCOD, 5'-AAAATATGGCTCAGGAGAC-3' and RZCOD, 5'-CAAAGGAAAATGGCCATGC-3'. CF3 (333 bp) is the 3'UTR of TTP (Accession # M57422). A near full-length cDNA of TTP (1.7 kb) was obtained by RT-PCR from adult skeletal muscle RNA with primers TTP5, 5'-AATACCGCGGTCTTTCACCAAGGCCATTC-3' and TTP3, 5'-CCCCGCGGTAGCAATATATTAATATATTATAGC-3'. CF4 (419 bp) is the 3'UTR of LIX (Accession # U27267). A near full-length cDNA encoding LIX (1.4 kb) was amplified from G0 myoblast RNA using primers LIX5, 5'-CACACCTCCTCCAGCAT-ATC-3' and LIX3, 5'-AGACACTATAAGATGTACAGGC-3'.

### RT-PCR analysis

Relative levels of CD34 mRNA were determined using RT-PCR. A 442 bp region common to both CD34 transcripts (exons 4-7) was amplified using primers described by Beauchamp et al. (Beauchamp et al., 2000). DNase-treated RNA samples (2.5  $\mu$ g) were reverse transcribed using the Advantage RT-for PCR kit (Clontech). Volumes of RT product were normalized to generate relatively equal amounts of PCR product for a control mRNA (L7). Each sample was then assayed in duplicate by RT-PCR for both CD34 (29 cycles) and L7 (24 cycles), separated on agarose gels, and bands quantified by Southern hybridization using CD34 and L7-specific probes and phosphorimager analysis (Fuji).

### Freeze injury of muscle in vivo and isolation of tissue

Animals were handled according to the guidelines of the CCMB Institutional Animal Ethics Committee. Balb/c and C57Bl/6 mice, ~3 months old, were anaesthetized by i.p. injection of 2.5% Avertin at a dose of 375  $\mu$ g/g. Freeze injury was performed as described previously (Dhawan et al., 1996; Pavlath et al., 1998). Briefly, the tibialis anterior (TA) muscle was exposed by a 2 mm incision in the overlying skin, and a small piece of dry ice was directly applied to the belly of the muscle for 15 seconds. The skin was sutured and mice were allowed to recover for varying periods of time (30 minutes to 14 days). Mice were sacrificed by cervical dislocation, the TA dissected free and frozen immediately in liquid nitrogen for RNA isolation or in embedding media (HistoPrep, Fisher Scientific) for histology. 20  $\mu$ m transverse cryosections were used for hematoxylin and eosin (HE) staining or in situ hybridization.

### RNA in situ hybridization

Detection of RNA in fresh cryosections of TA muscles was performed as described previously (Smerdu et al., 1994), but antisense and sense probes were labeled with digoxigenin-11-UTP (Roche) with a transcription kit (Stratagene) and detected with alkaline-phosphatase-coupled anti-digoxigenin antibody (Roche).

### Combined RNA in situ hybridization and immunodetection of the basal lamina

For co-detection of laminin protein and either Pax7 or TTP RNA, the

ISH protocol was modified as follows: protease treatment was reduced to 2 minutes, the anti-laminin polyclonal (Santa Cruz, 1:500) was included during anti-digoxigenin antibody incubation and detected with a goat anti-rabbit secondary antibody conjugated to AlexaFluor488 (1:500, Molecular Probes). Hybridized antisense RNA was then detected as described and nuclei were counterstained with Hoechst 33342. Secondary antibody controls showed no laminar staining.

### Image analysis

Autoradiographs and photomicrographs were scanned using a UMAX 3200 scanner and composites assembled using Adobe Photoshop 5.0.

## Results

### G0 arrest and synchronous activation of C2C12 myoblasts

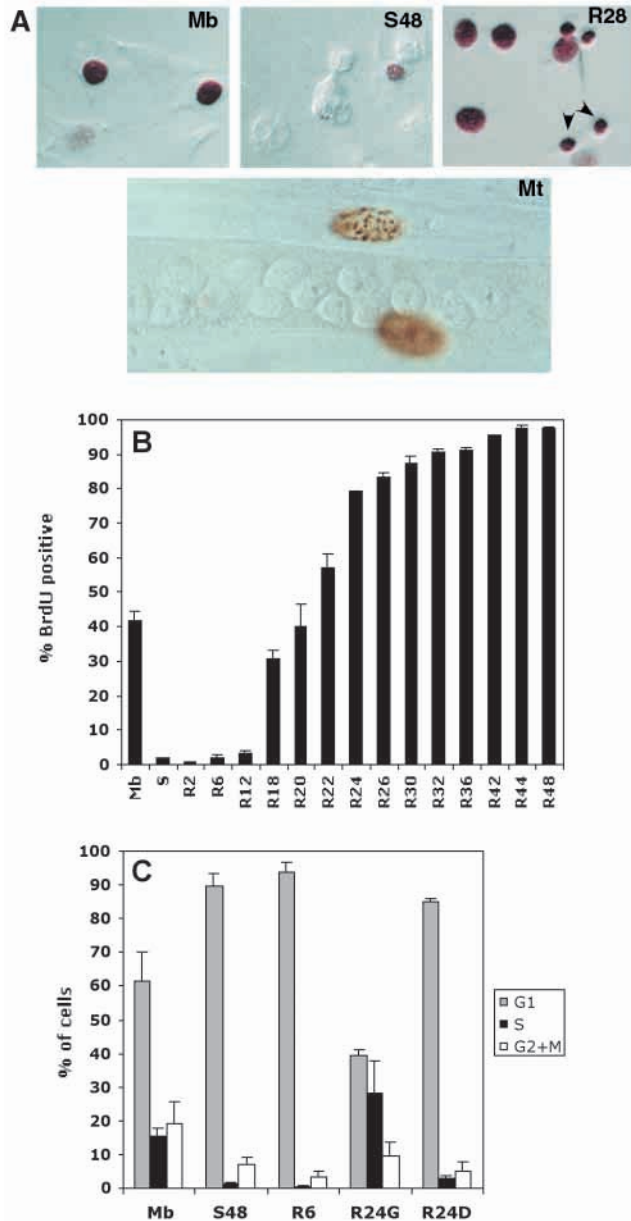
To identify events associated with cell cycle activation of myoblasts, synchronized cultures exhibiting strict growth regulation are essential. Anchorage dependence of proliferation in culture is the hallmark of stringent growth control (Stoker et al., 1968). Earlier studies had shown that C2C12 cells exit the cell cycle when cultured in non-adherent conditions (Milasincic et al., 1996). To eliminate the small but significant percentage of anchorage-independent cells (~5%) in the parent C2C12 line, we screened 22 subclones for their ability to arrest efficiently in suspension culture. Two subclones revealed strong anchorage dependence of growth: suspension culture for 48 hours resulted in a drastic inhibition of DNA synthesis ( $1.1 \pm 0.8$  [% cells  $\pm$  s.e.m.  $n=5$ ] labeled in a 15 minute pulse of BrdU compared with  $39.1 \pm 4.6$  in adherent cultures). Importantly, arrest was fully reversible: restoration of adhesive contacts by reattachment to culture dishes triggered entry into S phase in ~98% of cells (Fig. 1A,B). Significant DNA synthesis was observed only >12 hours after reattachment, and >75% of cells entered S phase between 12 and 24 hours after replating. Thus, the peak of S phase is broad, and entry into S may be semi-synchronous. Labeled mitoses were first seen 22 hours after replating and peaked at 28 hours (Fig. 1A, arrows), confirming that cells entering S phase completed the cell cycle. Primary myoblasts derived from SC were also found to survive suspension and enter G0 reversibly (data not shown), suggesting that this property is not exclusive to cell lines.

To ascertain the phase of the cell cycle in which C2C12 myoblasts had arrested, we performed a flow cytometric analysis of DNA content (Fig. 1C). By 48 hours in suspension >90% of arrested myoblasts possessed G1 DNA content, and replating for 24 hours in GM (20% FBS) activated >30% of cells into S phase with a proportionate decrease in G1. Since suspended cells possess a G1 DNA content, do not synthesize DNA and re-enter S phase with kinetics consistent with a G0-G1 transition (Fig. 1B), we conclude that anchorage deprivation arrests cloned C2C12 myoblasts in G0.

### Arrested C2C12 myoblasts downregulate myogenic regulators and do not differentiate

During the 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 we used western blot analysis (Fig. 2A). Both MyoD and Myf5 proteins were rapidly suppressed during arrest. During reactivation of growth, both MRFs were induced, although MyoD appeared within 6 hours (mid-G1) and Myf5 by 12-18 hours (late G1/early S). Consistent with their differing roles in myogenesis, MyoD was highly expressed in myotubes, but Myf5 was not. Actin levels varied with alterations in the growth/adhesive state, whereas levels of desmin, a muscle-specific intermediate filament protein did not.

To assess the differentiation status of reversibly arrested C2C12 cells we used northern blot analysis (Fig. 2B). Expression of the cell cycle dependent histone H2B confirmed efficient arrest and re-activation. Despite a high fusion index (~60%), differentiated cultures expressed the S phase marker as ~10% of nuclei (unfused cells, see Fig. 1A) incorporate BrdU. Muscle creatine kinase (MCK), a marker of differentiation, was abundantly expressed in myotubes but not

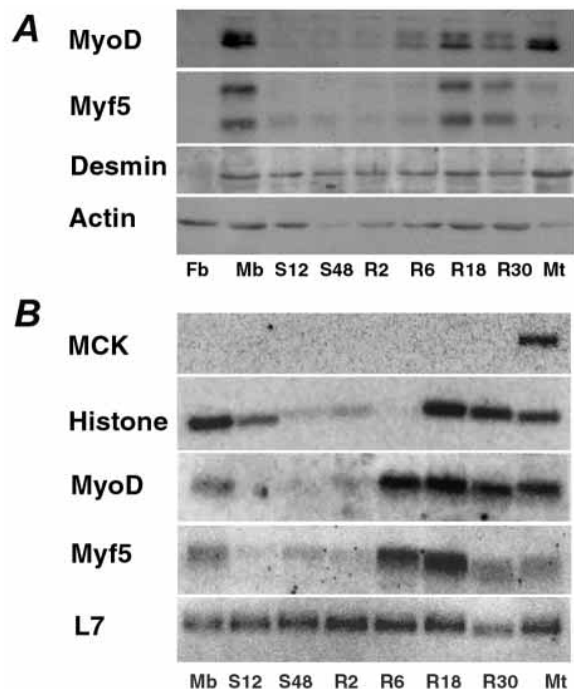
**Fig. 1.** (A) DNA synthesis in reversibly arrested C2C12 myoblasts. Immunodetection of BrdU in asynchronous myoblasts (Mb), cells synchronized by 48 hours in suspension (S48), activated cells 28 hours after replating (R28) and 3 day myotube cultures (Mt). Arrowheads in R28 point to a labeled mitosis (telophase). In differentiated cultures (Mt), residual cycling myoblasts incorporate label, whereas myotube nuclei do not. (B) All arrested myoblasts progress to S phase upon reactivation. Cumulative DNA synthesis in synchronized C2C12 myoblasts after labeling with BrdU for 2-48 hours of reactivation (R2-R48) shows that 98% of arrested cells re-enter the cell cycle. The extended G1 phase is consistent with inclusion of a G0-G1 transition phase. For comparison, note that asynchronous cells (Mb) labeled for 2 hours show high levels of DNA synthesis, whereas suspension-arrested cells (S) show <2% S phase cells despite labeling for 12 hours. Data represent the means  $\pm$  s.e.m. of duplicate samples per time point. Similar results were obtained with two independent experiments. (C) Synchronous activation of G0 myoblasts requires both adhesion and mitogens. FACS analysis of adherent C2C12 myoblasts (Mb) reveals a DNA content profile typical of an asynchronous population. 48 hours after suspension (S48), most cells show a G1 DNA content consistent with arrest in G0. Replating for 6 hours in GM (R6) or for 24 hours in DM (R24D) does not alter the profile, whereas by 24 hours in GM, adhesion- and mitogen-dependent signals synergize to return arrested cells to S phase (R24G). Data represent the means  $\pm$  s.d. of four independent experiments.

at any time during arrest and reactivation. MyoD and Myf5 mRNAs were suppressed in arrested cells and re-activated 6 hours after replating, well before the onset of DNA synthesis correlating well with the protein expression patterns (Fig. 2A). Thus, C2C12 myoblasts can be reversibly arrested in G0 in culture without activating the differentiation program, as observed in quiescent SCs in vivo (reviewed by Grounds, 1991; Seale and Rudnicki, 2000). The induction of MRFs in G1 also recapitulates their induction in activated SC (Grounds et al., 1992; Cornelison and Wold, 1997).

#### Expression of candidate SC regulators during arrest and re-activation

To examine the expression of other genes implicated in SC function we used northern blot analysis (Fig. 3). The c-met receptor, a marker of SC (Cornelison and Wold, 1997; Tatsumi et al., 1998), plays a role in their activation. As reported previously (Anastasi et al., 1997), transcripts encoding c-met were detected in C2C12 myoblasts and reduced in myotubes (Fig. 3A). However, c-met mRNA levels in asynchronous, arrested and activated myoblasts did not vary. Down-modulation of EGF and FGF receptors has been proposed as a mechanism for irreversible arrest of differentiated cells (Clegg et al., 1987; Olwin and Hauschka, 1988). Retention of c-met expression during reversible arrest may provide an option for activation when its ligand, HGF, is available.

HGF is a mitogen for SC (Allen et al., 1995; Tatsumi et al., 1998) that is also expressed by activated SC (Jennische et al., 1993; Cornelison et al., 2000). As with c-met, HGF mRNA has been reported to be expressed by C2C12 myoblasts and absent in myotubes (Anastasi et al., 1997). However, we did not detect HGF transcripts in either asynchronous or differentiated cultures (Fig. 3). Surprisingly, we found that HGF mRNAs were strongly expressed during G0 and early G1 but suppressed in late G1. Expression of both HGF and c-met



**Fig. 2.** (A) Myogenic specification factors are suppressed in G0 and activated in G1. Western blot analysis of total protein shows that both MyoD and Myf5 are present in asynchronous adherent cultures of C2C12 myoblasts (Mb) but not in C3H fibroblasts (Fb) as expected. 72 hour myotube cultures (Mt) strongly express MyoD but not Myf5. Neither MRF is expressed after 12 or 48 hours of suspension culture (S12, S48). Replating for 2 to 30 hours (R2-R30) causes induction of MyoD earlier (at R6) than Myf5 (at R18). Data are representative of three independent experiments. (B) Synchronization in G0 does not induce differentiation. Northern blot analysis of RNA isolated from asynchronous, arrested, reactivated or differentiated C2C12 cells. The differentiation marker MCK is only detected in myotubes (Mt), but not in asynchronous myoblasts (Mb), at 12 or 48 hours in suspension (S12, S48) or at 2-30 hours after replating (R2-R30). Histone mRNA indicates the extent of DNA synthesis. MyoD and Myf5 mRNAs correlate with the protein data in Fig. 2A. Data are representative of three independent experiments.

transcripts by synchronized C2C12 myoblasts implies that autocrine mechanisms may contribute to growth control as suggested previously (Anastasi et al., 1997). However, as suspended C2C12 myoblasts are quiescent despite expression of transcripts encoding both receptor and ligand, additional controls may be involved.

SC in uninjured muscle also express M-cadherin (M-cad), a muscle-specific adhesion molecule (Irintchev et al., 1994), but not all SC are positive for this marker (Cornelison et al., 1997; Beauchamp et al., 2000). We found that M-cad mRNA showed a marked cell cycle dependence, as it was abrogated in G0, reactivated during G1 and its peak was coincident with the onset of S phase (Fig. 3A).

We also examined the expression of PEA3, an *ets*-domain transcription factor reported in activated SC (Taylor et al., 1997). Consistent with its expression in vivo, PEA3 mRNA was not expressed in G0 myoblasts but was induced in G1 (Fig. 3A). Moreover, PEA3 induction was dependent on growth activation since cells replated in DM did not express this transcript at the levels seen in GM.

Recently, a marker of hematopoietic stem cells, CD34, has been localized to SC and by its expression during arrest and early activation, suggested to regulate the G0-G1 transition (Beauchamp et al., 2000). Using semi-quantitative RT-PCR, we estimated the relative levels of CD34 mRNA during reversible arrest (Fig. 3B). Synchronization in G0 led to a ~five-fold induction of CD34 transcripts relative to the levels seen in asynchronous C2C12 cells, declining rapidly during the G0-G1 transition but staying above basal levels at S phase. Thus, CD34 also appears to show cell cycle regulation in culture.

Taken together, these results show that synchronization of C2C12 myoblasts in culture reveals cell cycle dependent regulation of the MRFs and other genes implicated in SC function in vivo.

#### Isolation of genes induced in synchronized C2C12 myoblasts using differential display PCR

To isolate other genes induced in synchronized C2C12 cells, DD-PCR was used to simultaneously compare RNA from asynchronous, arrested and differentiated cultures. RNA samples were first tested for appropriate expression of markers of proliferation and differentiation (histone H2B and MCK, respectively) (data not shown). Fig. 4A shows a representative gel used for identification and isolation of differentially expressed cDNA fragments. Of 36 fragments isolated, four cDNAs (CF1-4) hybridized to single transcripts specifically detected in G0 synchronized myoblasts but not in either asynchronously growing or differentiated cells (Fig. 4B-E).

Sequence analysis of these cDNA fragments revealed that all four are ~100% identical to sequences in GenBank. CF1 is identical to a region of the 3'UTR of the mRNA coding for Matrilin-2, an extracellular matrix protein (Deak et al., 1997). CF2 spans the junction of the coding and 3'UTR region of the mRNA for Znf216, a zinc finger protein of unknown function (Scott et al., 1998). CF3 aligns completely with a portion of the 3'UTR of the mRNA coding for TTP, a zinc-finger protein that binds RNA and is implicated in mRNA decay (Carballo et al., 1998; Carballo et al., 2000). CF4 is identical to part of the 3'UTR of LIX, a chemokine first isolated from LPS-stimulated fibroblasts (Smith and Herschman, 1995). Interestingly, both LIX and TTP mRNAs contain multiple AU-rich elements (AREs) characteristic of labile transcripts (Shaw and Kamen, 1986).

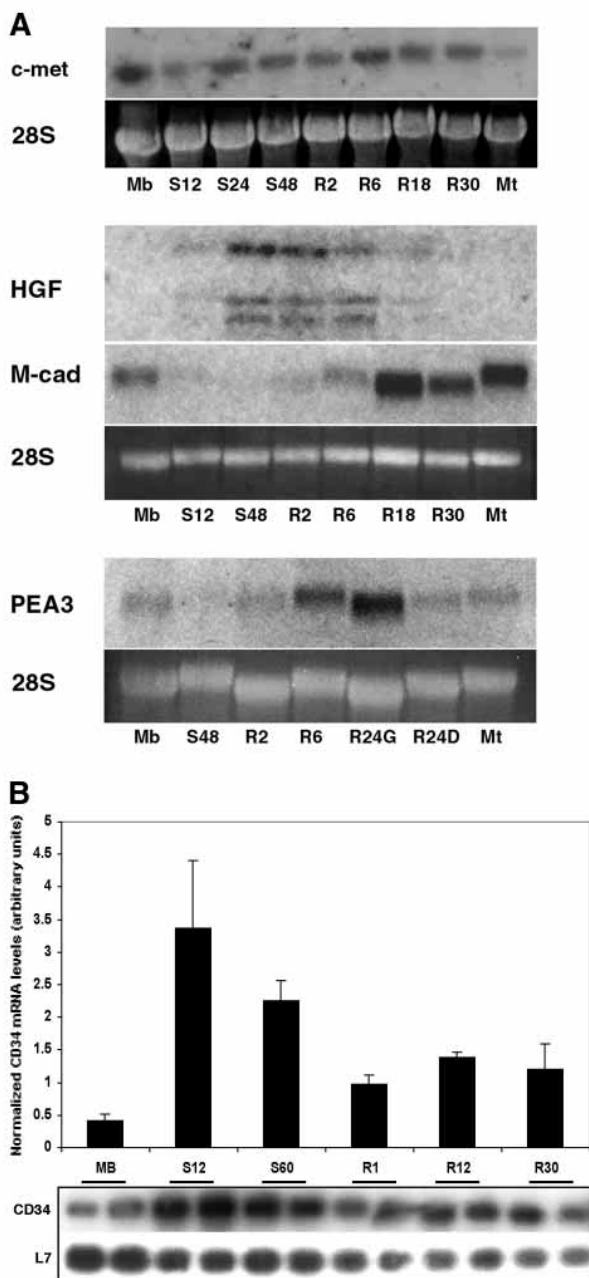
#### Dynamic expression of transcripts detected by differential display PCR products during synchronous activation of G0 C2C12 myoblasts

To examine the expression patterns detected by the cDNAs isolated from synchronized myoblasts, a time course of cell cycle activation was analyzed by northern blotting (Fig. 5). DNA synthesis monitored in parallel confirmed efficient arrest and activation (not shown). LIX mRNA was not detected in either asynchronous or differentiating cells. Although upregulated during G0, activation into G1 led to further induction of LIX transcripts after 2 hours followed by a rapid extinction, consistent with the behavior of labile ARE-containing mRNAs of immediate early genes (Chen and Shyu, 1994).

As with LIX, TTP mRNA was only detected in synchronized cultures. Although induced during G0, maximum levels of TTP

transcripts were detected at 1 hour after activation, followed by suppression later in G1. The rapid extinction of both LIX and TTP transcripts in activated C2C12 myoblasts coupled with the presence of AREs led us to examine the effect of translation inhibition on their expression. Cycloheximide treatment of replated cells led to a marked stabilization of both LIX and TTP transcripts (data not shown), suggesting that rapid turnover may account for their suppression in G1.

Matrilin-2 mRNA showed induction and decay kinetics similar to TTP in arrested and activated myoblasts. Differences in expression of Znf216 mRNA among asynchronous, arrested and differentiated cultures were less than that of LIX, TTP and Matrilin-2, and though induced after replating, Znf216 transcript levels declined with slower kinetics. Thus, all four transcripts identified by synchronization of C2C12 myoblasts were induced during cell cycle activation *in vitro*.



### LIX and TTP are rapidly and transiently induced in response to muscle injury *in vivo*

To determine whether the cDNAs we identified are expressed during regeneration, the tibialis anterior (TA) muscle of adult mice was subjected to focal injury and analyzed for molecular and histological changes. Freeze injury reproducibly leads to the degeneration and regeneration of 20-30% of the cross sectional area of the TA. We monitored histological changes by HE staining of muscle sections (Fig. 6). Damage is rapidly followed by inflammatory cell infiltration within hours, macrophage activity and MPC proliferation peaking at 2-3 days, appearance of new myotubes by 3 days and their maturation over the next 2 weeks into centrally nucleated regenerated myofibres (Pavlati et al., 1998).

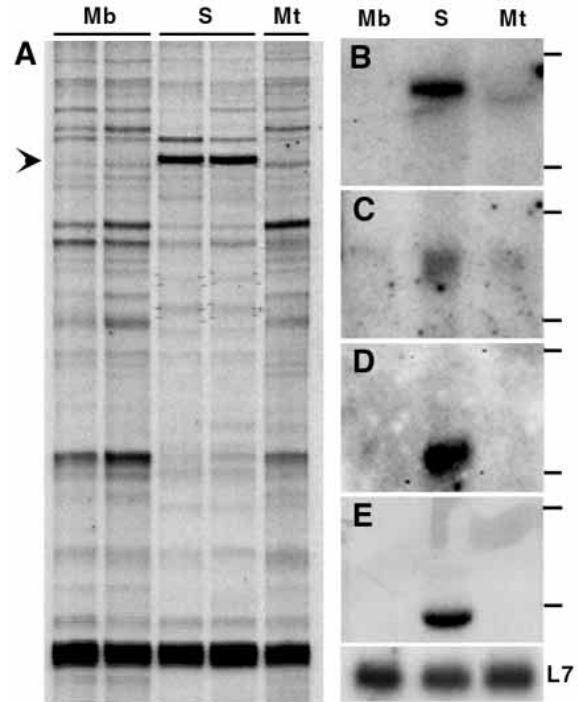
RNA isolated from time courses of induced regeneration in adult C57Bl/6 and Balb/c mice was analyzed by northern blotting (Fig. 7). Matrilin-2 mRNA was not detected in uninjured muscle or during regeneration. Znf216 mRNA was expressed at high levels in uninjured muscle but did not fluctuate markedly during regeneration. By contrast, LIX and TTP mRNAs were not detected in uninjured muscle but were rapidly and transiently induced in response to injury. Expression of LIX was activated at 6 hours and remained high until 3 days post injury (PI), a time when proliferation in the recovering tissue is maximal, as shown by histone H2B expression. Expression of TTP was seen within 30 minutes of injury followed by an acute downregulation between 3 and 6 hours PI. The timing of induction of TTP RNA in damaged muscle precedes that of MyoD, the first MRF expressed in activated SC (Grounds, 1992; Cooper et al., 1999). Although their expression in G0 myoblasts was not mirrored by uninjured muscle, the rapid and transient induction of both LIX and TTP during growth stimulation occurred similarly *in vitro* and *in vivo*. Thus, two of the four cDNAs isolated from synchronized C2C12 myoblasts in culture are part of the acute response to muscle damage *in vivo*. Interestingly, both are labile mRNAs encoding molecules implicated in inflammatory processes.

**Fig. 3.** (A) Cell cycle dependent expression in cultured C2C12 myoblasts of genes involved in muscle regeneration. (A) RNA analysis of adherent myoblasts (Mb), suspension cultures at 12, 24 or 48 hours (S12, S24, S48), replated cultures at 2-30 hours (R2-R30) and myotubes (Mt). c-met transcripts are expressed throughout the cell cycle but are downregulated during differentiation. HGF transcripts are undetectable in asynchronous or differentiated cultures but expressed in synchronized cells during arrest and early activation. M-cad and PEA-3 mRNAs show classic cell cycle dependence: suppression in G0 and activation in G1 (PEA-3) and G1-S (M-Cad). Ethidium bromide staining of rRNA [28S] indicates equal loading. Data are representative of three independent experiments. (B) 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 (upper panel) represent the means  $\pm$  s.e.m. of duplicate assays for CD34 RNA normalized with respect to L7 control RNA for each sample, shown in the Southern blot (lower panel). Similar results were obtained with two independent time course experiments.



**Fig. 4.** Identification of genes expressed by arrested undifferentiated C2C12 muscle cells. (A) Differential display PCR analysis.

Expression profiles of RNA from asynchronous (Mb) suspension-arrested (S) and differentiated (Mt) C2C12 cell cultures displayed by differential display PCR. (Mb and S were analyzed in duplicate.) The arrowhead indicates a fragment representing a cDNA expressed in arrested myoblasts but not in either asynchronous or differentiated cultures (see Fig. 4E). (B-E) Transcripts detected by differential display PCR fragments are enriched in synchronized C2C12 cells. Fragments excised from differential display PCR gels were labeled and used to probe 10 µg of total RNA from asynchronous myoblasts (Mb), arrested myoblasts (S) and myotubes (Mt). Each fragment (CF1-4) detected a unique transcript; all were expressed preferentially in synchronized cells. The sizes of the transcripts (B-E) are 3.5 kb (CF1/matriline2), 2.2 kb (CF2/Znf216), 1.7 kb (CF3/TTP) and 1.4 kb (CF4/LIX). Positions of 28 and 18S rRNA are marked (-). L7 demonstrates equal loading of RNA.



#### LIX and TTP transcripts show a spatial distribution similar to MyoD in injured muscle

To identify the cellular source of the mRNAs detected in regenerating muscle we used RNA in situ hybridization. LIX transcripts localized to a few mononucleated cells at 6 hours PI, with no signal in the myofiber cytoplasm (Fig. 8A). As expected from northern analysis, LIX transcripts were not detected in uninjured muscle (Fig. 8a). The timing and distribution of LIX mRNA are similar to that of MyoD mRNA (Fig. 8B), which is expressed only in activated SC of damaged muscle at this time (Grounds et al., 1992) (reviewed in Seale and Rudnicki, 2000). As with LIX, TTP mRNA was not detected in uninjured muscle (Fig. 8c). However, at 2 hours after injury, approximately one third of the cross-sectional area of the TA muscle was dotted with TTP-positive mononucleated cells (Fig. 8C,D).

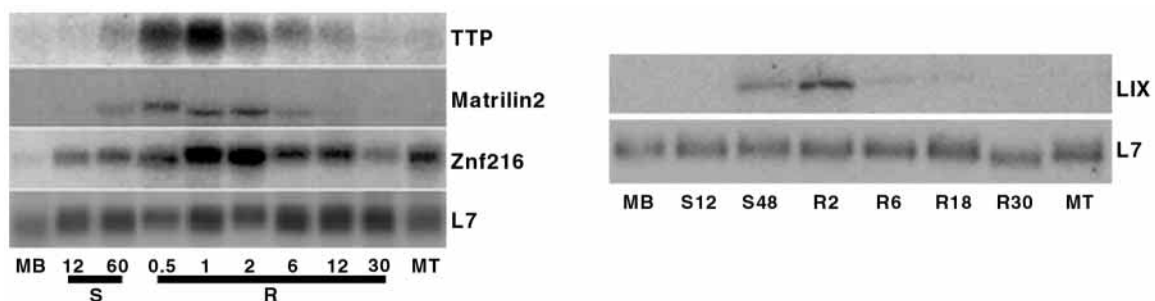
#### TTP transcripts are associated with presumptive satellite cells in injured muscle

Prior to their division following activation, SC contain sparse cytoplasm surrounding a condensed nucleus (Bischoff, 1994). To determine if TTP transcripts co-localized with nuclei, sections were counter-stained with Hoechst 33342 following RNA in situ hybridization. As seen in Fig. 9, the hybridization signals localize to a subset of nuclei at the myofiber periphery. The absence of signal away from these nuclei suggests that the

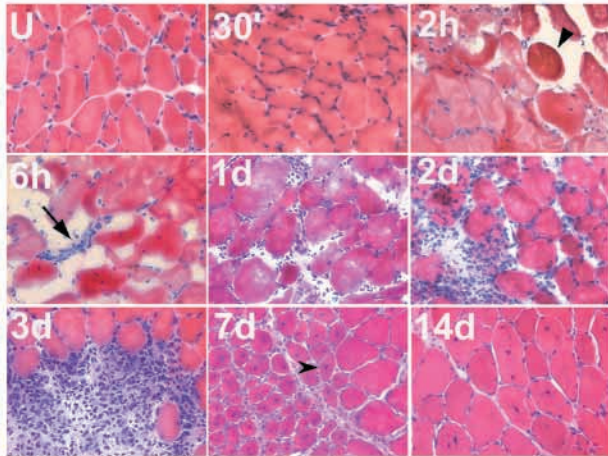
transcripts are physically constrained from diffusing into the myofiber interior, consistent with their location in mononucleated cells and distinguishing them from myonuclei.

To determine if TTP-positive mononucleated cells are found in a sublaminar rather than an interstitial location, we combined RNA in situ hybridization with immunodetection of the basal lamina (Fig. 10). TTP-positive cells were found beneath a laminin sheath at the myofiber periphery, as were cells expressing the SC marker Pax7. Taken together, these results suggest that TTP-positive cells are mononucleated and located below the basal lamina, features typical of SC.

Since neither LIX nor TTP is muscle specific, we cannot rule out their expression by mononucleated cells in addition to presumptive SC. However, along with their expression in cultured myoblasts, the timing and location of LIX and TTP mRNAs in vivo suggests that activated SC are among the mononucleated cells that express these transcripts in response to muscle injury.



**Fig. 5.** LIX and TTP are induced during C2C12 myoblast activation in culture. RNA isolated from a time course of cell cycle activation was probed with each fragment described in Fig. 4. Mb, asynchronous myoblasts; S12, S48, S60, cells held in suspension for 12, 48 or 60 hours, respectively; R 0.5-30, cells replated for 0.5 to 30 hours after arrest in suspension; Mt, myotubes. All four transcripts are detected in G0 synchronized cells and further induced transiently in newly activated cells. Data are representative of three independent experiments.



**Fig. 6.** Time course of muscle regeneration induced by focal freeze injury. Transverse cryosections from TA muscles of 3-month-old C57 mice subjected to focal damage *in vivo* were analyzed by HE staining. Uninjured muscle (U) shows a typical pattern of evenly sized myofibers with peripheral nuclei. 30 minutes after injury no histological changes are evident, whereas hyper-contracted fibers (arrowhead) and infiltrating mononuclear cells (arrow) appear by 2 and 6 hours, respectively. Mononucleated cells (inflammatory cells and myoblasts) increase in number in the lesion over the next 3 days. By 7 days post injury, myoblast fusion results in centrally nucleated regenerating fibers (arrowhead) of a smaller caliber than the adjacent uninjured fibers. The cross-sectional area of regenerating fibers matures to that of undamaged fibers over the next week (14d).

## Discussion

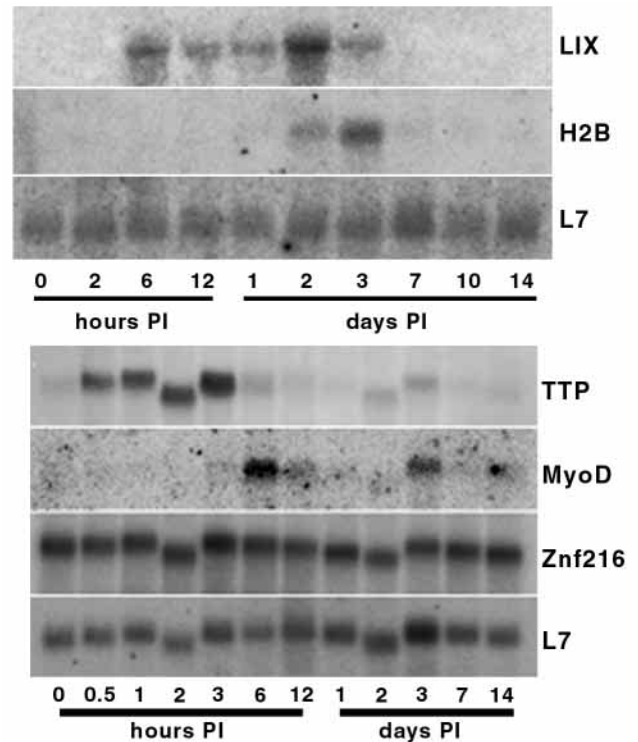
In this report, we show that genes isolated from synchronized C2C12 myoblasts in culture are expressed in regenerating muscle with kinetics and a location that suggest expression by activated satellite cells.

### Stringent growth control in synchronized C2C12 myoblasts

In non-tumorigenic cells, loss of adhesion triggers arrest despite the presence of mitogens (Benecke et al., 1978; Dike and Farmer, 1988; Milasincic et al., 1996). Anchorage-dependence of proliferation is the best *in vitro* correlate of strict growth control (Freedman and Shin, 1974). Therefore, we used adhesion-dependent subclones of C2C12 myoblasts to analyze synchronous cell cycle activation. Two lines of evidence suggest that cells arrest in G<sub>0</sub>. Firstly, FACS analysis shows that >90% of cells arrest with a 2 N DNA content. Secondly, the kinetics of return to S phase are consistent with the correspondence of the lag period to the G<sub>0</sub>-G<sub>1</sub> transition.

### MRF expression is suppressed in G<sub>0</sub>

Differentiation is accompanied by MRF expression and irreversible cell cycle exit (Andres and Walsh, 1996). By contrast, in quiescent SC, neither MyoD nor Myf5 are expressed (Grounds et al., 1992; Cornelison and Wold, 1997; Cooper et al., 1999), suggesting that MRF expression is incompatible with reversible arrest. Similarly, we find that expression of MyoD and Myf5 proteins is extinguished in suspension-arrested C2C12 myoblasts as in 'reserve' cells of



**Fig. 7.** LIX and TTP are rapidly induced in response to muscle injury. RNA was isolated from uninjured adult mouse skeletal muscle (0) and at different times post injury (PI) and probed for TTP and LIX expression. LIX is induced at 6 hours and peaks prior to the peak of proliferation (assessed by Histone H2B mRNA). TTP was activated by 30 minutes after injury, well before the activation of MyoD. Each lane represents pooled RNA (20  $\mu$ g) from TA muscles of two to three mice. Data are representative of three independent experiments.

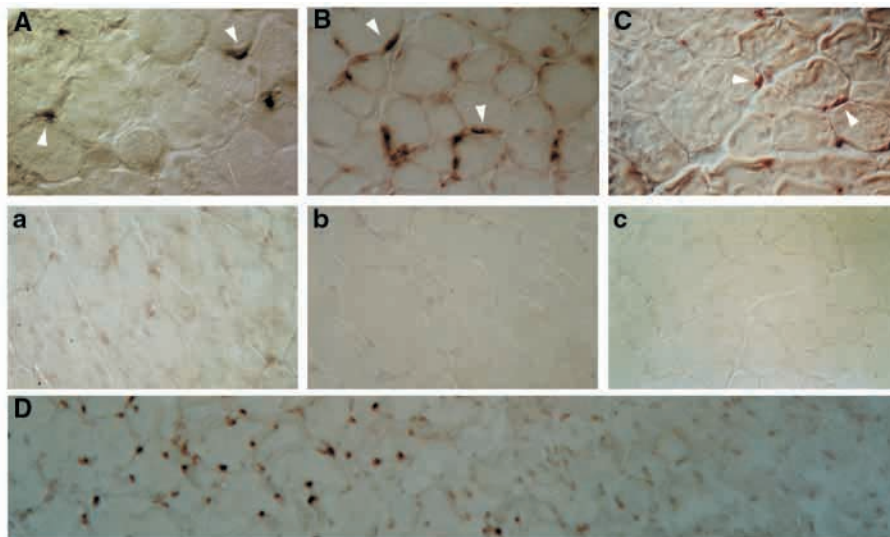
differentiating cultures (Yoshida et al., 1998). MyoD inhibits the cell cycle independently of its myogenic activity (Crescenzi et al., 1990), but as it is not detected in G<sub>0</sub> myoblasts, suspension-arrest may be independent of this MRF. Indeed, absence of MyoD in G<sub>0</sub> may be necessary for arrest to be reversible (Yoshida et al., 1998), and absence of the cyclin-dependent kinase inhibitor p21, a target of MyoD during irreversible arrest (Halevy et al., 1995; Guo et al., 1995), in G<sub>0</sub> arrested cells supports this hypothesis (J.D., unpublished). Unlike resting SC *in vivo*, C2C12 myoblasts induced to enter G<sub>0</sub> continue to express desmin, perhaps reflecting the greater stability of this cytoskeletal protein relative to the labile transcription factors MyoD and Myf5.

### Cell cycle dependent expression of candidate SC regulators in synchronized C2C12 myoblasts

Several genes detected in SC have been implicated in muscle regeneration (reviewed by Seale and Rudnicki, 2000). Conceivably, genes involved in SC activation may be cell cycle regulated, but genes specifying SC identity may not. Consistent with this idea, MyoD is induced during SC activation *in vivo* (Grounds et al., 1992; Cornelison and Wold, 1997) and is cell cycle responsive in culture (Kitzmann et al., 1998). By contrast, both resting and activated SC express Pax7 (Seale et al., 2000), suggesting that this specification factor may not be



**Fig. 8.** LIX and TTP transcripts are distributed in a pattern similar to MyoD in injured skeletal muscle. RNA in situ hybridization to frozen sections (20  $\mu$ m) of injured TA muscle using digoxigenin-labeled antisense probes to LIX, MyoD and TTP transcripts (A, B and C, respectively). Absence of staining in the myofiber interior suggests sequestration of the transcripts in mononuclear cells at the myofiber periphery. The same probes show no hybridization to uninjured muscle (a, b and c). LIX and MyoD were detected 6 hours post injury and TTP at 3 hours post injury. (D) shows TTP-positive cells located in the lesion; an adjacent uninjured area is devoid of TTP transcripts. Data are representative of three independent experiments each involving multiple cryosections from two mice.

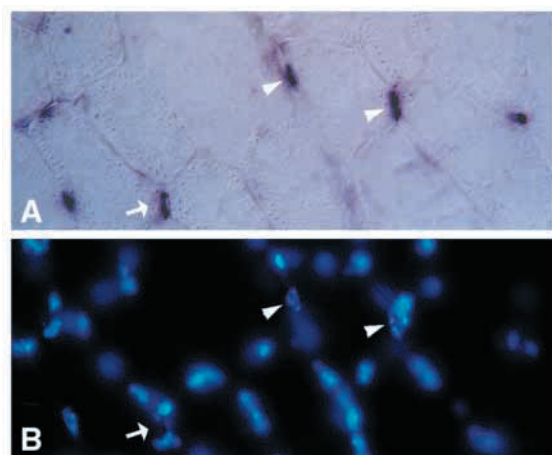


cell cycle regulated. Myf5 expression during G0 in C2C12 myoblasts (Kitzmann et al., 1998; Yoshida et al., 1998), in SC on single fibers (Beauchamp et al., 2000; Cornelison and Wold, 1997; Cornelison et al., 2000) and quiescent SC in vivo (Cooper et al., 1999; Beauchamp et al., 2000) remains controversial. In adhesion-dependent arrest, we find Myf5 to be absent in G0, but activated during G1, consistent with previous observations (Yoshida et al., 1998; Cornelison and Wold, 1997; Cooper et al., 1999).

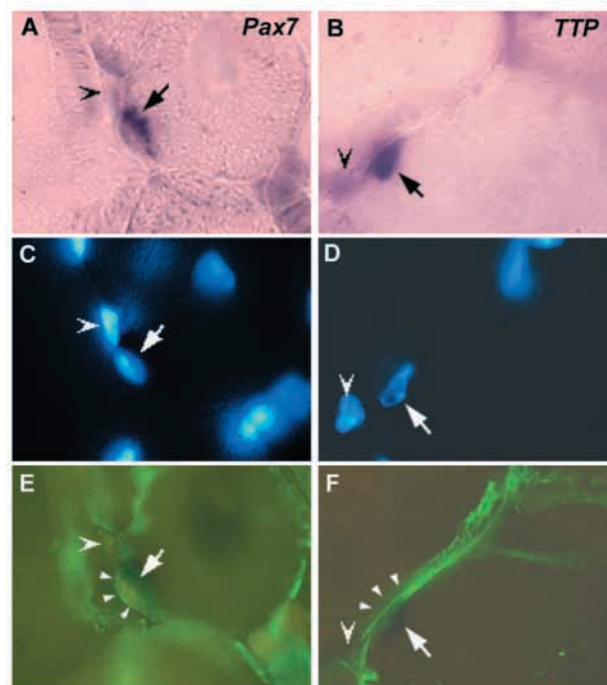
The regulation of other genes implicated in SC function has not been previously analyzed in synchronized myoblasts. Our data show that HGF, PEA-3, M-Cad and CD34, are all cell cycle dependent in culture.

The c-met receptor and its ligand HGF play a key role in SC activation. In uninjured muscle, whereas c-met has been detected on SC, HGF is found at the myofiber periphery

(Tatsumi et al., 1998). Immunodepletion of HGF from crushed muscle extracts results in a loss of SC mitogenic activity (Tatsumi et al., 1998). Thus, it is thought that HGF sequestered in the ECM is released by damage and stimulates resting SC in a paracrine fashion. HGF mRNA is not detected in SC until after their activation (Jennische et al., 1993; Cornelison et al.,



**Fig. 9.** TTP transcripts colocalize with a subset of nuclei in injured muscle. TTP transcripts were visualized by RNA in situ hybridization at 2 hours post injury (A) and nuclei counter-stained with Hoechst 33342 (B). There is a close correlation of TTP transcripts with a small proportion of the nuclei in the damaged area (arrowheads). The arrow indicates the occasional intense digoxigenin signal that quenched the Hoechst fluorescence of the underlying nucleus.



**Fig. 10.** TTP is expressed in mononucleated cells that lie beneath the myofiber basal lamina. Co-detection of laminin with Pax7 (A,C,E) or TTP (B,D,F) transcripts in cryosections of TA muscle 1 hour after injury using combined immunofluorescence (E,F) and in situ hybridization (A,B); nuclei are counterstained with Hoechst 33342 (C,D). Pax 7 RNA (A) and TTP RNA (B) are associated with nuclei that lie below a laminin sheath (arrowheads in E,F). The same sub-laminar cell (A,C,E or B,D,F, respectively) is indicated by arrows. Interstitial cells that do not express Pax7 or TTP RNA are indicated by 'V' arrowhead.

2000) and may serve to amplify the activating signal. Both *c-met* and HGF transcripts were detected in asynchronous C2 myoblasts, suggesting that autocrine activation may also play a role (Anastasi et al., 1997). In synchronized cultures, we find that *c-met* transcripts are maintained in G0 as well, raising the possibility that quiescent cells retain responsiveness to a ligand whose expression/activity is regulated. Strikingly, HGF transcripts are only detected during G0 and early G1. Although we have not assessed the levels of HGF protein, it must be absent or inactive in arrested myoblasts, as autocrine/paracrine effects would be readily detected as BrdU-positive cells.

The transcription factor PEA3 is expressed by activated SC but not by resting SC (Taylor et al., 1997). The rapid induction of PEA3 during cell cycle re-entry of G0 C2C12 myoblasts suggests that the activation process *in vivo* and in culture show some similarities in gene expression.

M-Cad expression by SC *in vivo* is heterogeneous: 20% of SC in uninjured muscle do not express either M-cad or CD34 and are proposed to comprise a minor stem-cell-like compartment that gives rise to the lineage-restricted marker-positive majority (Beauchamp et al., 2000). Further, whereas <20% of SC on freshly isolated single fibers are M-cad positive, 100% are positive after 96 hours, consistent with an induction of M-cad in activated SC (Cornelison and Wold, 1997). In this context, it is interesting that we find suppression of M-cad mRNA in G0 myoblasts and its reactivation during G1 in culture. Heterogeneity of M-Cad expression *in vivo* may also reflect cell cycle position.

CD34, a marker of hematopoietic progenitors is routinely used for their clinical isolation for transplantation (reviewed by Krause et al., 1996). Although the function of this cell surface glycoprotein is obscure, its presence on dermal (Nickoloff, 1991), liver (Omori et al., 1997) and muscle precursors (Beauchamp et al., 2000) in the adult strongly suggests a role in regeneration. Our data demonstrate that expression of CD34 mRNA is high in G0 and downregulated in activated C2C12 myoblasts, supporting the suggestion (Beauchamp et al., 2000) that it is regulated at the G0/G1 transition.

#### Differential display PCR reveals genes expressed specifically in synchronized C2C12 myoblasts

Since reversible arrest is at the core of SC function, we used synchronized cultures to search for cDNAs expressed during arrest and activation but not in differentiated cells. Interestingly, all four transcripts identified (LIX, TTP, Matrilin-2 and Znf216) are further induced during activation of arrested myoblasts, but as with other early response genes, the induction is short lived. The lack of expression of these genes at early times in suspension suggests that they are induced as a consequence of cell cycle synchrony caused by prolonged suspension culture and not as a stress response to non-adherent conditions. Our screen was not designed to isolate muscle-specific factors but cell cycle dependent genes, and those we identified show a wide tissue distribution, but are rapidly induced in response to a number of growth-activating stimuli (Varnum et al., 1989; Smith and Herschman, 1995). Such genes might also be expected to display cell cycle dependent expression during SC activation *in vivo*.

Both LIX and TTP transcripts contain multiple instability elements (AREs), hallmarks of labile cytokine and oncogene

mRNAs (reviewed by Chen and Shyu, 1995). Such transcripts are stabilized by the inhibition of translation, and indeed both LIX and TTP mRNAs are stable in cycloheximide-treated cells (C.S., unpublished). The pronounced suppression of protein synthesis accompanying quiescence (Benecke et al., 1980) may account for their induction in suspension. Since stable ARE-positive mRNAs are often not translated (Chen and Shyu, 1995), LIX and TTP proteins may not be synthesized in arrested cells despite the presence of their mRNAs. As translation is dramatically stimulated during the G0-G1 transition (J. Dhawan, PhD thesis, Boston University, 1991), a burst of synthesis of LIX and TTP proteins may precede the decay of both transcripts after activation into G1.

#### LIX and TTP are induced in response to muscle damage *in vivo*

Regeneration of damaged muscle is a complex process involving many cell types and the interplay of a number of growth factors, cytokines, chemokines, extracellular matrix components and signaling molecules (reviewed by Grounds, 1991; Seale and Rudnicki, 2000). Uninjured muscle containing quiescent SC is devoid of LIX transcripts, in contrast to quiescent C2C12 myoblasts in culture. 6 hours after injury, LIX mRNA is strongly induced and peaks at 48 hours, prior to the peak of proliferation in SC (and infiltrating cells). LIX was first isolated in a screen for LPS-inducible glucocorticoid-attenuated genes in fibroblasts (Smith and Herschman, 1995). This small, secreted protein contains a CXC motif preceded by an ELR sequence characteristic of chemokines with neutrophil-attracting activity (Wuyts et al., 1998). LIX is also induced during injury of the spinal cord (McTigue et al., 1998) and injury of cardiac myocytes *in vivo* and is a potent neutrophil chemoattractant (Chandrasekhar et al., 2001). In muscle, neutrophils enter a lesion 1 to 3 hours after damage (Orimo et al., 1991), before LIX is detected, but this chemokine could contribute to the massive inflammatory influx seen 6 to 24 hours PI. The presence of LIX mRNA at 3 days, after neutrophils have withdrawn from the site of injury (Orimo et al., 1991), may suggest that LIX plays additional roles. CXC R2, a receptor of ELR-positive chemokines has been found on endothelial cells (Addison et al., 2000), implicating these proteins in angiogenesis and raising the possibility that LIX promotes revascularization during tissue repair. Cytokines released by inflammatory leukocytes have been proposed to activate SC (Jesse et al., 1998). Conversely, regulators of inflammation such as LIX and TTP expressed by activated SC may affect the function of infiltrating and resident cells in regenerating muscle.

As with LIX, TTP RNA is not detected in uninjured muscle. Although uninjured muscle may contain rare activated SC, the transient expression of these early response genes may preclude their detection. Injury induces a dramatic increase of this transcript within 30 minutes, but the induction is transient and TTP is undetectable by 6 hours after damage. *In situ* hybridization shows that TTP transcripts are closely associated with a few nuclei at the myofiber margin and not with the myofibre interior. This sequestration is consistent with a barrier to diffusion such as the presence of a membrane and suggests that TTP transcripts are harbored in mononucleated cells at the myofiber periphery, thereby distinguishing them from myonuclei. Further, the visualization of TTP-positive cells



beneath the basal lamina in a location similar to Pax7-positive cells distinguishes these cells from interstitial cells. In addition, the frequency of TTP positive cells at 3 hours is similar to that of MyoD positive cells at 6 hours. Taken together, these data support the identification of TTP positive cells as SC.

The acute expression kinetics of TTP suggest that this zinc finger protein may perform a critical function in muscle immediately after injury. The lack of expression by myofibers suggests a specific early response of SC to muscle trauma. TTP is known to bind mRNAs encoding tumor necrosis factor (TNF $\alpha$ ) and granulocyte-macrophage colony stimulating factor (GM-CSF), triggering their decay. TTP<sup>-/-</sup> mice develop severe inflammation and cachexia (muscle wasting) (Taylor et al., 1996), symptoms that are largely attenuated by antibody neutralization of TNF $\alpha$ . The increased TNF $\alpha$  levels detected 24 hours after muscle injury (Collins and Grounds, 2001; Zador et al., 2001) have been attributed to infiltrating phagocytes. TTP's early induction in SC may involve regulation of TNF $\alpha$  expression, but it is also conceivable that as TTP binds to ARE instability motifs in other transcripts (Carballo et al., 2000), its targets may vary in different cell types. Thus, TTP could play a novel role in SC. Taken together with its localization, the induction of TTP expression by injury suggests that this transcript may serve as a marker for activated SC and could allow quantitative analyses of the extent of damage and/or repair.

From our observations, myoblast activation in vitro and in vivo appear to involve similar early events such as the induction of TTP and LIX. Identification of TTP in muscle within 30 minutes of damage suggests that SC respond to injury very rapidly. Thus, identification of the factors that control expression of TTP may lead to an understanding of acute signals released in muscle in response to injury. Further, as TTP is known to regulate the half-life of cytokine mRNAs, SC may be involved not only in the generation of new myoblasts but also in cell-cell signaling during injury and repair.

We are grateful to P. S. Manogaran and K. Kennady for assistance in flow cytometry, G. Swarnalatha for access to a cryostat and S. Tole for advice on in situ hybridization. We thank G. Pavlath and T. Vaidya for useful discussions and H. Blau, C. Birchmeier, G. Merlino and C. Peterson for reagents. C.S. and R.S. were supported by research fellowships from the University Grant Commission and the Council for Scientific and Industrial Research, respectively. This work was supported by grants from the Sir Dorabji Tata Trust and the Departments of Science and Technology and Biotechnology, Government of India, to J.D.

## References

- Addison, C. L., Daniel, T. O., Burdick, M. D., Liu, H., Ehlert, J. E., Xue, Y. Y., Buechi, L., Walz, A., Richmond, A. and Strieter, R. M. (2000). The CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR+ CXC chemokine-induced angiogenic activity. *J. Immunol.* **165**, 5269-5277.
- 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 Physiol.* **165**, 307-312.
- 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 on myogenic differentiation. *J. Cell Biol.* **137**, 1057-1068.
- Andres, V. and Walsh, K. (1996). Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. *J. Cell Biol.* **132**, 657-666.
- Beauchamp, J. R., Heslop, L., Yu, D. S., Tajbakhsh, S., Kelly, R. G., Wernig, A., Buckingham, M. E., Partridge, T. A. and Zammit, P. S. (2000). Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. *J. Cell Biol.* **151**, 1221-1234.
- Benecke, B. J., Ben-Ze'ev, A. and Penman, S. (1978). The control of mRNA production, translation and turnover in suspended and reattached anchorage-dependent fibroblasts. *Cell* **14**, 931-939.
- Benecke, B. J., Ben-Ze'ev, A. and Penman, S. (1980). The regulation of RNA metabolism in suspended and reattached anchorage-dependent 3T6 fibroblasts. *J. Cell Physiol.* **103**, 247-254.
- Bischoff, R. (1986). Proliferation of muscle satellite cells on intact myofibers in culture. *Dev. Biol.* **115**, 129-139.
- Bischoff, R. (1994). The satellite cell and muscle regeneration. In *Myology* (ed. A. G. Engel and C. Franzini-Armstrong), pp. 97-133. New York: McGraw Hill Inc.
- 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.
- Blau, H. M., Chiu, C. P. and Webster, C. (1983). Cytoplasmic activation of human nuclear genes in stable heterocaryons. *Cell* **32**, 1171-1180.
- Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E. and Arnold, H. H. (1989). A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. *EMBO J.* **8**, 701-709.
- Carballo, E., Lai, W. S. and Blakeshear, P. J. (1998). Feedback inhibition of macrophage tumor necrosis factor- $\alpha$  production by tristetraprolin. *Science* **281**, 1001-1005.
- Carballo, E., Lai, W. S. and Blakeshear, P. J. (2000). Evidence that tristetraprolin is a physiological regulator of granulocyte-macrophage colony-stimulating factor messenger RNA deadenylation and stability. *Blood* **95**, 1891-1899.
- Chandrasekar, B., Smith, J. B. and Freeman, G. L. (2001). Ischemia-reperfusion of rat myocardium activates nuclear factor-KappaB and induces neutrophil infiltration via lipopolysaccharide-induced CXC chemokine. *Circulation* **103**, 2296-2302.
- Chen, C. Y. and Shyu, A. B. (1994). Selective degradation of early-response-gene mRNAs: functional analysis of the AU-rich elements. *Mol. Cell Biol.* **12**, 8471-8482.
- Chen, C. Y. and Shyu, A. B. (1995). AU-rich elements: characterization and importance in mRNA degradation. *Trends Biochem. Sci.* **20**, 465-470.
- Clegg, C. H., Linkhart, T. A., Olwin, B. B. and Hauschka, S. D. (1987). Growth factor control of skeletal muscle differentiation: commitment to terminal differentiation occurs in G1 phase and is repressed by fibroblast growth factor. *J. Cell Biol.* **105**, 949-956.
- Collins, R. A. and Grounds, M. D. (2001). The role of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in skeletal muscle regeneration. Studies in TNF- $\alpha$ (-/-) and TNF- $\alpha$ (-/-)/LT- $\alpha$ (-/-) mice. *J. Histochem. Cytochem.* **49**, 989-1001.
- Cooper, R. N., Tajbakhsh, S., Mouly, V., Cossu, G., Buckingham, M. and Butler-Browne, G. S. (1999). In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle. *J. Cell Sci.* **112**, 2895-2901.
- Cornelison, D. D. 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.
- Cornelison, D. D., Olwin, B. B., Rudnicki, M. A. and Wold, B. J. (2000). MyoD(-/-) satellite cells in single-fiber culture are differentiation defective and MRF4 deficient. *Dev. Biol.* **224**, 122-137.
- Crescenzi, M., Fleming, T. P., Lassar, A. B., Weintraub, H. and Aaronson, S. A. (1990). MyoD induces growth arrest independent of differentiation in normal and transformed cells. *Proc. Natl. Acad. Sci. USA* **87**, 8442-8446.
- Davis, R. L., Weintraub, H. and Lassar, A. B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51**, 987-1000.
- Deak, F., Piecha, D., Bachrati, C., Paulsson, M. and Kiss, I. (1997). Primary structure and expression of matrilin-2, the closest relative of cartilage matrix protein within the von Willebrand factor type A-like module superfamily. *J. Biol. Chem.* **272**, 9268-9274.
- DeLisle, A. J., Graves, R. A., Marzluff, W. F. and Johnson, L. F. (1983). Regulation of histone mRNA production and stability in serum-stimulated mouse 3T6 fibroblasts. *Mol. Cell Biol.* **3**, 1920-1929.
- Dhawan, J., Rando, T. A., Elson, S. E., Lee, F., Stanley, E. R. and Blau, H. M. (1996). Myoblast-mediated expression of colony stimulating factor-1 (CSF-1) in the cytokine-deficient op/op mouse. *Somat. Cell Mol. Genet.* **22**, 363-381.
- Dike, L. E. and Farmer, S. R. (1988). Cell adhesion induces expression of



- growth-associated genes in suspension-arrested fibroblasts. *Proc. Natl. Acad. Sci. USA* **85**, 6792-6796.
- Freedman, V. H. and Shin, S. I.** (1974). Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium. *Cell* **3**, 355-359.
- Fuchs, E. and Segre, J.** (2000). Stem cells: a new lease on life. *Cell* **100**, 143-155.
- Garry, D. J., Yang, Q., Bassel-Duby, R. and Williams, R. S.** (1997). Persistent expression of MNF identifies myogenic stem cells in postnatal muscles. *Dev. Biol.* **188**, 280-294.
- Grounds, M. D.** (1991). Towards understanding skeletal muscle regeneration. *Pathol. Res. Pract.* **187**, 1-22.
- 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 Tissue Res.* **267**, 99-104.
- 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.
- Irintchev, A., Zeschnigk, M., Starzinski-Powitz, A. and Wernig, A.** (1994). Expression pattern of M-cadherin in normal, denervated, and regenerating mouse muscles. *Dev. Dyn.* **199**, 326-337.
- Jennische, E., Ekberg, S. and Matejka, G. L.** (1993). Expression of hepatocyte growth factor in growing and regenerating rat skeletal muscle. *Am. J. Physiol.* **265**, C122-C128.
- Jesse, T. L., LaChance, R., Iademarco, M. F. and Dean, D. C.** (1998). Interferon regulatory factor-2 is a transcriptional activator in muscle where it regulates expression of vascular cells adhesion molecule-1. *J. Cell Biol.* **140**, 1265-1276.
- Kitzmann, M., Carnac, G., Vandromme, M., Primig, M., Lamb, N. J. and Fernandez, A.** (1998). The muscle regulatory factors MyoD and myf-5 undergo distinct cell cycle-specific expression in muscle cells. *J. Cell Biol.* **142**, 1447-1459.
- Krause, D. S., Fackler, M. J., Civin, C. I. and May, W. S.** (1996). CD34: structure, biology, and clinical utility. *Blood* **87**, 1-13.
- Lassar, A. B., Skapek, S. X. and Novitch, B.** (1994). Regulatory mechanisms that coordinate skeletal muscle differentiation and cell cycle withdrawal. *Curr. Opin. Cell Biol.* **6**, 788-794.
- 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.
- Liang, P. and Pardee, A. B.** (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**, 967-971.
- Mauro, A.** (1961). Satellite cells of skeletal muscle fibers. *J. Biophys. Biochem. Cytol.* **9**, 493-494.
- McTigue, D. M., Tani, M., Krivacic, K., Chernosky, A., Kelner, G. S., Maciejewski, D., Maki, R., Ransohoff, R. M. and Stokes, B. T.** (1998). Selective chemokine mRNA accumulation in the rat spinal cord after contusion injury. *J. Neurosci. Res.* **53**, 368-376.
- Milasinic, D. J., Dhawan, J. and Farmer, S. R.** (1996). Anchorage-dependent control of muscle-specific gene expression in C2C12 mouse myoblasts. *In Vitro Cell Dev. Biol. Anim.* **32**, 90-99.
- Nickoloff, B. J.** (1991). The human progenitor cell antigen (CD34) is localized on endothelial cells, dermal dendritic cells, and perifollicular cells in formalin-fixed normal skin, and on proliferating endothelial cells and stromal spindle-shaped cells in Kaposi's sarcoma. *Arch. Dermatol.* **127**, 523-529.
- Olson, E. N.** (1992). Interplay between proliferation and differentiation within the myogenic lineage. *Dev. Biol.* **154**, 261-272.
- Olwin, B. B. and Hauschka, S. D.** (1988). Cell surface fibroblast growth factor and epidermal growth factor receptors are permanently lost during skeletal muscle terminal differentiation in culture. *J. Cell Biol.* **107**, 761-769.
- Omori, N., Omori, M., Everts, R. P., Teramoto, T., Miller, M. J., Hoang, T. N. and Thorgeirsson, S. S.** (1997). Partial cloning of rat CD34 cDNA and expression during stem cell-dependent liver regeneration in the adult rat. *Hepatology* **26**, 720-727.
- Orimo, S., Hiyama, E., Arahata, K. and Sugita, H.** (1991). Analysis of inflammatory cells and complement C3 in bupivacaine-induced myonecrosis. *Muscle Nerve* **14**, 515-520.
- Pavlat, G. K., Thaloer, D., Rando, T., Cheong, M., English, A. W. and Zheng, B.** (1998). Heterogeneity among muscle precursor cells in adult skeletal muscles with differing regenerative capacities. *Dev. Dyn.* **212**, 495-505.
- Scott, D. A., Greinwald, J. H., Jr, Marietta, J. R., Drury, S., Swiderski, R. E., Vinas, A., DeAngelis, M. M., Carmi, R., Ramesh, A., Kraft, M. L. et al.** (1998). Identification and mutation analysis of a cochlear-expressed, zinc finger protein gene at the DFN7/11 and dn hearing-loss loci on human chromosome 9q and mouse chromosome 19. *Gene* **215**, 461-469.
- Seale, P. and Rudnicki, M. A.** (2000). A new look at the origin, function, and "stem-cell" status of muscle satellite cells. *Dev. Biol.* **218**, 115-124.
- Seale, P., Sabourin, L. A., Girgis-Gabardo, A., Mansouri, A., Gruss, P. and Rudnicki, M. A.** (2000). Pax7 is required for the specification of myogenic satellite cells. *Cell* **102**, 777-786.
- Shaw, G. and Karner, R.** (1986). A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* **46**, 659-667.
- Sheehan, S. M. and Allen, R. E.** (1999). Skeletal muscle satellite cell proliferation in response to members of the fibroblast growth factor family and hepatocyte growth factor. *J. Cell Physiol.* **181**, 499-506.
- Smerdu, V., Karsch-Mizrachi, I., Campione, M., Leinwand, L. and Schiaffino, S.** (1994). Type IIx myosin heavy chain transcripts are expressed in type IIb fibers of human skeletal muscle. *Am. J. Physiol.* **267**, C1723-C1728.
- Smith, J. B. and Herschman, H. R.** (1995). Glucocorticoid-attenuated response genes encode intercellular mediators, including a new C-X-C chemokine. *J. Biol. Chem.* **270**, 16756-16765.
- Stoker, M., O'Niell, C., Barryman, S. and Waxman, B.** (1968). Anchorage and growth regulation in normal and virus transformed cells. *Int. J. Cancer* **3**, 683-693.
- Takayama, H., la Rochelle, W. J., Anver, M., Bockman, D. E. and Merlino, G.** (1996). Scatter factor/hepatocyte growth factor as a regulator of skeletal muscle and neural crest development. *Proc. Natl. Acad. Sci. USA* **93**, 5866-5871.
- 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.
- Taylor, G. A., Carballo, E., Lee, D. M., Lai, W. S., Thompson, M. J., Patel, D. D., Schenkman, D. I., Gilkeson, G. S., Broxmeyer, H. E., Haynes, B. F. and Blackshear, P. J.** (1996). A pathogenetic role for TNF alpha in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency. *Immunity* **4**, 445-454.
- Taylor, J. M., Dupont-Versteegden, E. E., Davies, J. D., Hassell, J. A., Houle, J. D., Gurley, C. M. and Peterson, C. A.** (1997). A role for the ETS domain transcription factor PEA3 in myogenic differentiation. *Mol. Cell Biol.* **17**, 5550-5558.
- Varnum, B. C., Lim, R. W., Sukhatme, V. P. and Herschman, H. R.** (1989). Nucleotide sequence of a cDNA encoding TIS11, a message induced in Swiss 3T3 cells by tumor promoter tetradecanoyl phorbol acetate. *Oncogene* **4**, 119-120.
- Weissman, I. L.** (2000). Stem cells: units of development, units of regeneration, and units in evolution. *Cell* **100**, 157-168.
- Wuyts, A., Haelens, A., Proost, P., Lenaerts, J. P., Conings, R., Opendakker, G. and van Damme, J.** (1996). Identification of mouse granulocyte chemotactic protein-2 from fibroblasts and epithelial cells. Functional comparison with natural KC and macrophage inflammatory protein-2. *J. Immunol.* **157**, 1736-1743.
- Wuyts, A., Proost, P. and van Damme, J.** (1998). Interleukin-8 and other CXC chemokines. In *The Cytokine Handbook* (ed. A. W. Thomson), pp. 271-281. London: Academic Press.
- Wuyts, A., D'Haese, A., Cremers, V., Menten, P., Lenaerts, J. P., de Loof, A., Heremans, H., Proost, P. and van Damme, J.** (1999). NH2- and COOH-terminal truncations of murine granulocyte chemotactic protein-2 augment the in vitro and in vivo neutrophil chemotactic potency. *J. Immunol.* **163**, 6155-6163.
- Yaffe, D. and Saxel, O.** (1977). Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature* **270**, 725-727.
- Yoshida, N., Yoshida, S., Koishi, K., Masuda, K. and Nabeshima, Y.** (1998). Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells'. *J. Cell Sci.* **111**, 769-779.
- Zador, E., Mandler, L., Takacs, V., de Bleecker, J. and Wuytack, F.** (2001). Regenerating soleus and extensor digitorum longus muscles of the rat show elevated levels of TNF-alpha and its receptors, TNFR-60 and TNFR-80. *Muscle Nerve* **24**, 1058-1067.