Myogenic Factor Expression and Quantitation in Satellite Cells Derived from Growing Rat Skeletal Muscle

Meng-Ling Ho, Greg Molnar, Vicky L. Funanage and Nancy A. Schroedl

Department of Clinical Science Nemours Research Programs, Alfred I. duPont Institute, Wilmington, USA

Abstract

Satellite cells express an intrinsic myogenic program that defines them as postnatal myoblasts. This myogenic program is best exemplified by the ability of satellite cells to proliferate, differentiate, and form contraction-competent myotubes *in vitro*. A class of muscle-specific transcription factors (including MyoD, myogenin, MRF4 and Myf5) have been implicated in the modulation of the myogenic program during muscle development. The purpose of this study was to determine the expression pattern for each myogenic factor and to accurately measure the level of each myogenic factor during the various stages of myogenesis using satellite cells isolated from growing rats. Reverse transcription-polymerase chain reaction (RT-PCR) and slot blot hybridization were used to determine the mRNA expression pattern for each myogenic factor. Several stages of myogenesis were characterized: proliferating cells (2 and 3 d post-plating), early myotube formation (5 d post-plating), later stages of myotube development (8 and 12 d post-plating), and mononucleated cells collected from 5 and 12 d cultures. Each of the myogenic factors was detected by RT-PCR in proliferating satellite cells and satellite cell-derived myotubes; however, the quantity of mRNA differed between, and within, specific stages. Myogenin was the most abundant myogenic factor at all stages and it increased dramatically during the period of myotube formation. MyoD levels were highest during proliferation of satellite cells and decreased during early myotube formation. Myf5 mRNA was not detectable by slot blot analysis during proliferation, but Myf5 mRNA was detectable at the onset of differentiation. MRF4 mRNA levels increased 2-3 fold during myotube maturation but remained 40-fold less abundant than myogenin.

Key words: myogenic factors, myogenesis, satellite cells.

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Satellite cells are postnatal myoblasts that provide nuclei to growing or regenerating muscle cells [32, 36]. During postnatal myogenesis, satellite cells exist as quiescent cells, as proliferating cells, or as cells in the process of differentiation [5, 21, 53]. The myogenic program of a satellite cell is maintained *in vitro* and is exemplified by the ability of satellite cells to proliferate, differentiate, and form contraction-competent myotubes [4, 57]. Since the majority of myofiber nuclei are derived postnatally from satellite cells [1], an understanding of the myogenic program of satellite cells and how this program changes during different developmental stages, would greatly improve our understanding of postnatal growth and repair in skeletal muscle.

Although a few markers that define a myogenic program in proliferating satellite cells have been described, including the intermediate filament desmin [2], H-36 [28] and β-enolase [41], these markers fail to account for different stages of myogenesis prior to differentiation [35, 42], or to account for the different populations of satellite cells based on proliferative and differentiative potentials [15, 34]. Following satellite cell differentiation, satellite cell-derived myonuclei express a myogenic program associated with contractile protein synthesis. This stage of myogenesis has been well characterized because of the dramatic increase in synthesis of contractile proteins following differentiation [24, 51], and the simultaneous formation of easily identifiable myotubes that can be monitored *in vitro*. However, the myogenic program of the myotube is complicated by its requirement to express the different contractile protein isoforms required by fast and slow myofibers [13, 27], and its ability to shift isoform expression in response to different physiological demands [20, 29]. Defining the transcriptional factors that control the myogenic program of proliferating satellite cells and satellite cell-

derived myonuclei could provide valuable information concerning the regulation of postnatal myogenesis.

Myogenin, MyoD, MRF4 and Myf5 belong to a family of muscle-specific transcription factors that are thought to regulate the myogenic program by controlling the activity of several muscle-specific genes [12, 39, 52]. Data from gene knockout experiments [37, 43] and *in vitro* cell culture studies [26, 35] provide a general scenario for each myogenic factor. MyoD and Myf5 seem to establish the identity of the myoblast (myogenic lineage). Myogenin apparently controls the differentiation of myoblasts into myotubes, and MRF4 may be related to myotube maturation. The majority of data characterizing the expression pattern of each myogenic factor has resulted from experiments using tissue or cells derived from embryonic or fetal stages of development. Since satellite cells are biochemically distinct from embryonic and fetal myoblasts [11,16, 23, 54], and have a different role in muscle development [8], it is important to establish the function of each myogenic factor during postnatal myogenesis.

MyoD and myogenin have been detected in mononucleated cells from regenerating skeletal muscle [17, 22]. In addition, both myogenin and MyoD were expressed in proliferating satellite cells isolated from adult murine skeletal muscle [30], or satellite cells sequestered within the basal lamina of isolated muscle fibers [55]. Primary myoblasts isolated from newborn rats expressed MyoD and myogenin during proliferation, but MRF4 and Myf5 expression was not detectable until myoblasts differentiated and formed myotubes [26]. Using reverse transcription-polymerase chain reaction (RT-PCR), no myogenic factors were detected in quiescent satellite cells collected from 9-month old rats; however, all factors were present in proliferating cells and in satellite cell-derived myotubes following differentiation [46].

Data describing the expression of myogenic factors in satellite cells are limited in scope because: 1) only MyoD and myogenin have been measured, 2) cells have been isolated from neonates, and therefore myogenic cells would be composed of both fetal and satellite myoblasts, or 3) the quantity of each myogenic factor has not been measured accurately. The purpose of this study was to determine the expression pattern for each myogenic factor and to quantitate the level of each factor for different stages of postnatal myogenesis using satellite cells isolated from young growing rats.

Material and Methods

Cell culture

Primary cultures of satellite cells were obtained from anterior tibialis muscles of 200-220 g male Sprague-Dawley rats (Hilltop; Scottdale, PA) using a combination of dispase (Collaborative Research; Bedford, MA) and collagenase (Worthington; Freehold, NJ) as previously described [13]. Experiments were conducted using Nunclon culture plates (Nunc; Roskilde, Denmark) coated with a 1:10 dilution of Matrigel (Collaborative Research) as previously described [19]. Culture media consisted of Dulbecco's Modified Eagle Medium (D-MEM) supplemented with 20% fetal bovine serum (FBS; Biocell, Rancho Dominquez, CA) and 30 U/ml of penicillin-streptomycin (GIBCO; Grand Island, NY); at confluency, 20% horse serum (HS; Biocell) was used in place of FBS. For each experiment, cells were inoculated at a density of 5×10^4 /100 mm diameter culture dish and RNA was extracted from six dishes at 3 d and one dish for the remaining time periods (5, 7, 10, and 12 d). At this inoculation density, satellite cells began to form myotubes at 5 d. For subpassage studies, mononucleated cells were liberated from 5 d and 12 d cultures using 0.25% trypsin/1mM EDTA and myotubes were removed by filtration through 20 µm Nytex (Tetco; Lancaster, NY). From this isolation, (10×10^6) mononucleated cells were immediately processed for RNA, or cells were replated for immunocytochemistry and RNA isolation. For some experiments, RNA was isolated from a subclone (L6D1) of the rat L6 cell line (gift from Dr. P.A. Benfield, Dupont Experimental Station, Wilmington, DE). In studies involving this subclone, cells were plated at 3×10^5 cells/100 mm diameter plate and RNA was collected 2 d after plating and 3 d after the initiation of myotube formation.

RNA isolation

Total RNA was isolated from individual 100 mm tissue culture dishes of satellite cells as well as from soleus, anterior tibialis, and cardiac muscles according to the RNAgents method (Promega, Madison, WI) [10]. All RNA samples were quantified spectrophotometrically [44]. RNA samples used for RT-PCR were treated with DNase according to the manufacturer's recommendation (GIBCO; Grand Island NY).

Reverse transcription PCR analysis

Expression of each myogenic factor mRNA was determined using RT-PCR as previously described [9]. MyoD and MRF4 primer pairs were designed according to published rat genomic sequences [25, 49]. Both primers spanned introns so that amplification from contaminating genomic DNA would be detected. Myogenin and Myf5 primer pairs were selected according to published mouse cDNA sequences [7, 18]. For myogenin, contaminating genomic DNA would generate a product of 1421 bp following PCR. Since a rat genomic sequence for Myf5 was not available, rat genomic DNA was amplified by PCR using Myf5 primers (results not shown); no signal less than 1000 bp was detected. Primer pairs for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed according to published sequence data [3]. Embryonic and neonatal myosin heavy chain primers were selected from the 3'-untranslated region of their respective cDNA sequences [38]. The specificity of RT-PCR products was confirmed by restriction enzyme digestion and by northern blotting.

Preparation of cDNA probes

Myogenic factor and myosin cDNAs generated by RT-PCR were ligated into the pCR™II vector using a TA cloning kit (Invitrogen, San Diego, CA). Ligated vectors were transformed into Epicurian Coli XL-1 Blue (Stratagene, La Jolla, CA). Colonies were selected on LB agar containing 50 µg/ml ampicillin and grown in LB liquid medium containing 50 µg/ml ampicillin. Plasmid DNA was isolated according to the rapid plasmid boiling mini-prep procedure [44], or by using a QIAGEN plasmid kit (QIAGEN, Chatsworth, CA). Plasmids were digested by *Eco*RI to confirm that cDNA inserts were of the appropriate size. cDNA inserts were separated using a 1% agarose gel and purified using a GeneClean kit (BIO 101, La Jolla, CA). Purified cDNAs were used as hybridization probes for northern and slot blots.

Northern blot

Northern blotting was used to establish binding specificity for each myogenic factor and myosin cDNA. RNA samples (20 µg) were fractionated by electrophoresis using 1% agarose/formaldehyde gels [44], transferred to Duralon-UV membrane (Stratagene, La Jolla, CA) by capillary diffusion, and immobilized by UV crosslinking. Membranes were prehybridized at 65°C overnight in HYBSOL [composed of 1.5 X SSPE (0.15 M NaCl, 0.01 M NaH_2PO_4, 0.001 M EDTA), 7% SDS, 10% polyethylene glycol (Nr 8000), 100 ng/ml denatured herring sperm DNA (Sigma), and 250 ng/ml heparin (Sigma)] [56]. Each cDNA was labeled with ^{32}P (dCTP) by random priming according to the manufacturer's direction (USB, Cleveland, OH) and added as 1 x 10^6 dpm/ml HYBSOL. Hybridization was carried out at 65°C overnight in fresh HYBSOL. Membranes were washed 4 times (15, 30, 30, and 15 min) at 65°C in 0.2 X SSC/0.1% SDS and exposed to Kodak XAR-5 film for 1-5 days.

Slot blot hybridization

Myogenic factor and myosin mRNAs were quantified by slot blot hybridization. Levels of myogenic factor mRNA were interpolated from a standard curve generated by measuring the amount of labeled myogenic factor probe bound to known concentrations of myogenic factor cDNA. The concentration of each myogenic factor cDNA was determined by spectrophotometer, and serial dilutions were made to establish each standard curve. Standard cDNA samples were mixed with 50 µl of 10X TE (10 mM Tris-HCL, 1 mM EDTA), 50 µl 3N NaOH and heated at 65°C for 15 minutes. Cardiac muscle RNA (10 µg), which does not express the myogenic factors, was added to each cDNA sample to mimic the amount of non-specific RNA in 10 µg RNA samples collected from satellite cell cultures. Standard samples were placed on ice, and 250 µl of 2 M sodium acetate was added; a volume of 350 µl from each sample was immediately vacuum-blotted onto Duralon membrane [44]. After the samples had been loaded onto the membrane, each well was washed twice with 400 µl of 10 X SSC.

Samples of RNA (10 µg) isolated from satellite cell cultures were brought to a constant volume with water and mixed with 20 µl of formamide, 7 µl of formaldehyde (37% v/v), and 2 µl of 20 X SSC. Samples were then heated for 15 min at 65°C and placed onto ice. A volume of 35 µl from each sample was vacuum-blotted onto Duralon membrane. After the samples had been loaded onto the membrane, each well was washed twice with 400 µl of 10 X SSC. The membrane was removed from the blotting apparatus and crosslinked with Stratagene's Stratalinker using the autocrosslink setting. Slot blots were prehybridized, hybridized, and washed using the conditions established for northern blots. An example of the methodology used to quantitate each myogenic factor is shown in Figure 1 for MyoD. To generate a standard curve, unlabeled MyoD probe was diluted to different concentrations (Figure 1A, lanes 1-6, respectively), and the amount of labeled probe bound was measured (Figure 1D). From the standard curve, the amount of probe bound to 10 µg of total RNA could be interpolated. Binding of the MyoD probe was shown to be specific for MyoD cDNA (Figure 1C, lane 2) as it did not cross react with myogenin, MRF4, or Myf5 cDNAs (Figure 1C, lanes 1, 3, and 5, respectively). For quantitation of slot blots, a separate autoradiogram for each myogenic factor was used. For each time period, 10 µg RNA samples from two separate experiments were loaded onto separate wells and analyzed. Each autoradiogram was scanned using an Apple Color One Scanner with Ofoto scanning software (Apple Computer, Cupartino, CA). Each scanned image was imported into the NIH Image (version 1.54) program and quantified (integrated density) using gel plotting macros.

Immunocytochemistry

Monoclonal antibodies MF-20 and D3, which have specificity for skeletal muscle myosin and desmin, respectively, were collected from hybridomas obtained through the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the NICHD. Hybridomas were grown according to the recommendation of the Developmental Studies Hybridoma Bank. For each experiment, a six-well plate (35 mm diameter/well) was inoculated with 2500 cells/well (three wells were used for desmin analysis and three wells for myosin analysis). At 48 h, cultures were fixed in methanol containing 0.6% hydrogen peroxide and stored at -20°C for 1 h. Each well was rinsed twice with PBS (pH 7.4) and incubated sequentially in 2% bovine serum albumin (BSA) diluted in PBS (pH 7.4) for 30 min, and then D3 or MF-20 supernatant diluted 1:1 in 2% BSA for 60 minutes. Wells were rinsed thoroughly with PBS to remove primary antibody and then horseradish peroxidase-

conjugated goat anti-mouse IgG (diluted 1:500 in PBS containing 0.1 % Triton X-100) was applied for 1 hr at room temperature. Culture wells were rinsed thoroughly with PBS to remove unbound secondary antibody, and the reaction products were detected using diaminobenzidine (Pierce Rockford, IL). The percentage of immunopositive cells was determined microscopically as the ratio of desmin- positive or myosin-positive cells to the total number of cells.

Results

Characterization of satellite cell proliferation and differentiation

Satellite cells derived from rat skeletal muscle proliferate and differentiate *in vitro* to form multinucleated myotubes. Since the objective of this study was to qualitatively and quantitatively determine the expression of myogenic factors during different stages of postnatal myogenesis, each stage of myogenesis was characterized. The percentage of satellite cells within the population of mononucleated cells was determined at 2 d by immunocytochemistry using a monoclonal antibody (D3) specific for the intermediate filament desmin, which is a known marker for satellite cells (Allen et., al 1991; Figure 2). Approximately 75% of the mononucleated cells were desmin-positive at 2 d. Previous studies (manuscript submitted) determined that proliferation of desmin-positive and desmin-negative populations was similar so that approximately 75% of the population remained desmin-positive until the formation of myotubes at 5 d.

Differentiation of satellite cells is biochemically characterized by the detection of numerous contractile proteins and morphologically characterized by the appearance of multinucleated myotubes. Monoclonal antibody MF-20 is specific for sarcomeric myosin heavy chain (MYHC), a subunit of the contractile protein myosin. In the current study, this antibody was used to identify differentiated mononucleated cells at 48 h in culture. Approximately 1% of the mononucleated cells were myosin-positive at 48 h (Figure 2). These results indicated that at least two populations of satellite cells were present at 48 h: a differentiated (myosin-positive, desmin-positive) population of cells representing approximately 1% of the mononucleated cells, and a desmin-positive, myosin-negative population of mononucleated cells representing approximately 74% of the cells. With regard to myogenic factor expression, these results suggested that at no time after 48 h is there a homogeneous population of satellite cells with respect to their stage of differentiation, and thus, caution must be used when attempting to demarcate distinct periods of myogenesis using primary cultures.

Myotubes were evident by morphological examination at 5 d. The number and size of myotubes increased between 5 and 7 d. The expression pattern of embryonic and neonatal MYHC mRNA was determined by slot blot hybridization; this technique was used to detect MYHC isoforms because it would also be used to quantify myogenic factors during the same time periods. Using this protocol, MYHC mRNAs were not detectable at 3 d in culture during the proliferative period of myogenesis. However, embryonic and neonatal isoforms were detected on the fifth day of culture (first day of myotube formation) and their levels increased between 5 and 10 d post-fusion. From 10-12 d, the expression of both isoforms decreased (Figure 3). However, since non-myogenic cells would represent a larger percentage of the total cell population at 12 d, relative to earlier time periods, the percentage of myosin RNA from myogenic cells should decrease.

Validation of RT-PCR

Reverse transcription-PCR was used to determine the pattern of myogenic factor expression during different stages of postnatal myogenesis in primary cultures of rat satellite cells. Since the accuracy of RT-PCR depends on specificity of base pair formation between the cDNA strand to be amplified during the PCR stage and the homology between forward and reverse primers, we validated our primer pairs. The results (Figure 4) demonstrated that primer pairs selected for each myogenic factor amplified a single cDNA of less than 1000 bp. Further, the amplified products contained the predicted number of base pairs estimated from either the genomic or cDNA sequences used to select each primer pair (Table 1). Restriction analysis confirmed that the RT-PCR products obtained corresponded to the myogenic factor in question. We also demonstrated that each primer pair was specific for cDNAs generated from mRNA expressed in both adult soleus and anterior tibialis muscle, but not in fibroblasts or cardiac muscle.

Myogenic factor expression during postnatal myogenesis in vitro

The expression pattern of myogenic factors was examined by RT-PCR. Myogenic factors were detected using RT-PCR during all stages of myogenesis *in vitro*. Detection of all myogenic factors during the proliferative and early differentiating stages of myogenesis was unexpected, and prompted us to determine if other muscle-specific mRNAs were present, and if this pattern of expression was unique to satellite cells. Towards these ends, expression of embryonic and neonatal MYHC isoforms was determined using RT-PCR, and expression patterns for myogenic factors and MYHC were compared between satellite cells and a subclone (L6D1) of the L6 cell line (Table 2). Satellite cells expressed the embryonic isoform of MYHC during all stages *in vitro*, but expression of the neonatal isoform was not detected until day 5 in culture. In L6D1 cells, MyoD and MRF4 were not detected during proliferation, but all myogenic factors were detected following myotube formation. L6D1 cells expressed the embryonic isoform of myosin during proliferation, but the neonatal isoform was only expressed following myotube formation. These results suggest that in contrast to L6D1 myoblasts, satellite cells express all myogenic factors during all stages of myogenesis *in vitro*.

Table 1. Primers and Products of PCR Amplification Reaction.

Gene	Primer Pairs		Product size (bp)
MyoD	AGA ATG GCT ACG ACG CCG CCT ACT AC	(F)	376
	CAT TTT TGG GGG GGC ATG AAG GAC CC	(R)	
Myogenin	GAG CGC GAT CTC CGC TAC AGA GG	(F)	380
	CTG GCT TGT GGC AGC CCA GG	(R)	
MRF4	CAG TGG CCA AGT GTT TCG GAT CAT TC	(F)	263
	AAT TCT GGG GTC TGT GAG CCC TAA TC	(R)	
Myf5	TGC CTG AAT GTA ACA GCC CTG TCT G	(F)	292
	AGG AGT GAT CAT CGG GAG AGA GTT C	(R)	
Myosin Embryonic	GAG CAT GTC CTC TTG GTG AG	(F)	101
	GCA TGT GGA AAG GGG TTA CG	(R)	
Myosin Neonatal	GTA AAC GCA TCT TGA GGA GG	(F)	126
	GGT TTT ATT CAG TCA GCA GTG	(R)	
GAPDH	GGT CGG TGT CAA CGG ATT TG	(F)	350
	GAG ATG ATG ACC CTT TTG GC	(R)	

Quantitation of myogenic factor expression

Table 2. Summary of the Expression Pattern of Myogenic Factors and Myosin Isoforms in Primary Cell Culture and the L6D1 Cell Line by RT-PCR.

Satellite Cells	MyoD	MyoG	MRF4	Myf5	myhc-emb	myhc-neo
2 day	+	+	+	+	+	-
3 day	+	+	+	+	+	-
5 day (fusion 1)	+	+	+	+	+	+
7 day (fusion 3)	+	+	+	+	+	+
10 day (fusion 6)	+	+	+	+	+	+
12 day (fusion 8)	+	+	+	+	+	+
L6D1Cells						
Myoblast	-	+	-	+	+	-
Myotube	+	+	+	+	+	+

The RT-PCR method used in the study was not quantitative. Therefore, RT-PCR products were subcloned and used as hybridization probes. Northern blots demonstrated that each myogenic factor cDNA used as a hybridization probe was specific for its respective mRNA. Since the level of myogenic factor mRNA was interpolated from standard curves (see Figure 1 in Materials and Methods), the level of each myogenic factor could be compared within a specific time period and between different time periods. Results obtained from quantitation of the myogenic factors indicate a dramatic difference in the level of expression that was not apparent using RT-PCR (Figure 5). During all stages of myogenesis, myogenin was the most abundant myogenic factor. During proliferation of satellite cells (3 d in culture), myogenin was 3-fold more abundant than MyoD and 21-fold more abundant than MRF4. Myf5 was not detectable by slot blot analysis at 3 d in culture. Between 3 d and the initiation of myotube formation at 5 d, myogenin levels increased 12-fold and the expression of the MyoD and MRF4 remained the same, however, Myf5 was now detectable and expressed at a similar level as MRF4. Thus, at the initiation of myotube formation, myogenin was 42-fold more abundant than MyoD, and approximately 200-fold more abundant than MRF4 and Myf5.

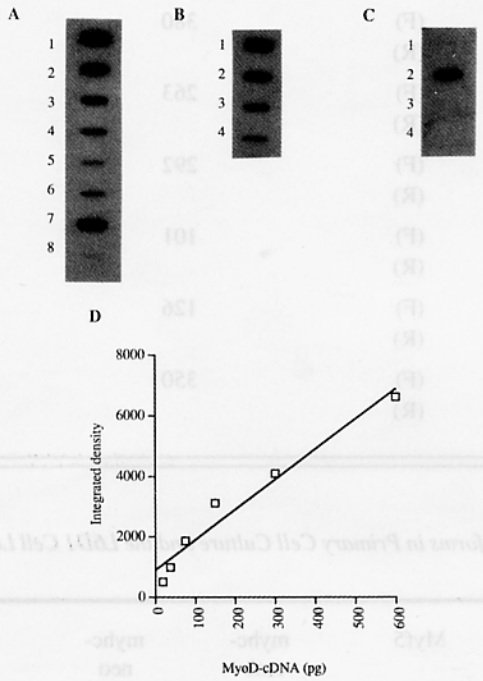

Figure 1. Methodology used to quantitate myogenic factor expression. Myogenic factor cDNAs derived from RT-PCR were subcloned, labeled with ^{32}P, and used as hybridization probes. (A) Binding of labeled probe (MyoD in this example) to serially diluted (600, 300, 150, 75, 19 and 38 pg; slots 1-6, respectively) unlabeled MyoD cDNA was used to establish a standard curve ($R^2 = 0.96$) as shown in D. Slot 7 contains 10 µg of total RNA collected from satellite cell-derived myotubes, and slot 8 contains 10 µg of RNA isolated from rat heart muscle. Responsiveness of the MyoD probe to different levels of total RNA (10, 5, 2.5 and 1.25 µg; slots 1-4 respectively) is shown in B. The lack of crossreactivity of labeled MyoD to other myogenic factor cDNAs (300 pg of myogenin, MyoD, MRF4, and Myf5, slots 1-4, respectively) is shown in (C).

From the initiation of myotube formation to the end of the experimental period (7 d later), myogenin, MyoD, and Myf 5 levels decreased from 2-4 fold. In contrast, during the same time period, MRF4 levels increased 2-3 fold. However, at the end of the experimental period, myogenin mRNA was still 40-fold more abundant than MRF4.

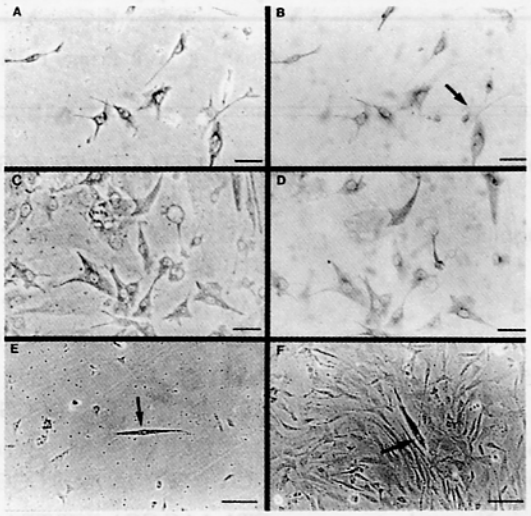

Figure 2. Desmin (A-D) and myosin (E,F) expression in mononucleated cells collected from anterior tibialis muscles of 6 week old rats. A,B represent phase contrast (A) and bright field (B) micrographs of cultures that were fixed and stained with anti-desmin (D3) antibody at 48 h. Arrow in (B) denotes a non-desmin staining cell. C, D represent phase contrast (C) and bright field (D) micrographs of mononucleated cells collected following Nytex filtration of 8 d post-fusion cultures; 25 % of mononucleated cells were desmin positive 48 h after subpassage. A small number (less than one percent) of mononucleated cells 48 h after inoculation (arrow in E), or 48 h after subpassaging 8 d post-fusion mononucleated cells (arrow in F), reacted positively with anti-myosin antibody (MF-20). In A, B, C, and D the bar represent 250 µm; for E and F the bar equals 100 µm.

Expression of myogenic factors in 5 d and 12 d mononucleated cells

Differentiation of satellite cells and the formation of myofibers is not a synchronous process *in vitro*, and after 12 d in culture there is still a small population of mononucleated satellite cells. Based on the expression of desmin (Figure 2), these satellite cells were similar to the satellite cells found immediately after isolation. Thus, it was of interest to determine if mononucleated cells collected 8 d (12 d of culture) following the initiation of myotube formation expressed myogenic factors in a similar pattern relative to mononucleated cells collected on the first day (5 d of culture) of myotube formation. Mononucleated cells collected on the first day of myotube formation, or 8 d after myotube formation, expressed myogenin and MyoD in ratios of 37:1 and 27:1, respectively; MRF4 and Myf5 mRNAs were not detected in mononucleated cells collected 8 d post-fusion (Figure 5). Although the concentration of myogenin and MyoD is decreased in mononucleated cells isolated 8 d after myotube formation, the ratio

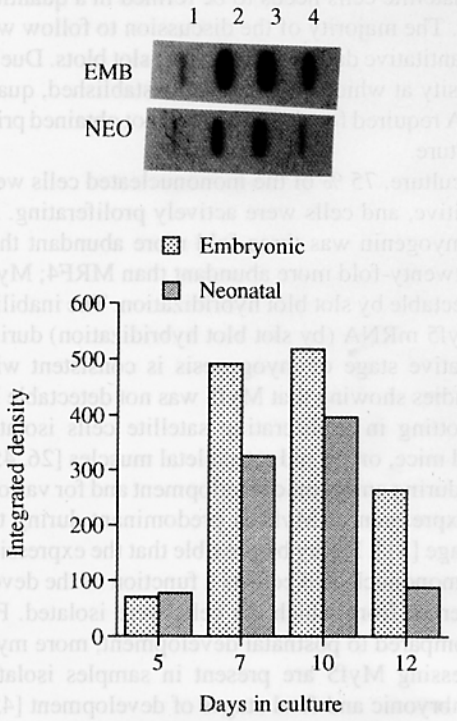

Figure 3. Expression pattern of embryonic (EMB) and neonatal (NEO) myosin heavy chain mRNA isoforms. (Top) Autoradiograms of slot blots from RNA isolated at 5 (day 1 of fusion), 7, 10 and 12 d of culture (lanes 1-4, respectively). Ten micrograms of total RNA was probed with embryonic and neonatal isoform-specific cDNAs. (Bottom) Integrated density from autoradiograms is shown graphically.

of myogenin to MyoD approximates the ratio found in mononucleated cells isolated at d 1 of myotube formation and is dissimilar to the ratio of 3:1 detected in proliferating satellite cells from 3 d cultures.

Discussion

Satellite cells retain a myogenic program that distinguishes them as postnatal myoblasts and endows them with the capacity to proliferate and differentiate during postnatal muscle development. Since the myogenic transcription factors MyoD, Myf5, MRF4 and myogenin regulate many aspects of muscle development, it was our objective to determine their expression pattern in satellite cells isolated from anterior tibialis muscles of growing rats. This study is unique because it is the first to accurately quantitate the levels of all myogenic factors in satellite cells or satellite cell-derived myotubes. Using RT-PCR, mRNA for all four myogenic factors was found to be present during proliferation and various stages of differentiation. By slot blot

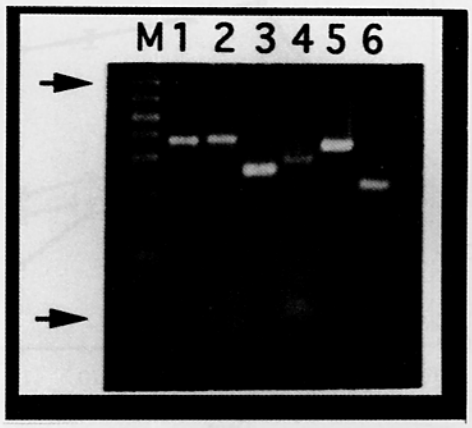

Figure 4. Validation of RT-PCR products of myogenic factors. RT-PCR products of myogenic factors showing single product formation: MyoD (376 bp), myogenin (380 bp), MRF4 (263 bp), Myf5 (292 bp), GAPDH (350 bp) and CKM (227 bp) (lanes 1-6, respectively). Molecular weight markers (M) include 1000 (arrow), 700, 500, 400, 300, 200, 100 and 50 (arrow) bp.

analysis, the concentrations of myogenic factor mRNAs differed within a specific stage of myogenesis and between different stages of myogenesis. Myogenin was the most abundant myogenic factor during all stages of myogenesis. The level of myogenin peaked at differentiation and subsequently declined. Expression of MyoD was highest in proliferating cells and decreased as differentiation approached. During proliferation, Myf5 was not detectable (by slot blot hybridization) and its level increased slightly at d 1 of differentiation and remained low throughout. In contrast, MRF4 was detectable during proliferation of satellite cells and increased approximately three fold following myotube formation.

The expression pattern for all myogenic factors has been determined by RT-PCR in primary cultures of satellite cells isolated from non-growing rats [46]. These investigators reported that MyoD was expressed after 12 h in culture and seemed to be associated with activation of satellite cell proliferation. MRF4 and Myf5 expression was detected by 48 h. Expression of myogenin was detected at 72 h and coincided with the first stages of differentiation. In another study [45], satellite cells cultured from 1-d-old mice expressed all myogenic factor proteins *in vitro*. In this study, myogenin and MRF4 expression followed the expression of MyoD and Myf5 by 1 d, and coincided with myogenic differentiation. Our data obtained using RT-PCR agree with the aforementioned studies at the qualitative level and add support to the idea that primary cultures of satellite cells express all myogenic factors. However, since each myogenic factor has the potential to regulate itself, or

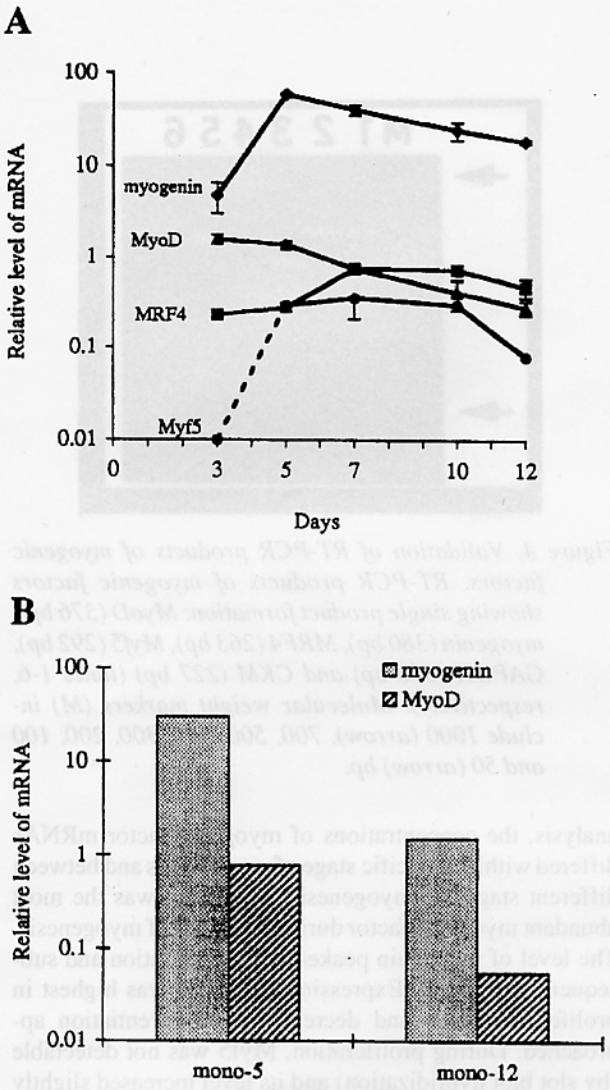

Figure 5. Quantitation of myogenic factor expression during different stages of satellite cell culture. (A) RNA from cells in culture was collected at days 3, 5, 7, 10, and 12. Samples (10 µg) were probed with ^{32}P-labeled myogenin, MyoD, MRF4, and Myf5 cDNA. Values were interpolated from standard curves generated by measuring the amount of labeled myogenic factor probe bound to known concentrations of myogenic factor cDNA. Data are expressed as the relative level of mRNA on a logarithmic scale. Results are presented as the mean ± standard error from two separate experiments. The dotted line indicates that Myf5 was not detectable at 3 days in culture. (B) Mononucleated cells were collected from day 5 (mono-5) and day 12 (mono-12) cultures (myotubes were removed by filtration through 20 µm Nytex). Myogenin and MyoD mRNA expression were compared for each population of mononucleated cells.

regulate the expression of other myogenic factors [6], and since the ability to act as transcription factors may depend upon the level of a particular myogenic factor [40], the notion that all myogenic factors are present in primary cultures of satellite cells needs to be refined in a quantitative manner. The majority of the discussion to follow will focus on quantitative data obtained using slot blots. Due to the low density at which cultures were established, quantities of RNA required for slot blots were not obtained prior to 3 d in culture.

At 3 d in culture, 75 % of the mononucleated cells were desmin-positive, and cells were actively proliferating. At this stage, myogenin was three-fold more abundant than MyoD and twenty-fold more abundant than MRF4; Myf5 was not detectable by slot blot hybridization. The inability to detect Myf5 mRNA (by slot blot hybridization) during the proliferative stage of myogenesis is consistent with previous studies showing that Myf5 was not detectable by northern blotting in proliferating satellite cells isolated from 1 d old mice, or 1 d old rat skeletal muscles [26, 45]. In contrast, during embryonic development and for various cell lines, expression of Myf5 is predominant during the myoblast stage [31]. It may be possible that the expression of Myf5 in mononucleated cells is a function of the developmental period from which the cells were isolated. For example, compared to postnatal development, more myoblasts expressing Myf5 are present in samples isolated from the embryonic and fetal stages of development [45]. Since formation of skeletal muscle occurs in distinct stages from discrete populations (embryonic, fetal and satellite myoblasts) of myogenic cells [11, 16, 47, 55], it may not be surprising that differences in the expression of myogenic factors could occur between different populations of myoblasts.

Expression of MRF4 during the proliferative stage of myogenesis is paradoxical in light of the many reported studies that suggest expression of MRF4 occurs during, or following, differentiation of myoblasts [26, 35, 45]. Although RT-PCR and immunocytochemistry demonstrated that myosin was being produced by a small (approximately 1%) population of satellite cells, it seems unlikely that MRF4 expression in this population would be greater than embryonic or neonatal MYHC isoforms which were not detected by slot blot hybridization. In a population of satellite cells devoid of any differentiated cells, MRF4 was expressed during proliferation [46]; however, MRF4 was not detected in proliferating satellite cells isolated from 1 d old mice [45], or in primary cultures from 1 d old rats [26]. Thus, satellite cells collected from adult skeletal muscle may differ in their expression of MRF4 relative to satellite cells collected immediately following birth. Since neonatal skeletal muscle contains a combination of fetal and satellite cell myoblasts, the inability to detect MRF4 during this period could result from a dilution effect caused by the fetal myoblasts which may not express MRF4, or which may express MRF4 at lower levels relative to satel-

lite cells. MRF4 expression may therefore, be a characteristic of mononucleated satellite cells.

The ratio of myogenin to MyoD shifted from approximately 3:1 to approximately 37:1 as satellite cells began to differentiate and form myotubes. This shift in the ratio of myogenin to MyoD in mononucleated cells resulted mainly from an increase in the expression of myogenin. Similar results (myogenin levels > MyoD levels) were previously derived in 2 d cultures of satellite cells isolated from different strains of mice [30]. By immunocytochemistry, it has recently been documented that rat satellite cells associated with an isolated muscle fiber can co-express both MyoD and myogenin, and that MyoD expression occurs approximately 24 h prior to the expression of myogenin [55]. Taken together, these studies indicate that satellite cells shift their pattern of myogenic factor expression (from MyoD to myogenin) at both the mRNA and protein level as differentiation approaches whether a satellite cell is associated with or independent of its basal lamina.

Following myotube formation on day 5, MyoD and myogenin levels decline at a time when the expression of MYHC and the number of myotubes was increasing both in number and size. This decreased expression of myogenin and MyoD likely coincided with the conversion of a satellite cell into a satellite cell-derived myofiber nucleus. However, myogenic factor expression following myotube formation must be interpreted with the knowledge that non-myogenic cells will continue to proliferate and represent a greater percentage of cells with increased culture age. Thus, decreased expression of MyoD and myogenin will in part result from the increased percentage of non-myogenic cells in the culture.

MRF4 levels increased following differentiation, a pattern that is characteristic of this myogenic factor [26, 45]. However, at its highest level of expression, MRF4 was still 53-fold less abundant than myogenin. Since MRF4 was the most abundant myogenic factor in the adult anterior tibialis muscle [50] from which the satellite cells were derived, it seems logical that satellite cell-derived myotubes lack the appropriate signals to downregulate myogenin and/or upregulate MRF4. Myogenin expression is known to be repressed by the electrical activity associated with innervation [14], and is increased following denervation [33]. The lack of muscle/nerve interactions *in vitro* may explain the imbalance of myogenic factors in satellite cell-derived myotubes. Also, satellite cell-derived myotubes fail to express numerous contractile protein isoforms characteristic of adult skeletal muscle [48]. This lack of maturation *in vitro* could directly result from the imbalance of myogenic factors and indirectly result from the lack of innervation. We have initiated studies to determine the effect of innervation on myogenic factor expression, and subsequent maturation of satellite cell-derived myotubes *in vitro*.

In this study we have begun to define the myogenic program of satellite cells. The expression pattern and quantity of myogenic factors thought to be responsible for regulating many aspects of the myogenic program have been determined. From this study it is clear that satellite cells have a program of myogenic factor expression distinct from established myoblast cells lines, or expression during embryonic development. Based on the level of expression, myogenin should have the greatest impact on the myogenic program of satellite cells *in vitro*.

Understanding what factors modulate the expression of myogenic factors and subsequently alter the myogenic program has important implications towards understanding maturation of myofibers, growth of myofibers, and repair of myofibers.

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Address correspondence to:

Greg Molnar, Ph.D., Department of Clinical Science, Nemours Research Programs, Alfred I. duPont Institute, P.O. Box 269, Wilmington, DE, USA 19899, tel. 302 651 6815, fax 302 651 6897, e-mail Molnar@helios.medsci.udel.edu.

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