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Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation

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Running Title: Mechanism of myostatin function
Summary

Myostatin, a member of TGF-β superfamily has been shown to be a negative regulator of myogenesis. Here we show that myostatin functions by controlling the proliferation of muscle precursor cells. When C2C12 myoblasts were incubated with myostatin, proliferation of myoblasts decreased with increasing levels of myostatin. FACS analysis revealed that myostatin prevented the progression of myoblasts from the G1 to S-phase of the cell cycle. Western analysis indicated that myostatin specifically up-regulated p21\(^{\text{Waf1, Cip1}}\), a cyclin-dependent kinase inhibitor (CKI) and decreased the levels and activity of Cdk2 protein in myoblasts. Furthermore we also observed that in myoblasts treated with myostatin protein, Rb was predominately present in the hypophosphorylated form. These results suggests that, in response to myostatin signaling, there is an increase in p21 expression and a decrease in Cdk2 protein and activity thus resulting in an accumulation of hypophosphorylated Rb protein. This, in turn, leads to the arrest of myoblasts in G1-phase of cell cycle. Thus, we propose that the generalized muscular hyperplasia phenotype observed in animals that lack functional myostatin could be as a result of deregulated myoblast proliferation.
Introduction

The Transforming Growth Factor-β (TGF-β) superfamily of genes encode secreted factors that are important for regulating embryonic development and tissue homeostasis in adults. Recently, McPherron et al. (1) described a new member of this family, myostatin, that is expressed in developing and adult skeletal muscle. Myostatin-null mice show a dramatic and widespread increase in skeletal muscle mass due to an increase in number of muscle fibers (hyperplasia) and thickness of fibers (hypertrophy) (1). Subsequently, we (2) and others (3,4) reported that the Belgian Blue and Piedmontese breeds of cattle which are characterized by an increase in muscle mass (double-muscling), have mutations in the myostatin coding sequence. Hence the function of myostatin as a regulator of muscle mass is very well established.

Myostatin shares several features with other members of the TGF-β superfamily: 1) a hydrophobic core of amino acids near the N-terminus that function as a secretory signal; 2) a conserved proteolytic processing signal of RSRR in the C-terminal half of the protein; 3) nine cysteine residues in the C-terminal region to facilitate the formation of “cysteine knot” structure. Myostatin protein is synthesized in skeletal muscle as a 375 amino acid pro-peptide, which is proteolytically processed at the RSRR (263-266) site to give rise to a 26 kD active processed peptide (5). This processed mature peptide binds to receptor to elicit biological function (6).

Myostatin gene expression appears to be developmentally regulated (1,2). Initially myostatin gene expression is detected in myogenic precursor cells of the myotome compartment of developing somites and the expression is continued in adult axial and paraxial muscles (1). Different axial and paraxial muscles have been shown to express different levels of myostatin (2). Although initial reports describing the expression of myostatin gene suggested that myostatin expression is exclusive to skeletal muscle, more recent publications
have shown that myostatin mRNA or protein is detected in other tissues. A report using myostatin specific antibodies indicates that myostatin protein is present in cardiomyocytes and purkinje fibers of heart (5), while Ji et al. (7) detected myostatin mRNA expression in the mammary gland.

Although the functional role of myostatin in control of muscle mass has been well documented by genetic models, the mechanism by which myostatin controls muscle fiber number is not known. Because myostatin expression is detected in somites during embryonic myogenesis, and its expression is continued in postnatal muscle, myostatin may very well play a role at all the stages of myogenesis. During myogenesis, myoblasts proliferate and withdraw from cell cycle at the first gap phase, G1, and commit to a differentiation pathway to form myotubes (8). Work over the past several years on myogenic differentiation has elucidated a hierarchical regulatory mechanism for the control of growth and differentiation of myoblasts. After stimulation in vitro by an appropriate environmental signal, such as serum deprivation, members of the basic helix-loop-helix transcription factor family, termed Myogenic Regulatory Factors (MRFs), initiate a cascade of events leading to the expression of muscle specific genes. Analysis of mice lacking individual MRFs have revealed a genetic hierarchy for their function. MyoD and Myf-5 play redundant roles in specifying a muscle lineage, that is, the formation of myoblasts (9-11). Myogenin, by contrast, has been shown to be required for the differentiation of myoblasts (12,13), while MRF4 is thought to be involved in the maturation of myotubes (14). Coupled with the induction of muscle specific genes is the permanent withdrawal of proliferating myoblasts from the cell cycle to become terminally differentiated myotubes. The connection between myoblast cell cycle withdrawal and differentiation is established through regulation of cyclin-dependent kinases (Cdks), a family of enzymes that catalyze events required for cell cycle transitions. Primary targets for this regulation are the G1 cyclin/Cdk complexes, cyclin-D/Cdk4 and cyclin-E/Cdk2, which
cooperate to control the G1 to S transition through phosphorylation and inactivation of the retinoblastoma (Rb) protein (15). Cyclin-dependent kinase inhibitors (CKIs), of which there are two families, the p16 family and the p21 family, in turn, regulate the kinase activities of the Cdks. The p16 family specifically inhibits Cdk4 and Cdk6, whereas the p21 family inhibits all Cdks involved in G1/S transition (16).

The loss of functional myostatin leads to hyperplasia and hypertrophy of skeletal muscle. Since increased muscle fibers can result from increased myoblast proliferation and delayed differentiation, we investigated the role of myostatin in controlling myoblast proliferation and cell cycle progression. Using cultured C\textsubscript{2}C\textsubscript{12} myoblast cells and recombinant myostatin protein, we show here that myostatin indeed regulates the cell cycle progression of myoblasts by controlling the G1 to S-phase and G2 to M phase transition. We also demonstrate that myostatin accomplishes this by increasing the level of p21 and decreasing Cdk2 protein levels and activity, thereby rendering the Cyclin-E/Cdk2 complex inactive. This results in suppression of the Rb protein phosphorylation \textit{in vivo} and concurrent cell cycle arrest of myoblasts in the G1-phase. The increased number of myofibers seen in cattle and mice with heavy muscling thus appears to be the result of deregulated myoblast proliferation caused by the absence of functional myostatin.
Experimental Procedures

Subcloning and expression of myostatin in E.coli

The pET protein expression system (Novagen; Madison, WI, USA) was used to express and purify recombinant myostatin. A portion of bovine cDNA spanning amino acids 267-375 was amplified by PCR as BamHI fragment and cloned into the pET 16-B vector. The myostatin coding sequence was placed in frame with the 10 histidine residues according to the manufacturer’s protocol (Novagen). The resulting construct was used to transform the BL 21 bacterial strain. An overnight *Escherichia coli* culture harboring the recombinant myostatin expression vector was diluted and grown up to an OD of 0.8 (600nm) in 1 L of Lennox L Broth (LB) medium plus ampicillin (50 mg/L). The myostatin fusion protein was induced by adding 0.5 mM isopropyl thio-β-galactoside (IPTG) to the culture and the induction was continued for 2 hours. Bacteria were collected by centrifugation, resuspended in 40 ml of lysis buffer (6 M guanidine hydrochloride; 20 mM Tris pH 8.0; 5 mM 2-mercaptoethanol) and sonicated. The lysate was centrifuged at 10,000 g for 30 min and myostatin was purified from the supernatant by Ni-Agarose affinity chromatography (Qiagen, Valencia), according to the manufacturer’s protocol. Soluble fractions containing myostatin were pooled and dialyzed against two changes of 50 mM Tris-HCl (pH 8.0) containing 500 mM NaCl and 10% glycerol for 6 hours.

Bovine myoblast culture generation

The AgResearch Ruakura Animal Ethics Committee approved the animal manipulations described in this paper. Standard superovulation and embryo transfer techniques were used to generate foetuses with expected double- and normal-muscled phenotypes as previously described (2). Double-muscled foetuses were Belgian Blue, and normal-muscled foetuses were Hereford x Friesian crossbreed. Cows were slaughtered at the Ruakura abattoir when foetuses were at 160 days of gestation. The M. semitendinosus muscle was excised, cut into
small pieces, placed in Minimal Essential Media containing 20% Fetal bovine serum and 10% DMSO, and frozen in liquid nitrogen for subsequent myoblast culture generation.

Mixed cultures containing both myoblasts and fibroblasts were liberated from thawed M. Semitendinosus by mincing muscle and then digesting with 0.25% trypsin (Sigma Cell Culture Ltd., St Louis, MO) for 45 min at 37°C. Media for subsequent culture consisted of Minimum Essential Medium (MEM; Life Technologies, Grand Island, NY), buffered with 41.9 mM NaHCO₃ (Sigma) and gaseous CO₂. 7.22 nM Phenol red (Sigma) was used as a pH indicator. 1 x 10⁵ IU/L penicillin (Sigma), 100 mg/L streptomycin (Sigma) and 10% fetal bovine serum (Life Technologies Ltd.) were routinely added to media.

The method of O’Malley et al. (17) was used to enrich cultures for myoblasts. A total of 1 x 10⁶ liberated cells were added to Matrigel (Collaborative Biomedical Research, CBR) coated 10 cm dishes (Nunc, Roskilde, Denmark). Matrigel coated dishes were prepared by adding 10 ml of 5.0 ml/L Matrigel in Earle’s Balanced Salt Solution (EBSS; obtained as a 10X concentrate) (Life Technologies Ltd.) to each 10 cm dish and then incubating at 37°C for 16 hours. Cultures were grown on Matrigel for 3 days, then digested with 0.5 g/L Type 1-A collagenase (Sigma) for 10 minutes to preferentially detach fibroblasts. Cultures were washed 2 times with EBSS before, and three times after collagenase digestion. Myoblast cultures were then grown in MEM media containing 10% FBS for an additional 24 hours before trypsinization and seeding into the methylene blue cell proliferation assay. When these myoblast cultures were cloned and cultured under differentiation inducing conditions, more than 90% of clones stained positive for the muscle specific marker desmin, indicating a predominance of myoblasts.

\text{C}_{2}\text{C}_{12} \text{ myoblast proliferation}

\text{C}_{2}\text{C}_{12} \text{ myoblasts (18) were grown prior to assay in Dulbecco’s Modified Eagle Medium (Life Technologies Ltd.) with additives stated above. Cell proliferation assays were}
conducted in either uncoated ($C_{2}C_{12}$ cultures) or gelatin coated (bovine myoblast primary cultures) 96-well Nunc microtitre plates. Plates were gelatinized by the method of Quinn and Namaroff (19). $C_{2}C_{12}$ cultures were seeded at 1,000 cells/well and bovine myoblast cultures at a density of 3,000 cells/well in relevant media. After a 16-hour attachment period myostatin test media was added. This consisted of 10% fetal bovine serum media with 0-10 $\mu$g/ml of recombinant myostatin. The position of the samples on the plate was randomly assigned and all samples were run in replicates of eight. Results presented in this paper are representative of at least two independent experiments. Plates were then incubated in an atmosphere of 37°C, 5% CO$_2$ for a further 72 hours. After the incubation period proliferation was assessed using a methylene blue photometric endpoint assay as previously described (20). In this assay absorbance at 655 nm is directly proportional to final cell number. Results are presented here as the mean and standard error of eight replicates.

Reversibility of myostatin inhibitory effect on proliferation

$C_{2}C_{12}$ myoblasts were seeded into Nunc 96-well plates at a density of 1,000 cells/well. After an overnight attachment period (time zero) plates were washed once with DMEM/10% FBS and test media added. One set of eight replicate wells received DMEM/10% FBS media without myostatin (control wells), whereas wells due to be subsequently ‘rescued’ from myostatin received DMEM/10% FBS containing 4 $\mu$g/ml myostatin. After a further 24-hour incubation period all plates were washed once and DMEM/10% FBS without myostatin was added into control wells and those wells due to be rescued from incubation with myostatin at 24 hours. DMEM/10% FBS containing 4 $\mu$g/ml myostatin was added back to wells due to be rescued subsequently. All plates were washed at 24 hour intervals during the course of the experiment and relevant media added back to wells. Further cultures were ‘rescued’ from incubation with myostatin at 72 and 120 hour time-points as described above. Plates were
fixed at 24 hour intervals throughout the experiment and assayed for cell proliferation as described above.

**Myf-5 Immunocytochemistry, cell staining and photography**

Myoblast cultures derived from a 160-day bovine foetus were seeded (9,000 cells/cm²) onto coverslips (Nunc) and following overnight attachment, incubated with 0 or 8 ug/ml myostatin in growth media for 72 hrs. Cultures were then fixed with 20 parts 70% ethanol: 2 parts formaldehyde; 1 part glacial acetic acid for 30 seconds, followed by three rinses with PBS. Cells were blocked overnight at 4°C in TBS (0.05 M Tris-HCL, pH 7.6 (Sigma); 0.15 M NaCl) containing 1% sheep serum, then incubated with primary antibody (5 ug/ml anti-Myf-5 (Santa Cruz biotechnology, CA) in TBS-1% NSP) for 1 hr at room temperature. Rabbit IgG (5 ug/ml; Dako Co., CA,) was used as a negative control. Following primary antibody incubations, cells were washed three times (5 minutes each) with TBS-Tween (TBS; 0.05% Tween-20) and incubated with secondary antibody, 1:100 dilution of donkey anti-rabbit IgG (Amersham), in TBS-1% NSP for 30 minutes. Cells were then incubated with 1:100 dilution of streptavidin-biotin peroxidase complex (Amersham) for 30 minutes. Myf-5 immunostaining was visualised using 3,3-diaminobenzidine tetrahydrochloride (DAB; Life technologies) enhanced with 0.0375 % CoCl. Myf-5 immuno-stained cultures were lightly counterstained with Gill’s haematoxylin and photographed using an Olympus BX50 microscope (Olympus Optical Co., Germany) fitted with a DAGE-MTI DC-330 colour camera (DAGE-MTI Inc., IN, USA).

To visually assess the effect of myostatin on C2C12 proliferation, cells were seeded onto coverslips (3,000 cells/cm²) and allowed to attach overnight. Cultures were then incubated with or without 4 ug/ml myostatin for 24, 48, or 72 hours and fixed as above. Cells were then stained with 1:1 Gill’s haematoxylin followed by 1% Eosin. Cultures were photographed as above.
Flow cytometry

A method by Darzynkiewicz and Li was used that enabled the analysis of apoptosis in addition to cell cycle analysis. Mouse C2C12 myoblast cells were cultured as described above in 100 mm dishes with or without myostatin treatment for 48 hours. Cells (~3x10⁶) were harvested using trypsin followed by centrifugation and fixed in 800 μl 70% ethanol/PBS. The fixed cells were resuspended in 500 μl PBS + 500 μl DNA extraction buffer (200 mM NaHPO₄; 100 mM citric acid) for 10 minutes at room temperature. DNA extraction buffer was replaced with DNA staining buffer (50 μg/ml Propidium iodide; 50 μg/ml DNase-free RNase A in PBS), vortexed briefly to resuspend cells and incubated in the dark at room temperature for 30 minutes. Cells were then examined for propidium iodide fluorescence using a Becton-Dickinson FACScan® flow cytometer (Becton-Dickinson Immunocytometry Sys., CA) and analyzed using CellFit software (Becton-Dickinson Immunocytometry Sys.).

Western blot analysis

For quantitative CKI, Cdk and cyclin immunoblot analyses, mouse C2C12 myoblasts were cultured as described above in 100 mm dishes with or without myostatin treatment. Cells (~3x10⁶) were resuspended in 200 μl lysis buffer (50 mM Tris pH 7.6; 250 mM NaCl; 5 mM EDTA; 0.1% Nonident P-40; Complete™ (Boehringer Mannheim, Germany) protease inhibitor) and sonicated. The cell extracts were centrifuged to pellet the cell debris and the supernatants frozen at −80°C. Bradford reagent (Bio-Rad Laboratories, CA) was used to estimate total protein content to ensure equal loadings. Precisely 15 μg of total protein was separated by SDS-PAGE (12%) and transferred to nitrocellulose membrane by electroblotting. After blotting the gels were stained in Coomassie Blue to visually insure equal loadings. The membranes were blocked in TBST/5% milk at 4°C overnight, then incubated with the primary antibody for 3 hrs at room temperature. The following primary antibodies were used for immunoblotting; p15, 1:400 dilution of purified rabbit polyclonal
anti-p15 antibody (sc-613; Santa Cruz Biotechnology Inc., Santa Cruz, CA); p16, 1:400 dilution of purified rabbit polyclonal anti-p16 antibody (sc-1207; Santa Cruz Biotechnology Inc.); p21, 1:400 dilution of purified mouse monoclonal anti-p21 antibody (SX118; PharMingen, CA); p27, 1:400 dilution of purified mouse monoclonal anti-p27 antibody (sc-1641; Santa Cruz Biotechnology Inc.); Cdk2, 1:400 dilution of purified mouse monoclonal anti-Cdk2 antibody (sc-2648; Santa Cruz Biotechnology Inc.); Cdk4, 1:400 dilution of purified rabbit polyclonal anti-Cdk4 antibody (sc-601; Santa Cruz Biotechnology Inc.); cyclin-D1, 1:400 dilution of purified mouse monoclonal anti-cyclin-D1 antibody (sc-8396; Santa Cruz Biotechnology Inc.); cyclin-E, 1:400 dilution of purified rabbit polyclonal anti-cyclin-E antibody (sc-481; Santa Cruz Biotechnology Inc.); α-tubulin, 1:3000 dilution of purified mouse monoclonal anti-α-tubulin antibody (DM 1A; SIGMA). The membranes were washed (5 X 5 min) with TBST and further incubated with either anti-mouse IgG HRP conjugate, 1:2000 dilution (W402B; Promega Corp., WI), or anti-rabbit IgG HRP conjugate, 1:1000 dilution (P0448; DAKO, CA), secondary antibodies for 1 hr at room temperature. The membranes were washed as above, and HRP activity was detected using Renaissance® Western blot chemiluminescence (NEL104; NEN Life Science Products Inc., MA).

For pRb phosphorylation studies, rat L6 myoblasts (21) were cultured as described above in 100 mm dishes with or without myostatin treatment. Cells (5-6x10^6) were counted and lysed at 6x10^4 cells/µl in sample buffer (100 mM Tris pH 6.8; 4% SDS; 0.2% Bromophenol Blue; 20% (vol/vol) glycerol) that was brought to boil for 5 minutes prior to addition to the cells. Lysates were boiled for a further 5 minutes and frozen at -80°C. Dithiothreitol was subsequently added to 15 µl of lysate (final concentration, 0.2 M), boiled, fractionated by SDS-PAGE (7.5%) and transferred to PVDF Immobilon-P™ membrane (Millipore Corp., MA) by electroblotting. A purified mouse monoclonal anti-pRb antibody (G3-245; PharMingen), that recognizes both a hyperphosphorylated form and
hypophosphorylated form was used at a 1:200 dilution. Subsequent steps of membrane blocking, antibody incubations, washes and detection were performed as described above.

**Cdk2 immunoprecipitation and histone H1 assay**

For the Cdk2 immunoprecipitation-kinase assay, mouse C2C12 myoblasts were cultured as described above in 100 mm dishes with or without myostatin. Cells were resuspended in lysis buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 0.4% NP40; 2 mM EDTA; 50 mM NaF; 10 mM β-glycerophosphate, 1 mM ATP; 2 mM Sodium Vanadate; 2 mM DTT; Complete™ (Boehringer Mannheim, Germany) protease inhibitor). After 10 passages through a 21-gauge needle, cell lysates were cleared by centrifugation. Protein concentrations were determined using Bradford reagent (Bio-Rad Laboratories, CA). 200 µg of each extract were immunoprecipitated with 1:20 dilution polyclonal anti-Cdk2 (M2, sc-163; Santa Cruz Biotechnology Inc.) in 100 µl for 1 hr at 4°C. Protein A-Agarose (Gibco-BRL; 50 µl of 50% -washed twice with lysis buffer) was added for 1 hr at 4°C, followed by centrifugation to pellet immunoprecipitated complexes. After centrifugation, pellets were washed three times with lysis buffer, twice in lysis buffer containing 400 mM NaCl and twice in kinase buffer (25 mM Hepes pH 7.4; 25 mM MgCl; 25 mM β-glycerophosphate; 50 µM ATP; 0.1 mM NaVO₃; 2 mM DTT). Pellets were then resuspended in 20 µl kinase buffer containing 5 µCi of γ-(³²P)ATP (3,000 Ci/mmol; Amersham) and 2 µg histone H1 (Roche) for 40 min at room temperature. The kinase reactions were stopped by the addition of 4X NuPAGE™ sample buffer (Novex, CA) boiled for 5 min and 10 µl loaded and run on a 4-12% gradient NuPAGE™ gel.

**Northern blot analysis**

RNA was isolated from cultured cells using TRIZOL® Reagent (15596; Gibco-BRL, NY) according to the manufacturer’s protocol. Northern analysis was performed essentially as described by Sambrook et al. (22). Twelve micrograms of total RNA was fractionated by
0.66 M formaldehyde-1% agarose gel electrophoresis. RNA was transferred to Hybond N+ membrane (Amersham) by capillary transfer using 10X SSC. The membrane was prehybridized in Church and Gilbert Hybridization Buffer (0.5 M Na₂HPO₄ (pH 7.2); 7% SDS; 1 mM EDTA) at 60°C for 1 hr, followed by hybridization with ³²P-labelled p21 cDNA probe in fresh Church and Gilbert Hybridization Buffer at 60°C, overnight. The membrane was washed at 60°C for 15 min each with 2X SSC + 0.5% SDS, and then 1X SSC + 0.5% SDS.

The p21 cDNA was obtained by RT-PCR. First-strand cDNA was synthesized in a 20 µl reverse transcriptase (RT) reaction from 5 µg total RNA (from bovine skeletal muscle) using SuperScript™II pre-amplification kit (Gibco-BRL), according to the manufacturer’s protocol. PCR was performed with 2 µl of the RT reaction at 94°C for 20 s, 55°C for 20 s, and 72°C for 1 min for 35 cycles. This was followed by a single 72°C extension step for 5 min. The primers used for amplification were 5’-CTGTTAGGCTGGTCTGCCTC-3’ and 5’-GTCCGATCCTGGTGATGTCC-3’ (443 bp). The p21 cDNA was radioactively labeled using α³²P-(dCTP) (Amersham Life Science Ltd., UK) and Rediprime™II labeling kit (Amersham Life Science Ltd.), according to the manufacturer’s protocol.
**RESULTS**

**Myostatin protein is synthesized and proteolytically processed in Myoblasts**

Previous published results show that myostatin expression is detected very early in the myotome of the developing somites of mice and cattle embryos (1,2) and the expression continues into adult muscle. In addition, using myostatin specific antibodies, we recently demonstrated (5) that myostatin protein is synthesized and proteolytically processed in adult skeletal muscle. However the site of myostatin biosynthesis and its processing has not been demonstrated so far.

To characterize the site of synthesis, we performed Western blot analyses on protein extracts prepared from bovine primary myoblasts. Anti-myostatin antibodies specifically detect two bands on the Western blot (Fig. 1). These two bands correspond to the unprocessed full-length protein (52 kD) and the N-terminal LAP (latency-associated peptide) (40 kD). In addition to the full-length and LAP, the anti-myostatin antibodies also detected the mature processed myostatin (26 kD) in skeletal muscle extract. However, this 26 kD mature myostatin is not detectable in myoblast extracts. This data demonstrates that myostatin protein is synthesized in myoblasts and that the precursor myostatin is processed in myoblasts.

**Expression of recombinant histidine tagged myostatin protein**

Myostatin protein is proteolytically processed, possibly at a conserved RSRR (263-266) site (5), to give an active processed myostatin protein, which can elicit biological function. The pET system was used to express this processed portion of myostatin (267-375) in *E.coli*. The expressed histidine fusion proteins were purified on a Ni-agarose column and separated on SDS-gel to check for the purity.

As shown on a Coomassie blue stained gel (Fig. 2 A), a 15 kD myostatin fusion protein was purified to a high degree in a single step. A typical yield of 6 mg myostatin protein was
purified per liter of induced bacterial culture. Western blot analysis confirmed that the purified 15 kD protein is indeed myostatin since it is detected by myostatin specific antibodies (Fig. 2 B). Furthermore there appears to be no degradation of recombinant myostatin during purification (Fig. 2 A, B). The observed relatively low amount of higher molecular weight protein recognized by anti-myostatin antibodies is the non-reduced form of purified recombinant myostatin which is not seen in the control un-induced bacterial extract (data not shown).

**Myostatin inhibits the proliferation of myoblasts**

Inactivating mutations in the myostatin gene lead to a heavy muscling condition in both cattle and mice (1,2). This observed heavy muscle growth is due to hyperplasia of muscle fibers. Since the increased number of muscle fibers could be due to increased proliferation of myoblasts, we investigated if the underlying mechanism for negative regulation of muscle growth by myostatin is specified through regulating the proliferation rate of myoblasts.

To determine the effect of increased myostatin protein concentration on the proliferation of myoblasts, we cultured myoblasts in the presence of varying concentrations of myostatin and evaluated the proliferation of myoblasts by methylene blue assay. For these studies C2C12 transformed myoblasts and primary cultures of bovine myoblasts, from both normal- and double-muscled Belgian Blue (which carry a mutation in the myostatin gene) animals, were used.

Myostatin inhibited the growth of C2C12 myoblasts in a dose-dependent manner (Fig. 3 A) with half-maximal inhibition occurring at a myostatin concentration of approximately 2.5 \( \mu g/ml \) (Fig. 3 D). In addition, myostatin treatment also inhibited the growth of C2C12 in a time dependent manner and furthermore, did not result increased myotube formation and overt differentiation (Fig. 3 E a-f). To further confirm the growth inhibitory effect of myostatin on myoblasts, primary bovine myoblasts were cultured from both normal- and double-muscled
160 day fetuses. The purity of the primary bovine myoblasts culture is very high, as can be seen by Myf-5 immunostaining and Gill’s Haematoxylin counterstaining of the culture (Fig. 3 E h). These primary myoblast cultures also displayed a dose-dependent inhibition of growth in the presence of myostatin (Fig. 3 B and 3 C), with half-maximal inhibition occurring with a myostatin concentration of approximately 1.75 µg/ml (Fig. 3 D). The inhibition of primary bovine myoblasts by myostatin is shown in Figure 3 E h,i. Again, no overt differentiation by myostatin was noted in the primary myoblasts.

These results indicate that myostatin controls the number of myoblasts by either regulating the cell cycle progression or apoptosis of myoblasts. Furthermore, the endogenous levels of myostatin do not appear to affect the exogenous myostatin biological activity since both myoblasts cultured from normal- and double-muscled animals are inhibited by recombinant myostatin.

**Myostatin inhibition of myoblast proliferation is reversible**

Active processed forms of TGF-β and its family members bind to their respective receptors to trigger the biological response (6). Hence in an *in vitro* bioassay, once the ligand is removed, the biological response, such as cell growth arrest by TGF-β, is reversed resulting in normal growth.

To address the question of whether myostatin growth-inhibition is reversible, C2C12 myoblasts were incubated with a growth-inhibitory dose of myostatin (4 µg/ml in 10% FBS/DMEM media). After incubation in the presence of myostatin for 24, 72 or 120 hours, myostatin was removed and cells were incubated in the presence of 10% FBS/DMEM media. Proliferation of the myoblasts was assessed at 24 hour intervals by the methylene blue assay.

Results of this experiment show that when myoblasts were incubated in growth media without myostatin there was a steady increase in cell number (Fig. 4 A and B a). When myostatin was added (4 µg/ml), total cell number remained constant for the entire duration of
the experiment (Fig. 4 A and B b). Conversely, when myostatin was subsequently removed from these cultures at either 24, 72 or 120 hours after its initial addition, myoblasts resumed growth and hence total cell number increased (Fig. 4 A and B c,d). Results of this experiment thus show that myoblast growth is not irreversibly inhibited by myostatin but, in fact, myoblasts retain the ability to proliferate after it is removed.

**Myostatin arrests the growth of C₂C₁₂ myoblasts by interfering at G1- and G2/M-phases of the cell cycle**

In order to determine if the observed decrease in C₂C₁₂ myoblast proliferation is due to apoptosis or altered cell cycle regulation we performed TUNEL assay and flow-cytometry analysis on control and myostatin treated myoblasts. To perform TUNEL assay, actively growing C₂C₁₂ myoblast cells were seeded at a low density, cultured for 48 hours with or without recombinant myostatin protein in the media. TUNEL assays performed on the C₂C₁₂ myoblasts revealed no increase in apoptosis between the myostatin treated and non-treated cells (data not shown). To confirm that myostatin treatment did not cause an increase in apoptosis, as well as determine the cell cycle distribution FACS analysis was performed. For this analysis, actively growing C₂C₁₂ myoblast cells were cultured with or without myostatin (4 µg/ml) for 48 hours and harvested. After staining with propidium iodide, the cell cycle distributions were examined by flow-cytometry.

The results confirmed that the decrease in myoblast proliferation was not due to increased apoptosis, as seen by the percentage of cells with low DNA content (Fig. 5; Table 1). The FACS analysis also showed a dramatic decrease in the number of cells in S-phase (26.64±0.18% to 5.96±0.93%), accompanied by an increase in the percentage of cells in both the G1 (50.94±0.42% to 63.77±1.07%) and G2/M (22.43±0.43% to 29.74±0.93%) cell cycle phases (Fig. 5; Table 1). These results clearly suggest that myostatin controls the
proliferation of myoblasts by blocking the cell cycle at G1 and G2/M without affecting the apoptosis of myoblasts.

**Myostatin up-regulates p21/WAF1 expression**

Progression of cells through various phases of their cell cycle appears to be controlled by the action of Cdks, their associated cyclins and the cyclin/Cdk inhibitors (CKIs). Given that myostatin inhibits the G1- to S-phase progression of myoblasts, we speculated that myostatin may affect one or more of the cell cycle control proteins. Of particular interest was the cyclin/Cdk inhibitor p21, which has previously been shown to be involved in cell cycle arrest in both the G1- and G2/M-phases (23). In addition, growth arrest by TGF-β signaling has been shown to influence p21 expression (24).

To examine this possibility, actively growing C2C12 myoblasts were cultured with or without myostatin in the media for 6, 12, 18 and 24 hours before harvesting for total RNA and total protein. Northern blot analysis using a p21 cDNA probe to detect p21 expression revealed that p21 mRNA is up-regulated at the 12, 18 and 24 hour time points in response to myostatin treatment (Fig. 6A). Western blot analyses using anti-p21 antibodies confirmed that p21 protein was also up-regulated at the 12, 18 and 24 hour time points after treatment with myostatin (Fig. 6B).

**Myostatin does not alter the levels of other CKIs belonging to either the p21 or p16 families**

TGF-β signaling has also been shown to influence the levels of other CKIs, namely p15 (25) and p27 (26). We therefore wanted to see if myostatin up-regulated any other CKI, belonging to either the p21- or p16-families. Western blot analysis using anti-p15, -p16 and -p27 antibodies were performed on protein extracted from myostatin treated C2C12 myoblasts after 6, 12, 18 and 24 hours. Figure 6C shows that neither p15 nor p16, of the p16-family are up-regulated in response to myostatin treatment. Similarly, p27 and p57 (of p21-family)
levels were unchanged between the myostatin treated and non-treated C₂C₁₂ myoblasts (Fig. 6 C).

**Myostatin down-regulates Cdk2 protein levels and Cdk2 activity in C₂C₁₂ myoblasts.**

The progression from G1 to S-phase in mammalian cells is thought to be regulated by D-type cyclins associated with either Cdk4 or Cdk6 and by cyclin-E associated with Cdk2 (27). p21 is normally induced in proliferating myoblasts (28) and indeed is thought to promote the association of Cdk4 with D-type cyclins (29). However, changing the molar ratio of p21 to cyclin/Cdk results in inactivation of the cyclin/Cdk complex (30).

To determine if the elevated p21 levels seen in myostatin treated C₂C₁₂ myoblasts coincided with constant or down-regulated levels of cyclin/Cdks and therefore a change in the ratio of p21 to cyclin/Cdk complexes, we performed Western blot analyses. Figure 7 A shows that the level of Cdk2 appeared to be slightly down regulated at the 12, 18 and 24 hour time points in response to myostatin treatment.

To confirm that the observed up-regulation of the CKI, p21, and down-regulation of Cdk2 resulted in an inactive cyclin/Cdk2 complex, the effect of myostatin signaling on Cdk2 kinase activity was measured in C₂C₁₂ myoblasts. Anti-Cdk2 immunoprecipitation assays were performed on cellular lysates from control and myostatin treated cells using histone H1 as an *in vitro* substrate (Fig. 7 B). Cdk2 activity was markedly diminished following both 12 and 24 hours of myostatin treatment.

Western blot analyses on other cyclin/Cdks however, showed that the levels of cyclin-E, cyclin-D2 and Cdk4 all remain the same with myostatin treatment (Fig. 7 C).

**Myostatin decreases the phosphorylation of retinoblastoma protein**

Previous studies have shown that the retinoblastoma susceptibility gene product, Rb, is a major substrate of G1-Cdks (27). Rb, acts by binding to and repressing the activity of certain transcription factors, the best characterized of which is the heteromeric E2F/DP1
complex (31). Phosphorylation of Rb by CdkS physically releases E2F/DP1 from this negative constraint, allowing the transcription of S-phase-specific genes (32). To determine if the phosphorylation of Rb protein was altered by the inhibitory action of p21 on cyclin/Cdk complexes in response to myostatin signaling, Western blot analysis was performed using an antibody that detects both the hypo- and hyperphosphorylated forms of Rb. For this study, total protein, extracted from cultured L6 rat myoblasts incubated with or without myostatin protein for 24 hours, was used in the Western blot analysis. Figure 8 shows a decrease in the hyperphosphorylated form of Rb and a corresponding increase in the hypophosphorylated form, consistent with the up-regulation of the CKI, p21, the down-regulation of Cdk2 and the decrease in Cdk2 activity.
Discussion

Myostatin, a recently reported member of the TGF-β super-family, is a key regulator of skeletal muscle development and growth. In this paper, we have characterized the site of myostatin synthesis and the possible mechanism by which myostatin could negatively regulate muscle growth.

Myostatin protein is proteolytically processed

The TGF-β superfamily members are synthesized as precursor proteins. Subsequently they are proteolytically processed at the site of synthesis and the biologically active mature peptide is secreted into circulation (6). Since the myostatin primary structure has all the hallmarks of the TGF-β members the key issue we address here is whether myostatin is processed at its site of synthesis.

Myostatin specific antibodies detect the precursor (52 kD) and the LAP (40 kD) forms of myostatin (Fig. 1) in Western blot analyses performed on cultured bovine myoblast extracts. This indicates that myostatin protein is indeed synthesized and proteolytically processed in myoblasts. Despite being detected in the total skeletal muscle extracts, the anti-myostatin antibodies failed to detect the processed active form of myostatin in the myoblast extract (Fig. 1). Since processed myostatin is secreted into the circulation, it is possible that the low amount of remaining processed myostatin protein in myoblasts is undetectable by the Western blot technique.

Based on the primary structure and reported mechanism of proteolytic processing of TGF-β proteins, the processing of myostatin putatively occurs at the conserved RSRR residues (263-266). This should result in a processed peptide of 12 kD apparent molecular weight and a 40 kD unprocessed precursor myostatin protein. However, using Western blot analysis we observe the precursor, LAP and processed myostatin as 55 kD, 40 kD and 26 kD proteins respectively (Fig. 1) (5). The observed discrepancy in the molecular weight of
processed myostatin protein could be due to post-translational modifications. Indeed, Gonzalez-Cadavid et al. (33) have recently reported that the monomer form of human processed myostatin protein (26 kD) is glycosylated. In contrast to this, McPherron et al. (1) have reported that recombinant processed myostatin and precursor myostatin behave as 12 kD and 52 kD proteins, respectively, in CHO cells. The observed lower molecular weight for processed myostatin protein (12 kD instead of 26 kD), ectopically expressed in CHO cells, could be due to the absence of specific post-translational modifications that occur in muscle cells. It is unlikely to be a result of alternate proteolytic processing site usage in different cell types.

**Myostatin controls myoblast cell cycle progression**

Since mutations in myostatin can lead to increased muscle fibers (1,2), it is possible that myostatin functions by controlling myoblast number during development. To examine this possibility, we incubated myoblast cultures with increasing amounts of myostatin. The results indicate that the exogenous addition of myostatin indeed inhibits the proliferation of exponentially growing myoblasts (Fig. 3 A-E), without causing overt differentiation. The inhibition of myoblast proliferation by myostatin appears to be specific since mutant recombinant myostatin from the Piedmontese allele (2) fails to inhibit myoblast proliferation in a similar assay (Berry et al., in preparation). In addition, cell culture experiments also indicated that this inhibition of growth by myostatin is reversible by removing myostatin from cultures (Fig. 4 A, B).

Since the surviving myoblasts at the end of the assay is the net result of cell proliferation and apoptosis (34), we performed both TUNEL assay and flow-cytometric analysis to quantify the extent of apoptosis and assess the cell cycle progression in myoblasts treated with myostatin. Both of these analyses indicated that there is no increase in apoptosis (Fig. 5; Table 1). However, the FACS analysis showed that when myoblasts are incubated
with myostatin, an increased number of myoblasts are arrested in G1-phase, and therefore did not make the transition to the S- (DNA synthesis) phase (Fig. 5; Table 1). Furthermore, it is also observed that myostatin affected the G2 to M progression of myoblasts.

TGF-β inhibits cell cycle progression of many types of mammalian cells by arresting them in the G1-phase of the cell cycle (24). This arrest is thought to be mediated through the many elements of the complex cell cycle machinery. The progression through the first gap phase (G1) and initiation of DNA synthesis (S-phase) during the mammalian cell division cycle is cooperatively regulated by several classes of cyclins and cyclin-dependent kinases (Cdk) whose activities are in turn are constrained by Cdk inhibitors (CKIs). The G1 Cyclin/Cdk complexes that play important roles in G1 to S-phase progression include, cyclin-E/Cdk2, cyclin-D/Cdk4 and cyclin-D/Cdk6, while their inhibitors (CKIs) include members of the p16- and p21-families, p15, p16, p21, p27 and p57. TGF-β growth suppression of cells is mediated by inhibiting the kinase activity of the G1-Cdks by up-regulating most of the CKIs (35). However, myostatin mediated growth arrest appears to be specifically regulated through p21, since only p21 levels are up-regulated as a result of myostatin treatment in myoblasts (Fig. 6 A, B). No change was observed in the levels of the other CKIs; p27, p57, p15, or p16 (Fig. 6 C). In addition, the increased expression of p21 correlated with a slight decrease in Cdk2 protein (Fig. 7 A). This would further increase the ratio of p21 to Cdk2 resulting in the observed reduction of Cdk2 activity (Fig. 7 B).

It is now widely accepted that cyclin/Cdk complexes are needed during the G1-phase of the cell cycle to phosphorylate the retinoblastoma susceptibility gene product, Rb. Hypophosphorylated Rb binds to transcription factors such as E2F/DP1 required for transcription activation of S-phase genes (31). Thus, phosphorylation, and hence inactivation, of the Rb protein by G1-Cdks is thought to release these S-phase transcription factors, allowing the passage through this restriction point (32). Conversely, the accumulation of the
hypophosphorylated form of Rb, through G1-Cdk inactivation, either by increasing the concentration of CKIs and/or decreasing the protein concentration of Cdk, will lead to G1 arrest of cells (27,30). In myoblasts treated with myostatin there is indeed an increase in p21 (Fig. 6 B), a decrease in Cdk2 (Fig. 7 A) and a decrease in Cdk2 activity (Fig. 7 B). In addition, we have also observed reduced phosphorylation of Rb (Fig. 8). We therefore propose that myostatin causes G1 growth arrest of myoblasts by the hypophosphorylation of Rb, via cyclin-E/Cdk2 inactivation by p21. The observed accumulation of myoblasts at the G2/M cell cycle transition is currently under investigation, however, it is noteworthy to mention that p21 has also been implicated in cell cycle arrest at the G2/M boundary (23,36).

Thus we propose the following model (Fig. 9) for the role of myostatin in myoblast proliferation regulation. In response to myostatin signaling, p21 expression is up-regulated inhibiting cyclin-E/Cdk2 activity causing the hypophosphorylation of Rb protein and G1 arrest. The increase in muscle fiber number (hyperplasia) seen in Belgian Blue cattle and myostatin-null mice is thus the result of deregulated (increased) myoblast proliferation.
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Figure Legends

Figure 1. Detection of myostatin protein in skeletal muscle and myoblast protein extracts by Western blot analysis. Fifteen micrograms of total protein from Bovine M. biceps femoris and 70 day primary bovine myoblast cultures, was resolved by 12% SDS-PAGE and myostatin protein was detected with rabbit anti-myostatin antibodies. Precursor, processed and Latency-Associated Peptide (LAP) forms of myostatin are indicated. Molecular weights of the Western positive bands are indicated.

Figure 2. Purified recombinant myostatin. (A) Coomassie stained gel showing purified myostatin. 3 µg of Ni-Agarose purified recombinant fusion myostatin (MSTN) protein was resolved by 4-12% gradient SDS-PAGE. Myostatin protein and molecular weight markers are indicated. (B) Western blot analysis showing the detection of purified myostatin (MSTN) by specific anti-myostatin antibodies. 0.3 µg purified myostatin was resolved by 4-12% gradient SDS-PAGE. Myostatin and molecular weight markers are indicated.

Figure 3. Inhibition of myoblast growth by myostatin. Myoblasts were grown in the presence of increasing concentrations of myostatin (0 to 10 µg/ml) for 72 hours and proliferation was monitored by methylene blue assay. Optical density (at 655nm) and increasing concentration of myostatin are shown on the Y- and X-axes respectively. (A) C₂C₁₂ myoblasts, (B) Normal muscled myoblasts and (C) double muscled myoblast cultures were used in different experiments. (D) Percentage of myoblast growth inhibition at increasing concentrations of myostatin is shown (●= C₂C₁₂, ○= normal muscled, ▽=double muscled). (E) C₂C₁₂ myoblasts were cultured for 24, 48, or 72 hours without myostatin (Panels a, b and c) or with 4 ug/ml myostatin (Panels d, e and f) in growth media. Cells were fixed and stained with Gill’s Haematoxylin and Eosin. Myoblasts derived from 160-day bovine foetuses were cultured for 72 hours without myostatin (Panel h) or with 8 µg/ml
myostatin (Panel i) in growth media. Cultures were fixed and immuno-stained using 5 µg/ml Rabbit anti-myf-5 antibody and lightly counterstained with Gill’s Haematoxylin. Arrows in h indicate fibroblast cells. Panel g is a micrograph of 160-day bovine myoblasts immunostained with control Rabbit IgG (5 ug/ml). Bar, 50 µM.

**Figure 4.** Proliferation of rescued C2C12 myoblasts. (A) C2C12 cells were incubated with 4µg/ml myostatin for either 24 hours (○), 72 hours (▼) or 120 (▽) hours and myostatin was removed from the culture media. Proliferation of myoblasts was measured (OD) by methylene blue assay at 655 nm and is represented on Y-axis. Hours of myostatin treatment are showed on X-axis. Proliferation of myoblasts with no myostatin (●) in the media and the myoblasts incubated with myostatin (■) throughout the experiment are also shown. (B) C2C12 myoblasts were cultured without (Panel a) or with 4 µg/ml myostatin (Panel b) in growth media for 96 hours. In other cultures 4 µg/ml myostatin was added to growth media for 24 (Panel c) or 72 (Panel d) hours after which myostatin was removed for the remainder of the 96 hour experiment. All cultures were then fixed, stained with Gill’s Haematoxylin and Eosin. Bar, 50 µM.

**Figure 5.** Effect of myostatin on C2C12 cell cycle progression. C2C12 cells, either treated with (Plus) or without (Minus) myostatin, were stained with propidium iodide and were analyzed on Flow-cytometer. Ten thousand cells for each treatment were analyzed and distributed graphically into apoptotic or G1, S and G2-M phases of cell cycle, based to the DNA content.

**Figure 6.** p21, but not other CKIs, is induced by myostatin in C2C12 myoblasts. (A) Northern analysis of the induction of p21 mRNA by myostatin. Total RNA was extracted from myoblasts that were either treated with (+) or without (-) myostatin for 6, 12, 18 or 24 hours. 15 µg of the total RNA was loaded in each lane for analysis. 28S and 18S rRNA bands of each lane is shown. (B) Western analysis of the induction of p21 protein by
myostatin. Protein extracts were made from myoblasts treated with (+) or without (-) myostatin for 6, 12, 18 or 24 hours. 15 µg of total protein was resolved by 12% SDS-PAGE. Monoclonal anti-p21 (PharMingen) and anti-tubulin (Sigma) were the respective primary antibodies used in the Western blot. (C) Myostatin does not induce other CKIs, including p15, p16, p27 and p57 in C2C12 myoblasts. Western analyses were performed on protein extracts made from myoblasts treated with (+) or without (-) myostatin for 6, 12, 18 or 24 hours. 15 µg of total protein was resolved by 12% SDS-PAGE. Primary antibodies include polyclonal anti-p15, polyclonal anti-p16, and monoclonal anti-p27 (Santa Cruz).

Figure 7. Myostatin down-regulates Cdk2 protein and Cdk2 activity, but does not alter the levels of Cdk4, cyclin-D1 or cyclin-E in C2C12 cells. (A) Western analysis for Cdk2 on protein extracts made from myoblasts treated with (+) or without (-) myostatin for 6, 12, 18 or 24 hours. 15 µg of total protein was resolved by gradient 4-12% SDS-PAGE. Monoclonal anti-Cdk2 (Santa Cruz) and monoclonal anti-tubulin (Sigma) were the respective primary antibodies used in the Western blot. (B) Anti-Cdk2 immunoprecipitation-kinase assay from myoblasts treated with (+) or without (-) myostatin for 18 and 24 hours. 200 µg of total protein was immunoprecipitated with polyclonal anti-Cdk2 antibodies (Santa Cruz) and histone H1 was used as in vitro substrate. 7.5 µg of protein extracts were also resolved by gradient 4-12% SDS-PAGE and probed with monoclonal anti-tubulin antibodies (Sigma) to show equal protein concentrations were calculated and used in the kinase assay. The two controls for the assay either lack protein (a) or lack primary anti-Cdk2 antibody (b). (C) Western analyses for Cdk4, Cyclin-D1 and Cyclin-E on protein extracts made from myoblasts treated with (+) or without (-) myostatin for 18 and 24 hours. 15 µg of total protein was resolved by 12% SDS-PAGE. Respective primary antibodies used in the Western blots include polyclonal anti-Cdk4, monoclonal anti-cyclin-D1 and polyclonal anti-cyclin-E antibodies (Santa Cruz).
Figure 8. Myostatin signaling causes retinoblastoma protein to accumulate in a hypophosphorylated form in L6 myoblasts. Western analysis was performed on protein extracts made from L6 myoblasts treated with (+) or without (-) myostatin for 24 hours. 15 µl of total protein was resolved by 7.5% SDS-PAGE. A monoclonal anti-Rb antibody (PharMingen) was used to detect the different phosphorylated forms of Rb protein. The hyperphosphorylated (pRb\textsuperscript{PP}) and hypophosphorylated (pRb) forms are indicated.

Figure 9. A model for the role of myostatin in muscle growth. A) During embryonic myogenesis, Myf-5 and MyoD specify cells to adopt the myoblast fate. Myoblasts then migrate and proliferate. In response to myostatin signaling, p21 is up regulated, inhibiting cyclin-E/Cdk2 activity causing Rb inactivation and G1 arrest. Thus, myoblast number and hence fiber number, following differentiation, is regulated (limited). B) In the absence of functional myostatin, the signal for p21 up-regulation is lost and Rb remains in a hyperphosphorylated form resulting in deregulated (increased) myoblast proliferation thus leading to increased fiber number.

Tables

Table 1. Cell cycle distribution of C\textsubscript{2}C\textsubscript{12} myoblasts after myostatin treatment.

Percentage of cells from 10,000 counts in apoptotic, G1, S, or G2-M phases of the cell cycle according to FACS-analysis (shown in Figure 5). Data are averages ± standard error of quadruplicate determinations.
|                      | Apoptotic Cells (%) | Cells in G1 (%) | Cells in S (%) | Cells in G2-M (%) |
|----------------------|---------------------|-----------------|----------------|------------------|
| Without Myostatin    | 0.20(±0.03)         | 50.94(±0.42)    | 26.64(±0.18)   | 22.43(±0.43)     |
| With Myostatin       | 0.58(±0.05)         | 63.77(±1.07)    | 5.96(±0.93)    | 29.74(±0.93)     |

Table 1
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

Minus Myostatin

Plus Myostatin

Cell Number (counts)

DNA Content

Apoptotic G1 S G2-M

Apoptotic G1 S G2-M
Figure 6
Figure 7

A

|       | 6 Hours | 12 Hours | 18 Hours | 24 Hours |
|-------|---------|----------|----------|----------|
| Myostatin | -       | +        | -        | +        |
| Cdk2   |         |          |          |          |
| Tubulin|         |          |          |          |

B

|       | 12 Hours | 24 Hours | Control |
|-------|----------|----------|---------|
| Myostatin | -       | +        | a       |
| Histone H1 |         |          | b       |
| Tubulin   |         |          |         |

C

|       | 18 Hours | 24 Hours |
|-------|----------|----------|
| Myostatin | -       | +        |
| Cdk4    |          |          |
| Cyclin-D1 |         |          |
| Cyclin-E |          |          |
Figure 8
A  With Functional Myostatin

Precursor → MyoD → Myf5 → Myoblasts → Myogenin → Proliferation → Committed Myoblasts → Differentiation → Myotubes

Myostatin → p21 → Cdk2

B  Without Functional Myostatin

Precursor → MyoD → Myf5 → Myoblasts → Myogenin → Proliferation → Committed Myoblasts → Differentiation → Myotubes

Myostatin → p21 → Cdk2

Figure 9