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

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ABSTRACT

Myostatin, a member of the transforming growth factor-b (TGF-b) superfamily has been shown to be a negative regulator of myogenesis. However, the molecular mechanism of myostatin function is, so far, not understood. Here we show that myostatin is synthesised and proteolytically processed in myoblasts and that myostatin functions by controlling the proliferation of muscle precursor cells. When actively growing C_2C_{12} myoblasts were incubated with myostatin, the proliferation of myoblasts decreased with increasing levels of myostatin. Furthermore, we show that the myoblast inhibition by myostatin is reversible, such that, myoblasts retain the ability to proliferate after myostatin protein is removed. FACS analysis revealed that myostatin inhibited myoblast proliferation through preventing the progression of myoblasts from the G1 to S-phase of the cell cycle. Thus, we propose that the generalised muscular hyperplasia phenotype observed in animals that lack functional myostatin could be as a result of deregulated myoblast proliferation.

Key Words: Myostatin; double muscling; hyperplasia; myoblast; myoblast proliferation assay; cell cycle.

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*. (1997) 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 fibres (hyperplasia) and thickness of fibres (hypertrophy) (McPherron *et al*., 1997). Subsequently, we (Kambadur *et al*., 1997) and others (McPherron and Lee 1997; Grobet *et al*., 1997) reported that the Belgian Blue and Piedmontese breeds of cattle, which are characterised 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 (McPherron and Lee, 1996). Myostatin protein is synthesised in skeletal muscle as a 375 amino acid pro-peptide that is proteolytically processed at the RSRR (263-266) site to give rise to a 26 kD active processed peptide (Sharma *et al*., 1999). This processed mature peptide binds to receptor to elicit biological function (McPherron and Lee, 1996).

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 fibre number is not known. Since increased muscle fibres 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_2C_{12} myoblast cells and recombinant myostatin protein, we show here that myostatin protein is synthesised and proteolytically processed in myoblasts. We also show that myostatin does indeed regulate myoblast proliferation and furthermore, achieves such by controlling the G1 to S-phase and G2 to M phase transition of the cell cycle. Hence, the increased number of myofibres 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.

MATERIALS AND METHODS

Subcloning and expression of myostatin in E.*coli*

The pET protein expression system (Novagen) was used to express and purify recombinant myostatin. Briefly, BL 21 *Escherichia coli* was transformed with bovine myostatin cDNA (aa 67-375) in pET 16-B vector. An overnight culture in 1 L LB; amp (50 mg/L) was grown to an OD of 0.8 (600nm). Expression of the myostatin fusion protein was induced by 0.5 mM IPTG for 2 hours. Bacteria was lysed in 40 ml lysis buffer [6 M guanidine hydrochloride; 20 mM Tris pH 8.0; 5 mM 2-mercaptoethanol] and sonicated. Myostatin was purified by Ni-agarose affinity chromatography (Qiagen), according to the manufacturer's protocol. Soluble fractions containing myostatin were dialysed against two changes of 50 mM Tris-HCl (pH 8.0); 500 mM NaCl; 10% glycerol for 6 hours.

Bovine myoblast culture generation

Standard superovulation and embryo transfer techniques were used to generate Belgian Blue (double muscled) foetuses and Hereford x Friesian crossbreed (normal muscled) foetuses as previously described (Kambadur *et al*., 1997). The M. semitendinosus muscle was excised from foetuses (day 160 gestation), and frozen in liquid nitrogen in Minimal Essential Media (MEM) (Life Technologies Ltd.); 20% Foetal bovine serum (FBS) (Life Technologies Ltd.); 10% DMSO. For use in experiments, the mixed cultures (myoblasts and fibroblasts) were thawed, minced and digested with 0.25% trypsin (Sigma) for 45 min at 37˚C.

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Primary cells were cultured in MEM; 10% FBS; 41.9 mM $NaHCO_3$ (Sigma); 7.22 nM Phenol red (Sigma); 1 x 10^5 IU/L penicillin (Sigma); 100 mg/L streptomycin (Sigma); 5% CO_2. The method of O'Malley *et al.* (1996) was used to enrich cultures for myoblasts. Briefly, 1 x 10^6 cells were added to Matrigel (CBR) coated 10 cm dishes (Nunc) and grown for 3 days, then digested with 0.5 g/L Type 1-A collagenase (Sigma) for 10 minutes to preferentially detach fibroblasts. Desmin staining of enriched myoblasts under differentiation inducing conditions showed a myoblasts population of 90%.

C_2C_{12} myoblast proliferation

C_2C_{12} myoblasts (Yaffe and Saxel, 1977) were grown prior to assay as stated above in either un-coated (C_2C_{12} cultures) or gelatin coated (bovine primary cultures) 96-well Nunc microtitre plates. Plates were gelatinised by the method of Quinn and Namaroff (1983). C_2C_{12} cultures were seeded at 1,000 cells/well and bovine cultures at 3,000 cells/well. After a 16-hour attachment period myostatin test media (MEM; 10% FBS; 0-10 mg/ml myostatin) was added. Proliferation was assessed after 72 hour incubation using a methylene blue photometric endpoint assay as previously described (Oliver *et al*., 1989). In this assay absorbance at 655 nm is directly proportional to final cell number.

Reversibility of myostatin inhibitory effect on proliferation

C_2C_{12} myoblasts were seeded into Nunc 96-well plates at a density of 1,000 cells/well. After an overnight attachment period (time zero) one set of eight replicates received DMEM/10% FBS media without myostatin (control wells). 'Rescued' replicates received DMEM/10% FBS containing 4 mg/ml myostatin. After 24-hour incubation period all plates were washed and DMEM/10% FBS without myostatin was added into control and "24 hour rescue" replicates, while DMEM/10% FBS containing 4 mg/ml myostatin was added other replicates. This method was continued for 72 and 120 hour replicates. Plates were fixed at 24-hour intervals throughout the experiment and assayed for cell proliferation as described above.

Flow cytometry

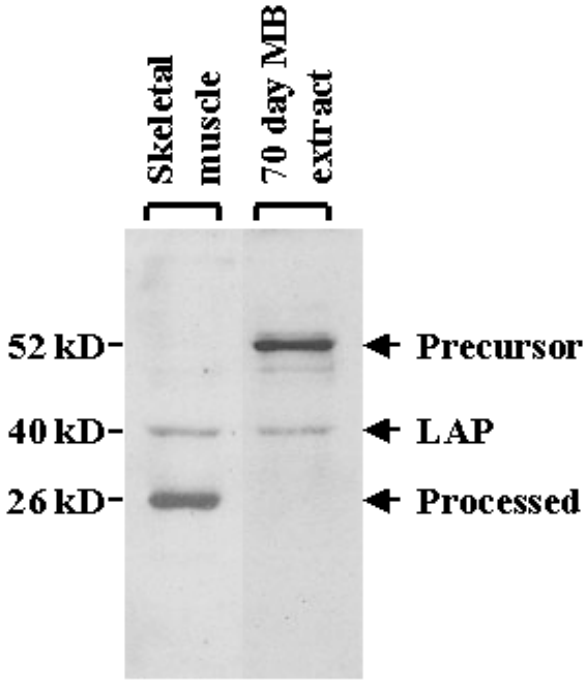
A method by Darzynkiewicz and Li was used that enabled the analysis of apoptosis in addition to cell cycle analysis. C_2C_{12} cells were cultured as described above in 100 mm dishes with or without myostatin for 48 hours. Cells (~3x10^6) were harvested, fixed in 800 ml 70% ethanol/PBS then resuspended in 500 ml PBS + 500 ml DNA extraction buffer [200 mM $NaHPO_4$; 100 mM citric acid] for 10 minutes at room temperature. DNA extraction buffer was replaced with DNA staining buffer [50 mg/ml Propidium iodide; 50 mg/ml DNase-free RNase A in PBS], cells resuspend and incubated at room temperature for 30 minutes, in the dark. Cells were then examined for propidium iodide fluorescence using a Becton-Dickinson FACScan® flow cytometer and analyzed using CellFit software.

RESULTS

Myostatin protein is synthesised and proteolytically processed in Myoblasts

Myostatin expression is detected very early in the myotome of the developing somites of mice and cattle embryos and the expression continues into adult muscle (McPherron *et al*., 1997; Kambadur *et al*., 1997). However the site of myostatin biosynthesis and its processing has not been demonstrated so far. To characterise the site of synthesis, Western blot analyses on protein extracts prepared from bovine primary myoblasts were performed. Anti-myostatin antibodies specifically detected two bands on the Western blot corresponding to the unprocessed full-length protein (52 kD) and the N-terminal LAP (latency-associated peptide) (40 kD). In skeletal muscle extract, the anti-myostatin antibodies detected the mature processed myostatin (26 kD) which is not detectable in myoblast extracts.

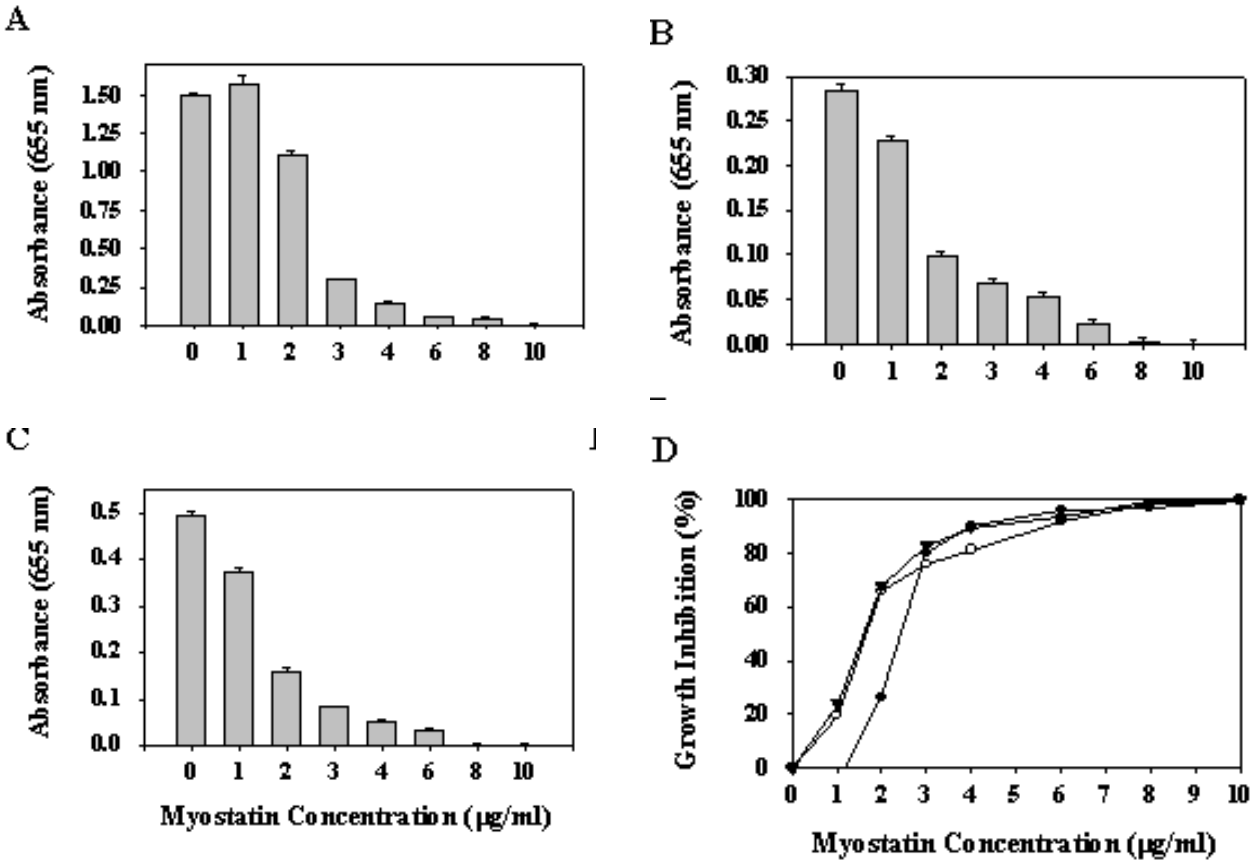
FIGURE 1 Detection of myostatin protein in skeletal muscle and myoblast protein extracts by Western blot analysis. Fifteen micrograms of total protein from Bovine M. biceps femoris and 70 day primary bovine myoblast cultures, was resolved in each lane and myostatin protein was detected with rabbit anti-myostatin antibodies. Precursor, processed and LAP forms of myostatin are indicated. Molecular weights of the Western positive bands are indicated.



Myostatin inhibits the proliferation of myoblasts

Inactivating mutations in the myostatin gene lead to a heavy muscling condition in both cattle and mice (McPherron *et al*. 1997; Kambadur *et al*. 1997). This observed heavy muscle growth is due to hyperplasia of muscle fibres. Since the increased number of muscle fibres could be due to increased proliferation of myoblasts, we investigated if recombinant myostatin affected the proliferation rate of myoblasts. To this end, C_2C_{12} myoblasts and primary bovine myoblasts (from normal and double muscled cattle) were cultured in the presence of varying concentrations of myostatin and their proliferation was evaluated the by methylene blue assay. Myostatin inhibited

FIGURE 2 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. C_2C_{12} (panel A), Normal muscled myoblasts (panel B) and double muscled myoblast cultures (Panel C) were used in different experiments. Percentage of myoblast growth inhibition at increasing concentrations of myostatin is represented in Panel D (ℵ= C_2C_{12}, ℵ= normal muscled, ❑=double muscled).

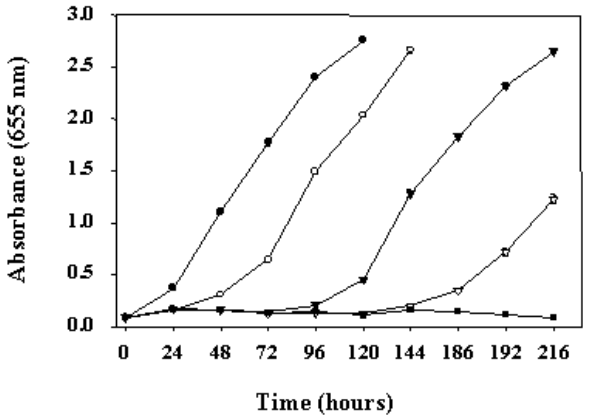


the growth of C_2C_{12} myoblasts in a dose-dependent manner with half-maximal inhibition occurring at approximately 2.5 mg/ml myostatin. Myoblast cultures from both normal and double muscled 160-day foetuses also displayed dose-dependent inhibition of growth, with half-maximal inhibition occurring with a myostatin concentration of approximately 1.75 mg/ml (Fig. 2d).

Myostatin inhibition of myoblast proliferation is reversible

Active processed forms of TGF-β and its members bind to their respective receptors to trigger the biological response (McPherron and Lee 1996). 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 see if myostatin growth-inhibition is reversible, C_2C_{12} myoblasts were incubated with a growth-inhibitory dose of myostatin for 24, 72 or 120 hours before myostatin withdrawal and proliferation assessed at 24 hour intervals by the methylene blue assay. Myoblasts incubated without myostatin showed a steady increase in cell number. When myostatin was added, total cell number remained constant for the duration of the experiment (Fig. 3). When myostatin was removed from the cultures at either 24, 72 or 120 hours, myoblasts resumed proliferation and total cell number increased (Fig. 3).Myostatin arrests the growth of C_2C_{12} myoblasts by interfering at G1- and G2/M-phases of the cell cycle

FIGURE 3 Proliferation of rescued C_2C_{12} myoblasts. C_2C_{12} cells were incubated with 4mg/ml myostatin for either 24 hours (ᔓ), 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 (ℵ) in the media and the myoblasts incubated with myostatin (O) throughout the experiment are also shown.



In order to determine if the observed decrease in C_2C_{12} myoblast proliferation is due to apoptosis or altered cell cycle regulation we performed FACS analysis on control and myostatin treated myoblasts. Flow-cytometry analysis of actively growing C_2C_{12} myoblast cells were cultured with or without myostatin for 48 hours showed no increased apoptosis, as seen by the percentage of cells with low DNA content (Fig. 4; Table 1). However, the FACS analysis 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.

Figure 4 Effect of myostatin on C_2C_{12} cell cycle progression. C_2C_{12} 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.

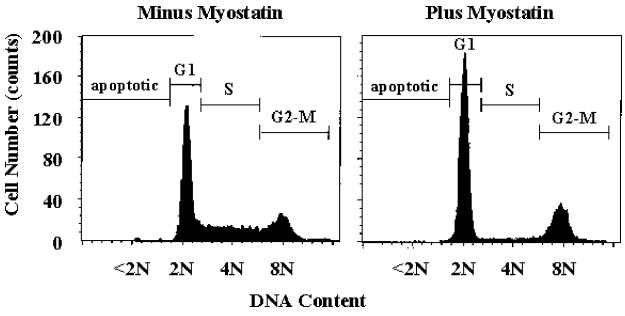


Table 1 Cell cycle distribution of C_2C_{12} myoblasts after myostatin treatment. Percentage of cells from 10,000 counts in apoptotic, G1, S, or G2-M phases of the cell cycle according to FACS-analysis (shown in Figure 5). Data are averages ± standard error of quadruplicate determinations.

	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)

DISCUSSION

The TGF-b superfamily members are synthesised as precursor proteins. Subsequently they are proteolytically processed at the site of synthesis and the biologically active mature peptide is secreted into circulation (McPherron and Lee, 1996). 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 synthesised 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) (Sharma *et al*., 1999). The observed discrepancy in the molecular weight of processed myostatin protein could be due to post-translational modifications. Indeed, Gonzalez-Cadavid *et al*. (1998) have recently reported that the monomer form of human processed myostatin protein (26 kD) is glycosylated. In contrast to this, McPherron *et al*. (1997) 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.

Since mutations in myostatin can lead to increased muscle fibres (McPherron *et al*., 1997; Kambadur *et al*., 1997), 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. 2a,b,c). The inhibition of myoblast proliferation by myostatin appears to be specific since mutant recombinant myostatin from the Piedmontese allele (Kambadur *et al*., 1997) fails to inhibit myoblast proliferation in a similar assay[i]. In addition, cell culture experiments also indicated that this inhibition of growth by myostatin is reversible by removing myostatin from cultures (Fig. 3).

Since the surviving myoblasts at the end of the assay is the net result of cell proliferation and apoptosis (Wang and Walsh, 1996), 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. 4; 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. 4; Table 1). Furthermore, it is also observed that myostatin affected the G2 to M progression of myoblasts.

Myogenic regulatory factors (MRFs) such as MyoD and Myf-5 have been shown to specify the formation of myoblasts (Rudnicki *et al*., 1992; Braun *et al*., 1992). Once specified, myoblasts continue to proliferate until they receive a differentiation signal. Based on the results shown here and the fact that there is delayed differentiation in myoblasts that lack functional myostatin (Quinn *et al*., 1990), we propose that during embryonic myogenesis Myf-5 and MyoD specify cells to adopt the myoblast fate.

[i] Berry *et al*., Unpublished result.

Myoblasts then migrate and proliferate. In response to myostatin signalling, myoblast G1 to S-phase cell cycle progression is inhibited, resulting in decreased or inhibition of myoblast proliferation. Myoblasts are then able to fuse and undergo differentiation forming mature muscle fibres. Thus the increases in muscle fibre number (hyperplasia) seen in Belgian Blue cattle and *myostatin*-null mice, where myostatin signalling is absent, are the result of deregulated (increased) myoblast proliferation and delayed differentiation.

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REFERENCES

Braun, T., M.A. Rudnicki, H.H. Arnold and R. Jaenisch. 1992. Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. *Cell*. **71:** 369-382.

Darzynkiewicz, Z. and X. Li. Measurements of cell death by flow cytometry. In *Techniques in Apoptosis: A User's Guide* (ed. T. G. Cotter and S. J. Martin). Princeton University Press, London.

Gonzalez-Cadavid, N.F., W.E. Taylor, K. Yarasheski, I. Sinha-Hikim, K. Ma, S. Ezzat, R. Shen, R. Lalani, S. Asa, M. Mamita, G. Nair, S. Arver and S. Bhasin. 1998. Organization of the human myostatin gene and expression in healthy men and HIV-infected men with muscle wasting. *Procedings of National Academy of Science. USA*. **95:** 14938-14943.

Grobet, L., L.J.R. Martin, D. Poncelet, D. Pirottin, B. Brouwers, J. Riquet, A. Schoeberlein, S. Dunner, F. Menissier, J. Massabanda, R. Fries, R. Hanset and M. Georges. 1997. A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle. *Nature Genet*ics **17:** 71-74.

Kambadur, R., M. Sharma, T.P.L. Smith and J.J. Bass. 1997. Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. *Genome Research* **7:** 910-915.

McPherron, A.C., A.M. Lawler, S. Lee. 1997. Regulation of skeletal muscle mass in mice by a new TGF-b superfamily member. *Nature*. **387:** 83-90.

McPherron, A.C. and S. Lee. 1996. The transforming growth factor-b superfamily. *Growth Factors Cytokines Health Disease*. **1B:** 357-393.

McPherron, A.C. and S. Lee. 1997. Double muscling in cattle due to mutations in the myostatin gene. *Procedings of National Academy of Science. USA* **94:** 12457-12461.

Oliver, M. H., N. K. Harrison, J. E. Bishop, P. J. Cole and G. J. Laurent. 1989. A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors. *Journal of Cell Sci*ence **92:** 513-518.

O'Malley, J. P., I. Greenberg, and M. M. Salpeter. 1996. The production of long-term rat muscle cell cultures on a Matrigel substrate and the removal of fibroblast contamination by collagenase. *Methods in. Cell Science* **18:** 19-23.

Quinn, L. S. and M. Nameroff. 1983. Analysis of the myogenic lineage in chick embryos. *Differentiation*. **24:** 111-123.

Quinn, L.S., D.O. Luan and R.A. Roeder. 1990. Paracrine control of myoblast proliferation and differentiation by fibroblasts. *Developmental Biology* **140:** 8-19.

Rudnicki, M.A., T. Braun, S. Hinuma, and R. Jaenisch. 1992. Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. *Cell*. **71:** 383-390.

Sharma, M., R. Kambadur, K.G. Matthews, W.G. Somers, G.P. Devlin, J.V. Conaglen, P.J. Fowke and J.J. Bass. 1999. Myostatin, a transforming growth factor-b superfamily member, is expressed in heart muscle and is upregulated in cardiomyocytes after infarct. *Journal of Cellular Physiology*. **180:** 1-9.

Wang, J. and K. Walsh. 1996. Resistance to apoptosis conferred by Cdk inhibitions during myocyte differentiation. *Science*. **273:** 359-361.

Yaffe, D. and O. Saxel. 1977. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature*. **270:** 725-727.