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Proteolytic processing of myostatin is auto-regulated during myogenesis

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Abstract

Myostatin, a potent negative regulator of myogenesis, is proteolytically processed by furin proteases into active mature myostatin before secretion from myoblasts. Here, we show that mature myostatin auto-regulates its processing during myogenesis. In a cell culture model of myogenesis, Northern blot analysis revealed no appreciable change in *myostatin* mRNA levels between proliferating myoblasts and differentiated myotubes. However, Western blot analysis confirmed a relative reduction in myostatin processing and secretion by differentiated myotubes as compared to proliferating myoblasts. Furthermore, *in vivo* results demonstrate a lower level of myostatin processing during fetal muscle development when compared to postnatal adult muscle. Consequently, high levels of circulatory mature myostatin were detected in postnatal serum, while fetal circulatory myostatin levels were undetectable. Since Furin proteases are important for proteolytically processing members of the TGF- β superfamily, we therefore investigated the ability of myostatin to control the transcription of furin and auto-regulate the extent of its processing. Transfection experiments indicate that mature myostatin indeed regulates furin protease promoter activity. Based on these results, we propose a mechanism whereby myostatin negatively regulates its proteolytic processing during fetal development, ultimately facilitating the differentiation of myoblasts by controlling both furin protease gene expression and subsequent active concentrations of mature myostatin peptide.

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Introduction

Myostatin is a secreted growth factor and a member of TGF- β superfamily. While lack of myostatin leads to increased muscle growth (Kambadur et al., 1997; McPherron et al., 1997), systemic injection of myostatin leads to muscle wasting (Zimmers et al., 2002) indicating that myostatin acts as a potent negative regulator of skeletal muscle growth. Myostatin expression is detected in embryonic, fetal, and postnatal myogenic cells suggesting that it plays a role in all stages of myogenesis. It is first detected in the myogenic precursor cells of the myotome compartment of developing somites (McPherron et al., 1997). Myostatin

expression continues throughout myogenesis and is detected postnatally at varying levels in different axial and paraxial muscles (Kambadur et al., 1997; McPherron et al., 1997). Myostatin expression has also been detected at low levels in other mammalian tissues (Ji et al., 1998; McPherron et al., 1997; Sharma et al., 1999).

The primary structure of myostatin contains several features shared with all members of the TGF- β superfamily. These features include a hydrophobic core of amino acids near the N-terminus that functions as a secretory signal and a putative proteolytic processing site (RSRR) in the C-terminal half of the precursor protein (McPherron and Lee, 1996). The proteolytic processing of myostatin is carried out by a calcium-dependent serine protease called furin (Lee and McPherron, 2001). Furin belongs to a family of mammalian processing enzymes called proprotein convertases (PCs), of which seven members have so far been identified (Thomas,

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2002). All PCs have overlapping cleavage site specificity and tissue distribution (Seidah and Chretien, 1997; Steiner, 1998). In particular, furin is highly concentrated in the trans-Golgi network (Molloy et al., 1994, 1999). Importantly, several TGF- β superfamily precursor proteins have been shown to be cleaved by furin, including TGF- β 1 (Dubois et al., 2001) and BMP-4 (Constam and Robertson, 1999; Cui et al., 1998, 2001). The proteolytic processing of myostatin results in both a N-terminal Latency Associated peptide (LAP, also referred to as pro-peptide) and a C-terminal mature myostatin peptide (Thomas et al., 2000). The mature myostatin is secreted into circulation as a high molecular weight protein in association with various interacting proteins. Titin cap and human small glutamine-rich tetratricopeptide repeat-containing protein (hSGT) have been shown to associate with intracellular forms of myostatin (Nicholas et al., 2002; Wang et al., 2003). While follistatin, follistatin related gene (FLRG), and growth and differentiation factor-associated serum protein-1 (GASP-1) have been shown to bind to the extracellular circulatory form of myostatin (Hill et al., 2002, 2003; Lee and McPherron, 2001). Recently, it is shown that the circulatory latency myostatin protein is activated by the BMP-1/tolloid family of metalloproteinases (Wolfman et al., 2003) to release the mature myostatin peptide. The mature myostatin presumably elicits its biological function by binding to its receptor, the Activin type II B receptor (Lee and McPherron, 2001). While the mature myostatin can bind to the receptor, the N-terminal LAP appears to be required for the correct folding, dimerization, and secretion of the mature peptide (Gray and Mason, 1990).

Mechanistically, myostatin appears to function by regulating myoblast cell cycle progression and differentiation. During the active proliferation phase of myoblast growth, myostatin negatively regulates the G1 to S phase transition of the cell cycle through both Rb dependent and independent pathways (Langley et al., 2004; Thomas et al., 2000). In addition, excess myostatin also inhibits myoblast terminal differentiation by inactivating MyoD (Langley et al., 2002). Although the function of myostatin is well defined, the significance of myostatin processing and secretion during myogenesis has yet to be investigated. Thus, in this manuscript, myostatin expression, processing, and secretion during myogenesis is investigated. The results presented here indicate that myostatin processing and secretion is reduced during myogenesis. An auto-regulatory loop, whereby myostatin self-regulates its own converting enzyme, appears to be a possible mechanism for the regulation of myostatin processing during development.

Materials and methods

Cell culture

Comprehensive methods covering cell culture and primary myoblast extraction are detailed previously (Tho-

mas et al., 2000). Briefly, C₂C₁₂ myoblasts were grown prior to assay in proliferation media (Dulbecco's modified Eagle's medium (D-MEM; Gibco-BRL, USA) containing 10% fetal bovine serum (FBS, Gibco-BRL, USA)) on uncoated plates, while primary bovine myoblasts were grown in Minimum essential medium-proliferation media (MEM, Gibco-BRL, USA) containing 10% FBS on gelatin (Sigma, St. Louis, MO, USA) coated plates. Differentiation was induced in the C₂C₁₂ myoblasts by culture in differentiation media (D-MEM) containing 2% horse serum (HS, Gibco-BRL, USA) for 24, 48, or 72 h and in primary bovine myoblasts by culture in MEM-differentiation media (with 2% HS) for 24, 48, or 72 h.

Transfections and luciferase assays

For transfections, C₂C₁₂ cells were seeded at a density of 15,000 cells/cm² in 6-well plates (Nunc, Roskilde, Denmark) with D-MEM media containing 10% FBS. After a 24-h attachment period, the cells were transfected with 3 μ g total plasmid DNA (2 μ g of P1 Furin promoter (Blanchette et al., 2001) construct and 1 μ g of SV40- β -galactosidase (β -gal) control vector, pCH110 (Amersham Biosciences, UK)) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. The cultures were then incubated in an atmosphere of 5% CO₂/37°C for a further 18 h. The media was removed and replaced with either proliferation or differentiation media. Cultures were incubated in 5% CO₂/37°C for a further 24 h. Media was then removed and cells rinsed twice with PBS (pH 7.4) and lysed in 300 μ l of 1 \times Reporter lysis buffer (Promega, Madison, WI, USA). Lysates were collected and vortexed for 10 s. After a quick freeze-thaw the lysates were centrifuged at 12,000 \times g for 15 s, and 10 μ l of the supernatant was analyzed for luciferase reporter gene activity (Promega) in a Turner Designs Luminometer (Model TD-20/20). To control for variations in transfection efficiency, the transfections were performed in triplicate, and each experiment repeated a minimum of three times. The individual luciferase value for each assay was normalized against β -galactosidase expression.

Myostatin indirect immunofluorescence microscopy and photography

C₂C₁₂ myoblasts were grown prior to immunostaining in proliferation media and seeded on Thermanox coverslips (Nunc) at a density of 15,000 cells/cm² for proliferation studies or 25,000 cells/cm² for differentiation studies. Following a 16-h attachment period, media was changed to either proliferation media (for the maintenance of actively proliferating myoblasts) or differentiation media (for the induction of differentiation and formation of myotubes). After 72 h of incubation, cells were rinsed once with PBS and then fixed with 70% ethanol:formaldehyde:glacial acetic acid (20:2:1) for 30 s, rinsed three times with PBS,

and blocked overnight at 4°C in TBS containing 1% normal sheep serum (NSS). Cells were incubated with the specific primary antibody; 1:100 dilution anti-myostatin antibody (Sharma et al., 1999) in TBS containing 1% NSS for 1 h. Rabbit IgG (5 µg/ml; DAKO, Copenhagen, Denmark) was used as a negative control. Cells were washed (3 × 5 min) with TBS containing 0.1% Tween 20 (TBST) and incubated with secondary antibody; 1:150 dilution biotinylated donkey anti-rabbit IgG (RPN1004; Amersham, UK) in TBS/1% NSS for 30 min. Cells were washed as before and incubated with tertiary antibody; 1:100 dilution of streptavidin–biotin fluorescein isothiocyanate complex (FITC; RPN1232; Amersham, UK) in TBS/1% NSS for 30 min. Cells were again washed. Myostatin-immunofluorescence was visualized using an UV emission wavelength of 520 nm (U-MWIG) filter, BH2-RFL-T3 burner; Olympus Optical Co., Germany). Cell nuclei were visualized by the addition of 0.3 µM 4, 6-diamidino-2-phenylindole, dihydrochloride (DAPI, Molecular Probes, USA) to the cells for 5 min followed by TBST wash (3 × 5 min), and a UV emission wavelength of 420 nm (U-MWU2 filter, BH2-RFL-T3 burner; Olympus). FITC and DAPI fluorescence was photographed using an Olympus BX50 microscope (Olympus) fitted with a DAGE-MTI DC-330 color camera (DAGE-MTI) and Scion Image Capture software.

Detection of myostatin secreted into cell culture media

Mouse C₂C₁₂ myoblasts were seeded at the density mentioned above in 6-well plates and cultured in either proliferation media or induced to differentiate in differentiation media for 72 h. The formation of myotubes in differentiation media cultured cells was confirmed by light microscopy. After washing three times with PBS, the cultured myoblasts and myotubes were incubated with methionine-free Proliferation or differentiation media, respectively. After 30 min, the cells were washed followed by the addition of test media. Test media consisted of methionine-free proliferation or differentiation media supplemented with 0.5 mCi/ml of L-[³⁵S]-methionine (1 mCi = 37 MBq; Amersham). Cells were incubated in precisely 2 ml of this media for 24 h. Conditioned media was collected for the immunoprecipitation of proteins, while the cells were harvested for the quantification of DNA content (Hoechst assay).

Immunoprecipitation of myostatin from conditioned media

The conditioned media (300 µl) was precleared prior to myostatin immunoprecipitation by incubating with 0.3 µg of anti-rabbit IgG HRP conjugate (P0448; DAKO) for 30 min at 4°C. Conditioned media was then incubated with 100 µl of 50% Protein A-Agarose (Invitrogen), washed twice with lysis buffer for 1 h at 4°C, centrifuged, and the supernatant (precleared media) collected. The precleared media was then incubated with 4 µl anti-myostatin antibody for 1 h at 4°C incubated with 50% Protein A-Agarose (Invitrogen)

(washed twice with lysis buffer) for 1 h at 4°C and centrifuged to pellet the immunoprecipitated complexes. Pellets were washed five times with lysis buffer. Pellets were then resuspended in 20 µl 4 × NuPAGE sample buffer (Invitrogen). Western blot analysis for myostatin was performed according to the published protocol (Langley et al., 2004).

Hoechst assay for the quantification of DNA

After the removal of the conditioned media from the cultures, cells were washed in PBS and detached from the culture dishes by incubating with 0.25% Trypsin (Sigma) in PBS. Cells were washed again with PBS and transferred to eppendorf tubes with 800 µl phosphate buffered saline EDTA (PBSE; 40 mM Na₂HPO₄·H₂O, 10 mM NaH₂PO₄·H₂O, 2 M NaCl, 2 mM EDTA, pH 7.4) incubated on ice and sonicated twice for 15 s. In a 96-well plate (Nunc), 15 µl of each sample was added to 35 µl PBSE in triplicate. Standards (50 µl of 0 to 20 µg/ml DNA) were also added to the 96-well plate (Nunc). Freshly made Hoechst buffer (250 µl of 0.5 µg/ml Hoechst in PBSE) was added to each well, and the plates read using microplate reader (model 3550; BioRad, Hercules, CA, USA) at 360 nm excitation and 460 nm emission wavelengths.

Detection of circulating levels of myostatin

Bovine serum samples were obtained from fetal days 70, 90, 138, 150, 165, and 240 through to adult. 2 µl of serum from each sample was loaded onto a 4–12% Bis–Tris pre-cast gel (Invitrogen). Subsequent Western Blot analysis for myostatin was performed according to the published protocol (Langley et al., 2004).

Detection of myostatin in wild type and Myostatin null mice

One milliliter of lysis buffer (PBS, pH 7.2) with 0.05% IGEPAL detergent (Sigma Chemical Co. St. Louis, MO, USA) and an enzyme inhibitor (Complete, Roche Diagnostics NZ Ltd., Auckland, New Zealand) was added to 100 mg of muscle from each animal. Samples were homogenized on ice then centrifuged at 11,000 × g for 10 min. Supernatant was recovered, mixed with Laemmli loading buffer (Laemmli, 1970), boiled for 5 min, then stored at –20°C until analysis. The protein concentration of the supernatant was determined using the bicinchoninic acid assay (Sigma Chemical Co.).

Twenty micrograms of protein from each muscle sample was loaded and separated in a 10% SDS-polyacrylamide gel under reducing conditions then transferred to a nitrocellulose membrane. After transfer, membranes were stained with Ponceau S to verify transfer of protein. Membranes were blocked and incubated in 0.05 M Tris-buffered saline with 0.05% Tween 20 (TBST, pH 7.6), containing 0.3% BSA, 1% polyethylene glycol (3,350 Mw), 1% polyvinylpyrrolidone (10,000 Mw). Membranes were incubated with

either rabbit anti-myostatin (Sharma et al., 1999) or goat anti-myostatin (1:5000, sc-6884, Santa Cruz). Twenty-four hours later, membranes were washed in TBST then incubated for 2 h with either HRP-conjugated goat anti-rabbit (1:10,000, Sigma Chemical Co.) or HRP conjugated to rabbit anti-goat (1:5000, Dako) against respective primary antibodies then washed again in TBST. Bound HRP activity was detected with enhanced chemiluminescence and then blots were exposed to XOMAT AR film (Eastman Kodak Company, Rochester, NY).

Biological activity of circulating myostatin

The AgResearch Ruakura Animal Ethics Committee approved all animal manipulations described in this paper. Standard superovulation and embryo transfer techniques

were used to generate bovine Hereford × Friesian cross-breed fetuses as described by Kambadur et al. (1997). Cows were slaughtered at the Ruakura abattoir when fetuses were at gestational age 120, 210, and 260 days. Fetal blood was collected via cardiac puncture and allowed to clot at 4°C overnight. Serum was then separated from the clot via centrifugation at 4200 rpm for 20 min. Serum was then filtered through a sterile 0.22 μm membrane.

Serum was then added to test media at a concentration of 10%. This test media consisted of D-MEM media (Life Technologies, Grand Island, NY, USA), buffered with NaHCO₃ (41.9 mmol/l, Sigma Cell Culture Ltd, St. Louis, MO, USA) and gaseous CO₂. Phenol red (7.22 nmol/l, Sigma) was used as a pH indicator. Penicillin (1 × 10⁵ IU/l) and streptomycin (100 mg/l, Sigma) were also included in the media.

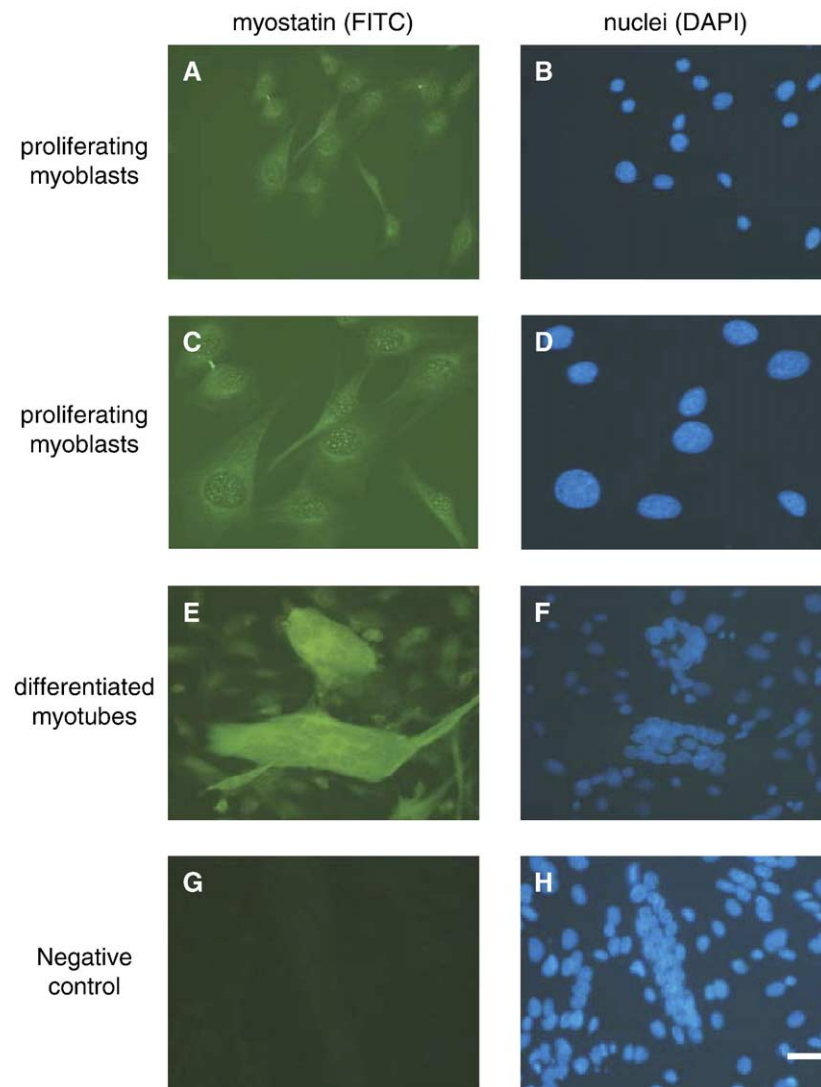


Fig. 1. Myostatin intracellular localization. Immunofluorescence microscopy showing myostatin localization in (A) proliferating C₂C₁₂ myoblasts and (E) differentiated C₂C₁₂ myotubes. Myostatin immunoreactive protein was detected using anti-myostatin antibodies and FITC staining. (G) Anti-rabbit IgG negative control on differentiated C₂C₁₂ myotubes. (B), (D), (F) and (H) DAPI stained cell nuclei of plates (A), (C), (E), and (G) respectively. Scale bar equals 50 μM. Panels (C) and (D) are larger inserts of panels (A) and (B) respectively.

Cell proliferation assays were then conducted to test the biological activity of the serum. C₂C₁₂ (Yaffe and Saxel, 1977) cultures were seeded at 1000 cells/well (3×10^3 cells/cm²) in Nunc 96-well dishes in proliferation media. After a 24 h attachment period, media was decanted and test media described above added back to the plates. Test samples were randomly distributed over the plate in order to avoid possible edge effects.

Plates wrapped in parafilm then incubated in an atmosphere of 37°C 5% CO₂. The plate was fixed at 72 h post media change and assayed for proliferation by the method of Oliver et al. (1989). Briefly, growth media was decanted and cells washed once with PBS then fixed for 30 min in 10% formol saline. The fixed cells were then stained for 30 min with 10 g/l methylene blue in 0.01 M borate buffer (pH 8.5). Excess stain was then removed by four sequential washes in borate buffer. Methylene blue was then eluted off the fixed cells by the addition of 100 µl of 1:1 (v/v) ethanol and 0.1 M HCL. The plates were then gently shaken for 30 s and absorbance at 655 nm measured for each well by a microplate photometer (BioRad model 3550 microplate reader, BioRad, Hercules, CA, USA). Absorbance is directly proportional to cell number in this assay.

Results

Intracellular localization of myostatin

Indirect immunofluorescence microscopy was performed on myoblasts and myotubes to investigate if there are any qualitative changes in myostatin distribution during myogenesis. C₂C₁₂ cell cultures were used for immunostaining. The immunofluorescence microscopy studies show that myostatin protein was detected in actively proliferating C₂C₁₂ myoblasts (Fig. 1A). Specifically, myostatin immunofluorescence was mainly localized in a perinuclear pattern, often showing the characteristic reticular morphology seen for TGF-β1 (Fig. 1C) (Miyazono et al., 1992; Mizoi et al., 1993; Roth-Eichhorn et al., 1998). In contrast to actively proliferating myoblasts, myostatin immunostaining in differentiated myotubes was mainly localized throughout the cytoplasm (Fig. 1E).

Myostatin processing is regulated during differentiation

Myostatin protein is synthesized and secreted from myoblasts, and it is shown that secreted myostatin regulates myoblast growth and differentiation systemically (Zimmers et al., 2002). Thus, processing and/or secretion of myostatin play a critical role in the myogenesis. To investigate if changes in either transcription or post transcription events regulate active levels of myostatin during myogenesis, the levels of *myostatin* mRNA and the level of myostatin processing were examined during the differentiation of myoblasts in a cell culture system. In this experiment,

bovine primary myoblasts were grown in either proliferation media or induced to differentiate in differentiation media for 24, 48, and 72 h before being harvested for total RNA and protein. Northern blot analysis revealed that *myostatin* mRNA (2.9 kb) was present in all of the total RNA extracts, with no appreciable change in the levels between myoblasts cultured in proliferation media and myoblasts undergoing differentiation in differentiation media (Fig. 2A). In the Western blot analysis using myostatin specific antibodies, no precursor myostatin was observed in the myoblasts cultured in proliferation media. In the myoblasts undergoing differentiation, however, a relatively high level of precursor

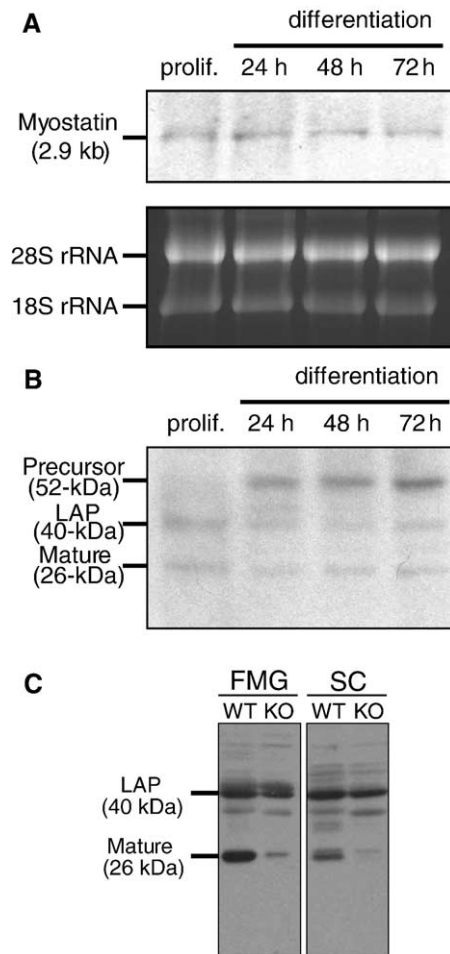


Fig. 2. Myostatin processing is regulated during myogenic differentiation. (A) Northern blot showing *myostatin* mRNA levels in primary bovine myoblasts cultured from fetal day 70 hindlimb muscle in proliferation media (prolif.) or differentiation media for 24, 48, or 72 h. The Northern blot was probed with a 514 bp *myostatin* cDNA probe. The 2.9 kb *myostatin* transcript is indicated. Ethidium bromide stained formaldehyde/agarose gel showing 28S and 18S rRNA is also included. (B) Western blot showing the levels of precursor, LAP, and mature myostatin in primary bovine myoblasts cultured from fetal day 70 hindlimb muscle in proliferation media (prolif.) or differentiation media for 24, 48, or 72 h. (C) Western blot showing the levels of LAP and mature myostatin between muscle samples obtained from wild type (WT) and myostatin null mice (KO). Two different antibodies were used, our in house anti myostatin antibody (FMG) and a commercially available GDF-8 antibody from Santa Cruz (SC).

myostatin was detected (Fig. 2B). In contrast to the precursor myostatin, the level of LAP and mature myostatin appeared to be slightly higher in actively proliferating myoblasts than in the differentiating myoblasts (Fig. 2B). In addition, we have performed a Western blot with both our in house myostatin specific antibody and a commercially available GDF-8 antibody (sc-6884, Santa Cruz) using muscle protein extracts from wild type and myostatin null mice. As shown in Fig. 2C, the myostatin specific antibodies fail to detect the strong 26 kDa protein band, which is observed in the wild type controls. Therefore, this supports our assignment of the 26 kDa band to mature myostatin.

In addition to the bovine primary myoblasts, mouse C₂C₁₂ myoblasts were cultured in either proliferation media or differentiation media for 24 h, and myostatin processing

was examined by Western blot analysis. In the C₂C₁₂ myoblasts cultured in proliferation media, the majority of myostatin protein that was detected were the 26-kDa mature and 40-kDa LAP forms. Only a relatively low level of precursor myostatin was detected (Fig. 3A). In myotubes by contrast, relatively high levels of the 52-kDa precursor myostatin are detected, while low levels of the 40-kDa LAP and no 26-kDa mature myostatin were detected (Fig. 3A). These results suggest that there is a change in the level of proteolytic processing of myostatin during myogenic differentiation.

Myoblasts and myotubes secrete myostatin protein

Northern and Western blot analyses performed on myoblast extracts indicated that, while there is no change

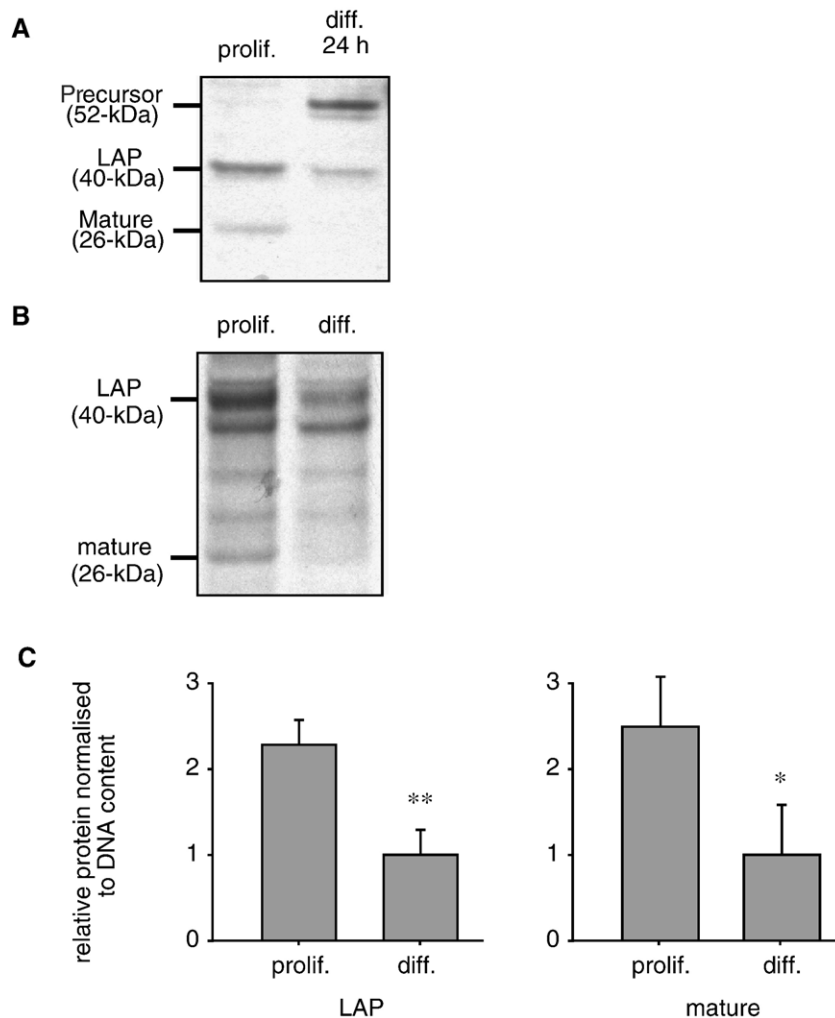


Fig. 3. Secretion of myostatin is regulated during myogenic differentiation. (A) Western blot showing the levels of precursor, LAP, and mature myostatin in C₂C₁₂ myoblasts cultured in proliferation media (prolif.) or differentiation media (diff.) for 24 h. Myostatin immunoreactive protein was detected using anti-myostatin antibodies. (B) Autoradiograph of metabolically labeled LAP and mature myostatin immunoprecipitated from the conditioned media of proliferating C₂C₁₂ myoblasts (prolif.) or differentiated C₂C₁₂ myoblasts (diff.). Myostatin immunoreactive protein was immunoprecipitated using anti-myostatin antibodies and fractionated by SDS-polyacrylamide gel electrophoresis. (C) Graph showing the ratio of myostatin secretion between proliferating and differentiated myoblasts for the LAP and processed forms. Myostatin secretion was measured by densitometric analysis of autoradiographs in panel (A), adjusted for background and normalized to total DNA content of the cells. Bars represent the average ratio of three replicates. Statistical differences (*P* values) determined by *t* test are indicated, ***P* < 0.01, **P* < 0.05 compared to proliferating sample.

in *myostatin* mRNA during myogenic differentiation, there is a dramatic difference in proteolytic processing of myostatin protein. Since proteolytic processing of the TGF- β superfamily members must occur for secretion, it is possible that more myostatin protein is retained in myotubes compared to myoblasts where the majority of myostatin is processed and presumably secreted. To investigate if the rate of myostatin secretion differs between myoblasts and differentiated myotubes, C₂C₁₂ myoblasts or myotubes were cultured for 72 h and myostatin protein was immunoprecipitated from the conditioned media. To distinguish between exogenous myostatin in the media serum (10% and 2% in proliferating and differentiated cultures, respectively) and endogenous myostatin secreted from the cells, the cultures were pulsed with S³⁵-methionine for a period of 24 h. Myostatin labeled with S³⁵-methionine therefore represented de novo synthesized and secreted myostatin. A Hoechst assay was also performed on the cultured cells to normalize the detected myostatin to DNA content.

As can be seen in Figs. 3B and C, less mature and LAP myostatin protein was detected, per DNA content, in the myotube conditioned media than in the proliferating myoblast conditioned media. This result supports the above observations that there is greater proteolytically processing of precursor myostatin to LAP and mature forms in proliferating myoblasts than in differentiated tubes. Thus, an increase in processing results in more secretion of myostatin by the myoblasts, while unprocessed precursor myostatin is retained intracellularly in the myotubes.

Myostatin processing is regulated during development

Previously, we have demonstrated that precursor myostatin protein is synthesized in myoblasts and proteolytically processed prior to secretion as a mature form (Thomas et al., 2000). Since mature myostatin is the active signaling peptide, proteolytic processing plays a significant role in the regulation of myostatin function during myogenesis. Although it has been shown that myostatin protein is present in myoblasts and adult muscle fibers, the pattern of myostatin processing during myogenic development is not yet known. Hence, Western blot analysis was performed on protein extracts from bovine fetal day 61 hindlimb, as well as *vastus lateralis* (*vl.*) and bovine *semitendinosus* (*st.*) muscles collected from various time points (fetal day 120 to postnatal day 14). Myostatin protein was detected in the precursor (52 kDa) and LAP (40 kDa) forms in nearly all of the bovine *vl.* muscle extracts (Fig. 4A). In the fetal day 61 muscle extract, a relatively high level of precursor myostatin was observed. No LAP was detected, even with a longer exposure of the Western blot to autoradiography film (Fig. 4A). As the fetal age increased (fetal days 120–260), a decrease in the amount of precursor myostatin and a corresponding increase in LAP was observed (Fig. 4A). Postnatally, the predominant form of myostatin present was

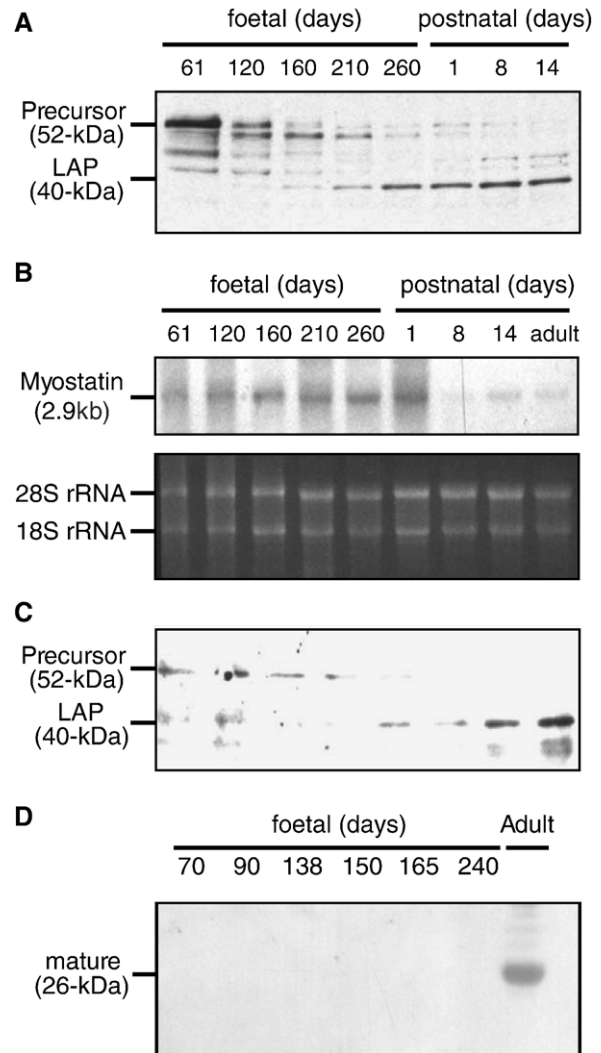


Fig. 4. Myostatin processing is regulated during development. (A) Western Blot showing the levels of myostatin precursor and latency-associated peptide (LAP) in *M. vastus lateralis*. (B) Northern blot showing *myostatin* expression in *M. vastus lateralis* from fetal days 61, 120, 160, 210, and 260 and postnatal 1, 8, 14, and adult cattle. The Northern blot was probed with a 514 bp *myostatin* cDNA probe. The 2.9 kb *myostatin* transcript is indicated. Ethidium bromide stained formaldehyde/agarose gel showing 28S and 18S rRNA is also included. (C) Western blot showing the levels of myostatin precursor and latency-associated peptide (LAP) in *M. semitendinosus* from fetal days 61, 120, 160, 210, and 260 and postnatal 1, 8, and 14 cattle. Myostatin immunoreactive protein was detected using anti-myostatin antibodies. (D) Western Blot showing the serum levels of mature myostatin from fetal days 70, 90, 138, 150, 165, 240 to adult. 2 μ l of total serum was loaded and analyzed for myostatin expression using specific anti-myostatin antibodies.

LAP, with very little precursor myostatin being detected (Fig. 4A). To determine the level of *myostatin* mRNA expression, Northern blot analysis was performed on total RNA extracted from the same source (Bovine hindlimb muscle, fetal day 61 and *vl.* muscle, fetal day 120 to postnatal day 14) used in the Western blot analyses. As shown in Fig. 4B, *myostatin* mRNA of 2.9 kb was detected in all of the bovine muscle samples from fetal day 61 through to postnatal day 14. At fetal days 61 and 120,

myostatin mRNA was relatively abundant. At fetal days 160 to 260 and full-term (280/postnatal day 1), the relative levels of *myostatin* mRNA were increased, peaking at the day 260 and full-term time points. Postnatally, *myostatin* mRNA levels dramatically declined, as seen by the relatively low level of *myostatin* mRNA in postnatal day 8 to adult (Fig. 4B). In addition to the vl. muscle, myostatin processing during development was also examined in *M. semitendinosus* muscle. A similar overall pattern of processing that is seen for vl. was also observed in the st. muscle (Fig. 4C). The observed increase in myostatin processing during gestation suggests that the extent of myostatin processing is regulated during embryonic and fetal bovine myogenesis.

Like other TGF- β superfamily members, proteolytic processing of the myostatin precursor molecule results in the formation of two distinct peptides, the LAP peptide and the C-terminal mature peptide (McPherron et al., 1997). Following processing, both the LAP and mature myostatin dimer are maintained in a circulatory latent complex (Lee and McPherron, 2001). Thus, to investigate if the extent of myostatin processing at the site of synthesis correlated with the amount of circulatory myostatin, we analyzed levels of myostatin in fetal blood during development. The results show that mature myostatin was virtually undetectable during early embryonic and fetal growth. However, postnatally high levels of 26 kDa mature myostatin were detected, confirming that reduced processing indeed results in low systemic levels of myostatin (Fig. 4D). These results, investigating the expression of myostatin, suggest that there is an increase in *myostatin* mRNA and an increase in the proteolytic processing of myostatin during fetal development. Postnatally, however, *myostatin* mRNA expression declines but a high level of processing is still maintained.

To determine if the increase in myostatin processing during fetal development is significant with respect to myostatin function, we have measured the biological activity of circulating myostatin. As shown in Fig. 5, C₂C₁₂ cells treated with serum obtained from day 260 bovine fetuses showed a 13% decrease in proliferation when compared to cells treated with serum obtained from earlier gestational time points. This decrease in proliferation coincides with the large increase in myostatin processing seen from day 260 onwards (Fig. 4).

Mature myostatin regulates furin promoter activity

Results shown above clearly indicate that differentiation somehow reduces processing of myostatin. Furin proteases are responsible for proteolytically processing TGF- β superfamily members; therefore, we reasoned that the observed reduction in the processing is perhaps due to reduced furin expression. To prove this, we transfected the P1 furin promoter-luciferase reporter construct into C₂C₁₂ myoblasts and measured the subsequent reporter activity during proliferating and differentiating conditions. Consistent with

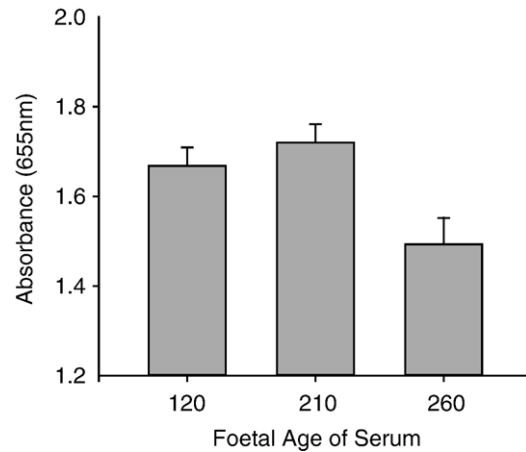


Fig. 5. Biological activity of circulating myostatin. The biological activity of myostatin in circulating fetal blood was assessed by collecting blood from known age bovine fetuses. This blood was then used to make serum and the biological activity of the serum assessed by its ability to promote the proliferation of C₂C₁₂ myoblasts in a 72 h methylene blue cell staining proliferation assay. Absorbance at 655 nm is directly proportional to cell number at the conclusion of the assay.

the reduction in processing of myostatin protein, furin promoter activity is also reduced in differentiated tubes as compared to actively growing myoblasts (Fig. 6A). This result indirectly suggested that furin gene expression could be regulated by myostatin. Thus, we measured the furin promoter-luciferase reporter activity in the presence and in the absence of myostatin. As shown in Fig. 6B, treatment of the P1 furin promoter with myostatin decreases the promoter activity in a concentration-dependent manner. This result indicates that the furin promoter is negatively regulated by myostatin.

Discussion

Previously published results have shown that *myostatin* is synthesized in both myoblasts and in muscle fibers, proteolytically processed, and secreted as a mature myostatin peptide (Thomas et al., 2000). However, the processing and secretion during myogenesis have not been widely investigated. In this communication, we show that the processing and secretion of myostatin is reduced upon differentiation or during fetal fiber formation stages. We also show that myostatin auto-regulates the extent of processing by regulating the gene expression of its converting enzyme, furin.

Myostatin expression during development

Northern blot analysis revealed that *myostatin* expression is developmentally regulated (Fig. 4B). *Myostatin* mRNA was abundant during early fetal development (days 61 and 160), increasing at fetal day 160. A relatively high level of *myostatin* mRNA was observed in each gestational

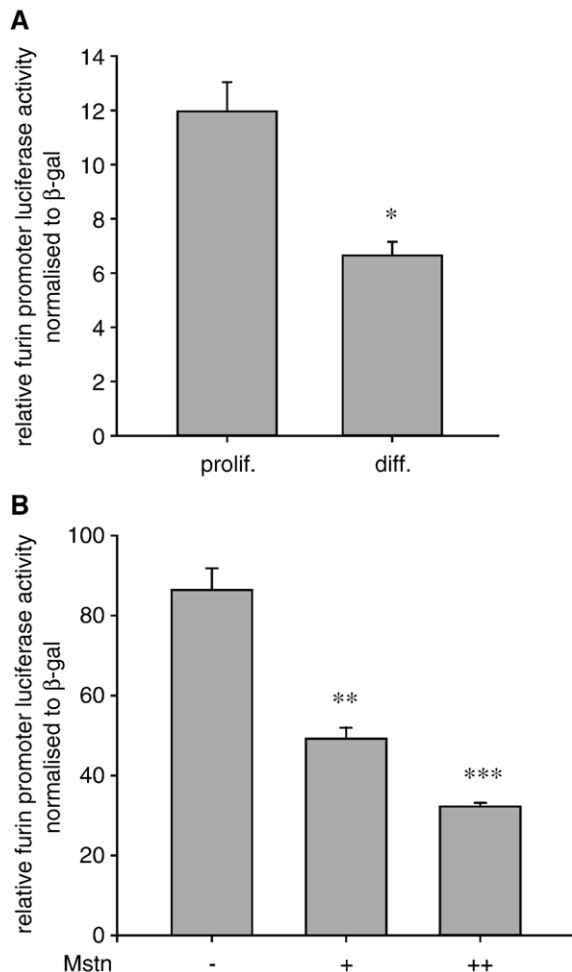


Fig. 6. Mature myostatin regulates furin promoter activity. (A) In vitro analysis of the furin promoter during proliferation and differentiation conditions. The furin promoter and β -galactosidase (β -gal) expression plasmids were transfected into C₂C₁₂ myoblasts. The cells were grown for a further 24 h in either proliferation or differentiation media after which luciferase and β -gal activity was measured. Results reflect relative furin promoter luciferase activity normalized to β -gal. Bars indicate means \pm standard error of three independent experiments. * $P < 0.05$ compared to prolif. control. (B) In Vitro analysis of the effect of myostatin on furin promoter activity during proliferation conditions. The furin promoter and β -galactosidase (β -gal) expression plasmids were transfected into C₂C₁₂ myoblasts. The cells were grown for a further 24 h in proliferation media after which luciferase and β -gal activity was measured. Results reflect relative change in furin promoter luciferase activity with increasing myostatin concentration (+0.5 μ g/ml, ++3 μ g/ml) as compared to the untreated control (–). Luciferase values were normalized to β -gal and the bars indicate means \pm standard error of three independent experiments. Statistical differences (P values) determined by t test are indicated, *** $P < 0.001$, ** $P < 0.01$ compared to un-treated control (–).

time point from day 160 onward, the peak expression being observed in the day 260 sample. After birth, the levels of *myostatin* mRNA declined dramatically and remained low in all the postnatal days (8, 14, and adult) observed. These results are predominantly in agreement with other results published from this laboratory in which *myostatin* expression is examined by RT-PCR in bovine semitendinosus muscle over the same time period (Oldham

et al., 2001). In the published results, a dramatic and transient increase in *myostatin* expression is also observed at fetal day 90, which lies between the two fetal time points, days 61 and 120, examined by Northern blot analysis in this study. This peak expression of myostatin coincides with the peak expression of MyoD. In addition, we have recently showed that the bovine myostatin promoter is a downstream target gene of MyoD (Spiller et al., 2002). Thus, it is quite possible that the developmental expression pattern of myostatin is predominately regulated by MyoD.

Myostatin indirect immunofluorescence microscopy experiments on myoblasts and myotubes confirmed that myostatin is synthesized in muscle cells (Fig. 1). Myostatin immunoreactive protein was detected in both proliferating myoblasts and nascent myotubes, localized in a perinuclear pattern (Fig. 1). In addition, myostatin was localized throughout the cytoplasm in the myotubes (Fig. 1). These findings are consistent with studies examining TGF- β in several cell types, whereby TGF- β 1 is localized to a perinuclear region, sometimes showing a characteristic reticular morphology (Miyazono et al., 1992; Mizoi et al., 1993; Roth-Eichhorn et al., 1998). In one of these studies, the subcellular localization of TGF- β 1 was determined by immunoelectron microscopy to be in the lumen of the rER and perinuclear cisternae of fibroblasts, macrophages, and endothelial cells, indicating the biosynthetic process of the protein (Mizoi et al., 1993). In another study, immunofluorescence microscopy of human erythroleukemia cells (HEL) revealed that TGF- β co-localizes with mannosidase II, an integral Golgi protein (Miyazono et al., 1992). Taken together, these studies indicate that TGF- β is synthesized and secreted via the endoplasmic reticulum and Golgi, respectively (Miyazono et al., 1992; Mizoi et al., 1993; Roth-Eichhorn et al., 1998). Based on these reports, it is possible that the perinuclear localization of myostatin reflects its synthesis and processing in the endoplasmic reticulum and Golgi. However, co-localization studies using Golgi-specific markers, such as mannosidase II, would be required to determine this for certain. In addition to the perinuclear localization of myostatin, a diffuse immunofluorescence was detected in the cytoplasm of the nascent myotubes supporting the observation that less myostatin is secreted in myotubes. Intracellular retention has also been observed for other members of the TGF- β superfamily, including Activin A, TGF- β 1 (Miyazono et al., 1992; Roth-Eichhorn et al., 1998), TGF- β 2 (Jakowlew et al., 1991; Roth-Eichhorn et al., 1998), and TGF- β 3 (Roth-Eichhorn et al., 1998). Latent TGF- β binding proteins (LTBPs) have been proposed to play a role in the cytoplasmic retention of TGF- β 1, - β 2, and β 3 (Oklu and Hesketh, 2000). It is unlikely that LTBPs would regulate myostatin secretion since yeast two hybrid and co-immunoprecipitation studies did not detect any interaction between LTBPs and myostatin (data not shown). However, previously published results from our laboratory suggest that the protein Titin-Cap (T-

cap) could regulate the intracellular levels of myostatin due to the fact that over-expression of T-cap leads to reduced secretion of myostatin (Nicholas et al., 2002). Further myostatin interacting proteins, including follistatin, FLRG, and GASP-1, appear to be unlikely candidates since they have been shown to interact with myostatin in circulation following secretion (Hill et al., 2002, 2003; Lee and McPherron, 2001).

Processing and secretion of myostatin during myogenic differentiation

Changes in the extent of myostatin proteolytic processing and secretion were observed between actively proliferating myoblasts and differentiated myotubes (Figs. 2 and 3). In actively proliferating mouse C₂C₁₂ and bovine primary myoblasts, the predominating forms of myostatin detected were the mature myostatin and/or LAP, suggesting that the myostatin precursor protein is being proteolytically processed. In differentiating and nascent myotubes by contrast, myostatin was observed predominantly in the precursor form. Consistent with the processing results, we find that during proliferation relatively more myostatin is secreted as compared to fully differentiated myotubes. Since the myostatin that was detected was metabolically labeled, it likely reflects the de novo synthesis from the cells and not myostatin introduced by media differences. Although the processing of myostatin is altered during myogenesis, no appreciable change in myostatin mRNA expression was observed in the bovine primary myoblasts. Levels of LAP and mature myostatin were measured between muscle tissues from wild type and myostatin null mice. Using two different specific myostatin antibodies, we have shown that there is a dramatic loss of the 26 kDa mature myostatin protein band in myostatin null mice (Fig. 2C), thus providing evidence for the validity of the protein bands we have assigned to myostatin. However, a faint band running at 26 kDa is observed in the myostatin null muscle tissue, this could be attributed to antibody cross reactivity with GDF11 as GDF11 shares a high homology with the mature region of myostatin.

Processing and secretion of myostatin during myogenesis

Lowered levels of myostatin processing occur during fetal muscle development when compared to postnatal adult muscle (Fig. 4). The primary observation in the examination of myostatin expression by Western blot analysis is the progressive shift in myostatin expression from precursor protein to LAP (Figs. 4A and C). At fetal day 61, most of the myostatin detected was present in the precursor form. Between fetal days 160 and 210, relatively more processing resulted in reduced precursor myostatin and increased LAP. From fetal day 260 onward, the majority of observed myostatin was present in the LAP form, suggesting that almost all of the synthesized myostatin is processed. Since

the processing of TGF- β superfamily members is required for secretion and moreover secretion occurs rapidly after processing (Miyazono et al., 1992; Roth-Eichhorn et al., 1998), this observation suggests that during late fetal development more myostatin is secreted. This increase in myostatin secretion, in conjunction with an up-regulation of *myostatin* gene expression, implies that there is a large increase in mature myostatin from day 210 in the developing fetus. This is indeed supported by Western analysis results of fetal systemic myostatin levels (Fig. 4D). While virtually all the secreted myostatin is undetectable during early fetal development, during postnatal adult stages, abundant levels of myostatin were present. Myostatin systemic expression was not detected during days 165 and 240 despite processing occurring. This could be due to the fact that myostatin circulatory levels during these time points were below the limits of detection for Western blot analysis. The dramatic increase in mature myostatin from day 210 in the fetus may result in the production of biologically active myostatin. To examine this, we performed a well-characterized bioassay for myostatin (Thomas et al., 2000). The results show that there is a 13% drop in C₂C₁₂ proliferation rate in cells treated with serum obtained from fetal day 260 cattle compared to cells treated with fetal day 210 serum (Fig. 5). This drop in proliferation rate is consistent with known myostatin function and interestingly coincides with the dramatic increase in myostatin processing observed during fetal day 260 (Fig. 4).

Low levels of circulatory myostatin during early fetal development may allow for the differentiation of primary and secondary waves of myoblasts to occur during myogenesis due to the fact that excess amounts of mature myostatin have been shown to cause a down-regulation of MyoD expression, resulting in the inhibition of myogenic differentiation of C₂C₁₂ myoblasts (Langley et al., 2002). Thus, to reduce the availability of biologically active myostatin and facilitate the process of post-mitotic differentiation, myoblasts could facilitate a reduction in processing of myostatin by an auto-regulatory mechanism. For such an auto-regulatory mechanism to be feasible, ligands involved in the functioning of TGF- β family members should be able to directly regulate proteolytic processing enzymes. Furin has been shown to cleave several TGF- β family members including bone morphogenetic protein-4 (BMP-4) and TGF- β 1 (Cui et al., 1998; Dubois et al., 2001). Previously, it has been shown that TGF- β up-regulates its own converting enzyme Furin (Blanchette et al., 1997). In contrast, the results presented here demonstrate that myostatin can negatively regulate furin protease promoter expression (Fig. 6B). Recently, it has been demonstrated that *myostatin* expression peaks at the onset of primary bovine myoblast fusion with a subsequent reduction during differentiation (Deveaux et al., 2003). Therefore, it is quite possible that the reduced processing of myostatin seen during differentiation is due to down-regulation of Furin protease activity by an increase in *myostatin* expression

prior to differentiation. In other words, a peak in myostatin levels during the onset of differentiation results in lowered furin protein expression and subsequent reduction in myostatin processing during differentiation. This novel mechanism of negative auto-regulation allows for timely myogenic differentiation during development leading to the formation of myogenic fibers.

An analysis of the Furin P1 promoter has identified several consensus E-Box sequences (data not shown), which could indicate the involvement of bHLH transcription factors in the regulation of furin gene expression. MyoD is an important bHLH transcription factor involved in regulating myogenesis. Previously, we have shown that myostatin can cause a down-regulation of MyoD expression resulting in an inhibition of C₂C₁₂ myoblast differentiation (Langley et al., 2002). Therefore, increased myostatin expression may cause a decrease in furin promoter activation by down-regulating MyoD expression.

Collectively our studies show that there is reduced proteolytic processing of myostatin protein during myogenesis and that myostatin negatively regulates the transcription of its own converting enzyme, furin. Given that myostatin is a negative regulator of myogenesis, we hypothesize that myostatin auto-regulates its processing, thereby regulating the process of myoblast terminal differentiation.

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