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Original article

Location of myostatin expression during bovine myogenesis in vivo and in vitro

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Abstract — Mutations in the *myostatin* gene lead to double-muscling in cattle indicating that it is a negative regulator of the total number of muscle fibres. Myostatin expression was analysed by RT-PCR in three developing bovine muscles. It decreased during differentiation in *Semitendinosus* and *Biceps femoris*, and increased in the late differentiating *Masseter* during gestation. A combination of in situ hybridisation and immuno-histochemical detection of myosin heavy chains (MHC) allowed us to locate the expression in myofibres containing only developmental MHC at different stages and in fast IIA fibres at the end of gestation. In vitro, myostatin was undetectable during proliferation, peaked at the onset of fusion and decreased during terminal differentiation. It was not detected in myotubes by in situ hybridisation. The inhibition of differentiation by BrdU prevented the decrease in expression. Our results show that the peak in myostatin expression coincides with early differentiation indicating a regulatory role in cattle myogenesis.

myostatin [GDF8] / cattle / myogenesis / myoblast culture / in situ hybridisation

1. INTRODUCTION

Myostatin (Growth/Differentiation Factor-8, GDF-8) is a member of the Transforming Growth Factor β superfamily [1]. Based on sequence homologies, myostatin shares structural features with the members of the family. It is produced as a precursor form that is proteolytically processed as are the other members of the superfamily. The bioactive form is probably a disulfide-linked dimer of the C-terminal domain [1]. Myostatin is highly conserved among species. Predicted proteins in humans, rats, mice, pigs and chicken exhibit 100% homology in their bioactive domain, whereas there is a 3 amino-acid difference in the mature sequence [2].

Transgenic mice lacking the *myostatin* gene exhibit enlarged skeletal muscles related to an increase in the number (hyperplasia) and in the area (hypertrophy) of the muscle fibres [2]. Double-muscling (DM) in cattle is also characterised by a greater

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muscle mass due primarily to hyperplasia [3]. Interestingly, DM animals display mutations in their myostatin gene [2, 4, 5] corresponding to the previously identified *mh* locus [4, 6]. These mutations lead to the production of an inactive peptide. For example, in the DM Belgian Blue breed systematically selected for double-muscling, an 11-bp deletion has occurred in the third exon in a region encoding the bioactive domain, generating a truncated inactive carboxy-terminal domain [2, 5, 7]. An increase in the total number of muscle fibres in double-muscling has been observed as early as the foetal period [8]. Cultured DM myoblasts display an increased proliferation thus supporting the latter [9, 10]. The combination of these data supports the hypothesis that myostatin acts as a negative regulator of muscle growth. In vitro studies have shown that recombinant myostatin reversibly inhibits myoblast proliferation [11–13]. Thomas et al. [13] have proposed a role for myostatin in the induction of myoblast withdrawal from the cell cycle, via an increase in cyclin-dependant kinase inhibitor p21 expression and a decrease in Cdk2 proteins and activity. Recently, in vitro experiments have also provided evidence that extra myostatin impairs C2C12 myoblast differentiation [14, 15], through the inhibition of MyoD1 expression and activity [14].

Myostatin is predominantly expressed in skeletal muscle. It has also been described in mouse adipose tissue [1], in mouse and sheep heart [16] and in sow mammary glands [17]. Its expression has been detected very early in the muscle of developing mice [1], cattle [7], pigs [17] and chicken [18]. It is decreased but just maintained after birth in skeletal muscles [1, 7, 19]. In the adult, muscle atrophy caused by hindlimb unloading is associated with increased myostatin expression [20–22]. Conversely, it has been shown that its expression is decreased during the early stages of muscle regeneration after injury [21, 23, 24]. The aim of this study was to follow the kinetics of myostatin expression during bovine muscle myogenesis in vivo and in vitro by RT-PCR. Moreover, it allowed us to identify the myostatin-expressing cells by combining in situ hybridisation and immuno-histochemical techniques.

2. MATERIALS AND METHODS

2.1. Animals

The study was carried out as a part of a research programme approved by the "Institut National de la Recherche Agronomique" (INRA, France) Ethical Committee. The cows were bred and slaughtered, and foetuses were collected, according to ethical guidelines concerning animal care. For the in vivo study 20 Charolais foetuses were produced by artificial insemination. Four foetuses were collected at 90, 110, 180, 230 and 260 days post-conception (dpc). Their *Masseter* (MA), *Biceps femoris (*BF) and *Semitendinosus* (ST) muscles were dissected and snap-frozen. For in vitro studies, four Holstein foetuses (tested for the absence of a myostatin mutation) were produced by artificial insemination. They were collected at the gestational age of 100 days, corresponding to a period of intense proliferation of the second myoblast generation [25].

2.2. Cell Cultures

Primary cultures of foetal myoblasts were performed according to Picard et al. [10]. Briefly, foetal skeletal muscles from the hindlimbs were dissected under sterile conditions. They were cut into small cubes in Dulbecco Modified Eagle Medium (DMEM; GibcoBRL, Life Technologies, France). Three incubations with 0.25% trypsin (GibcoBRL, Life Technologies, France) for 20 min allowed the cells to dissociate. After the first incubation, the sus pension (essentially composed of fibroblasts)

was used for seeding fibroblast cultures. Fibroblasts were plated at a density of 10^4 cells/cm^2 in 100-mm dishes. After the next two digestions, trypsin was removed by centrifugation of the cell suspension at 900 *g* for 10 min. Pelleted cells were then suspended in DMEM supplemented with 0.2 mM L-Glutamin, 5 μg·mL^{-1} gentamycin and 10% foetal calf serum (FCS; Sigma Aldrich, France). After 30 min pre-plating at 37 ºC, the cells were plated at a density of 2.10^4 cells/cm^2 in 100-mm dishes for mRNA studies. Labtek chamber slides (Nunc, Denmark) were used for in situ hybridisation studies. In order to study the influence of an inhibition of myogenic differentiation, some cultures were treated by 5 μg·mL^{-1} of 5-bromo-deoxyuridine (BrdU, [26]). All cultures were incubated at 37 ºC in a 5% CO_2 atmosphere, and were fed fresh medium 24 h after plating.

Primary cultures of normal satellite cells were prepared as described previously [27]. Briefly, the cells were isolated from cultured explants of *Longissimus thoracis* muscle derived from adult cows. They were seeded at a density of 10^4 cells/cm^2 in 100-mm gelatin-coated dishes and were grown in a feeding medium consisting of a mixture of DMEM and HAM-F12 (vol/vol) and of 15% FCS. At sub-confluence, the cells were shifted to a differentiation medium consisting of 2% Horse Serum in order to favour their differentiation.

2.3. RT-PCR

After extraction of total RNA using the thyocianate guanidium method derived from Chomczynski and Sacchi [28], first strand cDNA was synthesised from 2.5 μg of total RNA using a SUPERSCRIPT™ II RNase H$^-$ reverse transcriptase (GibcoBRL, Life Technologies, France), according to the manufacturer's protocol.

PCR reactions were performed using Taq DNA Polymerase (Promega, France) to monitor myostatin, myogenic regulatory factors and myosin heavy chain expression analyses. The primers were synthesised by Eurobio (France). Primer sequences and cycling conditions are listed in Table I. PCR were performed with 3 μL of the reverse transcription reaction. A fragment of the bovine TATA box-binding protein (TBP) cDNA was amplified to serve as a control for the in vivo studies whereas a fragment of the cyclophilin T cDNA served as a control for the in vitro studies. Statistical analysis confirmed that the expression of the TBP and cyclophilin T genes did not vary significantly during the developmental stages and time-course of culture respectively (data not shown). The PCR reaction started with one cycle consisting of 94 ºC for 5 min followed by 25/30 cycles of 94 ºC for 45 s, annealing temperature (Tab. I) for 45 s and 72 ºC for 45 s. PCR products were visualised on a 2% agarose gel stained by ethidium bromide and the intensity of the fragments was measured with the Chemilmager™ 5500 Fluorescence (Alpha Innotech Corporation) software Alpha ease FC™.

2.4. In situ hybridisation

The bovine myostatin cDNA was inserted into the pGEM®-11Zf(+) vector (Promega, France). After linearisation of the plasmid, anti-sense and sense (control) cRNA probes were synthesised respectively using T7 and SP6 RNA polymerase (RiboProbe® combination system SP6/T7, Promega, France). The probes were labelled with fluorescein-UTP (Roche Diagnostics, France).

Transverse sections of 10 microns thick and cell culture slides were fixed using a 4% paraformaldehyde solution in PBS for 15 min and dehydrated in three successive ethanol solutions (85%, 95% and 100%, 5 min each); then the slides were stored at –80 ºC until their utilisation. Hybridisation was performed using the method adapted from Fontaine-Pérus et al. [29]. Briefly, the probes were diluted in ULTRAhyb™ buffer

Table I. Primer sequences and cycling conditions used in RT PCR experiments.

Primers	Sequences (forward and reverse)	Annealing temperature/ cycle number	Length	Origin
Cyclophilin	5' – CCT GCT TTC ACA GAA TAA TTC CAG – 3' 5' – CAT TTG CCA TGG ACA AGA TGC CAG – 3'	58 °C / 25 cycles	154 bp	Ovine
TBP	5' – TCT ATT CTG GAG GAG CAG CAA– 3' 5' – GGA CGT CGA CTG CTG GAC – 3'	60 °C / 30 cycles	114 bp	Bovine
MRF4	5' – ACT GTG GCC AAC CCC AAC CAG – 3' 5' – GAA TGA TCG GAA ACA CTT GGC C – 3'	56 °C / 30 cycles	224 bp	Human
Myostatin	5' – GTC TCA TAC CAT GGC TGG AAT – 3' 5' – GGT AAT TGG CAG AGT ATT GAT – 3'	55 °C / 30 cycles	513 bp	Bovine
myf5	5' – GTC TGC CCT TGT TAA TTA CCA G – 3' 5' – CAT CAG AGC AAC TTG AGG TGG – 3'	56 °C / 35 cycles	635 bp	Bovine
myoD	5' – GCT TTG CCA GAC CAG GAC – 3' 5' – CCT GCC TGC CGT ATA AAC AT –3'	58 °C / 28 cycles	94 bp	Bovine
Myogenin	5' – CTG AGC TCA GCC TGA GCC CTG – 3' 5' – CAG GCG CTC TAT GTA CTG GAT G – 3'	60 °C / 30 cycles	284 bp	Bovine

(Ambion, Clinisciences, France) and were laid on the slides, which were then incubated at 42 °C overnight. After three washes in 1× SSC, 50% formamid and 0.1% Tween-20 solution and two washes in 0.1 M maleic acid, 0.2 M NaOH, 0.15 M NaCl and 1% Tween-20 solution, the slides were treated for 2 hours in 2% Reagent blocking (Roche Diagnostics, France) solution. Then they were incubated with a conjugated anti-fluorescein antibody coupled to alkaline phosphatase (AP) (Roche Diagnostics, France) at room temperature overnight. The revelation of the AP activity was performed with BM purple AP substrate (Roche Diagnostics, France). The slides were then mounted in PBS-glycerol and analysed under an inverted light microscope (Labophot-2, Nikon).

2.5. Muscle fibre-type determination

Myosin heavy chain (MHC) isoform content was revealed at development stages by immuno-histochemical staining using the following monoclonal antibodies (MAB) purchased from Biocytex (Marseille, France): MAB S, F36 5B9, specific to slow MHC; MAB R, F113 15F4, specific to fast MHC (MHC 2a and 2b, 2x); MAB F, F158 4C10, specific to foetal MHC; MAB I+IIb+IIx, S5 8H2, specific to both slow and fast 2b and 2x MHC (without any cross-reactivity with

2a MHC); MAB α, F88 10C2, specific to developmental α-cardiac MHC. The reactivity of all these antibodies has been tested on bovine muscle [25, 30].

Serial sections of those used for in situ hybridisation were incubated directly with the first monoclonal antibody for 30 min at 37 °C. Then they were washed in PBS and incubated with a second antibody (rabbit anti-mouse IgG labelled with dichlorotriazinlaminofluorescein, Interchim) diluted 1/30 in PBS for 30 min at 37 °C. After washing in PBS, the sections were mounted with mowiol (Calbiochem). Immunofluorescent staining was analysed under the microscope with a fluorescent light (excitation filter 450–490 and stop filter 520).

2.6. Western blot analyses

Cell cultures were homogenised in buffer containing 2% SDS, 10% glycerol, 63 mM Tris pH 6.8. The samples were spun at 8 000 rpm for 20 min at 4 °C and fractions of the supernatant were aliquoted and stored at –80 °C. Protein concentrations were determined according to Markwell et al. [31]. Fifteen micrograms of protein extract were mixed with Laemmli buffer [32], heated at 95 °C for 5 min and separated in a sodium dodecylsulfate-polyacrylamide gel electrophoresis (10%). The proteins were electroblotted to polyvinyl membrane (Immobilon, Millipore) and subsequently incubated with blocking buffer containing 3% gelatin in TBST (20 mM Tris pH 8, 137 mM NaCl , 0.1% Tween 20) and then with the following primary antibodies diluted in TBST: (1) a monoclonal anti-Desmin (D 33, Dako, 1:2000) or (2) a monoclonal anti-Myogenin (generously provided by V. Mouly from UMR 7 000, Paris, Jussieu, 1:1 000). Following incubation with the secondary antibody diluted in TBST (horseradish peroxidase-conjugated anti-mouse IgG, Amersham), the proteins were visualised by the enhanced chemiluminescence detection method (ECL, Amersham).

2.7. Statistical analysis

Myostatin data were analysed using the GLM procedure [33] in a model that contained TBP expression levels as covariables and the effects of age separately within muscles. Differences in LSMeans between ages were further separated by the PDIFF option. TBP was chosen as a reference gene according to [34] and was used as a covariable rather than a ratio normalisation term in order to avoid statistical bias [35].

3. RESULTS

3.1. Myostatin expression during in vivo myogenesis

The kinetics of myostatin expression was followed by semi-quantitative RT-PCR during foetal development in three different muscles. The *Masseter* (MA) was chosen because it is not hypertrophied in DM cattle. Conversely, the *Biceps femoris* (BF) and the *Semitendinosus* (ST) muscles were chosen because they are hypertrophied in DM cattle [36]. The temporal pattern of expression differed between muscles. In ST (Fig. 1A), the expression was high at 110 dpc, decreased between 110 and 180 dpc ($P < 0.05$), and thereafter did not significantly vary until 260 dpc. In BF (Fig. 1B), the expression significantly increased between 110 and 180 dpc ($P < 0.05$) and thereafter decreased ($P < 0.05$ between 180 and 260 dpc). In MA (Fig. 1C), myostatin expression gradually increased all through gestation and was significantly higher in the third term than at 110 dpc ($P < 0.05$ between 110 and 230 dpc, $P = 0.01$ between 110 and 260 dpc).

The location of myostatin expression in foetal skeletal muscle was possible by combining in situ hybridisation and immunological detection of MHC isoforms on transverse

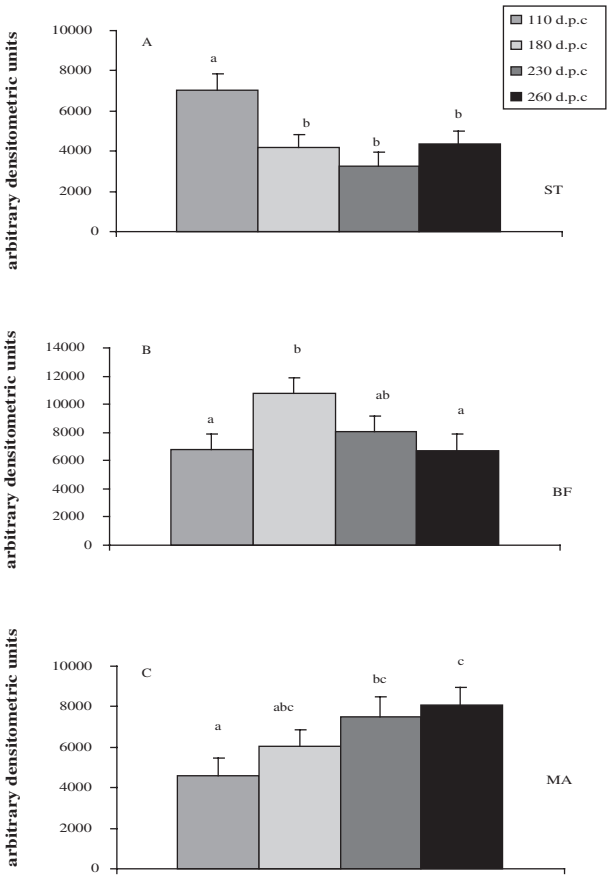

Figure 1. Myostatin expression during foetal development in *Semitendinosus* (A), *Biceps femoris* (B) and *Masseter* (C) muscles. RT-PCR were realised using specific primers for bovine sequences (see Tab. I). RT-PCR data are expressed in arbitrary densitometric units and were analysed in a model containing TBP levels as covariables. ST, *Semitendinosus*; BF, *Biceps femoris*; MA, *Masseter*. [abc] Mean values with different superscript letters are significantly different between groups. The statistical significances (*P*) of the effect of age were 0.049, 0.06 and 0.08 in ST, BF and MA respectively.

serial sections. In situ hybridisation revealed that myostatin mRNA was mainly located in bundles of fibres and only sparsely in the interstitial tissue (Fig. 2). Moreover, whatever the developmental stage, only a small proportion of cells expressed myostatin (Fig. 2). No specific staining was observed with the sense probe (Fig. 2). At each stage, no myostatin mRNA was detected within the slow MHC-expressing first myogenic generation whose differentiation is most advanced. At 90 dpc (33% of gestation), the myostatin-expressing cells were stained using antibodies raised against developmental α-cardiac and foetal MHC, but not with those raised against adult slow and fast MHC (Fig. 3A). The myostatin-expressing cells were small cells corresponding to the second myogenic generation according to Picard et al. [30]. At 180 dpc (66% of gestation), the expression was also located in the less differentiated cells (Fig. 3B) characterised by a restricted expression of developmental MHC as in the preceding stage [30]. At 260 dpc (96% of gestation), the myostatin-expressing cells no longer contained any developmental MHC but were positive for adult fast MHC. An antibody recognising both slow and fast (2b, 2x but not 2a) MHC did not stain the myostatin-expressing cells. According to Picard et al. [37], these fibres were identified as IIA fibres (Fig. 3C).

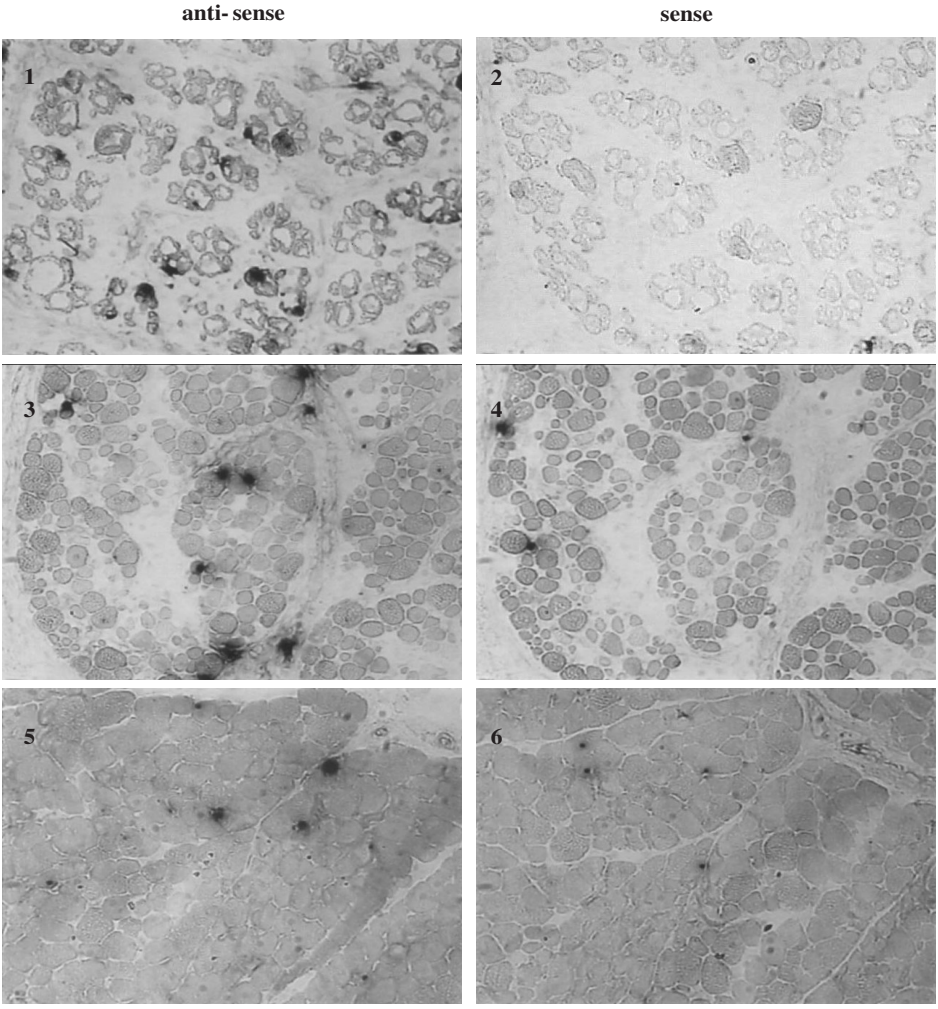

Figure 2. The location of myostatin expression during foetal development in ST muscle. Myostatin mRNA were detected by in situ hybridisation at 90 dpc (microphotograph 1), 180 dpc (microphotograph 2), and 260 dpc (microphotograph 3). Control hybridisations performed with sense cRNA (microphotographs 4, 5 and 6) are shown at 90 dpc, 180 dpc and 260 dpc, respectively. Magnification ×330.

3.2. Myostatin expression during in vitro myogenesis

In a second study, we investigated myostatin expression in primary myoblast cultures isolated from 100-day-old normal foetuses. The cells proliferated for 3 days until they aligned at confluence and began to fuse into myotubes. The cells were harvested at these different stages and total RNA was prepared for RT-PCR analysis of myogenic markers. MyoD and Myf-5 were expressed early during the culture, starting with the proliferation phase. Their expression

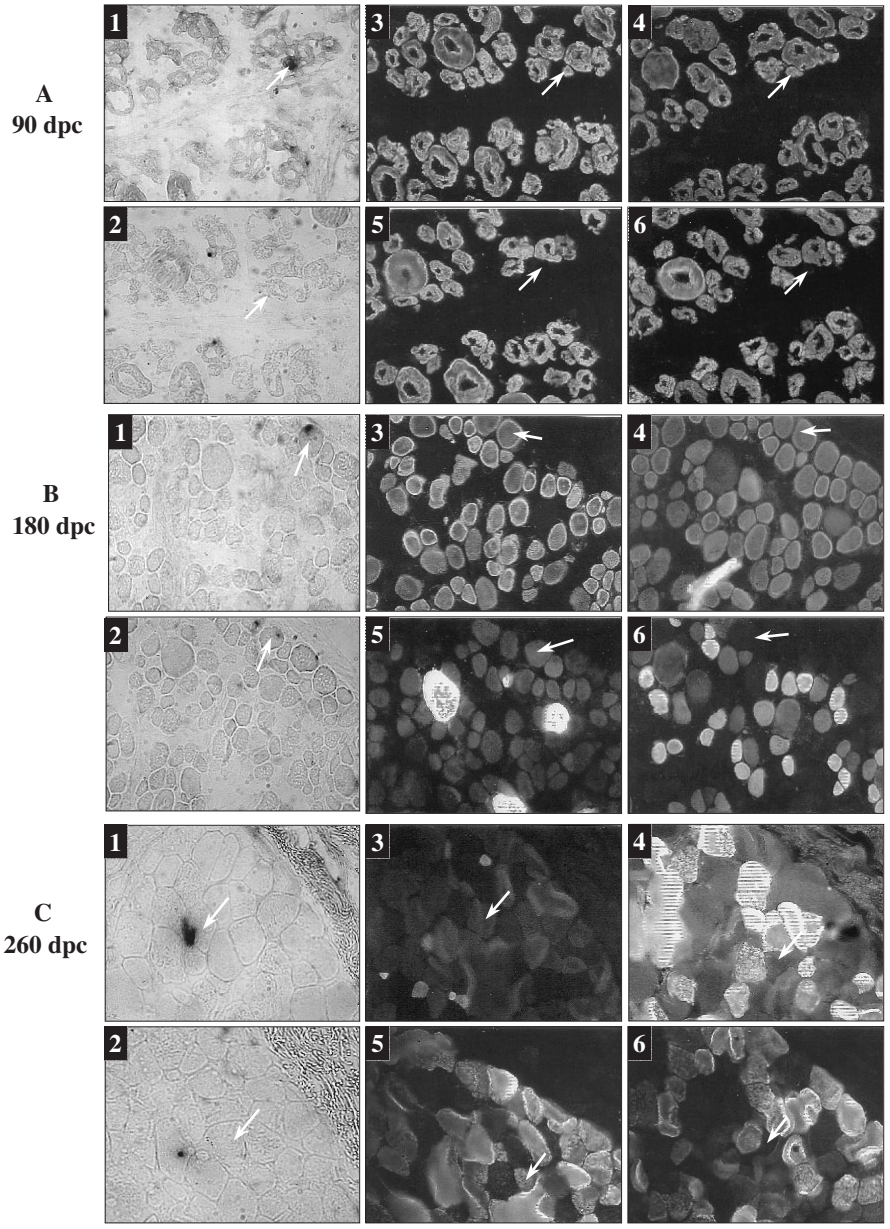

Figure 3. The location of myostatin expression during foetal development in ST muscle. Myostatin mRNA (microphotographs 1) were detected by in situ hybridisation at 90 dpc (**A**), 180 dpc (**B**), 260 dpc (**C**). Control in situ hybridisations were performed with sense cRNA (microphotographs 2) in serial sections. The immunological detection of MHC isoforms was performed on the next serial sections: developmental α-cardiac MHC (microphotographs 3), developmental foetal MHC (microphotographs 4), adult slow MHC (microphotographs 5), and adult fast MHC (microphotographs 6). Magnification ×480.

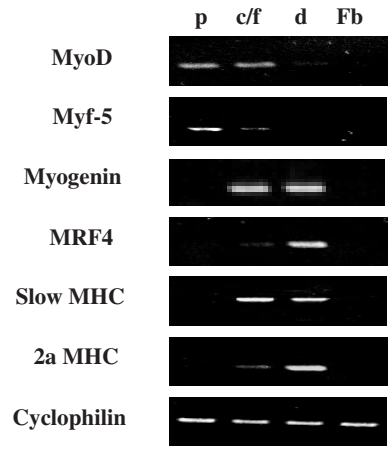

Figure 4. MRF and MHC expression in myoblast cultures. p, proliferation; c/f, confluence/onset of fusion; d, differentiation. Fb: confluent muscle fibroblast cultures. RT-PCR were realised using specific primers (see Tab. I).

was maintained until confluence/onset of fusion (Fig. 4). Neither factor was expressed any longer during differentiation (Fig. 4). Myogenin was expressed from the beginning of fusion and during differentiation (Fig. 4). MRF4 began to be slightly expressed at the onset of fusion and was expressed at the highest level during differentiation (Fig. 4). Expression of slow and fast 2a MHC began with the confluence/onset of fusion (Fig. 4) and increased during differentiation (Fig. 4). The detection and time-course of myogenic specific marker expressions confirmed that our primary cultures were mainly constituted of myogenic cells (90% of Myf-5-expressing cells, data not shown). As expected, none of these markers was expressed in control fibroblast cultures (Fig. 4).

We could not detect any myostatin expression by Northern blot analysis, except at

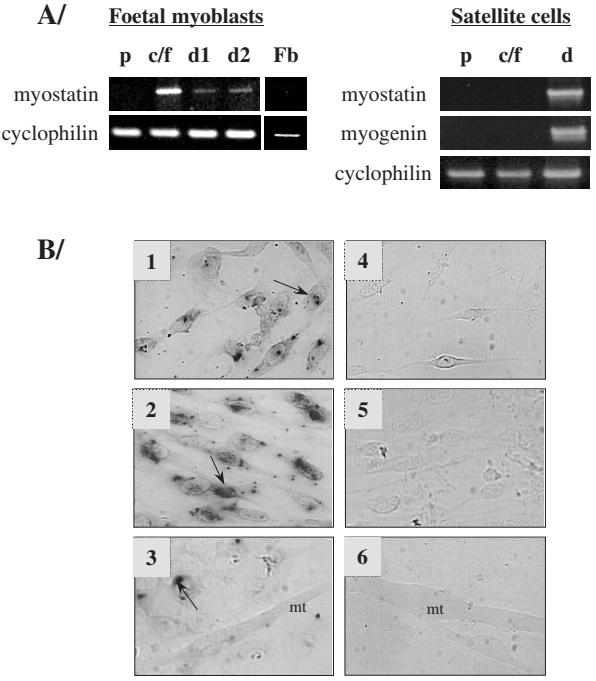

Figure 5. In vitro myostatin expression. (**A**) Foetal myoblast and satellite cell cultures were monitored for myostatin and myostatin/myogenin expression respectively. p, proliferation; c/f, confluence/onset of fusion; d, d1, d2, differentiation stages. Fb: confluent fibroblast cultures. RT-PCR were realised using specific primers (see Tab. I). (**B**) The location of myostatin expression in myoblast cultures. Microphotographs 1 to 3: Myostatin mRNA detected by in situ hybridisation. Microphotographs 4 to 6: in situ hybridisation controls performed with sense cRNA. 1 and 4: proliferation. 2 and 5: sub-confluence. 3 and 6: differentiation. Mt: myotubes. Arrows indicate examples of stained cells (Magnification ×440).

cell confluence (data not shown). Thus, myostatin expression was followed by RT-PCR (Fig. 5A). Myostatin mRNA was first detected in confluent cultures before the formation of myotubes and concomitantly with myogenin mRNA (Fig. 5A). In differentiating cells, myostatin expression was markedly decreased but remained detectable by RT-PCR (Fig. 5A). Myostatin expression was also found in differentiating bovine satellite cells, isolated from adult muscles. Myostatin expression, similar to myogenin expression, was undetectable during proliferation and was up-regulated once the cells had been switched to a differentiation medium (Fig. 5A). Interestingly, Myostatin mRNA was not detected by RT-PCR in muscle fibroblast cultures (Fig. 5A) clearly indicating that the expression was restricted to myogenic cells. As expected, no differences were detected in the housekeeping gene cyclophilin T levels of expression.

In situ hybridisation experiments confirmed the temporal pattern of myostatin expression in foetal myoblast cultures. During proliferation, only a few cells weakly expressed myostatin (Fig. 5B). At sub-confluence, a high percentage of mononucleated cells was positive for myostatin mRNA (Fig. 5B). The staining was more intense indicating a higher level of expression. In differentiating cultures, myostatin expression was located in a few mononucleated cells and was not detected in myotubes (Fig. 5B). No staining was detected with a myostatin sense probe demonstrating the specificity of the hybridisation (Fig. 5B). Thus, myostatin expression was restricted to non-fused cells in primary myoblast cultures. This finding indicated that it was closely linked to the differentiation state of the cells.

In order to test if the fall in myostatin expression was a consequence of myoblast differentiation, we induced experimental inhibition of myoblast fusion. The cells were treated with 5 $\mu g \cdot mL^{-1}$ of BrdU and compared to control cells grown in the absence of BrdU. The cells were harvested at confluence/onset of fusion, 24 h after confluence and differentiation respectively (Fig. 6A). The proteins and total RNA were prepared from the cultures. As expected, myoblast

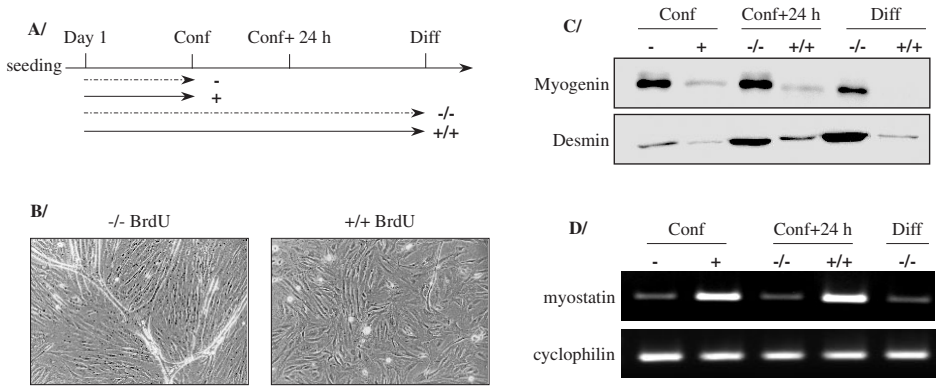

Figure 6. The effects of a BrdU-treatment on myostatin expression. (**A**) Treatment protocol (see material and methods section). Conf: confluence; Diff: differentiation. (**B**) Microphotographs of control (–/–) and treated (+/+) cultures. (**C**) Detection of Myogenin (53 KDa) and Desmin (54 KDa) protein accumulation by Western blot analyses. (**D**) Detection of myostatin expression by RT-PCR.

fusion was inhibited in BrdU-treated cells (Fig. 6B). The inhibition of differentiation was shown by the low accumulation of myogenin and desmin proteins (two markers of differentiation [10, 38]) in BrdU-treated cells compared to control cells whatever the stage (Fig. 6C). Interestingly, myostatin expression was higher in BrdU-treated than in control cells at confluence (Fig. 6D). This was still observed 24 h later and at a stage corresponding to differentiation in control cultures (Fig. 6D). Thus, the inhibition of myoblast fusion induced the maintenance of a high level of myostatin expression. These results strongly suggest that the decrease in expression observed during differentiation occurred following fusion and/or the onset of differentiation.

4. DISCUSSION

In this study, with a combination of RT-PCR and in situ hybridisation data we showed that myostatin expression is differentially regulated in developing muscles according to their kinetics of differentiation. We also found myostatin expression in myogenic cells corresponding to the less differentiated cells in vivo and in vitro. In particular, this expression was associated with fast IIA fibres at the end of gestation.

4.1. Differential expression between developing muscles according to their differentiation status

As in the report of Oldham et al. [39], myostatin expression in ST was high at 110 dpc, decreased thereafter, and was maintained at lower levels during the last trimester of gestation. Different kinetics of expression was observed in BF and MA compared to ST muscle. A peak in expression was detected at the end of the second gestation trimester (180 dpc) in BF. During the last trimester, the expression level was gradually restored to the one measured at 110 dpc. Conversely the expression steadily increased in MA during gestation and was higher at the end-term than at 110 dpc. Differences in the temporal pattern of myostatin expression may rely on the differential precocity of the muscles. In cattle, proliferation of muscle cells occurs during the first two trimesters of gestation, whereas their contractile and metabolic differentiations occur in the last trimester [25, 40]. However, muscles display a variability in the kinetics of differentiation. In particular, BF and MA differentiate later than ST muscle [25]. Interestingly, lower myostatin expression during the last trimester coincides temporally with the acquisition of contractile and metabolic properties of fibres in ST and BF. A decrease in myostatin expression with differentiation has also been reported in other species. In the chicken, it decreases at hatching [18] when the number of fibres is fixed [41]. In the pig, the highest level of expression detected before birth [17] also coincides with a fixed number of cells just after birth [42].

We confirmed the fall of myostatin expression along with the fusion and differentiation process in vitro. In cultured bovine myoblasts, no myostatin transcript was detectable in growing cells before differentiation started. We detected a transient peak of myostatin mRNA at the end of the proliferation phase. Myostatin expression was found in confluent cultures, e.g. before myoblast fusion and the appearance of myotubes. Several studies have described a similar expressional pattern respectively in chicken satellite cells and C2C12 myoblasts [11, 24, 43]. However, as in our satellite cell cultures, myostatin up-regulation was observed in C2C12 when fusion was already established [24]. Satellite cell cultures allowed us to confirm that the onset of myostatin expression was detected in post-mitotic cultures as shown by myogenin expression [10]. Unfortunately, we could not technically identify which type of cells (mononucleated vs. myotubes) expressed myostatin. Since foetal myoblasts and satellite cells

constitute distinct myogenic populations, this may account for the observed differences in their onset of myostatin expression. In particular, they display differences in their kinetics of titin, desmin and MHC accumulation [10, 27]. Moreover, the differentiation of satellite cells requires a switch to a differentiation medium which appears to delay the time-course of differentiation and the onset of myogenin expression compared to cultured foetal myoblasts.

As observed for MyoD and Myf-5 expression, myostatin expression decreased during terminal differentiation of foetal myoblasts and remained barely maintained. The expression was preserved when the fusion and terminal differentiation had been inhibited by BrdU. According to the finding that fusion-defective BC_3H_1 myocytes express myostatin under differentiation conditions [24], our results show that fusion is not a prerequisite for myostatin expression. Moreover, they indicate a link between the differentiation process and the decrease in myostatin expression. The fall of myostatin transcripts may occur consecutively to myogenin/MRF4 up-regulation and/or MyoD/Myf5 down-regulation during terminal differentiation. Myogenic transcription factor binding sites (E-boxes) have recently been found in the bovine myostatin promoter [44] indicating that the gene is at least a downstream target of MyoD1. However, in our differentiating cultures, myostatin levels were decreased in spite of high myogenin levels. Conversely, high myostatin levels were detected in spite of a decrease in myogenic factors induced by BrdU. All together, these results suggest that these E-boxes may not have a major role in the induction or maintenance of myostatin expression during terminal differentiation.

4.2. The location of myostatin expression within the less differentiated cells

Using in situ hybridisation and immunohistochemistry, we assigned myostatin expression within muscle bundles specifically to myogenic cells. We never observed myostatin expression in the first generation of differentiated myofibres at the stages we studied. However, myostatin may be expressed in the first generation at previous stages since expression has been found as early as in 30 dpc in bovine muscles [7] corresponding to the appearance of the first generation of myotubes. An explanation for the absence of detection of mRNA within the first generation can be that primary fibres are the most advanced in their differentiation at the stages we studied. In particular, differentiation is completed at 180 dpc [25, 40]. At 90 and 180 dpc, expression in ST was restricted to myotubes containing developmental MHC but not any adult MHC. At the end of gestation, myofibres positive for myostatin expression were fast IIA myofibres arising from the third generation. At this stage, IIA fibres contain a higher proportion of developmental MHC than I and IIX fibres and therefore are less differentiated [42]. From these results, myostatin expression appears to be restricted to the less differentiated myofibres at each developmental stage [42]. Thus, the decrease in myostatin expression during development may be related to the progressive maturation of myofibres of the second and third generations. This may explain why the level of expression was decreased earlier in ST than in the other muscles. In contrast, in MA in which differentiation is the latest and is not completed at birth [25], the highest myostatin expression was found at the end of gestation. Interestingly, this muscle also contains a high proportion of IIA fibres at this stage [25, 45] as compared to the others.

Contradictory data have been reported among species about the fibre type location of myostatin expression. In humans and in chickens, there is no difference between fast and slow muscles in terms of their myostatin expression in vivo and in vitro [43, 46]. In rodents, myostatin mRNA and protein levels are higher in fast than in slow muscles [20, 22]. In agreement with the l

e latter, we found that myostatin expression was localised within the fast fibres. However, Carlson et al. [20] demonstrated a significant positive regulation between MHC 2b content and myostatin mRNA abundance in normal muscle in mice. They hypothesised that muscle fibre type-specific gene expression could be modulated by myostatin and that myostatin may act to positively reinforce the fast phenotype. Unlike them, we showed that myostatin expression was associated with IIA fibres and we did not detect it in IIX fibres.

The observation that myostatin was expressed within the less differentiated cells in vivo was confirmed in vitro. In contrast with Artaza et al. [47] who located MSTN protein mostly in differentiated C2C12 myotubes, we found myostatin mRNA only in a few mononucleated cells and not in terminally differentiated myotubes in differentiating cultures. However, we cannot exclude that myostatin could be transiently expressed in early myotubes and that we have not detected it at the stages analysed in vitro. This would provide an explanation to the detection of expression within myofibres in vivo. Because no myostatin mRNA was detected in confluent muscle fibroblast cultures, and 90% of the cells were positive for Myf-5 at seeding, we can conclude that myostatin-expressing cells are mostly myogenic. These cells may constitute either myoblasts withdrawing late from the cell cycle and entering the differentiation process, or undifferentiated myoblasts protected from apoptosis [48]. The apparent contradiction between our results and those of Artaza et al. [47] may also be explained by the observation that the myostatin protein levels do not follow the changes of their transcripts and increase, whereas mRNA decrease during regeneration [24]. In such a hypothesis, in situ myostatin synthesis may increase in myotubes along with differentiation. Immunocytochemical detection of myostatin protein in our cultures may provide further elements to support this proposal.

4.3. Physiological significance of the myostatin expression-window

In this study, we observed that in developing bovine muscles, myostatin is expressed in a spatial and temporal sequence controlling their fibre numbers and terminal differentiation. Moreover, it is transiently expressed at the onset of differentiation in cultured myoblasts derived from foetal muscles. Based on these observations, myostatin appears to play a crucial role in the transition between proliferation and differentiation. Several studies have reported that myostatin acts as an inhibitor of proliferation [11–13]. It has also recently been shown that this factor inhibits myogenic differentiation in vitro [14, 15] and in vivo [49] by down-regulating the expression of transcription genes associated with myogenic differentiation such as MyoD1. However, a recent study has demonstrated that endogenous myostatin does not exert its inhibitory influence through the regulation of myoD1 expression [48] but through down-regulation of myogenic RNA and protein levels. Thus, myostatin is suspected to control myoblast numbers in the developing muscles by negatively regulating both their proliferation and differentiation although through distinct molecular pathways [50] as confirmed in vitro [13, 14]. In DM cattle, production of a non-functional protein could be responsible for the deregulation of the myoblast proliferation/differentiation process subsequently leading to (1) an increase in the myoblast pool resulting in fibre hyperplasia, and (2) increased myogenesis resulting in hypertrophic muscle masses. However, there is a discrepancy with the observation that differentiation is delayed both in vivo and in vitro in DM animals [10, 51].

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REFERENCES

[1] McPherron A, Lawler AM, Lee SJ. Regulation of skeletal muscle mass in mice by a new TGFβ superfamily member. Nature 1997, 387: 83–90.

[2] McPherron A, Lee SJ. Double muscling in cattle due to mutations in the myostatin gene. Proc Natl Acad Sci USA 1997, 94: 12457–12460.

[3] Holmes JH, Ashmore CR. A histochemical study of development of muscle fiber type and size in normal and "double muscled" cattle. Growth 1972, 36: 351–372.

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

[5] Grobet L, Poncelet D, Royo LJ, Brouwers B, Pirottin D, Michaux C, Ménissier F, Zanotti M, Dunner S, Georges M. Molecular definition of an allelic series of mutations disrupting the myostatin function and causing double-muscling in cattle. Mamm Genome 1998, 9: 210–213.

[6] Charlier C, Coppierters W, Farnir F, Grobet L, Leroy PL, Michaux C, Mni M, Schwers A, Vanmanshoven P, Hanset R, Georges M. The mh causing double-muscling in cattle maps to bovine chromosome 2. Mamm Genome 1995, 6: 788–792.

[7] Kambadur R, Sharma M, Smith TP, Bass JJ. Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. Genome Res 1997, 7: 910–916.

[8] Swatland HJ, Kieffer NM. Fetal development of the double muscled condition in cattle. J Anim Sci 1974, 38: 752–757.

[9] Deveaux V, Cassar-Malek I, Picard P. Comparison of contractile characteristics of muscle from Holstein and Double-Muscled Belgian Blue foetuses. Comp Biochem Physiol 2001, 131: 21–29.

[10] Picard B, Depreux F, Geay Y. Muscle differentiation of normal and double-muscled bovine fœtal myoblasts in primary culture. Basic Appl Myol 1998, 8: 197–198.

[11] Ríos R, Carneiro I, Arce VM, Devesa J. Myostatin regulates cell survival during C2C12 myogenesis. Biochem Biophys Res Commun 2001, 280: 561–566.

[12] Taylor WE, Bhasin S, Artaza J, Byhower F, Azam M, Willard DH Jr, Kull FC Jr, Gonzalez-Cadavid N. Myostatin inhibits cell proliferation and protein synthesis in C2C12 muscle cells. Am J Physiol Endocrinol Metab 2001, 280: E221–E228.

[13] Thomas M, Langley B, Berry C, Sharma M, Kirk S, Bass J, Kambadur R. Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation. J Biol Chem 2000, 275: 40235–40243.

[14] Langley B, Thomas M, Bishop A, Sharma M, Gilmour S, Kambadur R. Myostatin Inhibits Myoblast Differentiation by Down-regulating MyoD Expression. J Biol Chem 2002, 277: 49831–49840.

[15] Ríos R, Carneiro C, Arce V, Devesa J. Myostatin is an inhibitor of myogenic differentiation. Am J Physiol Cell Physiol 2002, 282: C993-C999.

[16] Sharma M, Kambadur R, Matthews KG, Somers WG, Delvin GP, Conaglen JV, Fowke PJ, Bass JJ. Myostatin, a transforming growth factor-β superfamily member, is expressed in heart muscle and is upregulated in cardiomyocytes after infarct. J Cell Physiol 1999, 180: 1–9.

[17] Ji S, Losinsky RL, Cornelius SG, Frank GR, Willis GM, Gerrard DE, Depreux FF, Spurlock ME. Myostatin expression in porcine tissues: tissue specificity and developmental and postnatal regulation. Am J Physiol 1998, 275: R1265-R1273.

[18] Kocamis H, Kirkpatrick-Keller DC, Richter J, Killefer J. The ontogeny of myostatin, follistatin and activin-B mRNA expression during chicken embryonic development. Growth Dev Aging 1999, 63: 143–150.

[19] Jeanplong F, Sharma M, Somers W, Bass J, Kambadur R. Genomic organization and neonatal expression of the bovine myostatin gene. Mol Cell Biochem 2001, 220: 31–37.

[20] Carlson CJ, Booth FW, Gordon SE. Skeletal muscle myostatin mRNA expression is fiber-type specific and increases during hindlimb unloading. Am J Physiol 1999, 277: R601–R606.

[21] Sakuma K, Watanabe K, Sano M, Uramoto I, Totsuka T. Differential adaptation of growth and differentiation factor 8/myostatin, fibroblast growth factor 6 and leukemia inhibitory factor in

overloaded, regenerating and denervated rat muscles. Biochim Biophys Acta 2000, 1497: 77–88.

[22] Wehling M, Cai B, Tidball JG. Modulation of myostatin expression during modified muscle used. FASEB J 2000, 14: 103–110.

[23] Kirk S, Oldham J, Kambadur R, Sharma M, Dobbie P, Bass J. Myostatin regulation during skeletal muscle regeneration. J Cell Physiol 2000, 184: 356–363.

[24] Mendler L, Zador E, Ver Heyen M, Dux L, Wuytack F. Myostatin levels in regenerating rat muscles and in myogenic cell cultures. J Muscle Res Cell Motil 2000, 21: 551–563.

[25] Gagnière H, Picard B, Geay Y. Contractile differentiation of cattle muscles: intermuscular variability. Reprod Nutr Dev 1999, 39: 637–655.

[26] Bischoff R, Holtzer H. Inhibition of myoblast fusion after one round of DNA synthesis in 5-Bromodeoxyuridine. J Cel Biol 1970, 44: 134–145.

[27] Cassar-Malek I, Langlois N, Picard B, Geay Y. Regulation of bovine satellite cell proliferation and differentiation by insulin and triiodothyronine. Domest Anim Endocrinol 1999, 17: 373–388.

[28] Chomczynski P, Sacchi N. Single-step method of RNA isolation by guanidium thyocianate-phenol-chloroforme extraction. Anal Biochem 1987, 162: 156–159.

[29] Fontaine-Pérus J, Halgand P, Cheraud Y, Rouaud T, Velasco ME, Cifuentes Diaz C, Rieger F. Mouse-chick chimera: a developmental model of murine neurogenic cells. Development 1997, 124: 3025–3036.

[30] Picard B, Robelin J, Pons F, Geay Y. Comparison of the foetal development of fibre types in four bovine muscles. J Muscle Res Cell Motil 1994, 15: 473–486.

[31] Markwell MA, Haas SM, Tolbert NE, Bieber LL. Protein determination in membrane and lipoprotein samples: manual and automated procedures. Methods Enzymol 1981, 72: 296–303.

[32] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970, 227: 680–685.

[33] SAS. STAT for personal computers. Version 6. Edition SAS Institute INC., Box 8000, Cary, North Carolina, 27511, 1985.

[34] Guernec A, Chevalier B, Duclos MJ. Estimation of myostatin mRNA expression in chicken muscle by real time RT-PCR: effects of nutritional state and genotype. In: Proceedings of the Franco-Polish Symposium, Paris, 2001, p C34.

[35] Hocquette JF, Brandstetter AM. Common practice in molecular biology may induce statistical bias and misleading biological interpretation. J Nut Biochem 2002, 13: 370–377.

[36] Boccard R, Dumont BL. Conséquences de l'hypertrophie musculaire héréditaire des bovins sur la musculature. Ann Genet Sel Anim, 6: 1974, 177–186.

[37] Picard B, Duris MP, Jurie C. Classification of bovine muscle fibres by different histochemical techniques. Histochem J 1998, 30: 173–179.

[38] Cusella-De Angelis MG, Lyons G, Sonnino C, De Angelis L, Vivarelli E, Farmer K, Wright WE, Molinaro M, Bouché M, Buckingham M, Cossu G. MyoD, myogenin independant differentiation of primordial myoblasts in mouse somites. J Cell Biol 1992, 116: 1243–1255.

[39] Oldham JM, Martyn JA, Sharma M, Jeanplong F, Kambadur R, Bass JJ. Molecular expression of myostatin and MyoD is greater in double-muscled than– normal muscled cattle fetuses. Am J Physiol Regul Integr Comp Physiol 2001, 280: R1488–R1493.

[40] Gagnière H, Ménissier F, Geay Y, Picard B. Comparison of foetal metabolic differentiation in three cattle muscles. Reprod Nutr Dev 1999, 39: 105–112.

[41] Rémignon H, Gardahaut MF, Marche G, Ricard FH. Selection for rapid growth increases the number and size of muscle fibres without changing their typing in chicken. J Muscle Res Cell Motil 1995, 16: 95–102.

[42] Picard B, Lefaucheur L, Berri C, Duclos MJ. Muscle fibre ontogenesis in farm animal species. Reprod Nutr Dev 2002, 42: 415–431.

[43] Kocamis H, McFarland DC, Killefer J. Temporal expression of growth factor genes during myogenesis of satellite cells derived from the biceps femoris and pectoralis major muscles of the chicken. J Cell Physiol 2001, 186: 146–152.

[44] Spiller MP, Kambadur R, Jeanplong F, Thomas M, Martyn JK, Bass JJ, Sharma M. The myostatin gene is a downstream target gene of basic helix-loop-helix transcription factor MyoD. Mol Cell Biol 2002, 22: 7066–7082.

[45] Picard B, Gagnière H, Geay Y. Contractile differentiation of bovine masseter muscle. Basic Appl Myol 1996, 6: 361–372.

[46] Gonzalez-Cadavid NF, Taylor WE, Yarasheski K, Sinha-Hikim I, Ma K, Ezzat S, Shen R, Lalani R, Asa S, Mamita M, Nair G, Arver S, Bhasin S. Organization of the human myostatin gene and expression in healthy men and HIV-infected men with muscle wasting. Proc Natl Acad Sci USA 1998, 8: 14938–14943.

[47] Artaza JN, Bhasin S, Mallidis C, Taylor W, Ma K, Gonzalez-Cadavid NF. Endogenous expression and localization of myostatin and its relation to myosin heavy chain distribution in

C2C12 skeletal muscle cells. J Cell Physiol 2002, 190: 170–179.

[48] Joulia D, Bernardi H, Garandel V, Rabenoelina F, Vernus B, Cabello G. Mechanisms involved in the inhibition of myoblast proliferation and differentiation by myostatin. Exp Cell Res 2003, 10 286: 263–275.

[49] Amthor H, Huang R, McKinnell I, Christ B, Kambadur R, Sharma M, Patel K. The regulation and action of myostatin as a negative regulator of muscle development during avian embryogenesis. Dev Biol 2002, 251: 241–257.

[50] Zhu X, Hadhazy M, Wehling M, Tidball JG, McNally EM. Dominant negative myostatin produces hypertrophy without hyperplasia in muscle. FEBS Lett 2000, 474: 71–75.

[51] Picard B, Gagnière H, Robelin J, Geay Y. Comparison of the foetal development of muscle in normal and double-muscled cattle. J Muscle Res Cell Motil 1995, 16: 629–639.