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TRANSFORMING GROWTH FACTOR-β1 UPREGULATES MYOSTATIN EXPRESSION IN MOUSE C2C12 MYOBLASTS

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Myostatin (MSTN) and transforming growth factor-β1 (TGF-β1) belong to the same TGF-β superfamily of proteins. They are involved in regulation of skeletal muscle growth and development as well as muscle catabolism. The aim of the present study was to investigate the relationship between MSTN and TGF-β1 expression in proliferating and differentiating mouse C2C12 myoblasts cultured in normal and catabolic conditions and to evaluate the effect of exogenous TGF-β1 as well as "knock down" of TGF-β1 receptor type II on MSTN expression in proliferating and differentiating myogenic cells. The direct effect of TGF-β1 on myostatin was also examined. Myostatin expression increased gradually with cell confluency in proliferating cultures, while the level of TGF-β1, detected in the form of a 100 kDa small latent complex diminished. Myostatin expression was accompanied by a partial cell cycle arrest. Three forms of myostatin were found: a 52 kDa precursor, a 40 kDa latency associated propeptide, and a 26 kDa active peptide. A decrease in myostatin and TGF-β1 levels was observed during the first three days of differentiation, which was subsequently followed by significant increase of their expression during next three to four days of differentiation. Catabolic state induced by dexamethasone significantly increased the level of all forms of myostatin as well as latent (100 kDa) and active (25 kDa) forms of TGF-β1 in differentiating myoblasts in a dose dependent manner. Exogenous TGF-β1 (2 ng/ml) significantly increased myostatin levels both in proliferating and differentiating C2C12 myoblasts, whereas silencing of the TGF-β1 receptor II gene significantly lowered myostatin level in examined cells.
The presented results indicate that TGF-β1 may control myostatin-related regulation of myogenesis through up-regulation of myostatin, predominantly in the course of terminal differentiation and glucocorticoid-dependent catabolic stimulation.

Key words: *myostatin (GDF-8), TGF-β1, dexamethasone, C2C12 myoblast*

INTRODUCTION

Myostatin (MSTN) and transforming growth factor-β1 (TGF-β1) are negative regulators of muscle growth that belong to the same TGF-β superfamily of proteins. Although they both show many similarities in their structure and function, the relationship between their expression and their mutual interactions are still unclear and require clarification.

The *mstn* gene was described by McPherron (1, 2). He found that *mstn* null mice showed a dramatic and widespread increase in skeletal muscle mass due to hyperplasia and hypertrophy of their muscle fibers. Mutations in the *mstn* coding sequence in Belgian Blue, Piedmontese and Marchigiana breeds of cattle cause the double-muscle phenotype (1-5). It has been also shown that MSTN is involved in skeletal muscle atrophy and regeneration at the molecular level, however, its role in these processes is not fully understood (6-8). The expression of *mstn* is specific for skeletal muscle, although its presence has also been described in cardiomyocytes and Purkinje fibers (9), the mammary gland (10) and blood serum (11). MSTN, like all members of TGF-β family, is secreted as a precursor protein comprising two identical 352 amino-acid polypeptides joined by disulphide bounds. In the course of its activation an N-terminal 243 amino-acid propeptide is created which matures and becomes an active disulfide-linked dimmer of 109 amino-acid (1, 2, 12-15). This latter is responsible for MSTN biological activity. The very stable amino-acid sequence of mature MSTN in many species: mouse, rat, chicken, turkey, pig and human suggests its crucial role in important biological processes. The MSTN propeptide is a potent inhibitor of the active MSTN and its overexpression results in a large increase (up to 200%) in skeletal muscle mass in mice, similar to that observed in myostatin knockout mice (15).

TGF-β1 is a regulatory peptide much more pleiotropic than the MSTN. It stimulates growth of the majority of the mesenchymal cells and inhibits growth of epithelial, lymphoreticular, hematopoetic and endothelial cells. TGF-β1, like a MSTN, is regarded as an inhibitor of myogenic cell proliferation and differentiation (16, 17).

Both MSTN and TGF-β1 possess common characteristic structural features: 1) the functional ligand is a disulfide-linked homodimer, 2) each monomer is expressed as the C-terminal part of a precursor propeptide, 3) the primary structure of the monomer contains a highly conserved seven-cysteine domain in the C-terminal region, 4) the mature peptide remains non-covalently associated with an N-terminal pro-domain as a small latent complex.

Receptors and transcription factors used by MSTN and TGF-β1 in the signal transduction pathways in myogenic cells belong to the same families. MSTN and TGF-β1 initiate signaling by binding directly to type II receptor, which leads to the recruitment of an appropriate type I receptor. Type II receptors for MSTN and TGF-β1 are ActRIIB and TβRII respectively, whereas the type I receptor- TβRI

(ALK 5) is common to both cytokines (18). Activated TβRI phosphorylates the receptor-regulated Smads (R-Smads): Smad 2 and Smad 3, that are associated with a common Smad 4. The R-Smad/Smad 4 complex translocates to the nucleus where it binds to one of many potential DNA-binding partners and regulates the transcription of target genes (19, 20).

The inhibition of myoblast proliferation by MSTN and TGF-β1 occurs through a similar molecular mechanism of inhibition of cdk2 activity based on up-regulation of p21 hypophosphorylation of the Rb protein and cell cycle arrest in the G1 phase (21-24). MSTN and TGF-β1 also inhibit terminal differentiation of myoblasts through the repression of the levels of myogenic regulatory factors: MyoD, Myf5 and myogenin as well as the inhibition of their transcriptional activity (22, 25-27).

Similarities in MSTN and TGF-β1 molecular structure and mechanism of action through receptor activation, signal transduction and expression of target genes, strongly suggests the close interaction between these two cytokines in the regulation of myogenesis.

The aim of the present study was to investigate the relationship between MSTN and TGF-β1 expression in proliferating and differentiating mouse C2C12 myoblasts. It was studied by: 1) comparision of MSTN and TGF-β1 expression patter in cultures stimulated to proliferate and differentiate in normal and catabolic conditions (induced by synthetic glucocorticoid - dexamethasone); 2) evaluation of the effect of exogenous TGF-β1 as well as "knock down" of TGF-β1 RII on MSTN expression in proliferating and differentiating myogenic cells.

MATERIALS AND METHODS

Media and reagents

DMEM with Glutamax, phosphate buffered saline (PBS) [pH 7.4], foetal bovine serum (FBS), penicillin-streptomycin, fungizone and gentamycin sulphate were from Gibco BRL (Paisley, Scotland). Dexamethasone (DEX) was from Sigma Chemicals Corp. (St. Louis, MO USA). Polyclonal chicken anti-mouse TGF-β1 and monoclonal rat anti-mouse MSTN (specific for recombinant mouse GDF-8) antibodies (both specific only for mouse proteins) were from by R&D Systems, Inc. (Minneapolis, USA). Specificity of the antybodies were evaluated in preliminary trials and it turned out to be superior when compared with other commercial antibodies. Mouse monoclonal anti-Tb-RII antibody was from Santa Cruz. Specificity of the antibodies Horseradish peroxidase-conjugated goat anti-chicken IgG and donkey anti-rat IgG from supplied by Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Alexa Fluor 488 secondary antibodies goat-anti chicken IgG, Alexa Fluor 488 secondary antibodies chicken anti-mouse IgG and Alexa Fluor 488 secondary antibodies chicken-anti rat IgG were purchased from Molecular Probes (Eugene, OR USA). Reagents for Western blotting were purchased from BioRad (Hercules, CA USA) and Western blotting detection reagents and Hyperfilm ECL were from Amersham Pharmacia Biotech (Little Chalfont, UK). Sterile conical flasks, Lab-teks (2/4 chambered slides), disposable pipettes were supplied by Nunc Inc.(Naperville, IL USA).

Cell culture

The mouse skeletal muscle cell line C2C12 (ATCC CRL-1772) was maintained in DMEM with Glutamax, supplemented with 10% (v/v) FBS (foetal bovine serum), 50 μg/ml gentamycin, 2.5 mg/ml fungizone, 50 IU/ml penicillin and 50 μg/ml streptomycin in an atmosphere of 5% CO_2 / 95% humidified air at 37°C, and subcultured every two days. For differentiation into myotubes, myoblasts were grown until they reached ~70-80% confluence, then the medium was changed to DMEM supplemented, as above, with 2% (v/v) HS (horse serum) instead of FBS. Myoblasts and myocytes at different stages of confluence and differentiation stage were used.

Experimental procedure

Cells growing exponentially were propagated onto Lab-Tek of two or four chamber slides (NUNC inc.) and cultured to different stages of confluency using 10% FBS/DMEM or allowed to differentiate, by being kept in 2% HS/DMEM over six days. Experimental media were supplemented with pharmacologic doses of TGF-β1 (0.5, 2, 5 ng/ml), or DEX (2, 10, 50 nM). The concentrations of dexamethasone and TGF-β1 were established on the base of the literature and pilot experiments.

Cells cultured in routine 10% FBS or 2% HS medium were used as controls. Each experiment was repeated at least three times.

Gene knock down

The commercial siRNA construct (GENSCRIPT inc. USA) was used to knock down of TGFß receptor II gene. C2C12 cells were transfected using lipofectAMINE Reagent and Reagent PLUS and TGFβ receptor II siRNA-vector. The cells were incubated in optimal conditions until 70% confluency. Then transfection solution was prepared: in one tube 2 μg of DNA and 4 μl of Regent PLUS diluted into 0.1 ml serum free medium, in the second tube 15 μl of lipofectAMINE Reagent diluted into 0.1 ml serum free medium. Both solutions were carefully combined and obtained mixture was incubated at room temperature for 30 min. The cells were then washed and overlaid with 0.8 ml of serum free medium and transfection mixture was added dropwise and incubated at 37°C, under 5% CO_2 atmosphere over the next 3h. Afterwards transfection medium was supplemented with FBS to 10% final concentration. After 48h transfected cells were incubated with zeocin® (800 μg/ml) to select the cells with TGFβ receptor II siRNA-vector. Experiment were conducted on polyclonal population of zeocin-resistant cells.

Immunofluorescence staining for Laser Scanning Cytometry

PBS-rinsed cells were fixed in 0.25% paraformaldehyde for 15 min, washed twice with PBS, suspended in ice-cold 70% methanol and stored at 4°C for 30 min. Then the methanol was aspirated and the samples were stored at -80°C until staining.

The cells were washed twice with PBS-1% w/v bovine serum albumin (BSA) and incubated for 1 h with the primary antibodies diluted 1:250 with PBS-1% BSA. After incubation the cells were washed twice with PBS-1% BSA and incubated for another 1 h with 1:500 fluorochrome-conjugated secondary antibodies. The cells were then washed twice in PBS-1% BSA and finally, incubated with a 5 μg/ml solution of 7-aminoactinomycin D (7-AAD) for 30 min to counterstain the DNA. Finally, the coverslips were mounted on microscope slides using ICN mounting medium (ICN Biomedicals inc., Aurora, OH USA)

Laser Scanning Cytometry

Probes were analyzed by LSC (CompuCyte Corp., Boston, MA USA). At least $5x10^3$ cells per slide were analyzed. Each experiment was repeated three times with three replicates. The

fluorescence excitation was provided by a 488 nm argon laser beam. A combination of dichroic mirrors and filters transmitting light at a 520 ± 20 nm for the green fluorescence of Alexa Fluor 488 antibodies, and of >650 nm for the far red fluorescence of 7-AAD, were used. Green fluorescence was measured separately over the nucleus (Nf) and the cytoplasm (Cf). The Nf was measured within area outlined by the 'integration contour' located 2 pixels outside the 'treshold contour' triggered by the far red fluorescence of 7-AAD. The Cf was measured within the rim of cytoplasm 10 pixels wide, located outside the 'integration contour'. The background green fluorescence was automatically measured within 2 pixels range outside 'peripheral contour' and subtracted from both nuclear and cytoplasmic green fluorescence, to obtain the final values of Nf and Cf, respectively.

The parameters measured the maximal pixel (MP) corresponding to the highest value of measured fluorescence in the cell, regardless of the cellular compartment and the green integral (GI) indicated the content of the measured protein in the cell. Based on DNA ploidy the cell cycle was evaluated. The results obtained were analysed by Microsoft® Excel 2003 software (Microsoft Corporation, Redmond, WA) and Prism version 2.00 software (GraphPad Software, San Diego, CA).

Western blot analysis

Cells were cultured in sterile conical flasks, or in Petri dishes in a routine culture medium (see Material and Methods), until they reached a stage of confluence. The medium was then removed and replaced with the experimental medium, as described earlier. 10% FBS or 2% HS supplemented medium was used as control.

The cultured cells were centrifuged at 2000g, for 5 min, at 4°C and the remaining pellet was used for following analyses. Samples were kept frozen at -80°C until the isolation of proteins. At the time of analysis the cells were suspended in ice-cold PBS. After centrifugation the supernatant was removed and the cell pellet was resuspended in 0.5 ml Lysis Buffer RIPA prepared according to the Research application from Santa Cruz Biotechnology, and incubated at 4°C for 30 min. The cells suspended in the buffer were centrifuged at 9000 g, 10 min, at 4°C, then the supernatant (containing total fraction of proteins) was carefully removed and passed six times through a 20-gauge syringe needle. The lysates were mixed 1:2 (v/v) with Laemmli sample buffer (BioRad) containing 2.5% 2-mercaptoethanol and boiled for 3 min.

The samples containing identical quantites of proteins were subjected to SDS-PAGE (12% gel) together with a Kaleidoscope Marker (BioRad). The electrophoresis was run for 1 hour at 100 V using Mini Protean II ™ cell (BioRad). After electrophoresis the separated proteins were electroblotted on a nitrocelulose membrane (Amersham Pharmacia Biotech) for 70 min at 110 V using the Mini Protean II ™ cell. The membranes were blocked overnight with 5% w/v solution of non-fat powdered milk in TBST (pH 7.5). The following day the membranes were rinsed three times for 10 min in TBST, at room temperature, and then incubated for 1 hour at room temperature with the primary antibodies diluted 1:400. The membranes were then rinsed four times for 10 min in TBST and incubated with diluted 1:2500 secondary antibodies conjugated with horseradish peroxidase for another 1 h at room temperature. Finally, the membranes were rinsed three times for 10 min in TBST, and labelled proteins were visualised using the ECL Western blotting detection reagent on a high performance chemiluminescence Hyperfilm ECL (Amersham Pharmacia Biotech). The image on hyperfilm was then analyzed with Kodak Edas System and the integrated optical density (IOD) was measured.

Statistical evaluation

The results were statistically evaluated by ANOVA and Tukey's multiple range tests using Prism version 2.0 software (GraphPad Software, San Diego, CA). pŁ 0.05 was regarded as significant and $p \leq 0.01$ as highly significant.

RESULTS

MSTN and TGF-β1 expression in proliferating and differentiating mouse C2C12 myoblasts

The confocal images of MSTN and TGF-β1 expression in proliferating C2C12 myoblasts were analyzed with a MicroImage System to calculate the integrated optical density of the overall MSTN- and TGFβ1-related fluorescence. Expression of MSTN was significantly higher and positively correlated with cell culture confluency, reaching a maximum value at 100% of confluence (*Fig. 1a*). Similar results were obtained using LSC (data not shown). Western blot analysis revealed the presence of three MSTN forms: a 52 kDa precursor, a 40 kDa latency associated propeptide (LAP) and a 26 kDa active peptide in 100% confluent myoblasts (*Fig. 1b*). Unlike MSTN, TGF-β1 was detected in proliferating C2C12 myoblasts only in the form of a 100 kDa small latent complex (*Fig. 1b*). Its cellular content was negatively correlated with cell confluency (*Fig. 1a*). Analysis of the cell cycle revealed that increasing cell confluency (and increasing MSTN expression) was accompanied by a decrease in number of cells in the G2/M phase, with a simultaneous increase in cells in the G1 phase of the cell cycle, shown as a significant increase in the G1/G2M ratio (*Fig. 1c*).

LSC analysis of mouse C2C12 myoblasts stimulated to differentiation by the 2% HS/DMEM medium, showed a decrease in MSTN and TGF-β1 expression in the first phase of the differentiation process (*Fig. 2*). MSTN and TGF-β1 expression was described by two parameters: 1) % of cells with high maximal pixel (MP), where MP value corresponds to the number of pixels with the highest cytokine-related fluorescence (*Fig. 2a*), and 2) cytokine-related fluorescence, reflecting the content of cytokine in the perinuclear area of the cell (*Fig. 2b*). The level of both analyzed cytokines remained low utill 2-3 day of differentiation. A subsequent significant increase of their expression was observed during the next 3-4 days of differentiation (*Fig. 2*). The considerable gain in the MSTN and TGF-β1 content in myoblasts during the 4^{th}, 5^{th} and 6^{th} day of differentiation was also evident on the confocal images (*Fig. 3*). Western blot analysis revealed that during differentiation the level of the 100 kDa small latent TGF-β1 complex increased. A new unidentified band of 70 kDa TGF-β1-related immunoreactivity was also visible on blots. The 25 kDa band indicating active TGF-β1 peptide was absent.

Effect of dexamethasone on MSTN and TGF-β1 expression in C2C12 myoblasts stimulated to differentiation

Synthetic glucocorticoid - dexamethasone (DEX) added to the culture medium in concentrations: 2, 10 and 50 nM significantly increased the level of all forms of MSTN (52, 40 and 26 kDa) in differentiating C2C12 myoblasts, as was shown by Western blot analysis (*Fig. 4a, 4b*). This stimulatory effect of DEX on MSTN expression was confirmed by LSC analysis (data not shown). The presence of

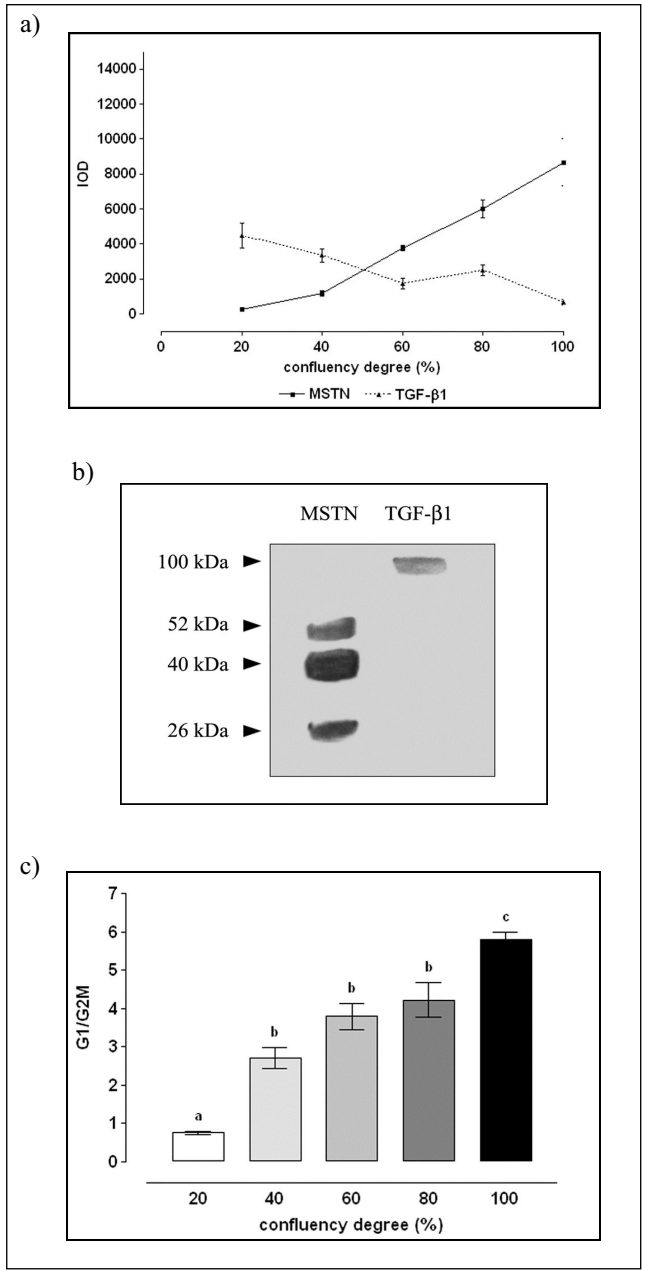

Fig. 1. Effect of degree of confluency on TGF-β1 and MSTN expression in proliferating cultures of mouse C2C12 myoblasts (a). The parameter measured was the integrated optical density (IOD) of TGF-β1 and MSTN-related fluorescence visualized by confocal microscopy. Results were presented as means ± SE from three separate experiments performed in triplicate. Western blot analysis of TGF-β1 and MSTN expression in a confluent proliferating culture (b). Three forms of MSTN: active (26 kDa), latent associated peptide (LAP) (40 kDa) and precursor (52 kDa) and only one form of TGF-β1: a small latent complex (100 kDa) were observed. Results were representative from three separate experiments. Effect of degree of confluency on G1/G2M ratio measured by LSC (c). Results were presented as means ± SE from three separate experiments performed in triplicate. Means described with different superscript letter differ significantly ($p \leq 0.05$).

DEX in the incubation medium increased the level of TGF-β1 in C2C12 myoblasts stimulated to differentiation in dose-dependent manner (*Fig. 4c*). It should be pointed out that the TGF-β1 response to DEX was not only quantitative

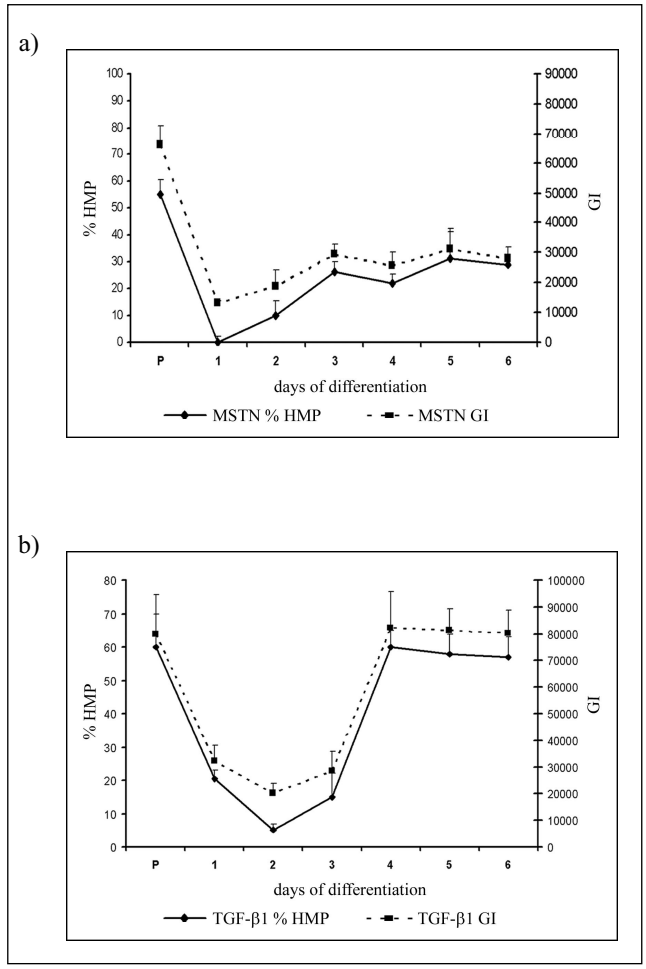

Fig. 2. LSC analysis of MSTN (a) and TGF-β1 (b) expression in proliferating C2C12 myoblasts (P) (10% FBS/DMEM enriched medium) and during six days of differentiation (2% HS/DMEM enriched medium). The parameters measured were: % HMP (percentage of cells with high maximal pixel - MP, where MP value corresponds to the number of pixels with the highest cytokine-related fluorescence) and GI (cytokine-related fluorescence, reflecting the content of cytokine in the perinuclear area of the cell). Results were presented as means ± SE from three separate experiments performed in triplicate.

but also qualitative since apart from higher level of 100 kDa small latent TGF-β1 complex, a 25 kDa active peptide was also found.

The influence of TGF-β1 on MSTN expression in proliferating and differentiating myoblasts

Administration of TGF-β1 in concentrations: 2 and 5 ng/ml to proliferating C2C12 myoblasts culture significantly increased the level of active (26 kDa) form of MSTN during the first 24 hours of treatment (*Fig. 5a*). After 48 hours of incubation a strong stimulatory effect of TGF-β1, particularly at 2 and 5 ng/ml concentrations was observed on the 52 kDa precursor, the 40 kDa LAP, and the 26 kDa active MSTN peptide. Exogenous administration of TGF-β1 in 2 or 5 ng/ml concentrations resulted in progressive decrease of the 100 kDa small latent

TGF-β1 complex after 24h and 48h of treatment (*Fig. 6*). TGF-β1 at 2 ng/ml, increased the MSTN level ($P<0.01$) in the differentiating C2C12 myoblasts, as shown by both LSC analysis (*Fig. 5b*) and Western blot (*Fig. 5c*). The above-described effect was evident on the 4th, 5th and 6th day of differentiation.

The upregulatory role of TGF-β1 in MSTN expression was ultimately confirmed by a „knock down" of TGF-β1 receptor II gene performed with RNAi

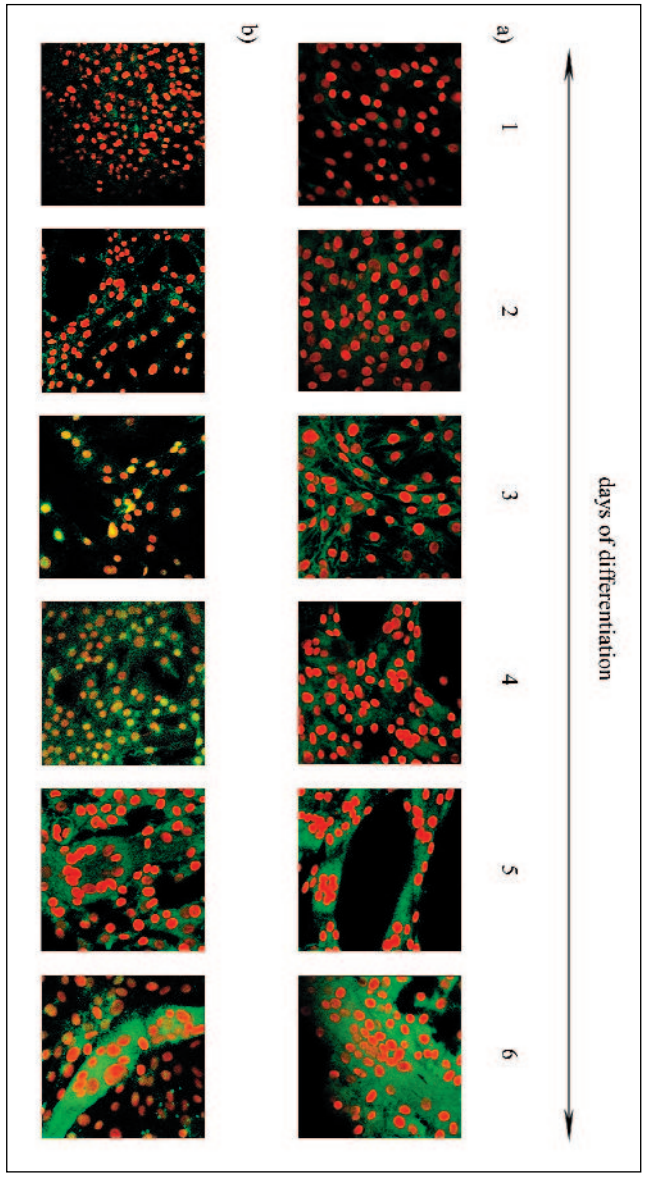

Fig. 3. Confocal images of MSTN (a) and TGF-β1 (b)-related fluorescence (green) in C2C12 myoblasts during six days of differentiation. DNA was stained with 7-AAD (red fluorescence). Images were representative of three separate experiments.

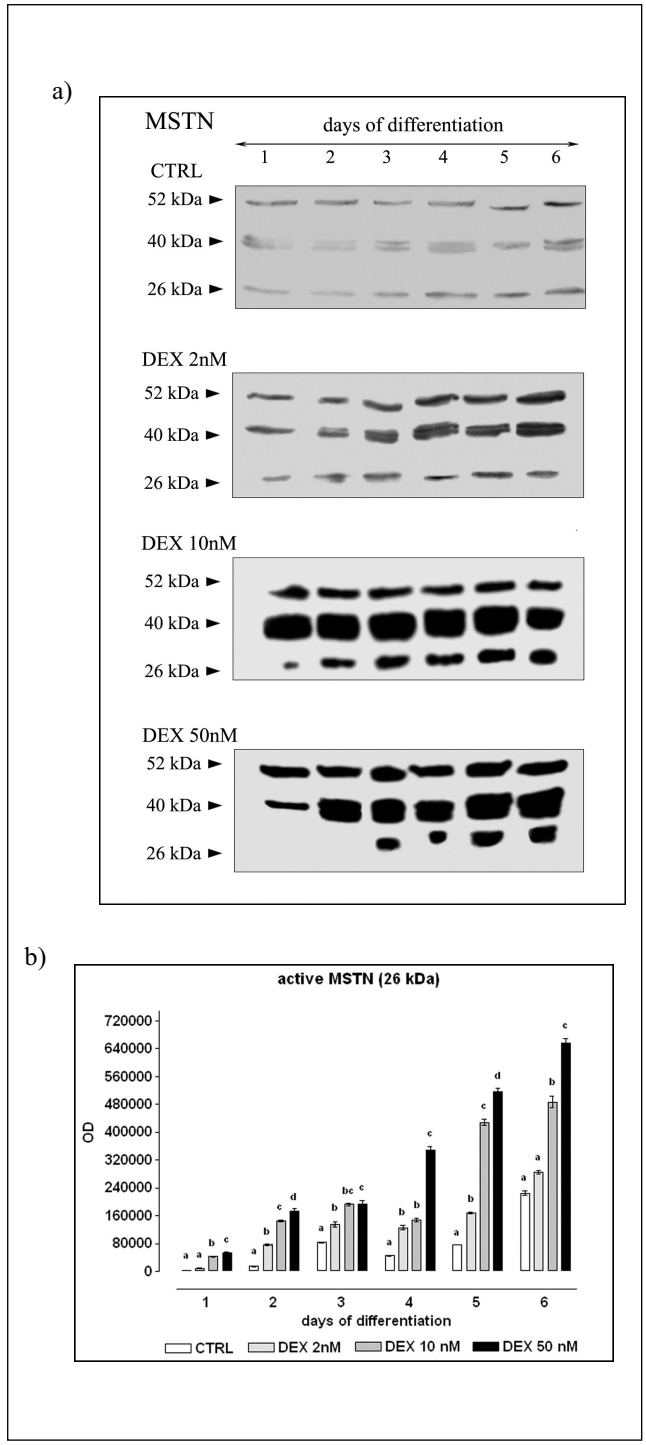

Fig. 4. Western blot analysis of MSTN expression (active peptide - 26 kDa, LAP - 40 kDa, and precursor - 52 kDa) during differentiation of C2C12 myoblasts (a). Expression of MSTN in control, untreated cells -and in cells treated with graded concentrations of DEX: 2 nM, 10 nM and 50 nM. (b) Optical density (OD) of the active form MSTN (26 kDa) was compared in control and DEX treated cultures. Results were representative of three separate experiments. Means (± SE), for the same day of differentiation, described with different superscript letter differ significantly ($p \leq 0.05$). (...)

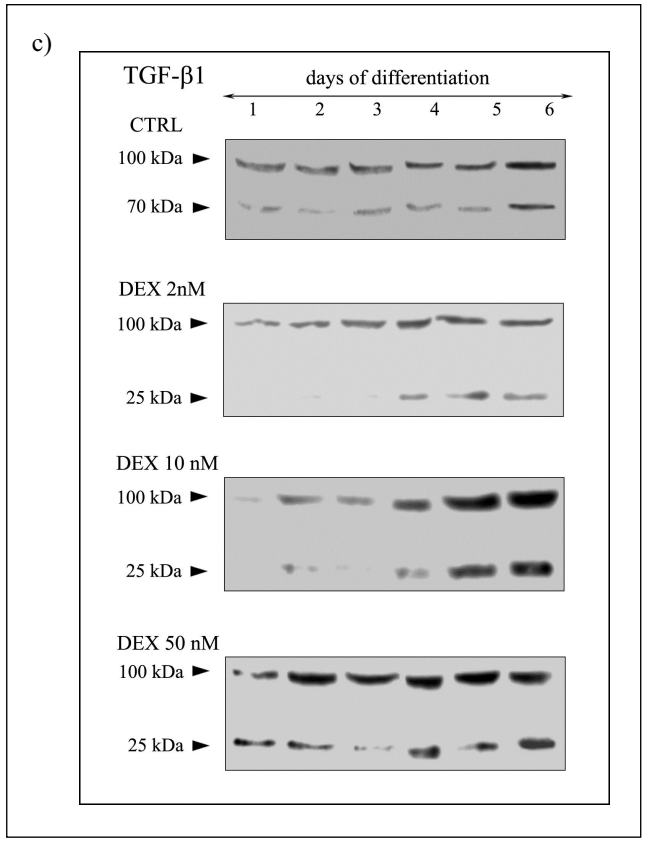

Fig. 4. (...) (c) Western blot analysis of TGF-β1 expression (active peptide - 25 kDa, unidentified peptide - 70 kDa, small latent complex - 100 kDa) during differentiation of control, untreated C2C12 myoblasts and myoblasts treated with graded concentrations of dexamethasone: 2, 10, and 50 nM (c). Results were representative from three separate experiments.

technique (see Materials and Methods). Silencing of TGF-β1 RII gene resulted in highly significant decrease of the level of endogenous TGF-β1 RII protein in examined cells and a highly significant decrease of the MSTN level in TGF-β1 RII (-) cells (*Fig. 7*). Silencing of Tβ-RII(-) led to a decrease of all three MSTN forms (*Fig. 7a*) through the whole period of myoblasts differentiation (*Fig. 7b*). Exogenous TGF-β1 administration did not result in an increase of MSTN expression suggesting 1) efficient silencing of TGF-β1 RII; and 2) direct relation between TGF-β1 and MSTN expression in skeletal muscle cells.

DISCUSSION

Our results indicate that MSTN and TGF-β1 may collaborate and interact in the regulation of skeletal muscle myogenesis. This was clearly shown by: 1) the similar pattern of changes in MSTN and TGF-β1 contents of mouse C2C12 myoblasts stimulated to differentiate (*Figs 2* and *3*); 2) the up-regulation of both cytokines in glucocorticoid-treated myoblasts (*Figs 4*); 3) the direct stimulatory

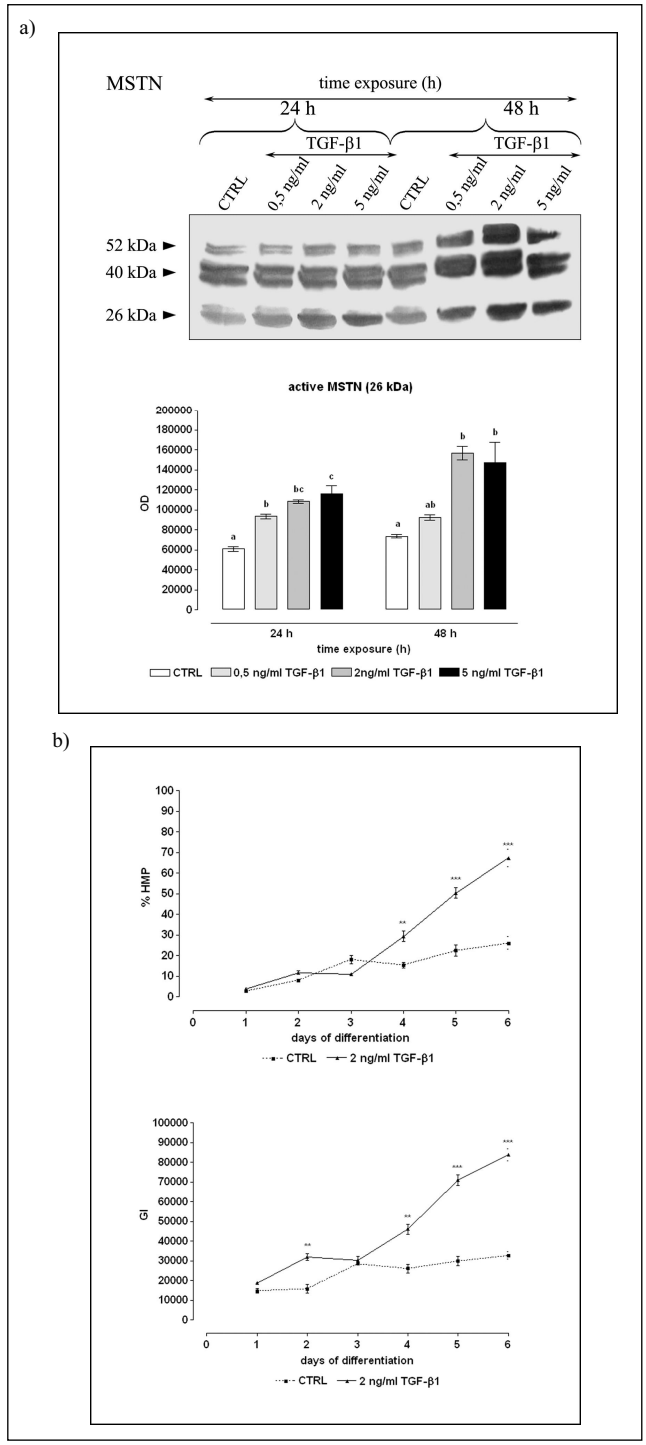

Fig. 5. (a) Effect of exogenous TGF-β1 (0.5, 2, and 5 ng/ml) on MSTN expression in proliferating C2C12 myoblasts (a). Results were representative from three separate experiments. Means (± SE) for the same time of cell exposure to TGF-β1 described with different superscript letter differ significantly ($p \leq 0.05$). (b) Effect of exogenous TGF-β1 (2 ng/ml) on MSTN-related fluorescence in C2C12 myoblasts stimulated to differentiation. The parameters measured were: % HMP (percentage of cells with high maximal pixel - MP, where MP value corresponds to the number of pixels with the highest cytokine-related fluorescence) and GI (cytokine-related fluorescence, reflecting the contents of cytokine in the perinuclear area of the cell). Results were presented as means ± SE from three separate experiments performed in triplicate. *-$p \leq 0.05$, **-$p \leq 0.01$, ***-$p \leq 0.001$. (...)

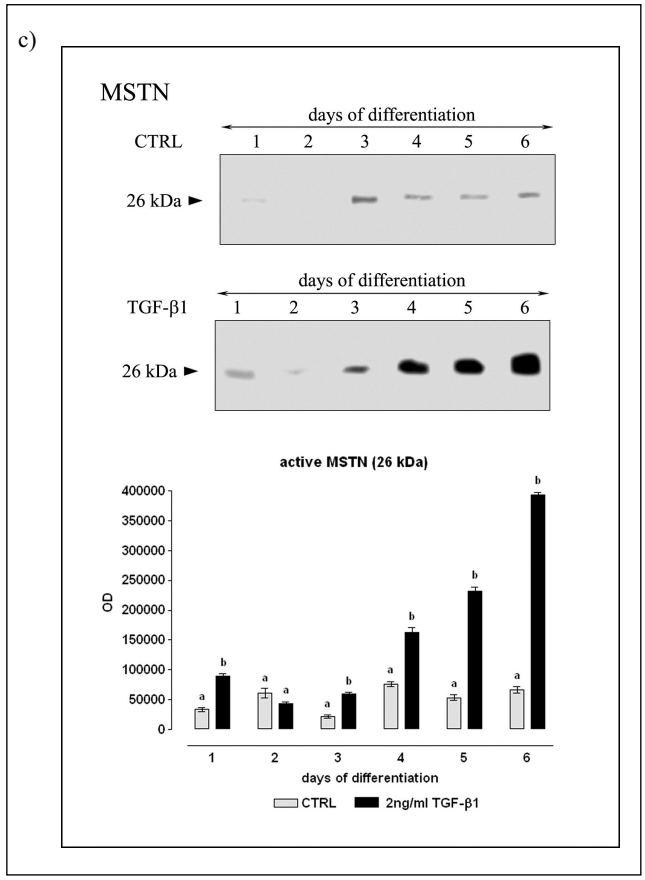

Fig. 5. (...) (c) Effect of exogenous TGF-β1 (2 ng/ml) on MSTN (26 kDa - active form) expression in C2C12 myoblasts stimulated to differentiation analyzed by Western blot. Results were representative from three separate experiments. Means (± SE), for the same day of differentiation, described with different superscript letter differ highly significantly ($p \leq 0.01$).

effect of TGF-β1 on MSTN expression in proliferating and differentiating myoblasts (*Figs 5*); and 4) the decreased MSTN level in TGF-β1 RII (-) myoblasts (*Fig. 7*).

In proliferating mouse C2C12 myoblasts the expression of MSTN was dependent on the degree of cell confluency. This expression peaked at 100% confluency (*Fig. 1a*). Comparable with the results of Thomas et al. and MacMahon et al., all three MSTN forms were present (*Fig. 1b*): the 52 kDa precursor, the 40 kDa LAP and the 26 kDa active peptide (8, 21). The gradual increase of MSTN synthesis with the increasing level of culture confluency is probably crucial for triggering the cascade of reactions leading to the inhibition of growth manifested by partial cell cycle arrest at the G1 phase (*Fig. 1c*). In G1 arrested C2C12 myoblasts up-regulation of MSTN expression depends on MyoD expression (28). The antiproliferative activity of MSTN manifests itself as an increase in the level of $p21^{Waf1,Cip1}$, a decrease in the level and activity of the cdk2 protein and a suppressesion of Rb protein phosphorylation (21, 23). The

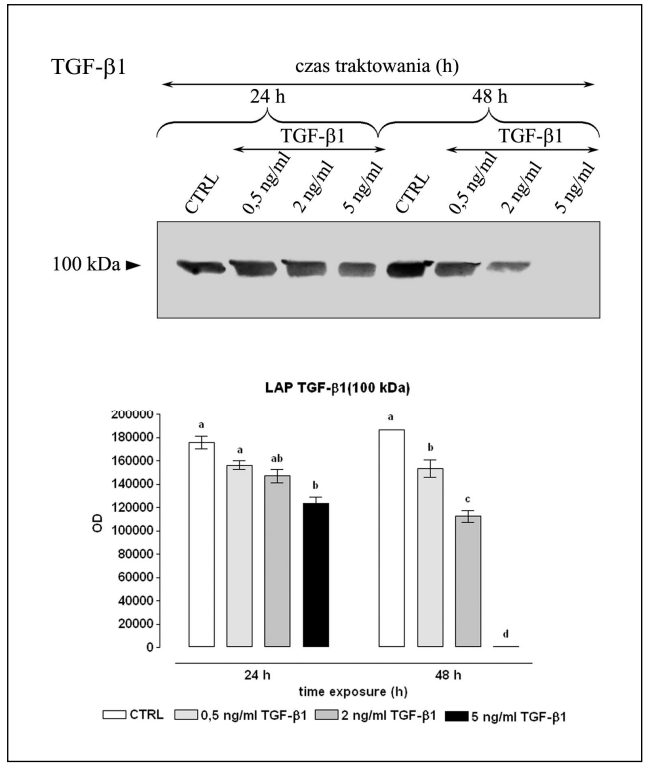

Fig. 6. Effect of exogenous TGF-β1 (2 ng/ml) on TGF-β1 expression in proliferating C2C12 myoblasts. Results were representative from three separate experiments. Means (± SE) for the same time of cell exposure to TGF-b1 described with different superscript letter differ significantly ($p \leq 0.05$).

induction of p21 by MSTN is mediated by transcriptional factors from the Smad family (6, 29) and eventually, Smad directly stimulates the mitogen-activated protein kinase pathway (30). The lack of a functional MSTN protein promotes myoblast proliferation, which results in hyperplasia and might explain the double muscle mass in cattle with the *mstn*$^{-/-}$ phenotype. Muscle hypertrophy has been also described in *mstn* knockout mice (1, 2), transgenic mice overexpressing the MSTN propeptide, follistatin and dominant-negative form of ActRIIB (15) and in certain breeds of *mstn* mutant cattle (3, 31). Experiments performed on pigs revealed that MSTN expression in skeletal muscle peaks prenatally and its greater expression is associated with low birth weight (32). MSTN antagonists could therefore be used in the treatment of muscle wasting and promotion of muscle growth in man and animals (31, 33, 34). The presence of TGF-β1 in the form of a 100 kDa small latent complex, in proliferating C2C12 cells (*Fig. 1b*) and the progressive decrease of TGF-β1 expression following the increasing cell confluency (*Fig. 1a*) supports the idea that MSTN, not TGF-β1, is responsible for growth inhibition in highly confluent myoblasts. Since TGF-β1 exerts an antiproliferative effect in myogenic cells (21), maintenance of the proper balance between MSTN and TGF-β1 levels could be the basis of the modulating mechanism for the rate of muscle growth. In contrast

to MSTN, TGF-β1 is produced by many types of cells and can be supplied to a developing muscles from other tissues. Our study provided evidence that the increased concentrations of a mature TGF-β1 peptide of exogenous origin enhanced MSTN levels in proliferating cultures of C2C12 myoblasts (*Fig. 5a*). This suggests that TGF-β1 is able to inhibit cell growth not only directly, but also indirectly through enhancement of MSTN expression. It should be noticed that in myoblasts exposed to the highest concentration of exogenous TGF-β1, (5 ng/ml) for 48 h, the endogenous synthesis of the small latent TGF-β1 complex was completely abolished (*Fig. 6*). This may indicate the presence of an auto/paracrine negative feedback loop controlling the formation and secretion of TGF-β1. A similar mechanism was observed in bovine mammary epithelial BME UV1 cells exposed to TGF-β1 (35).

The replacement of 10% FBS with 2% HS in the incubation medium resulted in a decrease in the overall MSTN and TGF-β1 levels in G1 arrested myoblasts (*Fig. 2*). This effect is probably critical for the promotion of the differentiation phase in arrested myoblasts, morphologically manifested by the fusion of myoblasts into multinucleated myotubes (*Fig. 3*). The progression of the differentiation process is coordinated by a muscle-specific basic helix loop. This loop includes helix transcription factors collectively referred to as myogenic

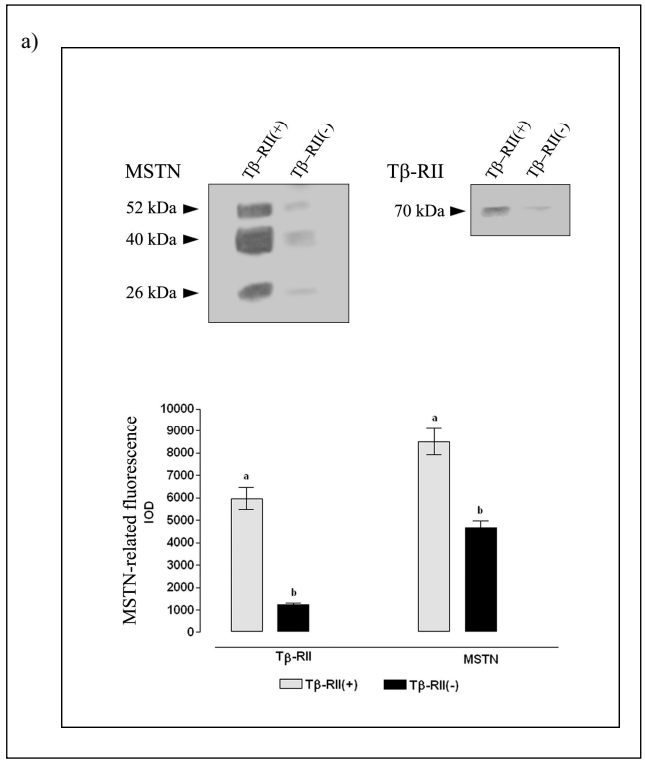

Fig. 7. Effect of TGF-β1 RII gene ,,knock down" (RNAi technique- see Materials and Methods) on TGF-β1 receptor II and MSTN expression in proliferating (a) and differentiating (...)

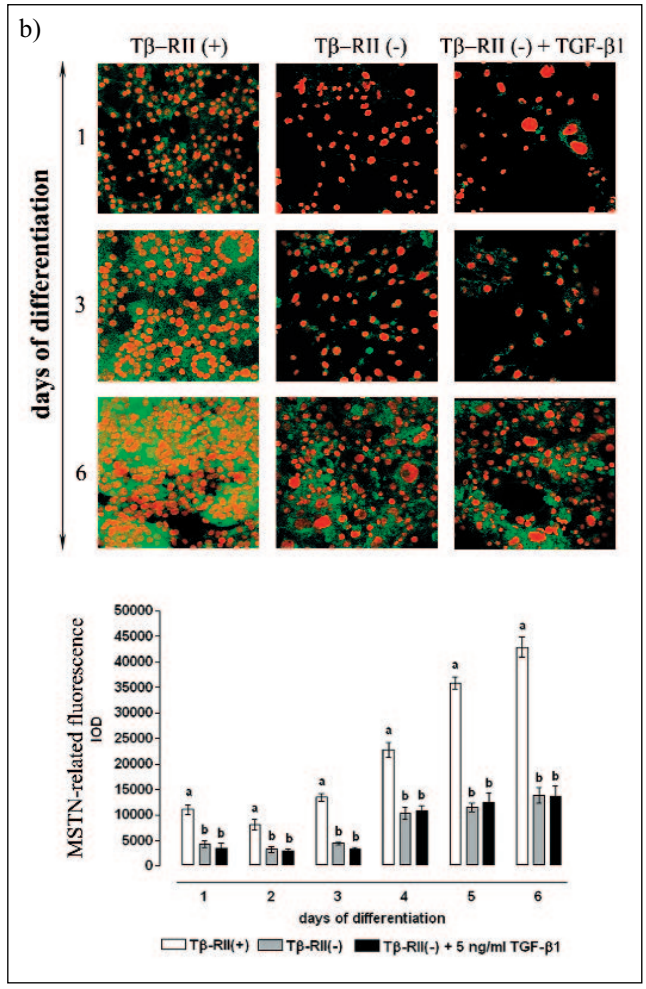

Fig. 7. (...) (b) C2C12 myoblasts. Results were presented as means ± SE from 6 experiments. On (a) means for the same parameter described with different superscript letter differ highly significantly ($p \leq 0.01$). On (b) means for the same day of differentiation described with different superscript letter differ highly significantly ($p \leq 0.01$).

regulatory factors (MRFs) that include MyoD, Myf5, myogenin and Mrf4. It has been shown that both MSTN (6, 27) and TGF-β1 (24, 36, 37) inhibit differentiation of myoblasts through the down-regulation of MRFs expression. We suggest that the decrease in MSTN and TGF-β1 expression leads to a lack of inhibitory influence of MSTN and TGF-β1 on MyoD, myogenin and other MRFs and triggers the process of myoblast differentiation. The down-regulation of MSTN and TGF-β1 was transient during the first two days of differentiation, but from the third day a subsequent increase in the expression of these cytokines was observed (*Fig. 2*). This increase in MSTN and TGF-β1 concentration was especially evident in the fusing myoblasts and multinucleated myotubes (*Fig. 3*), which could be associated with the ability of both cytokines to control the terminal differentiation and formation of myotubes. It has been shown that

C2C12 myoblasts cultured with exogenous MSTN did not differentiate morphologically or biochemically. The inhibition of myogenic cell terminal differentiation by MSTN is related to down-regulation of: MyoD, Myf5, myogenin and p21 expression (6). The stable transfection of *mstn* cDNA in C2C12 myoblasts effectively inhibits the formation of multinucleated myotubes, decreases the mRNA levels and the activity of MyoD, myogenin and its downstream target: creatine kinase (CK) (38).

Cloning and characterization of promoter region of the *mstn* gene revealed many putative glucocorticoid response elements (40), which enable binding and up-regulation of *mstn* gene expression by glucocorticoids. It has been postulated that muscle loss associated with dexamethasone administration is, at least partially, mediated by the up-regulation of *mstn* expression through a glucocorticoid receptor-mediated pathway (7). It has been suggested that a burn-induced increase in MSTN appears to be largely mediated by enhanced endogenous secretion of glucocorticoids, and is independent from changes in IGF-I, IGF-II or TNF-α levels (39). It seems possible that increasing synthesis of corticosteroids by the foetal adrenal cortex at the end of gestation may contribute to the increase of MSTN expression in myoblasts and in consequence, inhibition of myogenesis. In the present study we found a dose-dependent increase of MSTN expression during myoblast differentiation under the influence of DEX, as shown by Western blot (*Fig. 4a, 4b*). The increased content concerned all forms of MSTN: mature (25 kDa), LAP (40 kDa) and precursor (52 kDa) (*Fig. 4a*). These results correspond to those obtained during *in vivo* experiments, showing the increase of MSTN mRNA in rat muscle after an injection of DEX (39). However, it must be remembered that mRNA levels do not directly correlate with protein levels despite the differences between conditions in in *in vivo* and *in vitro* experiments. Our results indicate also that the inhibitory influence of dexamethasone on myogenesis may also be mediated by TGF-β1. The expression of this cytokine increased in the presence of DEX (*Fig. 4c*) and apart from the small latent complex (100 kDa) in the DEX-treated differentiating cultures of C2C12 myoblasts, the mature (25 kDa) form of TGF-β1 was also present. The presence of the latter should be pointed out since it may indicate an important role of TGF-β1 in muscle cells exposed to a catabolic state and suggests that glucocorticoids can act not only through MSTN but also TGF-β1 in inducing catabolism in skeletal muscle. The comparable response of MSTN and TGF-β1 under the influence of dexamethasone may indicate a collaboration between those two cytokines in the inhibition of myogenesis evolved by glucocorticoids.

Finally, we provided the evidence that TGF-β1 potentiated MSTN expression in differentiating C2C12 myoblasts (*Fig. 5b, 5c*). The biological effects of TGF-β1 in skeletal muscle cells are mediated by Smad proteins and the AP1 complex (36). Analysis of the 5'-regulatory region of the *mstn* gene revealed that, apart from the glucocorticoid response element, there are sequences responsible for binding of the transcription factors, such as Smads and AP1, which are involved

in the signal transduction pathway of TGF-β1. Finally the control of MSTN expression by TGF-β1 was confirmed by the experiment with ,,knock down" of TGF-β1 receptor II gene, showing a highly significant decrease of MSTN levels in TGF-β1 RII (-) myoblasts (*Fig. 7*). This decrease was observed in all three MSTN forms during the whole differentiation period suggesting that the presence of TGF-β1 RII is necessary for MSTN action. Exogenous TGF-β1 administration did not increased MSTN expression in TGF-β1 RII(-) cells showing 1) a good silencing of TGF-β1 RII; and 2) a direct relationship between presence of TGF-β1 signals and expression of MSTN. According to our best knowledge the direct realtionship between MSTN and TGF-β1 expression in muscle cells has been showed for the first time.

In conclusion, TGF-β1 may control MSTN-related regulation of myogenesis through the up-regulation of MSTN, predominantly during terminal differentiation of muscle cells and a glucocorticoid-dependent catabolic state when TGF-β1 expression is elevated.

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