Myostatin promotes a fibrotic phenotypic switch in multipotent C3H 10T1/2 cells without affecting their differentiation into myofibroblasts

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Abstract

Tissue fibrosis, the excessive deposition of collagen/extracellular matrix combined with the reduction of the cell compartment, defines fibroproliferative diseases, a major cause of death and a public health burden. Key cellular processes in fibrosis include the generation of myofibroblasts from progenitor cells, and the activation or switch of already differentiated cells to a fibrotic synthetic phenotype. Myostatin, a negative regulator of skeletal muscle mass, is postulated to be involved in muscle fibrosis. We have examined whether myostatin affects the differentiation of a multipotent mesenchymal mouse cell line into myofibroblasts, and/or modulates the fibrotic phenotype and Smad expression of the cell population. In addition, we investigated the role of follistatin in this process. Incubation of cells with recombinant myostatin protein did not affect the proportion of myofibroblasts in the culture, but significantly upregulated the expression of fibrotic markers such as collagen and the key profibrotic factors

transforming growth factor- β 1 (TGF- β 1) and plasminogen activator inhibitor (PAI-1), as well as Smad3 and 4, and the pSmad2/3. An antifibrotic process evidenced by the upregulation of follistatin, Smad7, and matrix metalloproteinase 8 accompanied these changes. Follistatin inhibited TGF- β 1 induction by myostatin. Transfection with a cDNA expressing myostatin upregulated PAI-1, whereas an shRNA against myostatin blocked this effect. In conclusion, myostatin induced a fibrotic phenotype without significantly affecting differentiation into myofibroblasts. The concurrent endogenous antifibrotic reaction confirms the view that phenotypic switches in multipotent and differentiated cells may affect the progress or reversion of fibrosis, and that myostatin pharmacological inactivation may be a novel therapeutic target against fibrosis.

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Introduction

Progressive scarring (fibrosis) is the main pathological process in fibroproliferative diseases. During advanced stages, these disorders are responsible for close to 45% of all deaths in the developed world (Wynn 2007). They often involve a relative loss of cells essential to normal tissue function. Fibrosis is analogous to abnormal wound healing occurring during tissue response due to chronic and sustained injury, microtrauma, oxidative stress, endogenous or exogenous insults, autoimmunity, and other factors. This process affects multiple organs in localized, multifocal, or disseminated forms, in conditions such as systemic sclerosis, liver cirrhosis, progressive kidney disease, cardiovascular disease, pulmonary fibroses, macular degeneration, and muscle dystrophies (Willis *et al.* 2006, Gharraee-Kermani *et al.* 2007, Henderson & Iredale 2007). Although chronic inflammation usually precedes fibrosis, it is neither necessary nor sufficient to trigger it, and as a result anti-inflammatory agents

are usually not effective against fibrosis. In fact, successful antifibrotic treatments are very rare (Wynn 2007).

At the cellular level, one of the main factors of fibrosis is the differentiation of a not well-defined progenitor (local mesenchymal stem cells; fibroblasts; or epithelial, smooth muscle, or stellate cells; or recruited exogenous cells such as pericytes or bone marrow fibrocytes) into myofibroblasts, the cells that share a fibroblast/smooth muscle phenotype (Kisseleva & Brenner 2006, Qi *et al.* 2006, Iredale 2007). In fact, myofibroblasts are usually absent from normal tissue. They accumulate after injury or the impact of a noxious factor, and synthesize collagen and extracellular matrix during tissue repair. They then normally disappear by apoptosis when the process is completed. An increase in the differentiation of fibroblasts from their progenitors, or the failure of myofibroblasts to be removed after sufficient collagen has been deposited, is probably the basis of many fibroses (Iredale 2007, Wynn 2007). However, the activation of already differentiated contractile cells – myofibroblasts,

fibroblasts, or smooth muscle cells – to a synthetic phenotype where collagen is produced intensively is also a key step in fibrosis progression. Counteracting this myofibroblast differentiation and/or activation is therefore the primary target of novel therapies, considering that tissue defense mechanisms against these processes may operate in the now accepted concept of spontaneous reversibility of fibrosis, as in cirrhosis of the liver and kidney (Iredale 2007, Wynn 2007).

Many members of the transforming growth factor- β (TGF- β) superfamily, particularly TGF- β 1 and activin A, are well-known profibrotic factors that play a role in most of the stages in this process in many organs. This occurs specifically in the stimulation of myofibroblast differentiation, the switch to the synthetic phenotype, and in the inhibition of apoptosis (Sulyok *et al.* 2004, Mauviel 2005, Ruiz-Ortega *et al.* 2007). They upregulate collagen synthesis, the expression of ancillary profibrotic proteins, and of each other, as in the case of PAI-1 or connective tissue growth factor. They also share the downstream Smad signaling pathway.

Smad3 is a well-known profibrotic agent, more than Smad2, whereas Smad6 and 7 are antifibrotic, similar to another upstream member of the TGF- β 1 family, bone morphogenic protein 7 (BMP-7; Wang *et al.* 2005, Ask *et al.* 2006, Liu 2006). Moreover, follistatin, a protein that binds and inactivates TGF- β 1 and activin A, and downregulates the expression of their mRNAs, is antifibrotic in a number of conditions, namely, lung, liver, and kidney fibrosis (Aoki *et al.* 2005, Patella *et al.* 2006).

Myostatin, the only known negative regulator of skeletal muscle mass, is also a member of the TGF- β superfamily (Lee 2004, Tsuchida 2004). It has been shown to modulate multipotent cell differentiation, specifically in the C3H10T1/2 mouse embryonic cell line of fibroblast origin, where myostatin stimulates adipogenic commitment while inhibiting myogenic lineage, whereas androgens exert the opposite effect (Singh *et al.* 2003, Artaza *et al.* 2005, Jasuja *et al.* 2005, Feldman *et al.* 2006). C3H10T1/2 is a well-known and widely used multipotent mesenchymal cell line that undergoes differentiation into several cell lineages (Taylor & Jones 1979, Atkinson *et al.* 1997, Fischer *et al.* 2002, Wilson & Rotwein 2006).

In addition, myostatin inactivation increases osteogenic differentiation in bone marrow stem cells (Hamrick *et al.* 2007). Recent indirect evidence suggests that myostatin acts in the same way as other members of the TGF- β family, by inducing fibrosis in the skeletal muscle (Engvall & Wewer 2003, McCroskery *et al.* 2005). This is likely to occur via the Smad pathway, since myostatin signals through Smad2–4, and phosphorylates Smad3 (Zhu *et al.* 2004). In addition, it triggers a feedback compensatory mechanism through the inhibitory Smad7 that inactivates myostatin promoter, and counteracts myostatin and TGF- β action (Zhu *et al.* 2004, Forbes *et al.* 2006, Kollias *et al.* 2006). In turn, Smad2–4 upregulate myostatin expression by activating its promoter (Zhu *et al.* 2004).

Similar to TGF- β 1 and activin A, myostatin is inhibited by follistatin, and also by the agents that induce follistatin

expression, such as deacetylase inhibitors that block myostatin negative action on the muscle (Lee & McPherron 2001, Amthor *et al.* 2004, Iezzi *et al.* 2004, Kocamis *et al.* 2004). However, it is not known whether myostatin's putative profibrotic action is exerted via the stimulation of myofibroblasts differentiation from their progenitor, or by inducing a switch of the differentiated cell to the synthetic phenotype producing extracellular matrix. It is also unclear whether follistatin neutralizes these effects.

In our current work, we examined the effects of recombinant and endogenous myostatin on a) the differentiation of the multipotent C3H10T1/2 cell line into myofibroblasts, b) the regulation of the expression of TGF- β 1 and other fibrotic-related genes, and c) proteins involved in the Smad signaling cascade and follistatin. In addition, we investigated whether a) follistatin counteracts the effect of myostatin, b) over-expression of myostatin mRNA mimics the paracrine effects of the exogenous protein, and c) this effect is blocked by the myostatin shRNA.

Materials and Methods

Cell culture

Mouse C3H10T1/2 multipotent cells (ATCC, Manassas, VA, USA) grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum at 37 °C were treated with or without 20 μ M 5'-azacytidine (AZCT) for 3 days to induce differentiation (Singh *et al.* 2003, Artaza *et al.* 2005). Cells were split in a 1:2 ratio; allowed to recover for 2 days; seeded onto 60–70% confluence in T75 flasks, eight-well chamber slides or six-well plates; and incubated with 4 μ g/ml recombinant 113 amino acid myostatin protein (R-Mst; Artaza *et al.* 2005) in DMEM–10% serum for 0.5, 1, 2, 3, and 24 h and for 3, 4, and 10 days to assess paracrine effects of myostatin (see the section below). Azacytidine-treated cells were also co-transfected at 60% confluence in six-well plates with a) a myostatin cDNA plasmid expressing the full-length 375 amino acid protein (pcDNA-Mst-375; Taylor *et al.* 2001) or b) the silencer RNAs for two different myostatin sequences (pSil-Mstno.4 and 326) (Artaza *et al.* 2005, Magee *et al.* 2006) (see the section below). A pcDNA3.1-EGFP (Invitrogen) was co-transfected separately to evaluate the transfection efficiency.

siRNA myostatin

siRNA myostatin was described by Artaza *et al.* (2005) and Magee *et al.* (2006). To prepare the silencer RNA construct for myostatin, we analyzed the mouse myostatin gene sequence (accession no. NM_010834) using the web-based siRNA target finder and design tool provided in the Ambion website (Ambion Inc., Austin, TX, USA). Five regions were initially targeted for likely inhibitory activity by siRNAs (nucleotide position target no.): 176(no. 4), 207(no. 8), 426(no. 26), 647(no. 45), and

1064(no. 72)). Double-stranded RNAs were transcribed *in vitro* using the Silencer siRNA Construction Kit. In addition, a control siRNA targeting glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and provided with the kit was also synthesized. Each siRNA was tested for inhibitory activity at 1, 10, and 100 nM concentrations by co-transfection of pCDNA3.1-Mst into human embryonic kidney cells (HEK)293 cell cultures using Lipofectamine 2000 (Invitrogen). After 48 h, cell lysates were collected in M-PER (Pierce Biotechnology Inc., Rockford, IL, USA) and western blot analyses were performed. Two siRNAs (growth differentiation factor (GDF)8 siRNA26 and siRNA4) were found to inhibit more than 95% of the myostatin gene expression (Magee *et al.* 2006).

Based on a GenBank Blast search, these sequences have homology not only to mouse but also to human, rat, rabbit, cow, macaque, and baboon. A short hairpin DNA sequence was synthesized and cloned into the pSilencer 2.1-U6 neo (Ambion Inc.), according to the manufacturer's instructions. The DNA sequence consists of a *Bam*HI DNA restriction site, sense strand, nine-nucleotide loop, antisense strand, RNA polymerase III terminator, and *Hind*III DNA restriction site 5' to 3' (Artaza *et al.* 2005, Magee *et al.* 2006). The pSilencer 2.1-U6 neo-GDF8 siRNA plasmid constructs were transfected (1 µg/well plate, six-well plate) into C3H10T1/2 cell cultures using Lipofectamine 2000 following the manufacturer's instructions as before.

Qualitative and quantitative immunocytochemical analyses

Cells grown in eight-well chamber slides were fixed in 2% *p*-formaldehyde, quenched with H₂O₂, blocked with normal goat or horse serum and incubated with specific antibodies (Artaza *et al.* 2002, Singh *et al.* 2003, 2006, Artaza *et al.* 2005, Jasuja *et al.* 2005). These consisted of a α -smooth muscle actin immunohistology kit (Sigma–Aldrich), and antibodies against pSmad2/3 (1:500); Smad3 (1:500), Smad4 (1:500) and Smad7 (1:500) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); PAI-1 (1:200; Abcam Inc.); TGF- β 1 (1:200) (Promega Corporation); collagen I (1:100) and collagen III (1:100; Chemicon International Inc., Temecula, CA, USA).

Detection was based on a secondary biotinylated antibody (1:200), followed by the addition of the streptavidin–horseradish peroxidase ABC complex (1:100), Vectastain (Elite ABC System, Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine and H₂O₂ mixture (Sigma). The cells were counterstained with Mayer's hematoxylin solution (Sigma). In negative controls, we either omitted the first antibody or used a rabbit non-specific IgG.

In all cases, the cytochemical staining was quantitated by image analysis using ImagePro-Plus 5.1 software (Media Cybernetics, Silver Spring, MD, USA) coupled to a Leica digital microscope bright field light fluorescence microscope/VCC video camera. After images were calibrated for background lighting, wherein integrated OD (IOD = area $\times$ average intensity) was calculated using at least ten pictures per treatment group

per well done in duplicate. These experiments were repeated at least thrice (Singh *et al.* 2003, Artaza *et al.* 2005, Jasuja *et al.* 2005).

Western blot and densitometry analyses

Cell lysates (40–80 µg protein) were subjected to western blot analyses (Artaza *et al.* 2002, Singh *et al.* 2003) by 4–15% Tris–HCl PAGE (Bio-Rad) in running buffer (Tris/glycine/SDS). Proteins were transferred overnight at 4 °C to nitrocellulose membranes in transfer buffer (Tris/glycine/methanol). The next day the non-specific binding was blocked by immersing the membranes in 5% non-fat dried milk and 0.1% (v/v) Tween 20 in PBS for 1 h at room temperature. After several washes with washing buffer (PBS Tween 0.1%), the membranes were incubated with the primary antibodies for 1 h at room temperature. Monoclonal antibodies were as follows: a) α -smooth muscle actin (1:1000; Calbiochem, La Jolla, CA, USA) and b) GAPDH; 1:10 000 (Chemicon International). Polyclonal antibodies were used for a) Smad3 (1:200), b) Smad7 (1:200; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), c) TGF- β 1 (1:1000; Promega Corporation), and d) myostatin (1:1000) (Chemicon International Inc.). The washed membranes were incubated for 1 h at room temperature with 1:3000 dilution (anti-mouse) or 1:2000 dilution (anti-rabbit) of secondary antibody linked to horseradish peroxidase respectively. After several washes, the immunoreactive bands were visualized using the emission of chemiluminescence (ECL) plus Western blotting chemiluminescence detection system (Amersham Biosciences). The densitometry analysis of the bands was done with the Scion Image software beta 4.0.2 (Scion Corp., Frederick, MD, USA).

Recombinant proteins

Myostatin recombinant protein was produced in *Escherichia coli* as a 16 kDa protein containing 113 amino acid residues of the human myostatin protein (BioVendor Laboratory Medicine Inc., Palackeho, Czech Republic). Recombinant follistatin protein was generated in Sf21 cells as a 31 kDa protein containing 289 amino acid residues (R&D Systems, Minneapolis, MN, USA). Recombinant human TGF- β 1 was produced in Chinese hamster ovary (CHO) cells as a 25 kDa protein containing 112 amino acid residues (Chemicon International Inc.).

Real-time quantitative PCR

Total RNA was extracted using Trizol reagent (Invitrogen) and equal amounts (2 µg) of RNA were reverse transcribed using a RNA PCR kit (Applied Biosystems, Foster City, CA, USA). The locations of forward/reverse PCR primers for real-time RT-PCR are as follows: Smad7 region 575–597/626–641 on BC074818.2 (67 bp); follistatin (Fst; 150 bp), PPM04451A on NM_008046 and GAPDH (152 bp), 606–626/758–738 on BC023196. Mouse gene PCR primer sets (RT2) were purchased from SuperArray Bioscience (Frederick, MD, USA). The Qiagenn Sybr Green

PCR kit with HotStar *Taq* DNA polymerase (Qiagen) was used with i-Cycler PCR thermocycler and fluorescent detector lid (Bio-Rad; Singh *et al.* 2006).

The protocol included melting for 15 min at 95 °C, 40 cycles of three-step PCR including melting for 15 s at 95 °C, annealing for 30 s at 58 °C, elongation for 30 s at 72 °C, with an additional detection step of 15 s at 81 °C, followed by a melting curve from 55 to 95 °C at the rate of 0.5 °C per 10 s. Fst annealing for primers, however, was at 55 °C and detection at 76 °C. We confirmed that inverse derivatives of melting curves show sharp peaks for *Smad7* at 82 °C, Fst at 84.5 °C, and *GAPDH* at 87 °C, indicating the correct products. Samples of 25 ng cDNA were analyzed in quadruplicate in parallel with *GAPDH* controls; standard curves (threshold cycle versus log pg cDNA) were generated by log dilutions of from 0.1 pg to 100 ng standard cDNA (reverse-transcribed mRNA from C3H10T1/2 cells in AM). Experimental mRNA starting quantities were then calculated from the standard curves and averaged using i-Cycler, iQ software, as described previously (Singh *et al.* 2003). The ratios of marker experimental gene (e.g. follistatin mRNA) to *GAPDH* mRNA were computed and normalized to control (untreated) samples as 100%.

DNA microarray analysis of TGF- β /BMP target genes

Pools of total cellular RNA from three different T75 flasks for each experimental condition were isolated with Trizol reagent from C3H10T1/2 cells undergoing differentiation with AZCT and treated with or without recombinant myostatin protein (4 μ g/ml) for 3 h and 3 days. Isolated RNA was subjected to cDNA gene microarrays (GEArray Q Series, TGF- β /BMP signaling pathway gene array (matrix microarray (MM)-023) and osteogenesis gene array (MM-026) analysis (SuperArray BioScience Corp). The microarray assays were performed in two separate experiments in each case. This series of mice TGF- β /BMP signaling pathway gene arrays are designed to study the genes involved in TGF- β /BMP signaling pathway (MM-023) and the osteogenic array (MM-026) contains collagens of some other fibrotic-related genes. Biotin-labeled cDNA probes were synthesized from total RNA, denatured, and hybridized overnight at 60 °C in GEHybridization solution to membranes spotted with TGF- β /BMP signaling pathway-specific genes, as well as with genes involved in the regulation of osteogenic differentiation. Membranes were then washed, and chemiluminescent analysis was performed as per the manufacturer's instructions. Raw data were analyzed using GEArray Expression Analysis Suite (SuperArray BioScience Corp). Fold changes in relative gene expression were presented after background correction and normalization with a housekeeping gene.

Confirmation of DNA microarray analysis by RT² profiler PCR array analysis of TGF- β /BMP target gene

RT² profiler PCR SuperArray analyses of TGF- β /BMP target genes were applied in order to confirm selected genes from the GEArray data. Aliquots of total cellular RNA isolated with

Trizol reagent from C3H10T1/2 cells undergoing differentiation with AZCT were treated with or without recombinant myostatin protein (4 μ g/ml) for 3 h and 3 days. They were then subjected to reverse transcription, and the resulting cDNA was analyzed by RT² profiler PCR mouse TGF- β /BMP signaling pathway (APM-035A) (SuperArray BioScience Corp). This series of mouse TGF- β /BMP signaling pathway gene array is designed to study the genes involved in and downstream of TGF- β /BMP signaling. Each array contains a panel of 84 primer sets related to the TGF- β /BMP signaling genes plus 5 housekeeping genes and two negative controls. Real-time PCRs were performed as follows: a) melting for 10 min at 95 °C, b) 40 cycles of two-step PCR including melting for 15 s at 95 °C, and c) annealing for 1 min at 60 °C. The raw data were analyzed using the $\Delta\Delta C_t$ method following the manufacturer's instructions (SuperArray).

Statistical analysis

All data are presented as mean $\pm$ S.E.M., and between-group differences were analyzed using ANOVA. If the overall ANOVA revealed significant differences, then pair-wise comparisons between groups were performed by Newman-Keuls multiple comparison test. All comparisons were two-tailed, and $P < 0.05$ were considered statistically significant. The *in vitro* experiments were repeated thrice, and data from representative experiments are shown. Specifically, the DNA microarrays tests were done twice and the results confirmed by qRT-PCR in triplicate.

Results

Myofibroblast generation from multipotent C3H10T1/2 cells occurs spontaneously, and is not affected by azacytidine or incubation with exogenous myostatin, but myostatin triggers a fibrotic phenotype associated with transcriptional regulation of fibrotic-related genes and the Smad cascade

C3H10T1/2 cells, treated or non-treated with AZCT, were tested for the presence of myofibroblasts (cells with a fibroblast/smooth muscle cell hybrid phenotype, which play a key role in fibrosis), and also to determine whether myostatin stimulates C3H10T1/2 commitment to this differentiation lineage. The cultures not treated with AZCT had some cells that stained positive for α -smooth muscle actin (ASMA), a marker that is common to myofibroblasts and smooth muscle cells (data not shown). They had the typical appearance of myofibroblasts with prominent actin filaments and lamellipodia. Treatment of these cultures with either AZCT alone or in combination with recombinant myostatin protein did not alter this morphology (data not shown), nor there was any apparent increase from these treatments in terms of myofibroblast number ($\sim 8\%$ of the total cell population) as confirmed by quantitative image analysis. Western immunoblot analysis of the cell extracts for ASMA compared with GAPH expression agreed with the qualitative

and quantitative immunocytochemistry observations. In addition, calponin, a marker for smooth muscle cells, which is not expressed in myofibroblasts, was not detected in the extracts (data not shown). This indicates that the ASMA⁺ cells in the C3H10T1/2 cultures are indeed myofibroblasts.

Although myostatin did not stimulate myofibroblast differentiation in the azacytidine-induced multipotent C3H10T1/2 culture, it switched these cells towards the synthetic fibrotic phenotype, as shown in Fig. 1. Starting with this experiment, cells were always treated with AZCT, even if this drug did not affect the number of myofibroblasts that originated from

C3H10T1/2 cells. This was done in order to facilitate comparisons with studies, including our previous results (Singh *et al.* 2003, Artaza *et al.* 2005, Jasuja *et al.* 2005) where azacytidine was routinely employed to stimulate multipotent stem cell differentiation (Schmittwolf *et al.* 2005). Incubation with recombinant myostatin protein clearly stimulated the intensity of immunocytochemical staining for the four selected fibrotic markers PAI-1, TGF- β 1, collagen I, and collagen III (Fig. 1A and B, left panels). Most of the cells had a very low basal level of expression in the absence of treatment, but the expression per cell was intensified by myostatin. This is reflected

Figure 1 Effects of recombinant myostatin protein on the expression of profibrotic genes and collagen in C3H10T1/2 cells. Representative pictures (200 $\times$) of cells treated with azacytidine (20 μ M) to induce differentiation and which, 2 days later, received myostatin (4 μ g/ml), or no myostatin, on eight-well removable chambers, for 3 days followed by immunostaining (left) with the corresponding antibodies. (A) Profibrotic genes: PAI-1 and TGF- β 1 and (B) collagen I and III. Mean $\pm$ S.E.M. corresponds to experiments done in triplicate of the integrated optical densities (IOD). * P <0.05, *** P <0.001 (200 $\times$).

on the quantitative image analysis (Fig. 1A and B, right panels) that shows a statistically significant increase in PAI-1, TGF- β 1, collagen I, and collagen III expression after incubation with recombinant myostatin (R-Mst) for 4 days.

The stimulation of fibrotic gene expression in the C3H10T1/2 cells by myostatin was also analyzed at the transcriptional level using mouse osteogenesis gene array (MM-026) and mouse TGF- β /BMP signaling pathway gene array (MM-023) (data not shown).

Figure 2 shows one of the two sets of membranes for DNA mouse osteogenesis gene array performed on total RNA extracted from cells subjected for 3 h (2A) and 3 days (2B) of incubation with or without recombinant myostatin. Some of the genes that showed differential RNA expression between the myostatin-treated and -untreated cells are indicated by circles (Fig. 2A and B) and were selected for the table shown in Fig. 2C, where the computer-generated ratios of spot intensities, normalized by housekeeping genes are tabulated for 3 days in the left column. This PCR microarray panel has only some of the fibrotic genes – essentially collagens, BMP members, and all the Smad genes – that transduce signals triggered by the members of the TGF- β 1 family. For this reason, other genes selected from the mouse TGF- β /BMP panel are also included. The change of expression of some selected genes at 3 days was ultimately confirmed by real-time PCR using the RT² profiler PCR SuperArray set of primers and procedures. The ratios for triplicate determinations are shown on the right column. The agreement between the ratios obtained by DNA microarrays and RT-PCR is in general adequate and provides a reasonable assessment of up- and downregulation. From both the columns and the quantitative microarray data for 3 h (not shown), it was found that the stimulation of the mRNA expression of collagen I α , collagen IX α 1, Smad3, 4, and 7, BMP 3, BMP 6, BMP7, and v cam occurred early, whereas it took longer to enhance the levels of the mRNAs for collagen I α 1, collagen IV α 4, collagen IV α 6, and MMP8. The increase in Smad7 mRNA by myostatin remained considerable at 3 days, whereas that for Smad3 and 4 mRNAs was negligible.

The early transcriptional stimulation of Smad3 mRNA expression by myostatin was demonstrated at the protein level by immunocytochemistry, as shown in the time course depicted in Fig. 3A. TGF- β 1, the main profibrotic factor and the member of the TGF- β superfamily that includes myostatin, signals through the Smad pathway, and was therefore used as positive control. There was an early and dramatic increase in the intensity of Smad3 staining (Fig. 3A) that peaked at 1 h, and was later downregulated reaching normal values at 24 h. The 3-h expression was confirmed by western blot analysis, which showed that myostatin was nearly as effective as TGF- β 1 (Fig. 3B).

The time course of exogenous myostatin induction of Smad proteins shows an early expression and phosphorylation of the Smad2–4 genes followed by a later upregulation of the inhibitory Smad7

Smad3 was not the only Smad protein modulated by myostatin. C3H10T1/2 cells were incubated with recombinant myostatin;

a very early stimulation of the phosphorylated Smad2/3 proteins was observed at 30 min, peaking at 1 h. This decayed at 2 h and normalized at 24 h (Fig. 4A). A similar process after 1 h occurred with the expression of Smad4 protein (Fig. 4B).

Consistent with the results shown in Fig. 2, Smad7 was expressed early. However, the immunostaining remained high even at 6 h (Fig. 5A). Western blot analysis confirmed the early Smad7 expression induced by myostatin. This was even higher than that induced by TGF- β 1 (Fig. 5B). On the other hand, real-time RT-PCR showed that the stimulation of Smad7 mRNA expression by myostatin persisted even at 3 days, when the other Smad proteins had fallen to negligible levels (Fig. 5C).

Exogenous myostatin upregulates the expression of its inhibitory protein, follistatin, and the addition of follistatin downregulates the myostatin-induced upregulation of TGF- β 1

Since the activities of myostatin, TGF- β 1, activin, and other members of the TGF- β family are inhibited by follistatin through binding to these proteins, we investigated whether myostatin modulated the expression of follistatin in the cells that were undergoing a fibrotic phenotypic differentiation. Contrary to our initial assumption that follistatin levels would be downregulated by myostatin, and would thus boost myostatin effects, real-time RT-PCR (Fig. 6A) revealed an early threefold stimulation at 3 h of follistatin mRNA levels that remained remarkably high even at 3 days.

To determine whether this was a compensatory mechanism of the C3H10T1/2 cells to counteract the profibrotic effects of myostatin, we tested whether the addition of recombinant follistatin protein would block the myostatin stimulation of the production of profibrotic factors by these cells. We also investigated whether myostatin modulation of the fibrotic phenotypic differentiation is mediated by the upregulation of the expression of TGF- β 1, the main profibrotic factor. Figure 6B shows that at 4 days, myostatin stimulated TGF- β 1 expression, and that the addition of follistatin (0.5 μ g/ml) blocked this stimulation, whereas follistatin *per se* did not affect TGF- β 1 expression. These effects were confirmed by western immunoblot (Fig. 6C).

The profibrotic effects of myostatin on C3H10T1/2 cells can also be exerted autocrinely by over-expression of myostatin mRNA, or through the breakdown of myostatin mRNA by its shRNA

The preceding experiments indicated that exogenous myostatin regulated the fibrotic phenotype of C3H10T1/2 cells which contain myofibroblasts that originated from this multipotent cell culture. Questions remained, however, whether endogenously produced myostatin would cause the same effects, and whether blocking its expression at the protein level would inhibit the production of fibrotic factors. Figure 7 shows that transfection of these cells with plasmid constructs expressing myostatin increased myostatin expression, as evidenced by the western blot analysis (Fig. 7B, left) and the corresponding densitometry

Figure 2 Effects of recombinant myostatin protein on the transcriptional expression of Smad proteins, collagen, and other fibrotic genes in C3H10T1/2 cells. Cells were treated as shown in Fig. 1, but in 75 cm² flasks, for 3 h (A) and 3 days (B). Total RNA was isolated and subjected to DNA microarray analysis for genes related to extracellular matrix represented in the osteogenesis gene array. (A and B) Representative membranes of assays performed in two separate experiments. (C) In parallel reactions, total RNA was subjected to RT-real-time PCR by the RT²-PCR profiler TGF-β array and the ratio between the myostatin-treated versus myostatin-untreated cells corrected by GAPDH was calculated for the assays performed in triplicate.

Figure 3 Myostatin stimulates the early expression of Smad3 protein in C3H10T1/2 cells. Azacytidine-treated cells were incubated on eight-well removable chambers in a time course manner and, for the 3 h incubation, on six-well plates, with or without myostatin, for the indicated periods. TGF-β1 was used as positive control. (A) Representative pictures of the immunodetection for Smad3 are presented (left) (200×), as well as the quantitative image analysis (right). (B) Western blot analysis was performed for the extracts from the 3-h incubation (left) and the corresponding densitometry analysis (right). Control; R-Mst, recombinant myostatin protein and TGF-β1 as positive control. Ab, antibody. Mean ± s.e.m. corresponds to experiments done in triplicate. **P* < 0.05, ****P* < 0.001. (A) 200×.

analysis of the band intensities (Fig. 7B, right). The transfection efficiency was estimated at about 60% by co-transfection of the myostatin construct with a reporter vector, pcDNA-EGFP (Fig. 7A). The two different plasmid constructs for the shRNA against myostatin mRNA decreased the expression of the myostatin band, as evidenced by the western blot analysis and the corresponding densitometry analysis (Fig. 7B). In parallel, there was considerable upregulation of PAI-1, visualized by the

western blot analysis, and of the myostatin densitometry analysis, which was also blocked by the myostatin shRNA (Fig. 7C). A random RNA construct corresponding to the shRNA sequences for myostatin had previously been shown by our group to be inactive both *in vitro* (Artaza *et al.* 2005) and *in vivo* (Magee *et al.* 2006). This set of experiments confirmed that endogenous myostatin might act similarly to the exogenous myostatin protein.

Figure 4 Time course of recombinant myostatin effects on the expression of the phosphorylated form of Smad2/3 protein (pSmad2/3) and Smad4 protein in C3H10T1/2 cells. Azacytidine-treated cells were incubated on eight-well removable chambers with or without myostatin, for the indicated periods. TGF-β1 was used as positive control. Representative pictures of the immunodetection are presented in left panels (200×), as well as the quantitative image analysis in the right panels. (A) pSmad2/3, (B) Smad4. Ab, antibody; R-Mst, recombinant myostatin protein. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (200×).

Figure 5 Time course of recombinant myostatin effects on the expression of the Smad7 gene in C3H10T1/2 cells. Azacytidine-treated cells were incubated on eight-well removable chambers in a time course manner, and, for the 3 h, 6 h, and 3 days incubations, on six-well plates, with or without myostatin. TGF-β1 was used as positive control. (A) Representative pictures of the immunodetection are presented (left), as well as the quantitative image analysis (right). (B) Western immunoblot analysis was performed for extracts from the 6-h incubation (left) and the corresponding densitometry analysis (right). (C) Total RNA isolation followed by real-time RT-PCR was applied in other aliquots for the 3-h and 3-day incubations normalized by GAPDH housekeeping gene. Ab, antibody; R-Mst, recombinant myostatin protein. Mean ± S.E.M. corresponds to experiments done in triplicate. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (200×).

Discussion

The current study elucidates an important issue in the cell biology effects of myostatin, a protein that, in addition to its well-known role as a negative regulator of skeletal muscle mass and modulator of stem cell differentiation, acts as a

profibrotic in this tissue (Engvall & Wewer 2003, McCroskery *et al.* 2005). This is consistent with the effects of other members of the TGF-β family such as activin and TGF-β1 in various tissues (Wada *et al.* 2004, Yamashita *et al.* 2004, Verrechia *et al.* 2006). Using the multipotent mesenchymal embryonic C3H10T1/2 cell line, it was possible in our

Figure 6 Effects of recombinant myostatin on follistatin and TGF- β 1 expression, and modulation by follistatin of TGF- β 1 protein expression. (A) Total RNA from the experiment of Fig. 5 was subjected to real-time RT-PCR for follistatin mRNA. (B) In a separate experiment, azacytidine-treated cells were incubated with either recombinant myostatin (4 μ g/ml), TGF- β 1 (5 ng/ml) or follistatin (0.5 μ g/ml), for 4 days and subjected to immunocytochemistry and quantitative image analysis for TGF- β 1 (200 $\times$). (C) Western immunoblot analysis for TGF- β 1: R-Mst, recombinant myostatin protein; Fst, follistatin. ** $P < 0.01$, *** $P < 0.001$.

current work to demonstrate that myostatin did not affect paracrinely the differentiation of these cells into myofibroblasts (the typical fibrotic cells), and did not generate smooth muscle cells that are also potentially involved in extracellular matrix deposition when they switch to a fibrotic phenotype.

However, myostatin was found to induce this switch in the overall multipotent and myofibroblast cell populations, as indicated by the stimulation in most cells within the culture of the expression TGF- β 1 and another key profibrotic factor, PAI-1 (Eddy & Fogo 2006), and particularly by the ultimate

Figure 7 Effect of the modulation of the endogenous expression of myostatin on the expression of PAI-1. C3H10T1/2 cells were grown on six-well plates and transfected with either a reporter gene plasmid construct and a plasmid DNA construct encoding myostatin, or the latter construct or the one for myostatin shRNA, and analyzed after 4 days. (A) Fluorescent microscope, green filter (right), or regular light (left) of cells co-transfected with pcDNA-Mst and pcDNA-EGFP in order to check transfection efficiency (200 $\times$). (B) Western blot analysis of myostatin expression, with GAPDH as reference gene of cells transfected with: pcDNA-Mst-375; Mst siRNAs: pSiL-Mstno. 4 and pSiL-Mstno. 26 (sequences no. 4 and no. 26) (left), densitometry evaluation of band intensities (right). (C) Western blot analysis of PAI-1 expression, as shown in (B).

products that define fibrosis – collagens I, III, and other isoforms (Bhagal *et al.* 2005, Attallah *et al.* 2007).

These processes were associated with the upregulation of the expression of Smad3 and 4, and the phosphorylation of Smad2 and 3, as expected from a member of the TGF- β family that signals through this pathway (Zhu *et al.* 2004, Kollias *et al.* 2006). An antifibrotic process was simultaneously elicited, as evidenced

by the stimulation of the expression of a) the myostatin activity inhibitor, follistatin (Hill *et al.* 2002, Anthor *et al.* 2004, Kocamis *et al.* 2004), b) a Smad signaling inhibitor, Smad7 (Forbes *et al.* 2006), and c) a collagen breakdown inducer, matrix metalloproteinase 8 (MMP-8; Siller-Lopez *et al.* 2004). Follistatin did block the upregulation of TGF- β 1 expression by myostatin, as shRNA against myostatin (Magee *et al.* 2006) inhibited the

expression of PAI-1, which in turn was stimulated autocrinely by the forced over-expression of myostatin. It is also well known that PAI-1 expression is upregulated by TGF- β 1 (Otsuka *et al.* 2007).

An intriguing aspect of this study is that neither myostatin nor the well-known demethylating agent and differentiation inducer, azacytidine (Singh *et al.* 2003), affected the sizable number of C3H10T1/2 cells (7–8%) that expressed the typical myofibroblast marker ASMA, in the absence of any detectable expression of the smooth muscle cell marker, calponin. Myostatin acted differently from TGF- β 1 in this respect, and in its inability to induce (at least in 10 days) the smooth muscle cell lineage, since TGF- β 1 stimulates considerably the appearance of smooth muscle markers in this cell line at an earlier stage, acting through the Smad2/3 pathway (Sato *et al.* 2005, Chen *et al.* 2006).

The induction by exogenous recombinant myostatin protein of the expression of collagens I and III mRNA and protein is the hallmark of the acquisition of a fibrotic phenotype. This is likely to be mediated by the ActIIb receptor that binds myostatin and mediates its signaling, and which was detected in C3H10T1/2 cells (Artaza *et al.* 2007). The increase in the mRNA expression of other minor isoforms of collagen, particularly II and IV, and, to a lesser extent, collagen IX, by myostatin, agrees with what occurs in fibrotic conditions (Bhagal *et al.* 2005, Attallah *et al.* 2007). This may be due at least in part to the observed upregulation of TGF- β 1 (Liu *et al.* 2006). This factor is known to upregulate myostatin expression (Budasz-Rwiderska *et al.* 2005). Reports are not available on the induction by myostatin of the other main vascular fibrotic factor, PAI-1, and we assume that the considerable upregulation observed may be either a direct effect of myostatin or is mediated by TGF- β 1 (Otsuka *et al.* 2007).

In any case, the observed profibrotic effects of myostatin do not seem to be due to the appearance of a new specific cell lineage that would be very active in extracellular matrix deposition, and would differ from the rest of the cell population in this respect. Rather, profibrotic factor expression and collagen deposition may be mediated by the stimulation of the general multipotent fibroblast population to acquire this phenotype, with little conversion into myofibroblasts and none into smooth muscle cells. This would be similar to the differentiated smooth muscle cell transition from a 'contractile' to 'synthetic' phenotype (Budasz-Rwiderska *et al.* 2005). This transition has also been documented on myofibroblast cultures *in vivo* and *in vitro* (Hirose *et al.* 1999, Burstein *et al.* 2007, Darby & Hewitson 2007, Krieg *et al.* 2007), and myostatin should be considered, along with TGF- β 1, as a factor eliciting this response.

We believe that the most significant findings of our study are the detection of an early 'antifibrotic' response simultaneous to the profibrotic phenotype induced by myostatin, as evidenced by the observed upregulation of Smad7, follistatin, and MMP8. The upregulation of Smad7 by myostatin was already observed in an elegant study where myostatin induced the expression of Smad7 in C2C12 myoblasts. The latter in

turn inhibited myostatin promoter activity, thus suggesting that myostatin autoregulates its expression by feedback loop through Smad7 (Forbes *et al.* 2006), which involves the interaction of Smad2/3 with the Smad7 promoter (Zhu *et al.* 2004). The fact that Smad7 abrogates myostatin – but not TGF- β 1-mediated repression of myogenesis (Kollias *et al.* 2007) – raises the question of why myofibroblast differentiation was not triggered by the myostatin-induced TGF- β 1 expression in our experiments. It seems that either this expression was too low to trigger C3H10T1/2 cell differentiation, or the Smad7 induction by myostatin was blocked in these cells through counteracting the Smad2/3 upregulation. Myostatin does inhibit myoblast progression into myotubes in C2C12 myoblasts via Smad3 phosphorylation (Langley *et al.* 2002).

The upregulation of follistatin expression by myostatin does not seem to have been reported before, although TGF- β 1 through the Smad protein potentiates the stimulatory effects of tumor necrosis factor- α (TNF- α) on the activity of a follistatin-related gene promoter (Bartholin *et al.* 2007), and TNF- α is associated with fibrotic processes (Yoshimura 2006). TGF- β 1 also directly upregulates follistatin expression in bovine granulosa cells (Fazzini *et al.* 2006). As stated previously, follistatin in turn inactivates myostatin, and is a well-known antifibrotic agent (Sulyok *et al.* 2004, Wada *et al.* 2004). Our findings are consistent in terms of indicating that treatment with follistatin counteracted the upregulation of TGF- β 1 expression exerted by recombinant myostatin in our culture.

Smad7 and follistatin inhibit myostatin signaling and activity respectively, and therefore act as a feedback mechanism against myostatin profibrotic effects, demonstrated by the fact that the C3H10T1/2 cells reacted to myostatin by upregulating MMP-8. This metalloproteinase, like MMP-1 or MMP-13, cleaves collagen at a single site and renders it susceptible to degradation by other MMPs and proteases. An adenoviral construct for a cDNA encoding MMP-8 over-expressed the MMP-8 pro-collagenase in rat models of liver fibrosis, which was then endogenously activated and led to a reversion of the process (Siller-Lopez *et al.* 2004). The MMP-8 upregulation exerted by myostatin in our system therefore appears to be an attempt to degrade collagens I and III deposited as a result of incubation with myostatin. This is sort of a second stage of defense to complement the feedback mechanism that inactivates myostatin.

The interplay of profibrotic and antifibrotic processes observed in the current study with myostatin suggests that counteracting myostatin might be a potentially effective therapy against fibrosis, in addition to that based on the use of decorin (Li *et al.* 2004), Smad7 cDNA (Forbes *et al.* 2006), and follistatin (Aoki *et al.* 2005, Patella *et al.* 2006), against TGF- β 1/activin A/Smad signaling, or the use of agents such as deacetylase inhibitors that induce follistatin (Iezzi *et al.* 2004). This approach of targeting myostatin may aim to downregulate myostatin expression, like the shRNA against

myostatin applied in the current *in vitro* study, and that we previously used *in vitro* and *in vivo* to promote myogenesis (Magee *et al.* 2006). Further experimental work is needed on the potential application of these inhibitors to discriminate the relative contribution of myostatin, TGF- β 1, and activin to fibrotic processes. It is also important to elucidate whether the Smad pathway is the single downstream signaling for these effectors in the acquisition of the fibrotic phenotype by terminally differentiated cells, and if so, whether Smad7 indeed acts specifically on myostatin in this respect. In addition, despite our results with the C3H10T1/2 cells, it is uncertain whether myostatin *in vivo* triggers the differentiation of endogenous or circulating stem cells to myofibroblasts, as does TGF- β 1. If this would be the case, then the anti-myostatin strategy may also block an additional profibrotic mechanism operating through stem cell lineage commitment. However, the fact that myostatin stimulates the switch of myofibroblast to a fibrotic phenotype suggests that this by itself may be the main cellular target for its profibrotic effects, since myofibroblasts play such a fundamental role in fibrosis and scarring (Darby & Hewitson 2007).

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