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Abstract

Background & aims: Myostatin is mainly expressed in skeletal muscle, where it negatively regulates trophism. This myokine is implicated in the pathophysiology of nonalcoholic steatohepatitis, an emerg-ing cause of liver fibrosis. In this study we explored the effects of myostatin on the biology of hepatic stellate cells.

Methods: The effects of myostatin were assessed both in LX-2 and in human primary stellate cells. Cell migration was determined in Boyden chambers. Activation of intracellular pathways was evaluated by Western blotting. Procollagen type 1 secretion was measured by enzyme immunoassay. The role of c-Jun N-terminal kinase was assessed by pharmacologic and genetic inhibition.

Results: Activin receptor-2B was up-regulated in livers of mice with experimental fibrosis, and detectable in human stellate cells. Serum myostatin levels increased in a model of acute liver injury. Myostatin reduced HSC proliferation, induced cell migration, and increased expression of procollagen type1, tis-sue inhibitor of metalloproteinase-1, and transforming growth factor- _1. Myostatin activated different signaling pathways, including c-Jun N-terminal kinase and Smad3. Genetic and/or pharmacologic inhi-bition of c-Jun N-terminal kinase activity significantly reduced cell migration and procollagen secretion in response to myostatin.

Conclusions: Activation of activin receptor-2B by myostatin modulates the fibrogenic phenotype of human stellate cells, indicating that a myokine may be implicated in the pathogenesis of hepatic fibrosis.

Introduction

Myostatin, also known as growth differentiation factor-8, is ahighly conserved protein belonging to the transforming factor- _family. Expression of myostatin is detected primarily in skeletalmuscle, and is a negative modulator of muscle growth and trophism[1]. In fact, mutations or targeted deletion in mammalian speciescause muscle hypertrophy and hyperplasia [2]. Accordingly, myostatin has been implicated in the pathogenesis of muscle wastingin different conditions, including cancer cachexia [3]. The actionof myostatin is mediated by interaction with activin receptor-2B(ActR2B) [4] and results in activation of a Smad3/4 complex and toits translocation to the nucleus, where target genes are activated.Recent findings suggest a possible involvement of myostatin in theabnormalities associated with the metabolic syndrome. Myostatin-deficient mice have a significant reduction in fat accumulation,despite normal food intake, and show improved insulin sensitiv-ity in conditions of obesity [5,6]. In humans, elevated expressionlevels of myostatin have been reported in the muscle of patientswith type 2 diabetes, and myostatin expression is reduced in theskeletal muscle of obese individuals undergoing weight loss [1,7,8].Skeletal muscle is a relevant part of the network coordinatingmetabolism and participates in the complex alterations occurringin the metabolic syndrome and insulin resistance [9]. Nonalcoholic fatty liver disease (NAFLD) is the hepatic counterpart of this dis-order and is characterized by accumulation of fat, predominantly in the form of triglycerides [10]. NAFLD is associated with a higher risk of liver-related and all-cause mortality, due to the occurrence of nonalcoholic steatohepatitis (NASH), which may lead to the appear-ance of cirrhosis and its complications, including hepatocellular carcinoma [11]. Recent studies have indicated that the presence of fibrosis is a major risk factor for progression of NAFLD and mortality [12,13]. Thus, understanding the molecular and cellular mechanisms lead-ing to fibrosis in this setting is of crucial importance to design appropriate management strategies for patients with NASH. Com-pelling evidence in the past two decades has highlighted the pivotal role played by hepatic stellate cells (HSC) as the key effectors in the pathogenesis of fibrosis in different chronic liver diseases, including NASH [14,15]. Although fibrosis is the ultimate out-come of all chronic liver diseases, there is accumulating evidence for disease- and context-specific mechanisms that may modulate or drive hepatic fibrogenesis [16]. In this respect, no informa-tion is currently available concerning a possible contribution of myokines, i.e. cytokines predominantly expressed in skeletal mus-cle, on the cellular and molecular mechanisms of fibrogenesis. Here we show that myostatin is a novel modulator of the biology of HSC, differentially affecting proliferation, migration and expres-sion of extracellular matrix components, via activation of the c-Jun N-terminal kinase (JNK) pathway.

Materials and methods

Materials

Recombinant human myostatin and platelet-derived growthfactor-BB were from Peprotech (Rocky Hill, NJ). Monoclonal anti-bodies against vinculin and _-actin were from Sigma Chemical Co.(St. Louis, MO). Polyclonal antibodies against total- and phospho-rylated JNK were from Santa Cruz Biotechnology (Santa Cruz, CA).Phosphorylation-specific antibodies against ERK1/2, Akt (Ser473)and Smad3 were from Cell Signaling Technology (Danvers, MA).SP600125 was from Sigma Chemical Co. (Sigma Aldrich Spa, Milano,Italy). Procollagen type 1 C-peptide (PIP) EIA kit was purchasedfrom Takara Bio (Mountain View, CA). The anti-Activin ReceptorType IIB antibody – N-terminal (ab135635) was purchased fromAbcam (Cambridge, UK). Anti- _-smooth muscle actin antibodieswere from Sigma.

Cell cultures

LX-2 cells were a kind gift of Dr. Scott L. Friedman (MountSinai School of Medicine, New York, NY). Primary human HSCwere isolated as previously described in detail and used aftercomplete transition towards a myofibroblast-like phenotype [17].Both cell types were cultured in Iscove's modified Dulbecco'smedium supplemented with 2.0 mmol/L of glutamine, 0.1 mmol/Lof nonessential amino acids, 1.0 mmol/L of sodium pyruvate,antibiotic–antimycotic solution and 20% foetal bovine serum (allprovided by Gibco Laboratories, Grand Island, NY). Cells were serumdeprived for 48 h before all the experiments.MTT assayCell proliferation was determined measuring the cellularmetabolic activity using the 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay, as described elsewhere[18]. Serum-starved LX-2 cells were exposed to different concentra-tions of myostatin or 10 ng/ml PDGF-BB, used as positive control,for 24, 48, or 72 h. Cells were then treated with MTT (5 mg/ml inPBS and then with lysis solution (20% (w/v) SDS, 50% (v/v) N,N-dimethylformamide, 2% (v/v) acetic acid and 25 mM HCl, pH 4.7).Samples were read at 590 nm in a Multiskan FC plate reader.

Migration assay

These experiments were performed essentially as previouslydescribed, using Boyden chambers equipped with 8- _m poros-ity polyvinylpyrrolidone-free polycarbonate filters [17]. Wheninhibitors were used, cultured cells were treated with drugs to betested or with their vehicle for 15 min before trypsinization, andequal concentrations were added to both chambers of the Boydenapparatus.

RNA isolation and quantitative real time PCR

Total RNA was isolated using the Nucleo Spin RNA kit(Macherey-Nagel, Duren, Germany) and reverse-transcribed byMMLV reverse transcriptase (200 U) using random hexamers.Reverse transcribed products were amplified by RT-qPCR MasterMix (Life Technologies) and TaqMan assays (Applied Biosystems,Hammonton, NJ, USA) for each of the human genes tested. Actinwas used as a housekeeping gene. Relative gene expression wascalculated as 2−_Ct(_Ct = Ct of the target gene minus Ct of actin).

Western blot analysis

Confluent, serum-starved cells were treated with the appropri-ate conditions, quickly placed on ice, and washed with ice-coldphosphate-buffered saline. SDS-PAGE, transfer, and Western blot-ting were performed as described elsewhere [19].

Measurement of Procollagen type 1 secretion

Serum-deprived cells were treated with myostatin in the pres-ence or absence of the JNK inhibitor, SP600125 for 48 h. At the endof the incubation, conditioned media were collected and stored at−20◦C until assayed. Type 1 procollagen was measured using aC-peptide enzyme immunoassay kit (Takara Bio Inc., Otsu, Shiga,Japan).

RNA interference

Transfection of primary HSC was performed by Amaxa nucleo-fection technology (Amaxa, Koln, Germany) as previously described[20], using 100 nM small interfering RNA (siRNA) targeting JNK orTGF-B-B, or control non-targeting siRNA (all provided by Dharma-con Inc., Lafayette, CO). The efficiency of silencing was evaluatedby immunoblotting (JNK) or RT- PCR (TGF-B).

In vivo studies

C57Bl/6 mice, 8 weeks of age, were purchased from Charles RiverLaboratories (Calco, Italy). All animals received humane care andexperimental protocols were conducted according to Internationalguidelines (Guide for the Care and Use of Laboratory Animals, NIHpublication No. 86-23), after authorization by the local regulatoryauthorities. Two different models were employed.A first group of mice was administered a single intragastric dose(1 ml/kg) of CCl4diluted in mineral oil (1:5, v/v) and sacrificed after24, 48, or 72 h. Control mice received an equal volume of mineraloil. In a second model, mice were fed either a high-fat diet deficientin methionine and

choline (MCD), or a control diet supplementedwith methionine and choline (CD) for 10 weeks. Diets were fromLaboratori Dottori Piccioni (Milan, Italy). At the end of the experi-mental procedures, mice were euthanized by exsanguination under anesthesia, livers were rapidly dissected out, snap frozen in liquidnitrogen and stored at −80◦C for RNA extraction. At least five micefor each experimental condition were analyzed.

Immunohistochemistry

The source of human liver tissue was previously reported [21].In addition, two animal models of chronic injury were analyzed.Mice were fed a MCD diet, as described above, or treated withchronic CCl4administration. This latter set of animals was admin-istered 0.5 ml/kg CCl4two times a week for six weeks, and thensacrificed as indicated above. Staining was performed essentiallyas described elsewhere [22]. Antigen retrieval was performed byheating in citrate buffer, pH 6.0 at 98◦C for 30 min. Non-specificsignal was eliminated by peroxidase block for 10 min at room tem-perature. Primary antibody was incubated at room temperature(1 hour) at a 1:1000 dilution in a humidified chamber, followed byincubation with a Horseradish peroxidase (HRP)-conjugated sec-ondary antibody for 20 min at room temperature. Antibody bindingwas revealed by 3,30-diaminobenzidine and reaction was stoppedby immersion of tissue sections in distilled water once the browncolour appeared. Tissue sections were counterstained by hema-toxylin and mounted. All reagents for immunohistochemistry werefrom Dako.

Measurement of myostatin levels

Determination of the levels of myostatin in serum and in cytoso-lic fractions obtained from liver tissues was carried out in a mousemodel of acute liver damage induced by CCl4administration, asdescribed above. A mouse MSTN/GDF8/myostatin Elisa kit (LSBio,Seattle, WA, USA) was employed.

Statistical analysis

Data in bar graphs represent means ± SD from at least threeindependent experiments. Luminograms are representative of atleast three experiments with similar results. Statistical analysis wasperformed using Student’s t test. P values <0.05 were consideredsignificant).

Results

We first analyzed the expression of ActR2B, which binds myo-statin, in experimental models of liver injury and fibrosis. A singleadministration of carbon tetrachloride in mice induces an acutedamage associated with activation of a transient repair process sim-ilar to the one activated during chronic liver injury [23]. At all timepoints tested after CCl4administration, gene expression of ActR2Bwas markedly and significantly increased compared to mice treatedwith vehicle (Fig. 1A). We next evaluated expression of ActR2B in amodel of fibrosis associated with steatohepatitis, after administra-tion of a diet deficient in methionine and choline for 10 weeks [24].This model causes pericentral fibrosis similar to the one observedin nonalcoholic steatohepatitis. Expression of ActR2B was dramat-ically increased in mice administered the fibrogenic diet comparedto those receiving the control diet, supplemented with methionineand choline (Fig. 1B).We also tested the expression of ActR2B at the tissue levelusing immunohistochemistry, in two mouse models of chronic liverinjury (Fig. 1C). In mice chronically intoxicated with CCl4, increasedexpression of ActR2B was evident in areas of inflammation andformation of the fibrotic septum. In mice fed a methionine andcholine-deficient diet for 10 weeks, a diffuse increase in Act2Rexpression was evident, also involving areas of pericentral fibrosis.To examine whether HSC, the major cellular players in the fibro-genic process, contribute to expression of ActR2B, we evaluatedgene expression of this receptor in different preparations of pri-mary human HSC and in LX-2, a human immortalized HSC line. HSCexpressed detectable transcripts for ActR2B (Fig. 1D), which, how-ever, were not regulated by exposure to recombinant myostatinfor as long as 24 h (data not shown). To investigate the pattern ofActR2B expression in human liver tissue, samples from patientswith NASH-related fibrosis were stained with specific antibodies(Fig. 1E). Specific staining was diffusely present in hepatocytes andwithin the fibrotic septum. Staining of serial sections with anti-_-smooth muscle actin antibodies, which detect activated HSC,demonstrated partial co-localization of the immunohistochemicalsignal, indicating that fibrogenic HSC contribute to ActR2B expres-sion in the fibrotic liver (Fig. 1E). Collectively, these data indicatethat the receptor for myostatin is upregulated in conditions associ-ated with fibrogenic repair of liver tissue, and that HSC contributeto expression of ActR2B.

We then explored whether myostatin levels are modulated inconditions of experimental liver injury. In mice with acute liverinjury caused by CCl4administration (Fig. 2A), serum levels ofmyostatin were significantly increased at early time points aftertoxin administration. In contrast, no significant differences wereobserved when myostatin levels were assayed in liver tissue lysates(Fig.

2B).Having shown that HSC express ActR2B, we next investigatedwhether myostatin modifies the biology of these cells after bindingto its cognate receptor. Proliferation and migration of HSC are piv-otal actions which contribute to their pro-fibrogenic phenotype,although cytokines may differentially regulate these processes.Exposure of LX-2 to myostatin resulted in a significant reduction incell proliferation, as established measuring the cellular metabolicactivity (Fig. 3A). In contrast, PDGF-BB, used as a positive control,significantly increased proliferation. Migration of HSC is critical todetermine accumulation of fibrogenic cells in discrete areas of thehepatic acinus, resulting in deposition of scar tissue in differentregions, according to the etiology of chronic liver disease. Exposureto increasing concentrations of myostatin resulted in a signifi-cant increase in cell migration, which was similar, at the 50 ng/mlconcentration, to the one achieved in response to PDGF-BB, themost potent motogenic stimulus for this cell type (Fig. 3B–C). Ofnote, comparable results were observed in LX-2, an immortalizedhuman HSC line, and in primary HSC, albeit minor differences inthe response to myostatin were observed.

Increased expression of factors implicated in matrix turnover isanother characteristic of activated HSC, and is modulated by dif-ferent soluble factors. We first tested whether myostatin has theability to regulate the expression of type 1 procollagen, a fibrillarcomponent of extracellular matrix abundant in the fibrotic liver.Exposure to myostatin resulted in a significant increase in pro-collagen expression (Fig. 4A). Of note, myostatin was also able toinduce a significant increase in the secretion of procollagen 1 inthe culture supernatant (Fig. 6). The expression of TGF-B1, one ofthe most potent fibrogenic cytokine, was also significantly upreg-ulated when HSC were exposed to myostatin (Fig. 4A). Finally,myostatin upregulated TIMP-1 gene expression, indicating thatreduced matrix turnover could contribute to the fibrogenic effectsof this factor (Fig. 4C). Taken together, these data indicate a directprofibrogenic role of myostatin, via induction of factors implicatedin matrix turnover.ActR2B belongs to the TGF-B receptors superfamily, and theeffects of myostatin are similar to those exerted by TGF-B onHSC. Moreover, myostatin increases expression of TGF-B1 (Fig. 4B).To establish whether myostatin acts inducing an upregulation ofTGF-B secretion by HSC, we silenced TGF-B-1 with siRNA and incu-bated the cells in the presence or absence of myostatin (Table 1).The effects of myostatin on both migration and proliferation ofHSC were maintained after TGF-B-1 knockdown, indicating thatincreased expression of TGF-B1 is not necessary for the motogenicand anti-proliferative actions of myostatin.Modification of the biologic properties of HSC is dependent onspecific changes in different intracellular signaling pathways [25].We tested whether myostatin modifies the activation of kinasesFig. 3. Myostatin induces growth-inhibition and promotes migration in HSC. (A)Serum-deprived LX-2 were left untreated (white bars), exposed to different con-centrations of myostatin (50 ng/ml, grey bars, 100 ng/ml,

black bars) or 10 ng/mlPDGF-BB (crossed-hatched bars, positive control) for 24, 48, and 72 h. Cell prolif-eration was determined by MTT assay. Results are expressed as optical densityvalues. Data are mean ± SD of three independent experiments. *P < 0.05 vs Cnt. (B–C)Migration of LX-2 (B) or primary HSC (C) in response to indicated concentration ofmyostatin was evaluated using Boyden chambers. Data are the mean ± SD of fourexperiments. *P < 0.05 vs Cnt.implicated in the regulation of the fibrogenic phenotype of HSC.Exposure to myostatin resulted in a rapid and significant increasein the activation of JNK (Fig. 5A). In contrast, activation of ERK1/2,another member of the mitogen-activated protein kinase family,and of Akt (Fig. 5B), was only modestly upregulated by myostatin.We next analyzed the possible effects of myostatin on the acti-vation of Smad3, an intracellular protein critically implicated inthe regulation of extracellular matrix turnover. Phosphorylation ofSmad3 on activation-specific residues was dramatically increasedin cells incubated with myostatin (Fig. 5C). These results providea link between myostatin and activation of intracellular pathwayslinked to expression of collagen and other matrix components.Activation of JNK represents a pathway associated with a profi-brogenic phenotype of HSC [26]. Thus, we focused on the role of thiskinase in the processes triggered by myostatin in HSC. In the presence of SP600125, a specific inhibitor of JNK activation, the abilityof myostatin to induce migration of HSC was markedly and sig-nificantly blunted (Fig. 6A–B). Noteworthy, comparable data wereobtained in the LX-2 cell line and in primary human HSC. Moreover,SP600125 reduced secretion of procollagen type 1 in the culturemedium of myostatin-stimulated HSC (Fig. 6C–D). To further sup-port these findings, we performed genetic silencing of JNK usingspecific siRNAs. This strategy determined a 50% decrease in JNKabundance (Fig. 7A). In these conditions, exposure to myostatinwas significantly less effective in causing migration of HSC (Fig. 7B).Taken together, these data provide evidence that JNK activationis necessary to mediate the effects of myostatin on the fibrogenicphenotype of HSC.

Discussion

This study provides the first demonstration of a direct linkbetween myostatin, a myokine, and the process of hepatic fibrogen-esis. We found that myostatin modulates several biologic actions of HSC relevant for the fibrogenic process, and specifically cell migra-tion and expression of molecules implicated in matrix turnover, atthe gene expression and protein levels. Of note, the effects of myo-statin resembled those of TGF-B- _, a protein of the same superfamily.Similar to TGF-B- _, myostatin caused growth arrest of HSC, while itup-regulated several factors implicated in fibrogenesis [27]. Finally,increased procollagen secretion was detected in cells exposed

tomyostatin. While these actions resemble those elicited by TGF-B- _, myostatin appears to act in an independent fashion, as geneticknockdown of TGF-B- _1 in HSC did not result in any changes in theeffects of myostatin on cell migration or proliferation.Of note, dataobtained in LX-2, an immortalized HSC line, were replicated in pri-mary HSC, albeit with minor quantitative differences, providingadditional evidence to the present results.Data obtained in the past decade has highlighted the importantconcept that the fibrogenic process may be modulated by stimuligenerated outside the liver [28]. Adipokines, i.e. cytokines mainlyexpressed in the adipose tissue, include leptin and adiponectin,which regulate fibrogenesis and HSC biology in opposite fashions[29]. More recently, the possibility that alterations in the microbiotaresult in modulation of fibrosis has been experimentally proven[30]. Data provided herein provide the first, proof-of-concept evi-dence that myokines represent an additional family of factorspotentially modulating fibrogenesis from outside the liver.

Several recent studies have highlighted the possibility that themuscle-liver axis is very likely to play a relevant role in the patho-genesis of the metabolic syndrome. Sarcopenia in patients withNAFLD correlates with the degree of liver damage, and specificallywith fibrosis [31]. In addition, reduced muscle mass is a hallmarkof patients with alcoholic liver disease, and is common in cirrhosis[32]. In these conditions, where myostatin has been shown to beelevated in the systemic circulation, this factor could be a relevantmediator of accelerated fibrogenesis. Circulating myostatin levelsin healthy subjects are around 40 ng/ml [33], and therefore the con-centrations used in our in vitro experiments (50–100 ng/ml) are ina similar range. Remarkably, very similar levels were found in theserum of control mice, whereas myostatin concentrations almostdoubled in conditions of acute liver injury.Data obtained in this study also provide insight on the intra-cellular signaling pathways implicated in the transmission of thefibrogenic signal by myostatin in HSC. Primary HSC and LX-2 werefound to express ActR2B, the specific receptor for myostatin, iden-tifying HSC as a target of myostatin's action. HSC appeared tocontribute to ActR2B expression also in human liver tissue frompatients with NASH and fibrosis, although other cells were alsopositively stained for this receptor.We next focused on proteins of the MAPK family and on Akt,which have been shown to play relevant roles in mediating theeffects of different fibrogenic factors [25]. Myostatin only mod-estly activated Akt, or members of the MAPK family such as ERK1/2and p38MAPK. In contrast, a marked and consistent activation ofJNK was observed. JNK, and particularly JNK-1, has been shownto be implicated in the fibrogenic process, both in vitro and inanimal models [26]. In particular, several studies have indicatedthe ability of JNK activation in HSC to activate downstream path-ways, regulating multiple actions relevant for fibrogenesis, suchas cell proliferation, oxidative stress, and expression of profibro-genic factors [34,35]. We used both a

pharmacologic approach withthe specific inhibitor SP600125 and a genetic approach with JNK-1 knockdown by siRNA. In both cases, the ability of myostatin toinduce cell migration and to upregulate collagen secretion wassignificantly reduced, both in LX-2 and in primary HSC. Remarkably, only limited evidence had previously linked myostatin withactivation of JNK. In C2C12 murine myoblasts, exposure to myostatin activates JNK, which mediated the growth inhibitory signalinduced by this cytokine [36]. As myostatin has been linked to fibro-sis in other tissues, but not in the liver, data reported herein arethe first example of a myostatin-JNK-myofibroblast axis regulatingfibrogenesis.In other systems, myostatin has been linked to the biology offibrogenic cells. Overexpression of myostatin in cardiomyocytesresulted in altered cardiac function in older mice and in inter-stitial fibrosis, through activation of the p38MAPKpathway [37].Moreover, myostatin has been implicated in the pathogenesis offibrosis in skeletal muscle, where it induces fibroblast proliferationand secretion of extracellular matrix [38] and increases fibroblastresistance to apoptosis [39], via activation of Smad and p38MAPKsignaling. Myostatin also promoted the differentiation of musclefibroblasts into myofibroblasts, and induced expression of TGF-B- _ inmyofibers [40]. In vivo, induction of muscular damage in myostatin-deficient mice was associated with increased regeneration andreduced fibrosis. Nonetheless, the fibrogenic action of myostatindoes not appear to be limited to muscle tissue, as myostatin hasbeen involved in the pathogenesis of experimental Peyronie's dis-ease in the rat [41]. Myostatin was also found to be expressed inthe skin, and myostatin-deficient mice showed reduced healing ofcutaneous wounds due to impaired myofibroblast differentiation,resulting in delayed contraction of the wound [42]. The identifica-tion of myostatin as a mediator of fibrosis in other tissues supportsour finding of its profibrogenic activity in hepatic myofibroblasts.A few limitations of this study must be acknowledged. In particular, the relevance of the myostatin system in vivo was onlyexplored analyzing the expression of ActR2B in models of liverrepair and fibrogenesis. While the marked upregulation of the myo-statin receptor in conditions of fibrogenic liver injury argues infavor of a pathogenic role of this factor, it does not provide in vivomechanistic evidence. On the other hand, this is not easy to obtaindue to the limited availability of pharmacologic blockers of myo-statin's action and to the fact that myostatin-deficient mice havea complex phenotype that makes it difficult to single out a directprofibrogenic effect. Nonetheless, extension of these studies withexperiments modulating the actions of myostatin in vivo is war-ranted in the near future.In conclusion, we have shown that myostatin, a protein whichinhibits growth of skeletal muscle, modulates the biologic proper-ties of human HSC in a profibrogenic fashion, via activation of JNK.These data identify a novel muscle-to-liver pathway potentiallyimplicated in the pathogenesis of hepatic fibrosis in conditions suchas NAFLD and alcoholic liver disease.

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Tables

Table 1. Effects of TGF-B- B1 silencing on myostatin-induced proliferation and migration of HSC.

A

	48 h	72 h
TGF-β1 mRNA(-fold change over NT siRNA)	0.18 ± 0.18*	0.07 ± 0.18*

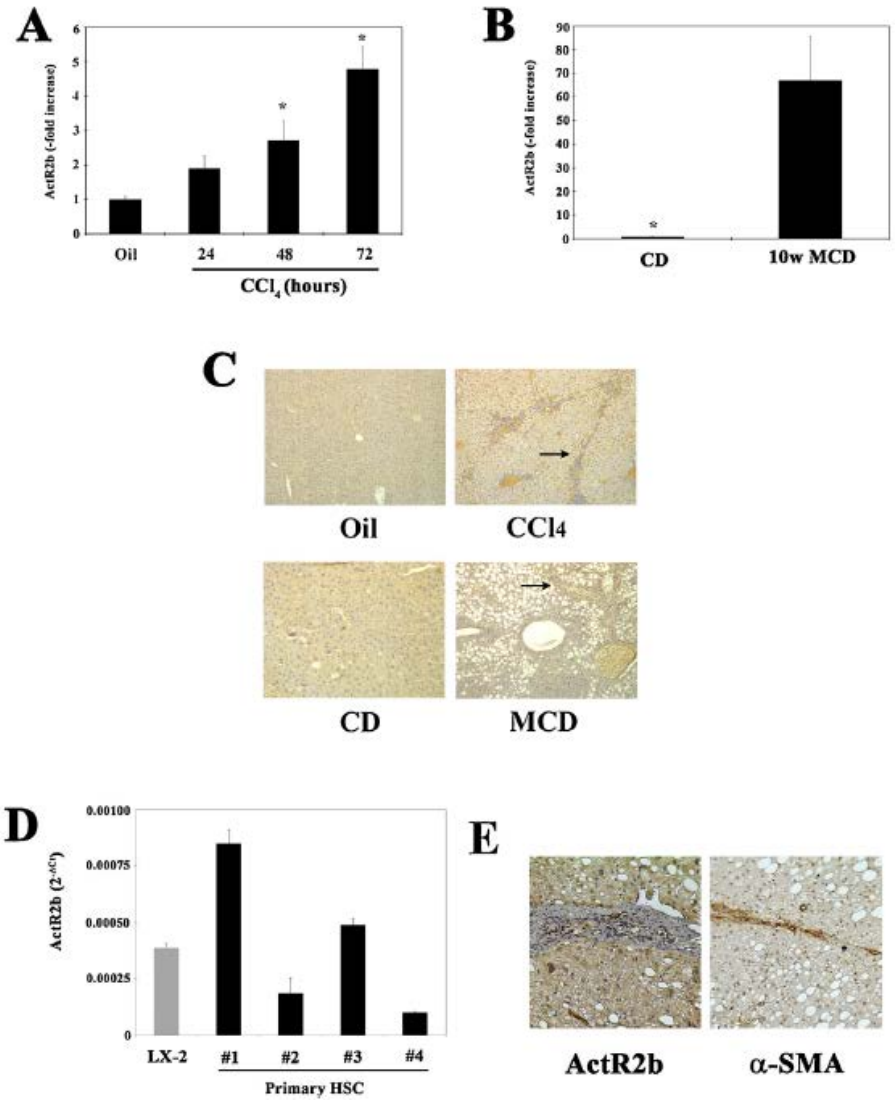
B

	Non-targeting siRNA		TGFβ1 siRNA	
	Control	Myostatin	Control	Myostatin
Migration (cells/HPF)	8.8 ± 5.5[a]	35.4 ± 12.5[§]	5.3 ± 2.4[b]	31.6 ± 19.2
Proliferation (MTT arbitrary units)	417 ± 138[a]	406 ± 140[c]	360 ± 90[d]	325 ± 87

(A): LX-2 cells were transfected with siRNA for the indicated time points, and gene expression of cells transfected with siRNA directed against TGF-B1 was compared to the one of cells transfected with non-targeting siRNA. (B) LX-2 cells were transfected with siRNA as indicated, and incubated in the presence or absence of 50 ng/ml myostatin. Analysis of cell migration and proliferation were performed as described in Materials and Methods. aP < 0.05 vs. myostatin and non-targeting siRNA.bP = 0.07 vs. myostatin and TGF-B1 siRNA.cP = NS vs. myostatin and TGF-B1 siRNA.dP < 0.05 vs. myostatin and TGF- B1 siRNA. HPF, high-power field.*P < 0.01.

Figures

Fig. 1. Hepatic expression of the myostatin receptor ActR2B in experimental liver injury, in human HSC, and in human liver tissue.



(A) C57Bl/6 mice (n ≥ 3) were orallyadministered CCl4(1 ml/kg) or vehicle (olive oil) for the indicated time periods. (B) C57Bl/6 mice (n ≥ 3) were fed a methionine and choline deficient diet (MCD) or a controldiet (CD) for 10 weeks. Total RNA was isolated from liver tissue and the expression of ActR2B was determined by qRT-PCR. Target gene expression was normalized to GAPDH.Data are expressed as −fold increase over control. *P < 0.05 vs. control. (C) Top panels: mice were treated with mineral oil or with CCl4for 6 weeks, as described in Materialsand Methods. Bottom panels: mice were fed with a control diet (CD) or a diet deficient in methionine and choline (MCD) for 10 weeks. Slides were stained with antibodiesagainst ActR2B. Arrows indicate areas of fibrogenesis. (D) Total RNA was isolated from the human HSC line, LX-2, and from different preparations of primary HSC. mRNAexpression of Act2Rb was evaluated by qRT-PCR. Relative gene expression was calculated as 2−_Ct(_Ct= Ctof the target − Ctof _-actin). (E) Serial

sections of liver tissuefrom a patient with NASH and fibrosis were stained with antibodies against ActR2b (left) or _-smooth muscle actin (right), a marker of activated HSC. Brown color showspartial co-localization between the two stainings in the fibrotic septum.

Fig. 2. Serum and hepatic levels of myostatin in conditions of liver injury. Mice were treated with mineral oil (control) or with a single intragastric administrationof 1.0 ml/kg CCl4and sacrificed after the indicated periods of time. Myostatin levelswere analyzed in serum (A) and in tissue lysates (B) as indicated in Materials andMethods. Data are mean ± SD of at least three mice per group. *P < 0.05 vs. control(Oil).

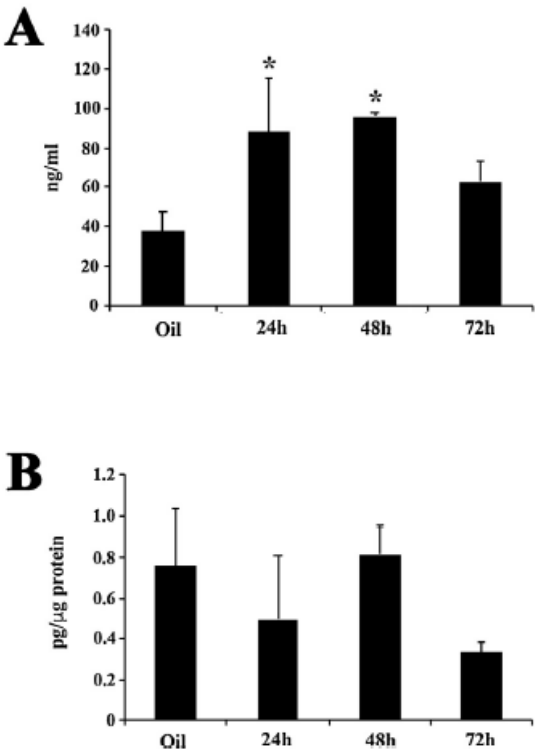
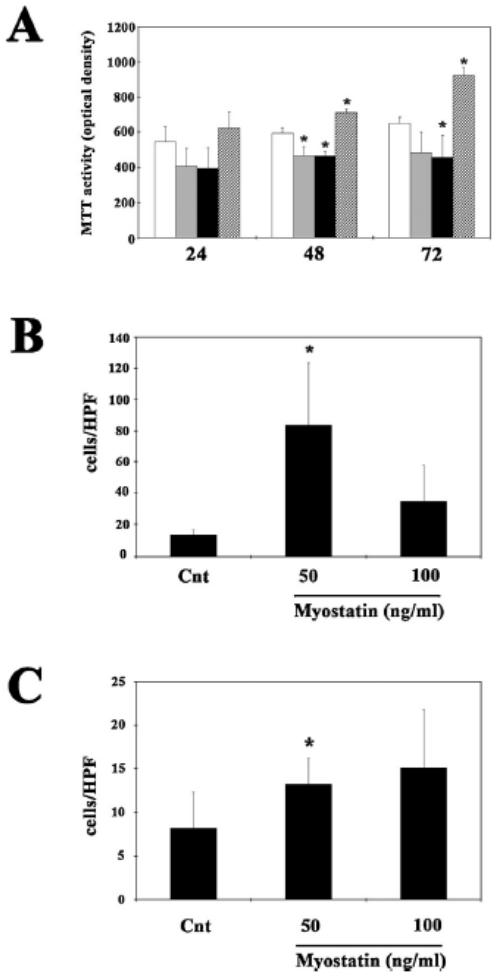
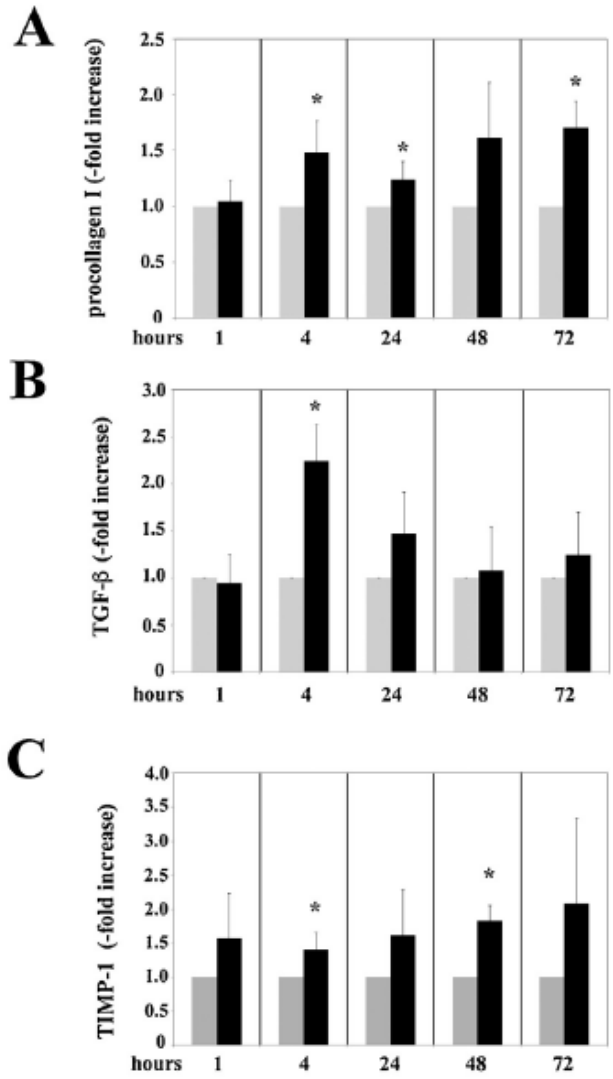


Fig. 3. Myostatin induces growth-inhibition and promotes migration in HSC.



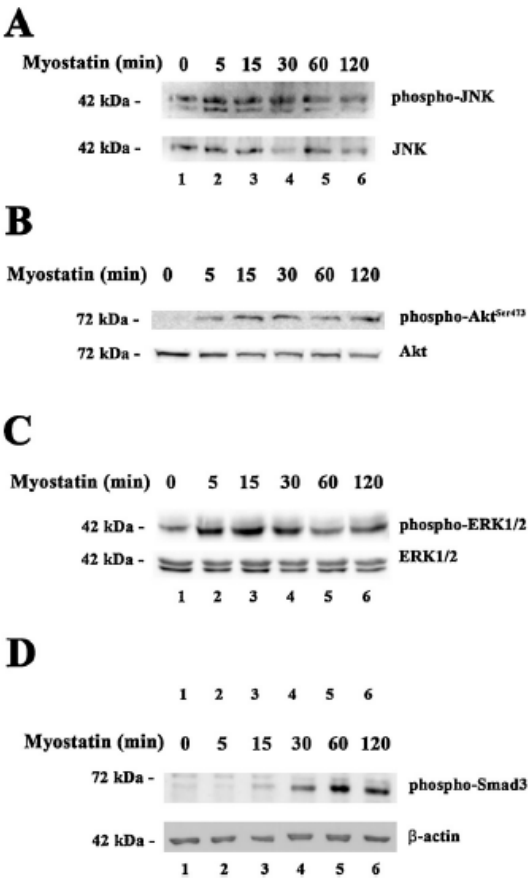
(A)Serum-deprived LX-2 were left untreated (white bars), exposed to different con-centrations of myostatin (50 ng/ml, grey bars, 100 ng/ml, black bars) or 10 ng/mlPDGF-BB (crossed-hatched bars, positive control) for 24, 48, and 72 h. Cell prolif-eration was determined by MTT assay. Results are expressed as optical densityvalues. Data are mean ± SD of three independent experiments. *P < 0.05 vs Cnt. (B–C)Migration of LX-2 (B) or primary HSC (C) in response to indicated concentration ofmyostatin was evaluated using Boyden chambers. Data are the mean ± SD of fourexperiments. *P < 0.05 vs Cnt.

Fig. 4. Myostatin positively modulates the expression of profibrogenic genes.



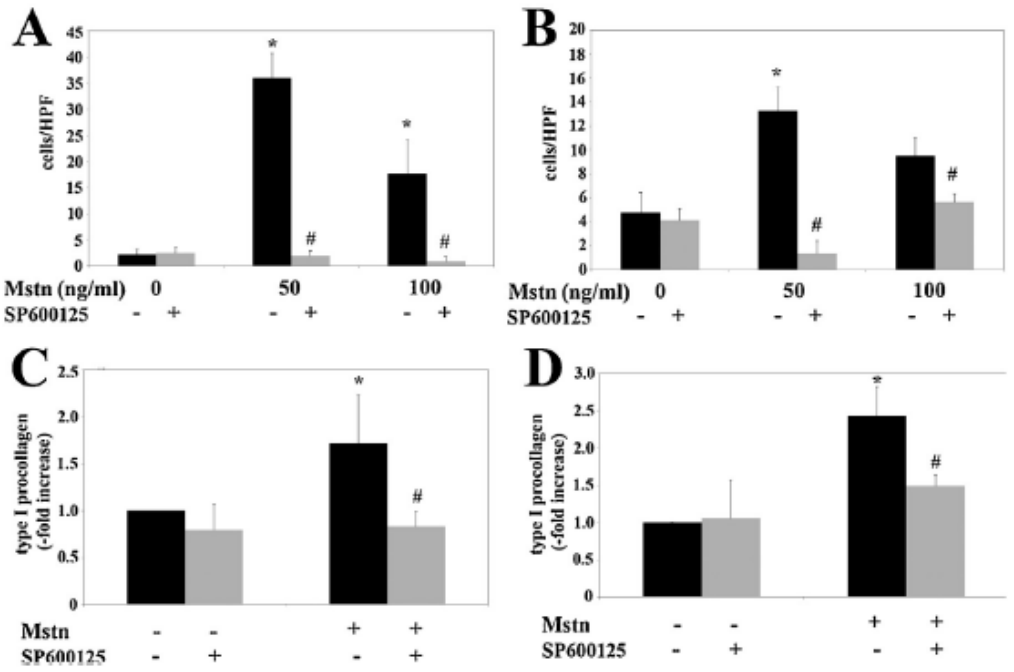
Serum-deprived LX-2 were incubated in the presence (black columns) or in theabsence (gray columns) of 50 ng/ml myostatin for the indicated time points. TotalRNA was extracted and the expression of procollagen 1 (A), TGF- _1 (B) and TIMP-1(C) was determined by qRT-PCR. Target gene expression was normalized to actin.Results, expressed as fold increase over control, represent the mean value ± SD ofthree independent experiments. *P < 0.05 vs the same time point in the absence ofmyostatin.

Fig. 5. Signaling pathways activated by myostatin.



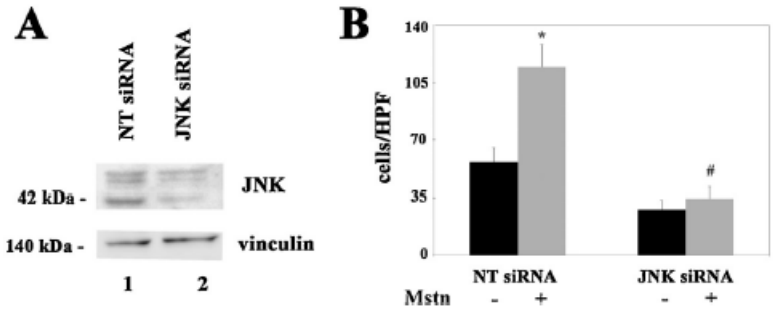
Serum-starved LX-2 cells wereexposed to 50 ng/ml myostatin for the indicated time points. Total cell lysates wereanalyzed by immunoblotting using antibodies directed against the phosphorylatedforms of JNK (A), Akt (B) ERK1/2 (C) or Smad-3 (D). Data from a single experimentrepresentative of at least three with similar results.

Fig. 6. Biologic effects of myostatin in HSC are blocked by a JNK inhibitor.



(A–B) Migration of LX-2 cells (A) or primary HSC (B) in response to myostatin (50–100 ng/ml)was evaluated in the presence or absence of the JNK inhibitor SP600125 (20 _M), using Boyden chambers. Data are the mean ± SD of three independent experiments. (C–D)Serum-starved LX-2 cells (C) or primary HSC (D) were treated with myostatin (50 ng/ml) in the presence or absence of 20 _M SP600125 for 48 h. Procollagen type I secretionwas determined in cell supernatants by EIA. Results were normalized for total protein content in cell lysates. Data are expressed as fold change over control and indicate themean ± SD of three independent experiments. *P < 0.05vs Cnt; #P < 0.05 vs myostatin (Mstn).

Fig. 7. JNK is required for myostatin-induced biological actions in HSC.



Knockdown of JNK in primary HSC was achieved by transfection of specific siRNA (JNK siRNA).Non-targeting siRNA (NT siRNA) were employed as control. (A) Transfection efficiency was evaluated by Western blotting using antibodies against JNK or vinculin, as controlfor equal loading. Data from a single experiment representative of three. (B) Cell migration in response to 50 ng/ml myostatin was tested in serum-starved HSC, 48 h followingtransfection. Data are the mean ± SD of three independent experiments. *P < 0.05 vs Cnt; #P < 0.05 vs NT siRNA + myostatin (Mstn).