Alterations in myostatin expression are associated with changes in cardiac left ventricular mass but not ejection fraction in the mouse

Jorge N Artaza^{1,2,3,*}, Suzanne Reisz-Porszasz^{2,3,*}, Joan S Dow⁴, Robert A Kloner⁴, James Tsao¹, Shalender Bhasin^{1,5} and Nestor F Gonzalez-Cadavid^{1,3}

¹Division of Endocrinology Metabolism and Molecular Medicine, ²Department of Biomedical Sciences and ³RCMI DNA Molecular Core, Charles R Drew University of Medicine and Science, Los Angeles, California 90059, USA

⁴The Heart Institute, Good Samaritan Hospital, Division of Cardiovascular Medicine of Keck School of Medicine at University of Southern California, Los Angeles, California 90017, USA

⁵Section of Endocrinology, Diabetes, and Nutrition, Boston Medical Center, Boston, Massachusetts 02118, USA

(Requests for offprints should be addressed to N F Gonzalez-Cadavid at Division of Endocrinology, Metabolism, and Molecular Medicine, Charles R Drew University of Medicine and Science, 1731 East 120th Street, Los Angeles, California 90059, USA; Email: ncadavid@ucla.edu)

*(J N Artaza and S Reisz-Porszasz are joint first authors)

(S Bhasin and N F Gonzalez-Cadavid contributed equally to this work)

Abstract

Myostatin (Mst) is a negative regulator of skeletal muscle in humans and animals. It is moderately expressed in the heart of sheep and cattle, increasing considerably after infarction. Genetic blockade of Mst expression increases cardiomyocyte growth. We determined whether Mst overexpression in the heart of transgenic mice reduces left ventricular size and function, and inhibits *in vitro* cardiomyocyte proliferation. Young transgenic mice overexpressing Mst in the heart (Mst transgenic mice (TG) under a muscle creatine kinase (MCK) promoter active in cardiac and skeletal muscle, and Mst knockout (Mst (−/−)) mice were used. Xiscan angiography revealed that the left ventricular ejection fraction did not differ between the Mst TG and the Mst (−/−) mice, when compared with their respective wild-type strains, despite the decrease in whole heart and left ventricular size in Mst TG mice,

and their increase in Mst (−/−) animals. The expected changes in cardiac Mst were measured by RT-PCR and western blot. Mst and its receptor (ActRIIb) were detected by RT-PCR in rat H9c2 cardiomyocytes. Transfection of H9c2 with plasmids expressing Mst under muscle-specific creatine kinase promoter, or cytomegalovirus promoter, enhanced p21 and reduced cdk2 expression, when assessed by western blot. A decrease in cell number occurred by incubation with recombinant Mst (formazan assay), without affecting apoptosis or cardiomyocyte size. Anti-Mst antibody increased cardiomyocyte replication, whereas transfection with the Mst-expressing plasmids inhibited it. In conclusion, Mst does not affect cardiac systolic function in mice overexpressing or lacking the active protein, but it reduces cardiac mass and cardiomyocyte proliferation.

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Introduction

Myostatin (Mst) is an endogenous negative regulator of skeletal muscle mass in rodents, cattle, other mammals, and fish (Lee 2004, Dominique & Gerard 2006, Tsao *et al.* 2006, Tsuchida 2006). The Mst knockout mouse (Mst (−/−)), where Mst expression is genetically blocked (McPherron *et al.* 1997), and other transgenic mice where its processing into an active product is inhibited (Szabo *et al.* 1998, Zhu *et al.* 2000), display a considerable increase in skeletal muscle mass with both hypertrophy and hyperplasia. A child with an inactivating mutation of the *Mst* gene has also been shown to be hypermuscular (Schuelke *et al.* 2004). In contrast, ectopic overexpression of Mst by *ex vivo* transduced non-muscle cells implanted into the mouse skeletal muscle (Zimmers *et al.* 2002), or by a transgenic mouse (Mst TG)

that was engineered to overproduce Mst exclusively in the skeletal muscle (Reisz-Porszasz *et al.* 2003), leads to a predominant reduction of muscle mass. Although alterations in Mst expression are associated with reciprocal changes in skeletal muscle mass, they do not affect the specific force of the skeletal muscle (Caiozzo *et al.* 2005).

The biological activity of Mst is not limited to the skeletal muscle; the genetic inactivation of its expression is associated with leanness (McPherron *et al.* 1997, Szabo *et al.* 1998, Zhu *et al.* 2000, Yarasheski *et al.* 2002), whereas overexpression is associated with increased fat mass (Zimmers *et al.* 2002, Reisz-Porszasz *et al.* 2003). Furthermore, Mst also appears to have profibrotic effects in both the skeletal muscle and the corpora cavernosa of the penis by stimulating extracellular matrix deposition and myofibroblast formation (Wagner *et al.* 2002, Ferrini *et al.* 2004, McCroskery *et al.* 2005, Parsons *et al.*

2006). This pleiotropic control of tissue composition may be related to the fact that Mst inhibits *in vitro* the myogenic differentiation of pluripotent cells and skeletal muscle-derived stem cells and stimulates their differentiation into fat cells and myofibroblasts (Ferrini *et al.* 2004, Artaza *et al.* 2005, 2006).

Although Mst was initially postulated to be expressed exclusively in the skeletal muscle and to a much lower extent in fat tissue (McPherron & Lee 1997, McPherron *et al.* 1997, Gonzalez-Cadavid *et al.* 1998), low levels of Mst have been detected in the myocardial tissues of sheep, fish, and cattle (Sharma *et al.* 1999, Ostbye *et al.* 2001, Gregory *et al.* 2004). Mst protein has been localized in cardiomyocytes in the ovine heart, more specifically in Purkinje fibers, and Mst expression is upregulated after experimentally induced myocardial infarction (Sharma *et al.* 1999). Myocardial Mst mRNA and protein expression were also upregulated in the rat model of volume overload heart failure (Shyu *et al.* 2006). The Mst precursor protein was also detected in the heart of fish, among other organs, by western blot (Ostbye *et al.* 2001, Gregory *et al.* 2004).

Overexpression of Mst in the skeletal muscle and heart of the Mst (+/+) male mouse was associated with lower heart weight (Reisz-Porszasz *et al.* 2003), despite the fact that a reduction in heart size had also been observed in animals not expressing active Mst, the Belgian blue bull (Sharma *et al.* 1999), and in an inbred line of mice where the Compact Mst mutation was introduced (Bunger *et al.* 2004). In line with the latter observations, in transgenic mice with cardiac-specific expression of activated Akt, which reduces cardiomyocyte death and induces cardiac hypertrophy, Mst expression in the heart was considerably induced (Cook *et al.* 2002).

However, the apparent paradox of Mst expression being associated with cardiac hypertrophy appears to be resolved by the demonstration that cyclic mechanical stretch, which causes cardiomyocyte growth *in vitro*, leads to Mst overexpression as a compensatory mechanism to insulin-like growth factor-I secretion induced by stretch (Shyu *et al.* 2005). This confirms the role of Mst as a chalone to control heart growth (Gaussin & Depre 2005), which has been further demonstrated in cardiomyocytes from the transgenic mouse with Akt activation, where Mst abrogates their *in vitro* growth caused by phenylephrine, through inhibition of p38 and Akt. Conversely, *in vivo* infusion of Mst (−/−) hearts with phenylephrine leads to considerable increases in p38 phosphorylation and Akt kinase activity (Morissette *et al.* 2006).

Thus, the effects of Mst on myocardial mass and function remain poorly understood and were the subject of this investigation. Accordingly, we determined the effects of alterations in Mst expression on myocardial mass and function in Mst transgenic mice that overexpress Mst and Mst knockout mice with diminished Mst expression. To obviate confounding factors due to the effect of aging and sexual maturation on myocardial size and function (Hacker *et al.* 2006, Rozenberg *et al.* 2006), we studied myocardial function in sexually mature, 7-week-old mice. We also considered the possibility that in transgenic mice that overexpress Mst, the alterations in myocardial size and function could result from endocrine effects of Mst

secreted by the skeletal muscle rather than the direct effect of Mst expressed in the myocardial tissue. Therefore, we evaluated the myocardial mass and function in transgenic mice in which Mst expression was restricted to the heart and skeletal muscle by using a MCK promoter. Since Mst inhibits proliferation of skeletal muscle precursor cells, we determined the effects of recombinant Mst protein on the proliferation of H9c2 cardiomyocytes *in vitro* and whether it affected cell size and apoptosis.

Material and Methods

Animals

We generated two sets of transgenic mice on a C57BL/6J background that overexpress Mst protein under either the muscle-specific creatine kinase (MCK) promoter (active in both skeletal muscle and heart), MCK-Mst TG, or the mutated MCK-3E promoter (active only in skeletal muscle), MCK-3E-Mst TG (Shield *et al.* 1996, Reisz-Porszasz *et al.* 2003). The strategy for the generation of these transgenic mice and their skeletal muscle phenotype has been described previously (Reisz-Porszasz *et al.* 2003). The enhanced green fluorescent protein (pEGFP) reporter mice included the C57BL/6J/MCK-pEGFP and the C57BL/6J/MCK-3E-pEGFP transgenic mice that express EGFP in BL/6J mice under either the MCK or MCK-3E promoter (Reisz-Porszasz *et al.* 2003, Caiozzo *et al.* 2005). Seven-week-old male mice were used in all cases ($n=6-11$ animals/group).

Mst knockouts, Mst (−/−), mice, provided kindly by Dr S J Lee, were bred in our vivarium (BalbC/Mst (−/−); McPherron *et al.* 1997). As reported previously by Dr Lee's group, these Mst null mice show greater muscularity and lower fat mass than age-matched wild-type (WT) controls.

Evaluation of heart function

Mice were anesthetized with an i.p. injection of ketamine and xylazine (200 and 10 mg/kg respectively). The neck was shaved to expose left jugular vein and a catheter was inserted for injection of contrast media (Optiray-320) and potassium chloride (KCl). Angiograms (Muller-Ehmsen *et al.* 2002) were performed using a XiScan (Xi Tec Inc., East Windsor, CT, USA) portable c-arm X-ray system, 30 min after anesthesia. With the animal positioned on its back or right side, and the XiScan running, media (0.2 ml per view) was rapidly injected, to capture images of the heart in the anterior-posterior and lateral views of the left ventricle (LV). Images were captured on half-inch super-VHS videotape at 30 frames per second. Video images were analyzed off-line. Three consecutive beats from each image (if available) was analyzed. For each beat, the LV cavity was traced in both diastole and systole. Measurements included circumference, area, length, and volume. Ejection fraction (EF) was calculated as: (end diastolic volume−end systolic volume)/end diastolic volume×100. These measurements were then averaged for each view. Mice were euthanized with an injection of KCl once the angiograms had been performed.

Cell culture

H9c2 rat embryonic cardiomyocytes (Gregory *et al.* 2004) were obtained from American Type Culture Collection (ATCC; Rockville, MD, USA), and were maintained in Dulbecco's modified Eagle's medium (DMEM, Cambrex Bio Science Walkersville Inc., Walkersville, MD, USA), supplemented with 10% fetal bovine serum (Gibco BRL), 4 mM glutamine, 4.5 g/l glucose, 100 IU/ml penicillin, and 100 µg/ml streptomycin (Life Technologies) and incubated at 37 °C in 5% CO₂, at 30–50% confluence. These cells are defined as embryonic by ATCC because they were obtained from rat embryos, and are acknowledged as such in the literature. The cells were transfected with Mst cDNA constructs expressing the full length 375 aa mouse protein, in the presence of lipofectamine (Invitrogen) using 2 µg total plasmid DNA at a 1:4 ratio of either pCMV/EGFP to pCMV/Mst or pMCK/EGFP to pMCK/Mst constructs (Reisz-Porszasz *et al.* 2003, Artaza *et al.* 2005, Magee *et al.* 2006). Transfection efficiency was followed by green fluorescence expression. Cells were harvested after 3 days of transfection and total RNA and protein were isolated. Alternatively, cells were replated and growth curves were performed by the cell proliferation assay.

For treatments with recombinant Mst protein, cells were incubated in 12-well plates with 4–8 µg/ml of the 16 kDa recombinant human Mst protein corresponding to the 110 aa (carboxy terminus; Artaza *et al.* 2005, 2006) or 3.3–33 µg/ml of our custom-made polyclonal anti-Mst antibody (Gonzalez-Cadavid *et al.* 1998, Artaza *et al.* 2005, 2006) in DMEM–10% serum for 2 days. The recombinant protein was tested for its biological activity in C₂C₁₂ cells (Taylor *et al.* 2001, Artaza *et al.* 2002). The recombinant Mst protein was obtained from BioVendor Laboratory Medicine Inc., Czech Republic.

Cell proliferation assay

It was determined in 96-well plates by the Formazan dye assay (Promega Corp.; Taylor *et al.* 2001). The cells were grown at an initial density of 4000 cells/well; then after 1 day, they were treated for 72 h with recombinant Mst protein in varying concentrations, with Mst polyclonal antibody in a 1/100 dilution, with IgG isotype and transforming growth factor (TGF) β1 recombinant protein at 10 ng/ml concentration as positive control. After 3 days of incubation, 100 µl of Formazan substrate buffer was added to the cultures for 3 h at 37 °C in 5% CO₂, and the absorbance at 492 nm was read by an ELISA plate reader. For cell counting, the cells were removed by trypsinization and the number of viable cells was counted in a hemocytometer with the use of Trypan blue staining.

Immunocytochemical analyses

For immunocytochemical characterization, cells were seeded at 60–70% confluence onto 8-well chamber slides, treated with recombinant Mst protein and fixed in 2% paraformaldehyde at different time points; quenched with H₂O₂, blocked with

normal goat or horse serum and incubated with specific antibodies (Schreier *et al.* 1990, Artaza *et al.* 2002, 2005, Magee *et al.* 2006). Mst polyclonal antibody 1/500 dilution; cardiac troponin T (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) dilution 1/500; myosin heavy chain (MHC) type I dilution 1/40 and MHC type II (1/40; Novocastra Laboratories Ltd, Newcastle, UK). 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 (Sigma). In the case of Mst monoclonal antibody generated by our group against the Mst-113 aa (mature fraction), we used the mouse on mouse (MOM; Vector Laboratories) immunodetection system, which provides a significant reduction of background staining on tissues or cells from mouse origin. The cells were counterstained with Meyer's hematoxylin. In negative controls, we either omitted the first antibody or used a rabbit nonspecific IgG.

Apoptosis. The apoptotic index was determined by the TdT-mediated dUTP nick end labeling (TUNEL) method, based on the ability of terminal TdT to catalyze addition of digoxenin-dUTP and dATP to 3'-OH ends of cleaved DNA. The cells were placed on removable 8-well chamber slides and incubated with or without Mst for 1–3 days and then fixed in 2% *p*-formaldehyde. The slides were treated with proteinase K and H₂O₂ followed by the addition of primary and secondary antisera. The chambers were stained with 3,3'-diaminobenzidine and sections were counter-stained with hematoxylin. Negative controls in the immunohistochemical detections were done by replacing the first antibody with IgG isotype. The negative control for TUNEL was by substituting buffer for the TdT enzyme (Taylor *et al.* 2001).

Quantitative image analysis

Quantitative image analysis was performed by computerized densitometry using the ImagePro 5.1 program (Media Cybernetics, Silver Spring, MD, USA), coupled to an Olympus BHS microscope equipped with an Olympus digital camera (Artaza *et al.* 2005).

For TUNEL determinations, the number of positive cells at 400× was counted and results were expressed as a percentage of positive cells/total cells. Negative controls without primary or secondary antibodies were performed to correct for antibody specificity and background intensity. In all cases, 20 fields at 400× magnification were selected at random and the apoptotic index of each field was calculated as the percent of TUNEL-positive cells. For cell size determination, cells were stained with Troponin A as above and the area covered by the stain was measured by quantitative image analysis and the average was expressed in µm².

Western blot analysis

Cell lysates (50–100 µg of protein) were subjected to western blot analyses by 7.5 or 12% gel electrophoresis

(Gonzalez-Cadavid *et al.* 1998, Taylor *et al.* 2001, Artaza *et al.* 2002, 2005, Reisz-Porszasz *et al.* 2003, Magee *et al.* 2006) using our monoclonal or polyclonal antibodies for Mst (1/500), as indicated, and polyclonal antibodies for p21 (cyclin kinase inhibitor; 1/500), cdk2 cyclin-dependent kinase 2 (1/500), and monoclonal antibody for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH; 1:10 000; all from Chemicon International, Temecula, CA, USA). The washed membranes were incubated with 1:1000 dilution, or 1/3000 for Mst, of secondary antibody linked to horseradish peroxidase. Immunoreactive bands were visualized by using the emission of chemiluminescence (ECL) plus western blotting chemiluminescence detection system (Amersham; Artaza *et al.* 2005).

RT-PCR

Two micrograms of total RNA, extracted from heart tissue or cardiomyocytes using the Trizol-Reagent (Invitrogen), was reverse transcribed, and cDNA was amplified for 35 cycles by PCR at 94 °C for 30 s, primer annealing at 58 °C for 30 s, and extension at 72 °C for 1 min (Gonzalez-Cadavid *et al.* 1998, Taylor *et al.* 2001, Artaza *et al.* 2002, 2005, Reisz-Porszasz *et al.* 2003, Magee *et al.* 2006). PCR products were analyzed in 1.5% agarose gels. The sequences of the Mst forward/reverse PCR primers are as follows: (i) P1 primers for WT Mst: forward: 5'-GACAAAACACGAGGTACTC, reverse: 5'-TGGATTACAGGCTGTTTG AGC (531 bp); (ii) P2 primer for transgene Mst: forward: 5'-GTCTCCCATTAA-TATGCTAT, reverse: 5'-GGGAGGTGTGGGAGGTTTT (175 bp); (iii) primers for GAPDH (152 bp): forward primer: 5'-ATCACTGCCACCCAGAAGACT, reverse primer: 5'-CATGCCAGTGAGCTTCC CGTT; and (iv) primers for Act R11b: forward 5'-GCCTCTCTCTCATCGTCCTG (545–564 bp), reverse 5'-GACTGCTTGTCTGAAGTGG (779–760 bp). The PCR fragment is 235 bp in length.

Statistical analysis

All data are presented as mean $\pm$ S.E.M., and between-group differences were analyzed by using ANOVA. If overall ANOVA revealed significant differences, then pairwise comparisons between groups were performed using Newman-Keuls multiple comparison test. All comparisons were two-tailed, and *P* values <0.05 were considered statistically significant. The *in vitro* experiments were repeated twice to thrice, and data from representative experiments are shown.

Results

Mst is an endogenous negative regulator of heart and LV size

As shown in Fig. 1A, Mst TG mice in which the MCK promoter directed Mst expression have consistently slightly lower body weights over the first 7 weeks of postnatal development, in comparison to the WT mice of the same

strain. Body weights in Mst TG mice tend to become more similar to the WT animals between 7 and 8 weeks of age (Reisz-Porszasz *et al.* 2003). The MCK promoter drives the expression of Mst not only in the skeletal muscle, but also in the heart. Tissue sections from the heart of transgenic mice that express the enhanced green fluorescent protein driven by the mouse MCK promoter demonstrate intense green fluorescence in the myocardial tissue, consistent with the expression of the transgene in the myocardium (B, left). In contrast, similar tissue sections from transgenic mice in which EGFP expression was driven by the mutated MCK promoter (MCK-3E; Reisz-Porszasz *et al.* 2003) showed EGFP expression in the skeletal muscle, but not in the myocardium, confirming the ability of MCK-3E, the mutated MCK promoter, to restrict gene expression to the skeletal muscle (B right).

The mean heart weights in male transgenic mice, in which Mst transgene was expressed in both the skeletal and myocardial muscles, were significantly lower (mean difference in heart weights 11%) than age-matched WT controls (Fig. 1C left). Significantly, the mean heart weights in transgenic mice carrying the MCK-3E skeletal muscle-specific promoter that restricted the expression of the Mst transgene to the skeletal muscle did not differ significantly from those of WT controls (data not shown). This suggests that Mst effects on heart weight are not endocrine due to circulating Mst secreted from the skeletal muscle, but rather autocrine or paracrine, as these effects were observed only when Mst was also expressed in the myocardium itself and not when its expression was confined to the skeletal muscle. The heart weights of the Mst (–/–) mice on a Balb c background were significantly higher than that of their respective age-matched WT controls (mean difference in heart weights 24%).

The reduction in the heart weight observed in the Mst TG mouse was mostly due to the lower left ventricular mass; even after correction by body weight, the left ventricular mass in male transgenic mice was 21% lower than in the corresponding WT controls (C, right).

To confirm that the differences in cardiac size correlated with Mst expression in the heart, RT-PCR reactions were performed on RNAs isolated from this tissue as well as from the Mst TG and Mst (–/–) animals. Figure 2A shows the two set of primers, P1 and P2, used for these reactions that respectively encompass the Mst coding region bridging two exons (to eliminate any potential DNA interference) or the 3' end and part of the SV40 polyA signal that is transcribed from the Mst cDNA construct used to generate the Mst TG mouse. As expected, the P1 primers did not amplify any DNA band in the RNA from four Mst (–/–) hearts, although the 531 bp band was clearly visible in the positive control from skeletal muscle (B). The intensity of this band was moderate but variable in the RNA from the hearts of the WT mice and more consistent in the Mst TG mice, thus indicating that Mst mRNA is endogenously expressed in the heart of WT animals, and overexpressed in the transgenic mice. The RNA reverse transcribed and amplified with the P2 set of primers showed an intense 175 bp band in the Mst TG hearts, and none in the Mst

Figure 1 Genetic blockade or overexpression of myostatin in the Mst ($-/-$) and Mst TG mice affects heart and left ventricle size. (A) Mst TG mice growth curves. (B) Heart tissue from 7-week-old MCK/EGFP and MCK-3E transgenic mice was excised, fixed, cut in 6 μ m sections, and examined under the fluorescent regular microscope. (C) Heart weights (left) and left ventricle weights corrected by body weights in mice. WT, wild-type (Mst $(+/+)$) mice; TG, transgenic overexpressing myostatin, Mst TG mice; KO, myostatin knockout (Mst $(-/-)$) mice; MCK and MCK-3E denote the respective promoters in the Mst TG mice. * $P<0.05$.

($-/-$), as expected (C), thus confirming the identity and expression of the exogenously transcribed Mst RNA.

Parallel to the RNA results, Mst protein was barely detectable in the hearts of WT mice on western blots using an anti-Mst monoclonal antibody that in the skeletal muscle detects a 50–52 kDa band that is assumed to be the monomer of the full length unprocessed precursor, and a 30–32 kDa

band assumed to be a glycosylated dimer of the carboxy terminus portion of the full length translational product (Gonzalez-Cadavid *et al.* 1998, Artaza *et al.* 2005; Fig. 3A, left). The 52 kDa band for the precursor was seen in the homogenates from the six Mst TG transgenic hearts, in some of them rather intense, and this band was absent, as expected, in the Mst ($-/-$) mice. The 32 kDa processed band was not

Figure 2 Relative expression of myostatin RNA in the heart of the Mst TG and wild-type mice, determined by RT-PCR. (A) Schematic of the P1 and P2 set of primers to detect both endogenous and recombinant myostatin (P1), or recombinant myostatin only (P2). (B) RNA was isolated from total cardiac tissue from BL/6 background mice and subjected to RT-PCR with the P1 set of primers, identifying amplified DNA bands by ethidium bromide staining after agarose gel electrophoresis. (C) Similar reactions were carried out with the P2 set of primers. For other symbols see Fig. 1.

evident on this gel. The densitometric comparison showed a twofold increase in the Mst TG when compared with WT (A, right). However, when double the protein was loaded in another run and western blotted and the X-ray was over exposed (B), a clear 32 kDa band was detected in the TG heart, which was very faint but visible in the WT, where the 52 kDa band was also clear. Both the 52 and 32 kDa bands were absent in the Mst (–/–) heart tissue, as expected.

Left ventricular EFs are normal in Mst transgenic mice

In spite of the differences in heart weight, and specifically in left ventricular weight, in parallel with the differences in cardiac Mst expression, the Mst transgenic mice had an EF in the anterior/posterior view (AP) that was not different from the one in their respective WT controls in the BL/6 background (Fig. 4A, left). The same situation occurred in the Mst (–/–) mice when compared with their respective WT controls in the Balbc background (A, right). The lateral view confirmed these observations (B left and right).

The end systolic volumes in the AP view reflected the differences in heart sizes (AP: Mst TG: 0.015 ± 0.001 versus WT: 0.018 ± 0.002 , and Mst (–/–): 0.021 ± 0.004 versus WT: 0.012 ± 0.003), and the same occurred with the end diastolic volumes (AP: Mst TG: 0.038 ± 0.002 versus WT: 0.044 ± 0.005 ; and Mst (–/–): 0.050 ± 0.003). The lateral view showed the same trend but with less marked differences (not shown). In other words, irrespective of the significant changes in heart size, and particularly in LV size, due to the opposite degrees of Mst expression, the EF did not differ significantly between the Mst transgenic and Mst knockout and their corresponding WT controls.

Mst inhibits replication of cardiomyocytes without affecting apoptosis or cell size

Since Mst effects on heart size and function in transgenic and knockout mice are probably cumulative over the whole gestational and early postnatal period until the 7 weeks of age we chose for measurements, it is unlikely that the changes in gene expression that may affect cardiac size would be so

Figure 3 Relative expression of myostatin protein in the heart of the Mst (+/+) and wild-type mice, determined by western blot. (A) Left: total protein was obtained from mouse hearts different from those on Fig. 2, and subjected to western blot analysis (20 μ g protein) for myostatin, using a monoclonal antibody and GAPDH as housekeeping reference protein. Right: densitometric analysis. (B) In a separate gel, the loading was 40 μ g and the X-ray was overexposed to intensify bands, so that the expression of myostatin in the wild-type heart was more evident.

significant, or even persist, as to be detected unequivocally at the time of killing. Therefore, we decided to study these possible effects of Mst *in vitro* in an accepted cardiomyocyte cell line of embryonic origin, the H9c2 cells. First, we aimed to detect Mst protein expression in these cells by immunocytochemistry with our polyclonal antibody. Figure 5A shows that it is localized predominantly in the nuclei, as previously seen (Artaza *et al.* 2002), thus confirming the previous observation in sheep (Sharma *et al.* 1999) that the modest expression of Mst seen in the heart of the WT mice occurs in the cardiomyocytes themselves. The immunocytochemical determination of the cardiomyocyte marker, cardiac troponin I (B) along with the lack of expression of MHC type I (C) and II (D) confirmed the purity of the H9c2 cell culture. Further confirmation of the endogenous production of Mst was obtained by RT-PCR with primers specific for Mst that shows the 531 bp DNA band reverse transcribed and amplified from the H9c2 RNA (E). This set of primers overlap two exons in the Mst DNA coding region, thus excluding contamination with genomic DNA. This band was also seen, as expected, in the reactions from C2C12 myotubes (Taylor *et al.* 2001) and C3H 10T (1/2) cells differentiated with 5'-azacytidine (Artaza *et al.* 2005).

In order to decide whether Mst in the heart, presumably translated from RNA in the cardiomyocytes, would act exclusively in an autocrine fashion or may also be secreted and

interact with membrane bound receptors, we investigated whether RNA for the Mst receptor, the ActRIIB, is also expressed in cardiomyocytes, in this case, the H9c2 cells. Figure 5F shows that this is the case since the corresponding 235 bp DNA band was detected after RT-PCR of the H9c2 cells RNA, as well as in the reaction from the C2C12 and C3H 10T (1/2) RNAs.

To test the hypothesis that Mst decreases cardiac muscle mass by inhibiting cardiomyocyte proliferation, H9c2 cells were transfected in the presence of lipofectamine with two types of constructs encoding the full length Mst cDNA under either the strong, tissue unspecific, CMV promoter, or the weak, skeletal muscle/heart-specific, MCK promoter.

A parallel test with similar constructs expressing the reporter gene EGFP in lieu of Mst showed that both promoters were activated in the cells as expected, and that the level of transfection were reasonable for both constructs (Fig. 6A). Western blot analysis for several proteins present in the cell extracts from non-transfected cultures and the cells transfected with the Mst plasmids is presented in Fig. 6B. The polyclonal antibody against Mst detected a faint expression of the 32 kDa band for the glycosylated dimer, thus confirming that the Mst mRNA in the H9c2 cells seen in Fig. 5 is indeed translated into protein. Transfection with the Mst cDNA constructs resulted in a much more intense 32 kDa band indicating Mst processing by the cardiomyocytes. The

Figure 4 Genetic blockade or overexpression of myostatin in the Mst (—/—) and Mst TG mice affects left ventricle size in the male animal, but not cardiac function. (A) Ejection fraction in mice under the anterior/posterior position determined by angiograms. Ejection fraction: (EDV-ESV)/EDV. (B) Ejection fraction under the lateral view. For other symbols see Fig. 1.

housekeeping gene, GAPDH, was virtually similar in the three extracts.

The expression of the cell cycle inhibitor p21 was virtually negligible in the untransfected H9c2 cells, and the reverse occurred with the cyclin Cdk2, as expected from proliferating cells (Fig. 6B). Mst overexpression by both Mst cDNA constructs led to much higher p21 levels and lower cdk2, thus suggesting that Mst induces the exit from the cell cycle that would reduce cell proliferation *in vitro* and tissue growth *in vivo*.

In order to determine whether Mst does indeed inhibit cardiomyocyte replication, H9c2 cells were incubated with increasing concentrations of the recombinant Mst protein corresponding to the 110 aa (carboxy terminus; Mst 110), the processed active Mst fragment, from 0 to 8 µg/ml, starting at a low confluence (20%) for 4 days, and proliferation was estimated by a formazan assay (Fig. 7A). Mst at 4 and 6 µg/ml induced a statistically significant reduction in cell number that increased only moderately with 8 µg/ml. The paracrine inhibitory activity of Mst was low in comparison to the one of TGFβ1 at 10 ng/ml. In contrast, incubation with the polyclonal rabbit IgG antibody against Mst (100 µg/ml) produced statistically significant stimulation of H9c2 cell proliferation, whereas the non-immune IgG at the same concentration did not have any effect.

When H9c2 cells were transfected with the two plasmid constructs expressing Mst under the CMV or MCK promoter, there was statistically significant reduction in cell proliferation measured as in the experiment with recombinant Mst, when compared with the controls either without transfection, incubated with lipofectamine only, or transfected with an empty pcDNA 3.0 CVM plasmid (Fig. 7B). There was no significant difference in cell proliferation among the control incubations.

To confirm that Mst may reduce cardiac size by down-regulating the number of cardiomyocytes mainly via the inhibition of cell replication and not through the induction of cell death, we repeated the incubations of H9c2 cells with recombinant Mst protein at 4 µg/ml for 3 days. Figure 8A and C left, shows with the TUNEL reaction followed by quantitative image analysis that there was no significant effect of Mst on the cardiomyocytes apoptotic index. The alternative possibility, namely that Mst would reduce cardiomyocyte size was excluded *in vitro*, since we did not find any significant difference in the presence or absence of Mst (B and C right).

Discussion

To our knowledge, this is the first demonstration that Mst overexpression in the heart of the Mst TG mouse is associated with decreased cardiac left ventricular mass, without a significant change in the fractional ejection volume. Similarly, decreased Mst expression in the Mst (—/—) mice was associated with increased cardiac mass without a significant functional change. By focusing on the *in vitro* effects of Mst on a widely used cardiomyocyte cell culture, in order to amplify molecular and cellular changes that may not be readily apparent *in vivo* due to the uncertainty on when Mst acts during gestational and postnatal development period in the mouse, we aimed to detect a potential mechanism of action that would explain the *in vivo* effects of Mst on cardiac size. We found that Mst inhibits proliferation of H9c2 cardiomyocytes *in vitro*, an effect that is blocked by anti-Mst antibody. Transfection of H9c2 cardiomyocytes with a Mst expression cDNA construct also is associated with decreased cell replication. Additionally, Mst upregulates the expression of p21, an inhibitor of cdk2 and downregulates cdk2 in cardiomyocytes, but does not affect apoptosis or cardiomyocyte size. Collectively, these data suggest that Mst reduces cardiac size during development mainly by inducing replicating cardiomyocytes to exit the cell cycle. It is unlikely that this would occur once cardiac growth is arrested in the animal along its lifespan or under conditions of cardiac hypertrophy in the mature heart.

The selective effects of Mst on cardiomyocyte mass but not on EF are analogous to those found in the skeletal muscle, where Mst-induced changes in muscle mass are not associated with changes in specific tension generation (Caiozzo *et al.* 2005). In other words, the contractile units in both tissues retain the same functional efficacy. The XiScan small imaging amplifier used in this study has previously been applied to

Figure 5 Cultures of H9c2 embryonic cardiomyocytes express both myostatin and its receptor. (A–D) H9c2 cells were plated onto 8-well removable chamber plates and subjected to immunocytochemistry as indicated. (E and F) Total RNA was isolated from H9c2 cells and subjected to RT-PCR with primers as indicated. RNA from C₂C₁₂ and C3H 10T(1/2) cells was used as reference.

image the hearts of both mice and rats, and in our opinion the visualization of the ventricular cavity is superior to that obtained by echocardiography. Since fluoroscopy was performed at the same time following anesthesia in all animals, the slowing effects of ketamine/xylazine on the heart affected them similarly. While no formal specific sensitivity/reproducibility studies have been performed with this procedure, the

data generated by this technique has yielded consistent data when studying rat myocardial infarcts (Muller-Ehmsen *et al.* 2002, Yao *et al.* 2003, Dai *et al.* 2005). This same technique has been used to analyze *in vivo* ventricular function of the normal and pressure overloaded right ventricle (Rockman *et al.* 1994).

We do not know whether inhibition of Mst by administration of Mst antagonists in humans would cause myocardial

Figure 6 Expression of recombinant myostatin by cultured cardiomyocytes affects the expression of cell cycle proteins. (A) H9c2 cardiomyocytes were transfected with plasmid vectors driven by the CMV or MCK promoters that express EGFP as reporter protein, and examined at 2 days under the fluorescent inverted microscope. (B) H9c2 cells were transfected with constructs encoding the myostatin full length coding region in the same vectors as in A, or left untreated (control), and cell homogenates were subjected to western blot analysis for the indicated protein, using GAPDH in each case as housekeeping protein.

hypertrophy, and whether this hypertrophic response is beneficial or deleterious. No morphometric histological analysis of the heart was performed in the current study. However, a very recent paper has addressed this issue in the Mst knockout mouse (Cohn *et al.* 2007), showing that no cardiomyocyte hypertrophy was evident in aged (2 years old) Mst (–/–) mouse versus the WT animal with Bl/6 background. Interestingly, the measurements performed by echocardiography measurements under ketamine/xylazine coincide with our corresponding results

with the XiScan small imaging amplifier, where no significant differences are apparent between Mst (–/–) and WT in both Bl/6 and Balb c backgrounds.

The maintenance of a constant functional efficacy per unit of cardiac or skeletal muscle mass irrespective on how the latter is affected by variations in Mst levels, is counterintuitive to the putative effects of Mst on tissue composition, derived from its effect *in vitro* where this protein not only inhibits myogenic differentiation from pluripotent cells (Artaza *et al.*

Figure 7 Cardiomyocyte cell proliferation is reduced by expression of either exogenous or endogenous recombinant myostatin protein, and increased by the inhibition of myostatin activity. (A) H9c2 cells were incubated for 4 days, as follows: control: no addition; R-Mst: increasing concentrations of recombinant myostatin protein corresponding to the 110 aa (carboxy terminus); Ab 1/100: polyclonal IgG antibody against myostatin, diluted to 0.1 µg/ml; TGFβ1: used as reference protein; IgG: control non-immune IgG diluted to 0.1 µg/ml. (B) H9c2 cells were transfected and maintained for 4 days, as follows: control: no transfection; Con ± Lipf: transfection with lipofectamine only; Con/pcDNA3.0: transfection with empty CMV-driven vector; pCMV/Mst: transfection with CMV driven construct of full length myostatin cDNA; pMCK/Mst: transfection with equivalent myostatin construct, but driven by the MCK promoter.

2005), but also promotes their adipogenic and fibrogenic conversion (Artaza *et al.* 2006, Tsao *et al.* 2006). Moreover, *in vivo* Mst expression is associated with skeletal muscle fibrosis both in normal and dystrophic mice (Wagner *et al.* 2002, McCroskery *et al.* 2005, Parsons *et al.* 2006). Therefore,

we hypothesized that connective tissue and fat infiltration would occur in the Mst TG heart in parallel to the reduction in organ size, and this would impair the fractional ejection volume. The absence of this functional alteration implies that during embryo development and early postnatal life, the factors that may act in conjunction with Mst to trigger lipofibrotic degeneration of the heart, such as TGFβ1 (Khan & Sheppard 2006) are absent or non-operative, or there is an intense level of cardiac repair maintaining the normal tissue composition.

Since our animals were only 7 weeks old, it may be worth to determine in future studies whether in aged animals, where cardiac fibrosis and dysfunction occur (Hacker *et al.* 2006, Rozenberg *et al.* 2006), the pro-lipofibrotic effects of Mst prevail and this translates into a further aggravation of these processes. In fact, although lipid composition was not analyzed, at 2 years of age, the heart of the Mst null mice displays normal histological features as assessed with Masson trichrome (Cohn *et al.* 2007). However, the converse situation that fibrosis may occur more intensively in mice overexpressing was not studied. Therefore, the possibility of Mst inducing fibrosis in the heart in certain conditions remains open, despite the findings of the authors that the absence of Mst did not prevent cardiac fibrosis in the Mst^{-/-}/mdx hybrid mouse.

Our data demonstrate that Mst inhibits proliferation of H9c2 cardiomyocytes. Western blot analysis confirmed that incubation of H9c2 cardiomyocytes with recombinant Mst protein upregulated p21, an inhibitor of cdk; additionally, cdk2 expression was downregulated. These findings were confirmed in separate experiments in which H9c2 cardiomyocytes were transfected with a Mst cDNA expression constructs which revealed that Mst inhibits G1 to S phase progression. These data on Mst effects on cardiomyocytes are analogous to those reported in cultures of skeletal myoblasts in which Mst also has been shown to inhibit cell replication, mainly by interfering with the progression into S phase of the cell cycle (Artaza *et al.* 2002, McCroskery *et al.* 2003, Sakuma *et al.* 2004, Shyu *et al.* 2005, McFarland *et al.* 2006, Tsao *et al.* 2006). Our results with the H9c2 cell line also coincide with a very recent study (McKoy *et al.* 2007) in primary rat embryonic cardiomyocytes showing that Mst is expressed faintly in these cells, and that recombinant Mst inhibits cell proliferation via upregulation of p21 and Smad2 phosphorylation, thus blocking the G1 to S phase progression.

Our finding that Mst is expressed, albeit at very low levels, in the heart of 7-week old WT mice, and that both Mst mRNA and protein and Mst receptor, the ActRIIB, are all detected in the H9c2 rat cardiomyocyte cell line, raises several questions. The precise functional role of endogenous Mst in the postnatal heart or of the circulating Mst secreted by the skeletal muscle (Gonzalez-Cadavid *et al.* 1998, Hill *et al.* 2002, 2003, Rios *et al.* 2004) that may reach the heart is unknown. It is possible that upregulation of endogenous Mst production may be involved in cardiac remodeling and scarring after myocardial infarction, as has been shown previously in sheep

Figure 8 Myostatin does not affect cardiomyocyte apoptotic index or size. H9c2 cells were incubated for 4 days, as follows: control: no addition, R-Mst: 4 μg/ml of recombinant myostatin protein corresponding to the 110 aa (carboxy terminus), as in Fig. 7. (A) Representative fields of cells stained by TUNEL. (B) Representative fields of cells stained for cardiac troponin I. (C) Quantitative image analysis of TUNEL stained sections (left) or of cell size (right).

(Sharma *et al.* 1999). In this respect, our Mst TG mouse, by moderately overexpressing Mst in the heart, may replicate this condition, not only in animals but also speculatively in humans too. Others have speculated that Mst may act as a chalone to regulate myocardial growth in response to hypertrophic or atrophic signals. Thus, Mst has been reported to be upregulated in states of cardiac hypertrophy induced by phenylephrine infusion and Akt hyperexpression (Gaussin & Depre 2005). This hypothesis merits further investigation.

The inhibition of cardiomyocyte proliferation by Mst observed *in vitro* may be responsible for the reduction in heart growth in the Mst transgenic mice that overexpress Mst. It is noteworthy that we did not observe any change of cell size or

multinucleation by Mst *in vitro*, upon reducing cell replication despite the fact that H9c2 can undergo hypertrophy under different stimuli, for instance oxidative stress caused by H₂O₂ (Chen *et al.* 2000), doxorubicin (Merten *et al.* 2006), TGF β (Huang *et al.* 2004), or vasopressin (Brostrom *et al.* 2001). Our observations that Mst did not affect apoptosis in H9c2 cultures are compatible with what we reported in skeletal myoblast cultures (Taylor *et al.* 2001). H9c2 cells do respond to cardiac-relevant apoptotic stimuli, such as hypoxia combined with serum deprivation (Tantini *et al.* 2006). In addition, it is notable that in Mst transgenic mice in which Mst expression was driven by MCK-3E reporter, which restricted the Mst transgene expression to the skeletal muscle, the cardiac mass

did not differ from that in WT controls. Taken together, these data suggest a paracrine or autocrine effect of Mst on the heart during gestational development or early postnatal growth rather than an endocrine effect.

In summary, we have shown that Mst overexpression in transgenic mice is associated with decreased cardiac and left ventricular mass but normal left ventricular EF. Mst inhibits cardiomyocyte proliferation by inhibiting progression through the S phase of the cell cycle, without affecting apoptosis or cell size. Long-term effects of Mst inhibition in adult and aged mammals on left ventricular mass and function need further investigation as they are relevant to the clinical application of Mst antagonists as anabolic therapies in humans.

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