Muscle phenotype of the myostatin mutant *Compact* mice and myostatin/IGF-I transcript levels in pathological human hearts

Summary of Ph.D. Thesis

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Publications

List of full papers related to the subject of the Thesis

I. Baán JA, Kocsis T, Keller-Pintér A, Müller G, Zádor E, Dux L, Mendler L (2013) The Compact mutation of myostatin causes a glycolytic shift in the phenotype of fast skeletal muscles. J Histochem Cytochem. 61:889-900.
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II. Baán JA[#], Varga VZ[#], Leszek P, Kusmierczyk M, Baranyai T, Dux L, Ferdinandy P, Braun T, Mendler L (2015) Myostatin and IGF-I signaling in end-stage human heart failure: a qRT-PCR study. Journal of Translational Medicine. 13(1):1-9. DOI: 10.1186/s12967-014-0365-0
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Introduction

Significance of myostatin

Myostatin (Mstn) is an important regulator of skeletal muscle growth. Mstn is a member of the TGF-β (transforming growth factor β) superfamily, mainly expressed in skeletal muscle, with less, but still significant expression in the heart and adipose tissues. Enhanced production of Mstn inhibits skeletal muscle growth and decreases muscle mass (e.g. in cachexia, muscle dystrophy or aging) while Mstn mutations cause hypermuscular phenotype in several different species. Furthermore, Mstn plays a crucial role in the regulation of cardiomyocyte growth as well as in the maintenance of oxidative metabolism and contractility of the heart. In heart failure patients myocardium produces and secretes Mstn protein into the circulation thereby inhibiting skeletal muscle growth leading to cachexia. Mstn secreted by skeletal muscles might also have an impact on the heart and adipose tissues. Its mechanism of action has not been clarified in its full complexity so far, although it might be a promising therapeutical target molecule in both metabolic and muscle diseases.

Mstn is synthetized as a precursor protein in muscle cells and after cleaveage and secretion, forms an inactive complex with its propeptide. Once activated by propeptide proteases, the active Mstn binds to activin type IIB receptor (ActRIIB), which dimerizes with and phosphorylates type I receptor (ActRI). Mstn acts through different intracellular pathways with Smads representing the classical way to transduce Mstn effects by inhibiting protein synthesis, myoblast proliferation and differentitation and by activating protein degradation.

Skeletal muscle development and adult muscle phenotype

Mstn was shown to play an important role in regulating skeletal muscle growth (hyperplasia: increase in cell number, hypertrophy: increase in cell size) and adult muscle phenotype. According to the ATPase activity of myosin heavy chains (MHC), slow type MHC I and fast type MHC II are distinguished giving rise to type I and type II fibers, respectively. Within type II fibers, oxidative MHCIIA, oxidative-glycolytic MHCIIX and glycolytic MHCIIB isoforms are identified, representing the dominating isoforms in type IIA, IIX and IIB fibers, respectively. Regulation of fiber types is only partly understood, but Mstn might be one of the candidates which may modulate oxidative versus glycolytic fiber type determination.

Myostatin mutations in skeletal muscle

The Mstn knock out (KO) mouse shows a hypermuscular phenotype similar to that of several different organisms (Piedmontese and Belgian Blue cattles, dog, pig, Texel sheep and human infant) carrying naturally occuring mutations in the biologically active part of Mstn. In contrast to aforementioned mutations, the hypermuscular *Compact (Cmpt)* mice which was originated from the Technical University of Berlin carry a 12-bp natural mutation in the Mstn propeptide, with additional modifier genes being responsible for the phenotype, however these genes have not been identified yet. To date, only few studies provided phenotypical or molecular analysis of the *Cmpt* mice although this line represents a complex and mainly unknown mechanism of Mstn-dependent hypermuscularity.

Role of cardiac myostatin, interplay between myostatin and IGF-I in the heart

Mstn has been identified as a key modulator of heart function by regulating growth, metabolism and contractility but its interaction with IGF-I has not been clarified in its full complexity. IGF-I was shown to play a pivotal role in cardiovascular physiology and aging. In concert with insulin itself, IGF-I proved to be a positive regulator of cardiac growth and contractility under both physiological and pathological conditions. Mstn might be a cardiac chalone of IGF-I, since cardiac growth induced by IGF-I was feed-backed by the overexpression of the negative growth regulator Mstn. According to the hypothesis, chalones are secreted by specific tissues and provide a negative feedback mechanism to control the size of tissue that produces it.

MicroRNAs (miRs) are small noncoding RNAs that negatively regulate cardiac gene expression under both physiological and pathological conditions with a potential impact on cardiac Mstn or IGF-I expression as well. Indeed, microRNA(miR)-208, has been reported to be a negative

regulator of Mstn expression and to play a crucial role in pathological cardiac hypertrophy and fibrosis.

No studies have systematically analyzed the relevance of the possible reciprocal regulation of Mstn and IGF-I at the gene expression level in healthy or failing human hearts, nor the potential role of miR-208 under these conditions. Previous investigations focused only on Mstn protein activation in hearts of dilated or ischemic cardiomyopathic patients (DCM or ICM, respectively). Moreover, no data exist in the literature about the expression pattern of Mstn in comparison with IGF-I and their receptors in various regions (i. e. left (LV) and right ventricles (RV)) of the human heart. Given the different functional requirements LV and RV should cope with, and the markedly different development of these regions, one could assume that the gene expression pattern of Mstn and IGF-I signaling might show remarkable spatial differences under both physiological and pathological conditions.

Aims

1. To describe the muscle phenotype of the Mstn mutant *Cmpt* mice using morphological assessments in order to uncover the combined effects of the unconventional propeptide *(Cmpt)* mutation in Mstn and the unknown modifier genes in the genetic background.
2. To analyze transcript pattern of Mstn versus IGF-I with their respective receptors in different regions of healthy and pathological human hearts and to reveal their spatial regulation in healthy hearts or potential transcript alterations in dilatative (DCM) versus ischemic cardiomyopathic (ICM) patients.

Materials and Methods

Mouse studies: Animals and experimental design

Male, 2.5-month-old male *Cmpt* (44-50,7 g) and BALB/c (24-28g) mice were used for the experiments (n=5-10). Typical hindlimb muscles (m. quadriceps (Quadr), m. tibialis anterior (TA), m. extensor digitorum longus (EDL) m. soleus (SOL) and m. gastrocnemius (Gastro)) of *Cmpt* and BALB/c strains were removed under intraperitoneal anaesthesia. For comparison of body and muscle weights as well as for TA muscle fiber analysis, older *Cmpt* male mice were also used. All muscles were weighed and frozen in isopenthane/liquid nitrogen and kept at -80°C until further use. TA, EDL and SOL muscles of BALB/c and hypermuscular *Cmpt* mice from the right leg were used for morphological and immunohistochemical analysis while RNA isolation followed by qRT-PCR was carried out from the contralateral TA counterparts. Animal experiments were approved by the

Institutional Animal Care and Use Committee at the University of Szeged in accordance with the U.S. National Institutes of Health guidelines for animal care.

Human studies: Patients and experimental design

Healthy human hearts were obtained from organ donor patients (CONT, n=5) whose hearts were explanted but due to technical reasons not used for transplantation. The donors did not present any important previous medical history or any abnormalities in ECG and echocardiography and had died from head trauma, cerebral or subarachnoid hemorrhage. Explanted end-stage failing hearts were obtained from patients with advanced heart failure of non-ischaemic (DCM) (n=5) or ischaemic aethiology (ICM) (n=5). Tissue samples of the right and left ventricular free walls (RV and LV, respectively) and the inter-ventricular septum (S) were taken at the time of explantation. The samples were rinsed immediately, blotted dry, frozen in liquid nitrogen and kept at − 80°C until further processing for RNA isolation and qRT-PCR analysis. All procedures were in accordance with the Helsinki Declaration of 1975. Informed consent was obtained from all patients for being included in the study according to the protocol approved by the Local Ethics Committee (IK-NP-0021-24/1426/14).

Morphological and morphometrical analysis of mouse skeletal muscles

10 μm serial cryostat sections were taken from the midbelly region of different muscles of both *Cmpt* and BALB/c mice followed by standard hematoxylin-eosin (HE) staining. By taking pictures from all microscopic fields the whole cross-sectional area (CSA) of each muscle was reconstructed and determined by counting all fibers in TA, EDL and SOL muscles with Digimizer software.

Myosin heavy chain (MHC) immunohistochemistry of mouse skeletal muscles

Sections were incubated with mouse monoclonal primary antibodies BA-D5 (1:25), sc-71 (1:25) and BF-F3 (1:5) specific for myosin heavy chain (MHC) I (slow oxidative), MHCIIA (fast oxidative) and MHCIIB (fast glycolytic), respectively. After incubation with the peroxidase-conjugated secondary antibody immunocomplexes were visualized by diaminobenzidine (DAB) staining with or without nickel enhancement. IIX fibers were considered as those not stained by any of the above antibodies. The size and distribution of muscle fibers were determined by analyzing at least 20-50% of fibers in SOL, EDL and TA muscles.

Quantitative RT-PCR analysis of mRNA transcripts in mouse skeletal muscles and human heart samples

Total RNA was isolated from TA muscles of *Cmpt* and BALB/c male mice (n=5) with TRI reagent or from the LV, S and RV samples of the CONT, DCM or ICM patients (n=5) with the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method, followed by reverse transcription. For the detection of transcript levels of mouse MHCI, MHCIIA, MHCIIX and MHCIIB as well as human Mstn, ActRIIB, IGF-I, and IGF-IR quantitative RT-PCR was carried out with SYBR GREEN master mix on a Light Cycler 1.5. As an internal control, hipoxanthine-guanine phosphorybosyltransferase (hprt/HPRT) has been used in our experiments. Cycle conditions were set as an initial denaturation step, followed by 45 cycles for template denaturation, annealing and extension phases. Specificity of the PCR products was confirmed by melting curve analysis and by agarose gel electrophoresis (1.5%). Primer pairs were designed to intron spanning exons by Primer 3 Input software and tested to avoid primer dimers, unspecific amplification and self-priming formation.

Quantitative RT-PCR analysis of miRNA transcripts in human heart samples

From the above detailed total RNA isolates of human heart samples, cDNA was synthetized and quantitative real-time PCR was performed with miRCURY LNA^{TM} Universal RT microRNA PCR kit (Exiqon, Denmark) on LightCycler®480 according to the manufacturer's instructions. The primers for both microRNA-208b and control microRNA-103a-3p were designed and prepared by using Exiqon's LNA^{TM} technology.

Statistical analysis

Statistical analysis was performed by unpaired t test and one- way/two-way ANOVA or non-parametric t-test (Welch test) using Prism software (GraphPad Software, Inc.; San Diego, CA), as appropriate. All data were expressed as means±SEM. The level of $p<0.05$ was considered significant. The individual p-values are indicated in the figure legends.

Results

Muscle phenotype of *Compact* mice

Body- and muscle weight

We compared 2.5-month-old Mstn mutant *Cmpt* mice with wild-type BALB/c mice in terms of body and muscle weight. *Cmpt* male mice were significantly larger than BALB/c regarding their body weight. Absolute weights of the different hindlimb muscles, such as Quadr, Gastro, TA, EDL

and SOL muscles, were significantly larger in *Cmpt* mice. Similarly, muscle weights normalized to body weights were significantly increased in *Cmpt* animals, with the exception of the oxidative SOL muscle. These results indicated a disproportionate increase in muscle masses in *Cmpt* mice. To assure that the 2.5-month-old *Cmpt* mice had already finished the intense growing phase, we analyzed older mice as well. Body weight was similar in young (2.5-month-old) and adult (4- and 7-month-old) animals, while 12-23-month-old mice had slightly higher weight measurements. However, no significant difference was found in muscle weights amongst any of the age groups, indicating that the 2.5-month-old animals have already reached muscle size typical of adult mice.

Muscle fiber number and fiber cross sectional area

To define whether the hypermuscularity of *Cmpt* mice is caused by hyperplasia or hypertrophy, we analyzed muscle fibers on HE- or MHC-immunostained serial cross-sections of different muscle types. Fiber number was significantly higher in all examined muscles of the 2.5-month-old *Cmpt* mice than that observed in wild-type mice. TA muscle revealed the most significant hyperplasia as compared with EDL and SOL muscles. However, fiber size did not differ in TA muscles between *Cmpt* and BALB/c mice. EDL and SOL muscles of *Cmpt* mice, on the other hand, showed clear evidence of hypertrophy as well. To control fiber parameter alterations in *Cmpt* mice along with aging, we analyzed TA muscles of 7-month-old *Cmpt* mice and found no differences compared with 2.5-month-old mice.

In summary, based on our results, hypermuscularity of *Cmpt* mice is characterized by fiber hyperplasia in TA muscle and by a combination of hyperplasia and different grades of hypertrophy in EDL and SOL muscles.

MHC composition and fiber size distribution

To analyze the possible effects of the *Cmpt* mutation on MHC composition, serial cryosections of TA, EDL and SOL muscles were immunostained using sets of monoclonal antibodies in order to differentiate type I, IIA, IIX and IIB fibers, respectively. In line with the literature, only MHCII isoforms were detected in TA and EDL muscles of both mouse lines, whereas no MHCIIB fibers were found in SOL muscle.

We noted that the fast-type TA muscles of *Cmpt* mice contained significantly more glycolytic IIB fibers while a decreased number of IIX and IIA fibers as compared with that in wild-type mice. Similar to TA muscles, the number of glycolytic IIB fibers was increased in the fast-type EDL muscles of *Cmpt* mice, while the number of IIX and IIA fibers was significantly lower as compared with that of wild-type mice. In contrast, the oxidative SOL muscles in *Cmpt* mice contained more slow-type I fibers and less fast-type IIA fibers than those in wild-type mice, while the number of IIX

fibers was very low and not different in the two mouse lines. Together, our findings clearly demonstrate a substantial shift toward a more glycolytic phenotype in the fast-type TA and EDL muscles but not in the mixed-type oxidative SOL muscles of *Cmpt* mice.

We could not detect specific hypertrophy for any of the IIB, IIX or IIA fiber types in TA muscles. In EDL muscles, however, fiber hypertrophy was exclusively found in IIB fibers. In contrast to fast muscles, both I and IIA fibers showed evidence of hypertrophy in SOL of *Cmpt* mice.

mRNA levels of MHC isoforms

According to qRT-PCR analysis of TA muscles, MHCIIB mRNA levels were significantly increased, while MHCIIX and MHCIIA transcript levels both decreased in *Cmpt* mice. The slow MHCI isoform was almost undetectable. These results are in line with those obtained by immunohistochemical analysis, suggesting that the fiber-type shift in the TA muscle is regulated at the level of MHC transcription.

Myostatin and IGF-I signaling in the human heart

Study patients

Both female and male patients were included in all groups. The age of ICM patients differed as expected significantly from both CONT and DCM, since ICM patients are usually diagnosed with end-stage heart failure later than DCM patients. Extra care was taken to exclude diabetic (insulin-treated) patients from the study to avoid possible modification of the IGF-I signaling by insulin treatment. All patients were managed with angiotensin-converting enzyme (ACE)-inhibitors, beta-blockers and diuretics, however, aspirin and statins were only used in case of ICM patients. CONT subjects received iv. treatment composed of very low catecholamine infusion whereas adequate fluid balance was maintained with intravenous fluids including colloids (e.g. Voluven - hydroxyethyl starch) and Desmopressin.

Myostatin and IGF-I signaling in healthy human control hearts

So far, no comprehensive study has been carried out to reveal gene expression of the growth regulators Mstn and IGF-I and their receptors in different regions of healthy human hearts. In the present work we detected no significant difference in Mstn and ActRIIB transcript levels nor in Mstn signaling index between septum (S), left ventricle (LV) and right ventricle (RV), however, Mstn levels showed decreasing tendency in the RV. In contrast, both IGF-I mRNAs and IGF-I signaling index followed an increasing tendency in RV accompanied with a significantly higher IGF-IR levels compared to those of LV.

As a consequence, the ratio of Mstn/IGF-I gene expression as well as those of ActRIIB/IGF-I receptors and finally, the ratio of Mstn to IGF-I signaling all showed significantly higher values in

LV/S as compared to RV. These data clearly demonstrate that Mstn signaling dominates over IGF-I in the LV more than in RV of healthy human hearts.

Myostatin and IGF-I signaling in DCM patients compared to healthy controls

In DCM patients we measured massive upregulation of Mstn mRNA associated with an increased Mstn signaling index in all heart regions compared to CONT, although ActRIIB levels remained relatively unchanged. Similar to healthy hearts, we found a significant upregulation of IGF-I transcripts as well as of IGF-I signaling index in RV of the failing hearts when compared to those of S, although IGF-IR expression did not show significant difference in either region nor in comparison to CONT hearts. Thus, the ratio of Mstn/IGF-I mRNA levels and the Mstn/IGF-I signaling index proved to be significantly higher in the left versus right side of the DCM hearts and showed much higher levels than those of the CONT regions. Since the ratio of the ActRIIB/IGF-I receptors did not change significantly we can conclude that Mstn was upregulated in all regions of failing hearts in DCM patients as compared to CONT. However, given the higher IGF-I levels in RV, left and right side of failing heart differed significantly from each other in regard to the ratio of Mstn/IGF-I signaling similar to those of healthy ones.

Myostatin and IGF-I signaling in ICM patients compared to healthy controls

In contrast to DCM patients, we could not detect any difference in either Mstn or ActRIIB transcript levels or in Mstn signaling index in any heart region of ICM patients compared to those of CONT. Similarly, IGF-I and IGF-IR levels as well as IGF-I signaling index did not differ in ICM heart regions; however, in comparison to CONT, IGF-I showed a decreasing tendency of expression in the RV, while increased expression of IGF-IR in the LV was present. Consequently, both ratios of Mstn/IGF-I and ActRIIB/IGF-IR were similar in all analyzed regions of ICM hearts. However, significantly higher Mstn/IGF-I ratios were revealed in RV due to decreased IGF-I levels as well as significantly lower ActRIIB/IGF-IR ratios in LV when compared to CONT hearts (due to increased IGF-IR levels). In summary, ICM hearts did not show significantly altered modulation of Mstn signaling in either heart region, whereas IGF-I signaling, in contrast to the healthy situation, seemed to be moderately induced in the LV, while inhibited in the RV.

Differences in Myostatin/IGF-I signaling between DCM and ICM patients

Based on our results, all regions of DCM hearts showed significantly higher Mstn levels as well as elevated Mstn signaling index than those of ICM hearts. Moreover, ActRIIB also revealed increased levels in LV of DCM vs. ICM patients. Nevertheless, we found no significant difference in IGF-I signaling on the left side of failing hearts, although significantly less IGF-I transcripts were evident on the right side of ICM hearts in comparison with that of DCM ones. As a consequence, all

parameters describing the ratio of Mstn to IGF-I signaling showed significantly increased values in the LV of DCM versus ICM hearts. Although in the RV we have revealed similar signaling ratio in both types of failing hearts the reason for that was an upregulation of Mstn signaling in DCM patients while a downregulation of IGF-I signaling in ICM heart samples.

miR-208 in relation to myostatin expression in DCM and ICM patients compared to healthy controls

In parallel with the massive upregulation of Mstn mRNA in the LV of DCM patients we measured a mild upregulation of miR-208b (1.505 fold change) compared to CONT. A similar but less pronounced upregulation was seen in ICM hearts (1.405 fold change) when compared to CONT, however, no significant difference was detected between DCM and ICM patients.

Discussion

Mstn belongs to the TGF-β family and is a negative regulator of skeletal muscle growth. However, it plays also a crucial role in governing heart growth, metabolism and contraction. Our aim was to describe different aspects of Mstn signaling in skeletal muscle and heart tissue. To this end, two different model systems have been used in our experiments, (i) the Mstn propeptide-mutant *Cmpt* mice as well as (ii) healthy and pathological human hearts (S, LV, RV samples of DCM and ICM patients). Muscle cellularity and fiber type distribution have been analyzed in the fast TA and EDL as well as the mixed-type SOL muscles of *Cmpt* mice and transcript levels of MHC isoforms were quantified by qPCR. On the other hand, gene expression of Mstn and IGF-I, the two major but mostly counter-acting regulators of cardiac tissue have been investigated in different regions of healthy or pathological human hearts by measuring transcript levels of Mstn, ActRIIB, IGF-I, IGF-IR and miR-208, the negative posttranscriptional regulator of Mstn.

Muscle phenotype of *Cmpt* mice

The *Cmpt* mouse line takes a special place in the group of hypermuscular animals carrying naturally occurring Mstn mutations, as the propeptide region, not the biologically active domain of Mstn is affected. It has also been shown that the *Cmpt* mutation of Mstn is an indispensable yet not satisfactory requirement for the full expression of the hypermuscular phenotype, pinpointing the potential significance of additional modifier genes.

Since the genetic background of the *Cmpt* mice is very complex and no appropriate genetic control line was available at the time of our experiments, we decided to analyze BALB/c mice as a control. for the following reasons: i) This inbred line was used for mapping the Mstn mutation and the modifier genes in *Cmpt* mice. ii) BALB/c mice are generally used as wild-type controls and their

muscle parameters as well as fiber composition have already been reported. iii) Muscle characteristics of BALB/c mice are similar to those of C57BL/6, another wild-type mice used as a genetic background for Mstn KO mice, suggesting that these lines might be comparable to some extent. In the present study we show, in accordance with others that body- and muscle weights of male *Cmpt* mice are higher than those of wild type BALB/c mice. Body mass is even higher in *Cmpt* mice (47,7g) than in male Mstn KO of the same age (38-41g), but represent a similar grade of muscularity. One exception was the SOL muscle, which seemed to be different in this regard, as its relative muscle-to body weight did not increase in *Cmpt* compared with the wild type mice. Indeed, it has previously been reported that the oxidative SOL muscle contained less Mstn transcript than the glycolytic EDL and, that the lack of Mstn had stronger effect on glycolytic muscles than on oxidative ones. Therefore, we analyzed three different hindlimb muscles: the fast-glycolytic TA and EDL as well as the oxidative SOL muscles in *Cmpt* mice. Based on extensive morphometrical analysis TA muscles were exclusively characterized by muscle fiber hyperplasia, with no obvious hypertrophy.

This observation may somewhat be surprising, as previous reports described both hyperplasia and hypertrophy in different Mstn KO animals. However, even in the original Mstn KO mouse study, the ratio of hyperplasia to hypertrophy varied in different muscles, *e.g*. the TA muscles of Mstn KO mice were dominated by hyperplasia, while only 14% fiber hypertrophy was documented. In contrast to TA, EDL muscles of *Cmpt* mice were characterized by a combination of hyperplasia (50%) and hypertrophy (20%), whereas EDL in the Mstn KO mice showed only a moderate hyperplasia and a stronger hypertrophy. We found that the moderate fiber hypertrophy in *Cmpt* EDL confined to the most glycolytic IIB fibers and was not present in IIX or IIA fibers. This is again in accordance with the previous reports on a stronger effect of Mstn on glycolytic muscles. We believe that the long separation of the ‘Berlin’ and Hungarian *Cmpt* lines, gender differences, the use of different controls as well as our detailed sample analysis may account for these differencies.

In the oxidative SOL muscle of *Cmpt* mice, low-grade hyperplasia (15%) and mainly hypertrophy (27%) accounted for the moderately higher muscle mass. Moreover, both the oxidative I and IIA fibers showed hypertrophy compared with the wild-type control. The changes in SOL are different from those of fast TA and EDL muscles in *Cmpt* line. Intriguingly, controversial results have been published regarding SOL in Mstn KO mice, where either 32% hyperplasia, or 20% fiber hypertrophy has been described. It is not known so far, how cellularity in different muscle types is regulated upon Mstn defect. However, prenatal hyperplasia seems to be the major effect of developmental Mstn deficiency in most muscles of mice and cattle. Based on the different ratio of hyperplasia to hypertrophy in various Mstn deficient muscles, Mstn might have a strong, but muscle-type dependent effect on proliferation of muscle precursor cells. Consequently, postnatal fiber

hypertrophy might be restricted to different grade. Rehfeldt and co-workers (2005) introgressed the Mstn mutant *Cmpt* allele into a special high growth mouse line (DUHi) and detected exclusively hyperplasia, similar to our results, in the predominantly fast rectus femoris and longissimus dorsi muscles. These data suggest that, at least in fast muscles, hyperplasia is even more pronounced in *Cmpt* than in Mstn KO mice. This difference might reside in the allelic variation of Mstn defect and/or in modifier genes influencing *Cmpt* phenotype.

Although TA and EDL muscles contain predominantly type II glycolytic fibers, we observed a significant glycolytic shift within type II fibers in *Cmpt* mice. This result is consistent with that of Rehfeldt et al. (2005) who found more glycolytic fibers in the rectus femoris muscle of the special *Cmpt*-DUHi line. A substantial glycolytic shift has also been described in EDL muscles of Mstn KO mice as well as in double-muscled cattle by applying different methods, which suggests that both metabolic and structural protein changes occur upon Mstn deficiency. However, immunohistochemical staining of MHC isoforms is more appropriate to distinguish between fiber types. We have detected significantly more IIB fibers and less IIX or IIA fibers in TA muscles of *Cmpt* mice than in the wild type. Transcript levels were in agreement with our immunohistochemical results; MHCIIB mRNA levels were significantly higher, while those of MHCIIX and IIA significantly lower in *Cmpt* TA muscles compared with those genes in wild type muscles. These findings suggest that the glycolytic fiber-type shift in fast muscles is supported by respective changes of MHC transcripts.

Along this line, we measured the same ratio of IIB/IIX/IIA fibers in EDL muscles of *Cmpt* by using immunohistochemical methods. Amthor and colleagues (2007) demonstrated similar consequencies in regard to glycolytic shift in Mstn KO mice. Altogether, these data suggest that the function of the bigger and more glycolytic muscles might be impaired upon Mstn defect.

However, the oxidative SOL muscle has not shown any glycolytic shift in *Cmpt* mice (53% slow-oxidative type I fibers, 44% fast-oxidative IIA fibers) as compared with BALB/c line (38% type I fibers, 60% type IIA fibers). In contrast, these fibers were even more oxidative than the controls contradicting previous reports on Mstn KO mice. To conclude, different mechanisms of Mstn deficiency -with or without the influence of modifier genes- may induce differential and muscle-specific effects.

In summary, *Cmpt* mouse, inspite of its complex genetic background, shows similarities (at least in fast muscles) to Mstn KO mice in terms of muscle cellularity and glycolytic muscle phenotype, suggesting that the lack of Mstn is responsible for these morphological/functional changes. However, based on the more pronounced hyperplasia in *Cmpt* fast muscles as well as the

different cellularity and oxidative phenotype of *Cmpt* SOL, additional studies are needed to elucidate the molecular mechanisms of Mstn inactivity and the possible role of modifier genes in *Cmpt* mice.

Myostatin and IGF-I signaling in the human heart

Mstn and IGF-I both have been shown to play a crucial role in the pathomechanism of human heart failure with upregulated Mstn protein in the failing myocardium. However, the exact mechanism and their possible interplay in the course of heart failure have not been clarified yet.

In the present comprehensive qRT-PCR study we have found that Mstn dominated over IGF-I signaling much more in the LV than in the RV of healthy human hearts, and that DCM hearts upregulated Mstn expression in contrast to ICM hearts. This is the first demonstration that Mstn/IGF-I signaling differs in LV and RV in healthy hearts and shows significant alterations in end-stage heart failure due to DCM and ICM.

Several studies exist in the literature, indicating the presence and/or de-regulation of both Mstn and IGF-I under different conditions in the heart, however, the majority of data were collected from whole hearts or separately, from the LV. Regarding Mstn expression in healthy hearts, only one study has been published so far on higher transcript levels of Mstn in LV as compared to RV in young piglet hearts. In line with these data we have demonstrated here an obvious reciprocal regulation of Mstn and IGF-I in LV compared to RV characterized by elevated ratios of both Mstn/IGF-I and ActRIIB/IGF-IR transcripts in healthy human LV. Septum (S) samples, as being part of LV from the functional point of view, revealed similar values to those of LV in most cases. We assume that cardiomyocyte growth in LV/S should be balanced more tightly by growth inhibitors (i.e. Mstn) than that of RV since LV is exposed to higher-pressure overload, while RV to a relatively higher-volume overload. In addition to Mstn and IGF-I, several other factors have been reported to be enriched in either LV or RV; their asymmetric expression might reflect a molecular predisposition of myocardium to LV-concentric or RV-eccentric remodeling during postnatal development. Similarly, higher expression levels in LV versus RV have been demonstrated for cytochrome c oxidases and PGC1α, both of which are known to contribute to maintain mitochondrial function and oxidative metabolism. Recently, it has been shown that Mstn plays an important role in the regulation of oxidative metabolism of the myocardium. Therefore our results support the idea that the elevated ratio of Mstn/IGF-I signaling is an important regulatory mechanism under physiological conditions maintaining higher workload and oxidative metabolism in the LV. These results are also in accordance with the glycolytic shift in skeletal muscles upon Mstn defect and suggest that Mstn exert similar metabolic effects in both skeletal muscle and cardiac tissues, at least in the LV.

In human failing hearts (in whole hearts or LV), Mstn protein activation was reported to be accelerated in both DCM and ICM patients. In parallel with this observation several groups found increased serum levels of Mstn protein in patients suffering from heart failure, although no correlation was demonstrated with the severity or type of cardiac disease. One should also consider that elevated serum Mstn levels in cardiomyopathic patients might be a combined effect of increased secretion from both cardiac and skeletal muscles. Nevertheless, exercise training lead to a reduction of Mstn levels only in skeletal muscles but not in serum of patients with chronic heart failure. Intriguingly, it has not been clarified, whether protein activation in failing heart is regulated either at the level of gene expression or posttranscriptionally. Here we show that DCM hearts are indeed characterized by upregulated Mstn transcripts in both LV/S and RV supporting the previous reports on protein activation in LV. However, we could not detect any significant elevation of Mstn transcript levels in ICM patients. We also tested the miRNA-dependent posttranscriptional regulation of Mstn. miR-208 have been reported to be a negative regulator of Mstn expression, and to be upregulated in various forms of cardiomyopathy and myocardial ischemia. In line with these, we detected a mild upregulation of miR-208b in the LV of DCM patients characterized by massive increase of Mstn transcripts that might suggest an adaptive counter-regulatory mechanism fine-tuning the expression of Mstn during heart failure. Since we have found no difference in miR-208b expression between DCM and ICM hearts, it is likely that increased Mstn mRNA in DCM is mainly regulated at the level of transcription. Although alterations in Mstn gene expression are followed by similar changes at the protein level in most cases, the intra- or extracellular proMstn-pool might be posttranslationally activated by the cleavage of the propeptide. Thus, ICM patients might have a Mstn activation at the protein level, however, not at the level of transcription, whereas DCM hearts react with significant upregulation of Mstn transcripts. Similar to the results of George et al. (2010) we detected lower level of ActRIIB in ICM than in DCM patients, but this occured only in the LV. On the other hand, IGF-I expression was shown to be dynamically regulated in the course of heart failure as an important compensatory mechanism; however, conflicting data exist in the literature with both down- and upregulated IGF-I levels in end-stage heart failure. We could confirm significant decrease of IGF-I signaling in the RV of ICM patients but not in LV or DCM patients. Moreover, DCM patients still maintained the physiological difference in Mstn/IGF-I signaling ratios in LV versus RV, whereas no asymmetric gene expression pattern was detected in ICM patients. The mechanisms leading to different regulation of growth factor signaling in DCM and ICM patients remain to be clarified, however, it might relate to the different pathomechanism of heart failure and/or alternative regulation of compensatory mechanisms. Indeed, Mstn upregulation detected in DCM hearts might be part of adaptation reactions since Biesemann et al (2014) have recently

reported that acute cardiac-specific deletion of Mstn in adult mouse hearts induces dilated cardiomyopathy followed by a massive compensatory up-regulation of Mstn in non-cardiomyocytes. It is known, however, that various cardiac disease conditions, i.e. hypoxia can result in an imbalance of chamber-associated gene expression in myocardium. Therefore, ICM hearts of ischemic origin might not be able to compensate as effective as do DCM patients by upregulating Mstn to maintain oxidative metabolism and by regulating their IGF-I signaling to counteract decreased contractility. However, further research is needed to elucidate the physiological and pathological relevance of the complex Mstn/IGF-I network in human heart.

Conclusions

We have described different aspects of Mstn signaling in skeletal muscle and heart tissue by applying two different model systems: (i) the Mstn mutant hypermuscular *Cmpt* mice and (ii) healthy and pathological human hearts.

1. We have morphologically analyzed different glycolytic (TA, EDL) versus oxidative (SOL) skeletal muscles of *Cmpt* mice. All of them were significantly larger characterized by fiber hyperplasia of various grade. Fiber hypertrophy was either not present (TA) or confined to a specific type (IIB fibers in EDL) or in contrast, present in all fibers (SOL). These findings imply for muscle-specific morphological effects of Mstn defect in *Cmpt* mice.
2. Fast-type glycolytic muscles (TA, EDL) of *Cmpt* mice contained significantly more glycolytic IIB fibers as well as increased MHCIIB transcript levels. According to previous reports, similar glycolytic shift was observed in Mstn KO mice suggesting that the lack of Mstn might be responsible for these changes in both *Cmpt* and Mstn KO mice. However, the genetic background (modifier genes) might also have an impact, since the oxidative SOL muscle in *Cmpt* mice did not display any glycolytic alteration.
3. According to our human heart study we have found, that Mstn dominated over IGF-I signaling much more in the LV than in the RV of healthy human hearts. The spatial asymmetry in the expression pattern of Mstn/IGF-I is likely to play a role in the different growth regulation of LV versus RV.
4. We identified Mstn as a massively up-regulated gene in DCM but not in ICM as part of potential compensatory mechanisms in the failing heart.
5. Mild upregulation of miR-208b in pathological human hearts might serve as a Mstn counter-regulatory mechanism. Since miR-208b expression was similar in DCM and ICM hearts, higher levels of Mstn mRNAs in DCM might be maintained through accelerated transcription.

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