ASSOCIATION OF MYOSTATIN AND DECORIN IN THE MUSCLE OF CATTLE

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***Abstract* - The objectives of this study were to investigate the expression, localization, and interaction of myostatin (MSTN) and decorin (DCN) in bovine skeletal muscle and to find associations with muscle fiber and adipocyte development. Samples of two muscles, known for a different fiber composition, namely longissimus muscle (LD) and semitendinosus muscle (ST), were obtained from 18 months old bulls of the F2 generation of a Charolais x Holstein cross. Individual muscle sections were stained for determination of size and type of muscle fibers and immunohistochemical detection of the proteins. The mRNA abundance and protein expression of MSTN and DCN were analyzed by real-time PCR and Western blot, respectively. As expected, the ST had more fibers of the fast type, less fibers of the intermediate and the slow type, and less intramuscular fat than the LD. The abundance of MSTN and DCN mRNA was not significantly different between both muscles. At the protein level, mature MSTN was similarly expressed in both muscles, whereas its inhibitors MSTN propeptide and DCN were higher expressed in ST. Myostatin propeptide was detectable in all muscle fibers of adult cattle muscle. The mature MSTN was detectable to a much lower extent and mainly in slow fibers. Furthermore, MSTN was localized in close proximity to DCN in intermyocellular space, suggesting a possible interaction between both proteins. Co-localizations of MSTN and DCN were often observed near slow fibers or in connective tissue near developing adipocytes. Despite a comparable MSTN expression in both muscles, its biological activity may be different due to different amounts of inhibitors and possible interactions, suggesting a muscle specific regulation of MSTN action. The role of MSTN and DCN as well as their interactions in the determination of muscle composition needs to be further elucidated.**

***Index terms* - cattle, decorin (DCN), muscle, myostatin (MSTN)**

I. INTRODUCTION

Myostatin (MSTN), a member of transforming growth factor type beta (TGF-β) superfamily of growth factors, is a negative regulator of skeletal muscle mass, leading to a significant decrease in muscle mass, muscle fiber cross-sectional area, and muscle protein content when overexpressed (Durieux et al., 2007). Myostatin is secreted into the extracellular matrix where it can interact with Decorin (DCN, Miura et al., 2006). Decorin is a small leucine-rich proteoglycan that modulates the activity of TGF-β and other growth factors and thereby influences the processes of proliferation and differentiation in a wide array of physiological and pathological reactions (Brandan, Retamal, Cabello-Verrugio, & Marzolo, 2006). Decorin interferes with muscle cell differentiation and migration and regulates connective tissue formation in skeletal muscle and mRNA expression is therefore higher in fetal skeletal muscle than in neonates and adults (Casar, McKechnie, Fallon, Young, & Brandan, 2004; Yoshida N., Yoshida, S., Koishi, Masuda, & Nabeshima, 1998). Recent studies showed: (1) DCN can bind to MSTN and inhibit MSTN activity (Miura et al., 2006); (2) DCN enhanced the proliferation and differentiation of myogenic cells by suppressing MSTN activity (Kishioka et al., 2008); (3) MSTN administered to proliferating satellite cells depress the synthesis of DCN (McFarland, Velleman, Pesall, & Liu, 2007); (4) MSTN inhibits adipogenesis in 3T3-L1 cells, but could not alter lipolysis in fully differentiated adipocytes (Stolz et al., 2008). However, the association between MSTN and DCN in adult muscle of cattle is still uncertain. The objectives of this study were to investigate the expression, localization, and interaction of MSTN and DCN in skeletal muscle of adult cattle and to find associations with muscle fiber and adipocyte development.

II. MATERIALS AND METHODS

A. Animals and sampling

A sample group of 11 bulls, selected from a F2 resource population generated from the founder breeds Charolais and German Holstein (Kühn et al., 2002), was slaughtered at 18 months of age in the research institute's experimental abattoir according to a standardized protocol. All animals were cared for and slaughtered according to German rules and regulations for animal care. The experiment was approved by the institutional authorities and by the responsible office of the State of Mecklenburg-Western Pommerania, Germany.

Muscle tissue from longissimus dorsi (LD) and semitendinosus (ST) was collected separately for RNA extraction and histology within 30 min after slaughter, immediately frozen in liquid nitrogen, and then stored at -70°C until use. Carcass and meat quality traits were recorded.

B. Histological analysis

Samples of LD and ST muscles were cryosectioned using a Leica CM3050 S (Leica, Bensheim, Germany) cryostat microtome. The sections (10 µm thick) were stained with hematoxylin and eosin, for measurement of muscle fiber and fat cell size. Fiber types were detected using actomyosin Ca^{2+} adenosine triphosphatase stability after alkaline preincubation (pH 10.4) and staining with azure II (Chroma-Gesellschaft, Schmid, Köngen, Germany) as described by Wegner, Albrecht, Fiedler, Teuscher, Papstein, & Ender (2000). The muscle fiber and fat cell traits in individual skeletal muscles were analyzed using an image analysis system equipped with a Jenaval microscope (Carl Zeiss, Jena, Germany), an Altra20 CCD camera (OSIS, Münster, Germany) and CELL^D image analysis software (OSIS, Münster, Germany; Add-In Muscle-Fiber-Analysis, MAS, Freiburg, Germany).

C. RNA isolation and real-time RT-PCR

Total RNA was isolated from LD and ST muscles using Trizol Reagent (Invitrogen, Karlsruhe, Germany) according to manufacturer's instructions. Concentration and quality of the extracted RNA were measured using a NanoDrop ND-1000 Spectrophotometer (Peqlab Biotechnologie, Erlangen, Germany). The iScriptTM cDNA Synthesis Kit (Bio-Rad Laboratories, Munich, Germany) was used to synthesize cDNA from 100 ng of total RNA from each sample according to manufacturer's instructions. A negative control, without reverse transcriptase, was processed for each sample. The abundance of mRNA for ribosomal protein S18 (RPS18), MSTN, and DCN was quantified by real-time RT-PCR (iCycler, BioRad Laboratories, Munich, Germany). PCR was performed in 40 cycles with 180 s at 94°C, 10 s at 94°C followed by 30 s at 60°C and 225 s at 70°C. The sequences of specific bovine primers used were as follows: RPS18 (GeneID: 326602) forward: 5'-CTTAAACAGACAGAAGGACGTGAA-3', reverse: 5'-CCACACATTATTTCTTCTT GGACA-3'; DCN (GeneID: 280760) forward: 5'-AACTCTTTTGCTTGGGCTGA-3', reverse: 5'-CCAGAAGCCTCA TCTTCCAG-3'; MSTN (GeneID: 281187) forward: 5'-GTGTTGCAGAACTGGCTCAA-3', reverse: 5'-TCATCACA ATCAAGCCCAAA-3'. The specificity of amplification was determined by melting curve analysis and agarose gel electrophoresis. The cDNA structure was checked by sequencing. Each cDNA was quantified in triplicate; the average value of each sample minus the corresponding negative control value was used to calculate the cDNA product corresponding to the abundance of mRNA. The values were normalized to RPS18 mRNA.

D. Western blotting

Total protein was extracted from LD and ST muscles using CelLytic MT lysis reagent (Sigma-Aldrich, Munich, Germany) with protease inhibitor according to manufacturer's instructions. Protein extract, 50 µg, was mixed with loading buffer and denatured by boiling for 5 min before loading on a 12.5% SDS-PAGE gel. After electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) for 1 h at room temperature. The membranes were than incubated with the respective primary antibodies against α-tubulin (T6074, Sigma, St. Louis, USA), MSTN propeptide (ab37254, Abcam, Cambridge, UK), MSTN (AB3239, Millipore, Schwalbach, Germany), or DCN (H00001634-M01, Abnova, Taipei, Taiwan) at 4°C overnight. After washing, membranes were incubated with the respective secondary antibodies, either mouse IgG TrueBlot (18-8817) or rabbit IgG TrueBlot (18-8816, eBioscience, Frankfurt, Germany). Antibody label was detected with chemiluminescence substrate (Super Signal West Pico or Femto, PIERCE, Rockford, USA) and quantified using a Chemocam HR-16 imager (INTAS, Göttingen, Germany).

E. Immunohistochemical analysis

Muscle sections were fixed in ice cold acetone for 10 min. Unspecific bindings of the secondary antibody were blocked using 10% secondary antibody serum in PBS-TritonX100 (PBST) for 15 min. Sections were incubated with the respective primary antibody against MSTN-propeptide, MSTN, or DCN (as used for Western blots), for 1 h at room temperature in a humidity chamber. Specific binding of primary antibodies was detected with the respective goat anti-mouse or rabbit IgG secondary antibodies labeled with Alexa Fluor 488 (Molecular Probes, Eugene, USA). In MSTN-DCN double labeling experiments, MSTN was detected by an Alexa Fluor 594 labeled goat-anti-rabbit IgG and the DCN was detected by an Alexa Fluor 488 rabbit-anti-mouse IgG. Nuclei were counterstained with 1 µg/ml Hoechst 33258 (Sigma-Aldrich, Munich, Germany). Slides were covered using MobiGLOW mounting medium (MoBiTec, Göttingen, Germany) and appropriate cover-slips. Negative controls were incubated omitting the primary antibody. No unspecific binding of the secondary antibody was detected. Immunofluorescence was visualized with a Nikon Microphot SA fluorescence microscope (Nikon, Düsseldorf, Germany) and an image analysis system equipped with CELL^F software and a CC-12 high resolution color camera (OSIS, Münster, Germany).

F. Immune-electron microscopy

Muscle samples were fixed in 1% paraformaldehyde, dehydrated and embedded in acryl resin (LRWhite, hardgrade, Plano, Wetzlar, Germany). Samples were cut using an ultramicrotome (Ultracut S, Leica, Wetzlar, Germany) and transferred to grids (Plano, Wetzlar, Germany) and sections were incubated concurrently with antibodies against MSTN and DCN (as used for IHC). For detection, secondary antibodies conjugated either to 10-nm (anti-rabbit, G7402, Sigma-Aldrich, Munich, Germany) or 5-nm (anti-mouse, G7527, Sigma-Aldrich, Munich, Germany) colloidal gold particles were used. The immunogold-labeled proteins were visualized using a transmission electron microscope Libra 120 (Zeiss, Oberkochen, Germany).

G. Statistical analysis

Statistical analysis was performed using the SAS statistical software (Version 9.2, SAS Inst. Inc., Cary, USA). For comparison of the two muscles, data were analyzed by ANOVA using the MIXED procedure with fixed factor muscle and random animal. The t-test was used as post-hoc test with $P \leq 0.05$ as threshold for significant differences.
Relationships between traits were calculated as Pearson's-correlation coefficients using the CORR procedure of SAS.

III. RESULTS AND DISCUSSION

The study was conducted to investigate MSTN and DCN expression in muscles showing different muscle fiber size and profile, different meat quality properties, and intramuscular fat deposition. Meat quality and muscle structure data showed the expected clear difference between LD and ST muscles. Color, water holding capacity, and shear force value were lower and intramuscular fat content was higher in LD (Table 1). Based on myofibrillar ATPase activity, ST displayed a larger proportion of fast fibers and a reduced proportion of slow fibers compared with LD (Table 1). The muscle fiber area was larger for all three types in ST. The LD had therefore a higher muscle fiber density than ST. Intramuscular fat cell size was not different between muscles. Nevertheless, the samples varied sufficiently to enable the detection of possible associations between these traits and MST and DCN expression.
The mRNA abundance of DCN and MSTN was not significantly different between the muscles. However at protein level, we found higher amounts of MSTN propeptide and DCN in ST. Decorin can affect morphogenesis of the intramuscular connective tissue that supports muscle fibers, which is important for the tenderness of meat (McCormick, 1999). An increased DCN level could affect the formation of collagen fibers and therefore negatively influence meat quality. We found a positive relationship between shear force value and DCN protein abundance in ST ($r = 0.86$).

Table 1: Meat quality, muscle structure, and gene expression of longissimus and semitendinosus muscle

Trait	Longissimus muscle	Semitendinosus muscle	SE	P-value
Meat quality				
Brightness, L*	36.2	38.6	0.7	0.023
Water holding capacity, %	31.4	37.1	2.0	0.023
Shear force 24 h, kg	15.9	22.2	1.0	<0.001
Shear force 14 d, kg	10.3	14.9	0.7	<0.001
Intramuscular fat content, %	5.0	2.4	0.6	0.003
Muscle fiber cross sectional area, µm²				
Total	2,802	4,889	317	<0.001
Fast	3,481	5,713	364	<0.001
Intermediate	2,462	3,452	290	0.022
Slow	1,949	3,719	305	0.001
Muscle fiber type area %				
Fast	56.7	72.1	2.2	<0.001
Intermediate	28.8	17.7	1.8	<0.001
Slow	14.5	10.1	1.3	0.014
Muscle fiber number per cm²	36,299	22,602	1,983	<0.001
Fat cell cross sectional area, µm²	3,585	3,115	369	0.389
Gene expression, arbitrary units				
MSTN mRNA	0.118	0.177	0.027	0.122
DCN mRNA	0.506	0.740	0.111	0.138
MSTN-propeptide protein	3.01	4.39	0.36	0.014
MSTN protein	0.595	0.560	0.154	0.874
DCN protein	0.519	0.908	0.133	0.001

It is well known that overexpression of MSTN elicits a significant decrease in muscle mass, muscle fiber cross-sectional area, and muscle protein content (Durieux et al., 2007). On the other hand, the myostatin-null genotype produces "double muscling" in mice (McPherron, Lawler, & Lee, 1997) and cattle (McPherron & Lee, 1997). The absolute amount of MSTN expressed in skeletal muscle is only one indicator for its biological activity. There are several inhibitors of MSTN activity among them the MSTN propeptide (Dickson, 2009), and DCN (Miura et al., 2006). Interactions between these proteins require a physical proximity. We therefore investigated the localization of mature MSTN, its propeptide, and DCN. Myostatin propeptide was detected in all muscle fibers, with varying intensities, but was never seen outside the muscle fibers. For DCN and the mature form of MSTN, immunohistochemical and electron microscopic studies showed a distinct distribution of the proteins and a partial co-localization (Figure 1). Both proteins were often detected in close proximity to slow muscle fibers in the intermyocellular space or in connective tissue in the neighborhood of developing adipocytes. The co-localization of MSTN and DCN was supported by electron microscopic findings. This is the first report of a co-localization in bovine skeletal muscle and may be indicative for an interaction. However, co-localization is only a first indicator for protein-protein interactions. Miura et al. (2006) demonstrated that

DCN binds to MSTN in rat skeletal muscle and thus, modulates its biological activity. If DCN binds to MSTN and sequesters it in a biologically inactive state (Kishioka et al. 2008) surrounding muscle fibers could exhibit a larger growth. Although the mRNA abundance did not differ for MSTN and DCN between the two investigated muscles and the MSTN protein amount was similar, we detected significant differences in the protein amounts of the MSTN inhibitors MSTN propeptide and DCN. This could be an indicator for a muscle specific regulation of the MSTN action and consequently contribute to the different fiber properties in LD and ST. Further investigations are necessary to elucidate the role of MSTN and DCN in muscle development and composition of cattle.

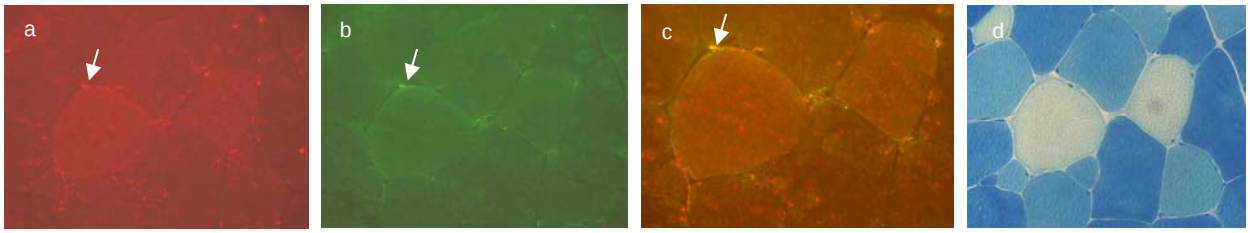

Figure 1: Immunohistochemical detection of MSTN (a: rabbit anti-MSTN and Alexa Fluor 594 goat anti-rabbit IgG) and DCN (b: mouse anti-DCN and Alexa Fluor 488 rabbit anti-mouse IgG) in muscle cross sections and the respective, magnified overlay (c) of double labeled section, and fiber typing in a serial section (d). Arrows show co-localization of MSTN and DCN.

IV. CONCLUSION

The study indicates that there are quantitative differences in the protein expression of MSTN inhibitors, MSTN propeptide and DCN, between muscles showing different muscle fiber sizes and different meat quality properties. Despite a comparable mRNA and protein abundance of MSTN in both muscles, its biological activity may be different due to different amounts of inhibitors. Our results provide first evidence for a similar mechanism of MSTN regulation in bovine skeletal muscle as previously described for the rat.

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