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Colocalization of myostatin and decorin in bovine skeletal muscle

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Abstract

The objectives of this study were to investigate the expression and localization of myostatin (MSTN) and decorin (DCN) in bovine skeletal muscle and to find associations with muscle fibre and adipocyte development. Samples of two muscles, known for differences in meat quality and fibre composition, namely *longissimus* muscle (LD) and *semitendinosus* muscle (ST), were obtained from 18 months old bulls of the F_2 generation of a Charolais×Holstein cross. Individual muscle sections were stained for determination of size and type of muscle fibres and immunohistochemical detection of the proteins. The mRNA abundance and protein expression of MSTN and DCN were quantified by real-time PCR and Western blot, respectively. As expected, the ST had more fast fibres, less fibres of the intermediate and the slow type, and less intramuscular fat than the LD. Despite these differences, the mRNA and protein abundance of MSTN was comparable in both muscles. The protein abundance of MSTN inhibitors, namely MSTN propeptide and DCN, was greater in LD, which may have affected the biological activity of mature MSTN. Myostatin propeptide was detected in all muscle fibres; however the mature MSTN was detected to a much lower extent and mainly in slow fibres. Furthermore, MSTN was localized in close proximity to DCN in intermyocellular space, suggesting possible interactions between both proteins and effects on muscle structure and meat quality. The role of MSTN and DCN as well as their interactions in the determination of muscle composition needs to be further elucidated.

Keywords: cattle, decorin (DCN), muscle, myostatin (MSTN)

Zusammenfassung

Co-Lokalisation von Myostatin und Decorin im bovinen Skelettmuskel

Ziel der Untersuchungen war es, die Expression und Lokalisierung von Myostatin (MSTN) und Decorin (DCN) im bovinen Muskel zu bestimmen und Beziehungen zur Muskelfaser- und Adipozytenentwicklung zu finden. Dazu wurden von 18 Monate alten Bullen der F_2 Generation eines Charolais×Holstein Kreuzungsexperiments Proben von zwei Muskeln (*M. longissimus dorsi* - LD und *M. semitendinosus* - ST) gewonnen, die für Unterschiede in der Fleischqualität und Faserzusammensetzung bekannt sind. Entsprechend gefärbte Serienschnitte wurden verwendet, um die Größe und das Typenprofil der Muskelfasern zu bestimmen sowie für den immunhistochemischen Nachweis der Proteine. Die mRNA- und Proteinmenge von

MSTN und DCN wurden außerdem mit real-time PCR und Western Blot quantifiziert. Wie erwartet hatte der ST mehr schnell kontrahierende Fasern und weniger intermediäre und langsam kontrahierende Fasern sowie weniger intramuskuläres Fett als der LD. Trotz dieser Unterschiede wurden in beiden Muskeln vergleichbare MSTN mRNA- und Proteinmengen gemessen. Im LD wurde jedoch mehr MSTN Propeptid und DCN Protein gefunden, die als MSTN Inhibitoren die biologische Aktivität beeinflussen können. Das MSTN Propeptid wurde in allen Muskelfasern nachgewiesen, dagegen die reife Form des MSTN in viel geringerer Menge und hauptsächlich in den langsam kontrahierenden Fasern. Weiterhin wurde MSTN zwischen den Muskelfasern in der Nähe von DCN lokalisiert, was auf mögliche Interaktionen zwischen beiden Proteinen und Effekte auf die Muskelstruktur und Fleischqualität hinweisen könnte. Die Rolle von MSTN und DCN sowie deren Interaktionen bei der Ausprägung der Muskelzusammensetzung bedarf weiterer Untersuchungen.

Schlüsselwörter: Rind, Decorin (DCN), Muskel, Myostatin (MSTN)

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 fibre 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 *et al.* 2006). Decorin interferes with muscle cell differentiation and migration and regulates connective tissue formation in skeletal muscle and mRNA expression is therefore higher in foetal skeletal muscle than in neonates and adults (Casar *et al.* 2004, Yoshida *et al.* 1998). Sun *et al.* (2010) reported associations between DCN gene polymorphisms and the birth weight of cattle. 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 *et al.* 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 fibre and adipocyte development.

Material and methods

Animals and sampling

A sample group of 11 bulls, selected from a F_2 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.

The calves were weaned from their mothers immediately after birth and received a milk replacer diet until day 121 *post partum*. This diet was gradually replaced by *ad libitum* access to hay and concentrates. Subsequently, the individuals were kept in a tie-stall barn on a daily *semi-ad libitum* feed ration, which was composed of chaffed hay and a concentrate (RM 2007; Vollkraft Mischfutterwerke, Rendsburg, Germany) with a hay-to-concentrate ratio of 1:3 and an energy content of 12.7 MJ ME/kg dry matter. The ration provided for a maximal average daily weight gain, while still being compatible with ruminant requirements.

Muscle tissue from *M. longissimus dorsi* (LD) and *M. semitendinosus* (ST) was collected separately for RNA extraction, protein extraction, and histology, immediately frozen in liquid nitrogen, and then stored at –70 °C until use. Carcass and meat quality traits were recorded. Brightness was measured with a Minolta CR 200 (Minolta GmbH, Ahrensburg, Germany) in triplicates on the freshly cut surface 24 h *post mortem* using the parameter L* (L=0 designates black and L=100 designates pure white). The water holding capacity was determined by the method descibed by Grosse *et al.* (1975). Shear force measurement was done 24 h and 14 days after slaughter as described by Otto and Stang (1975). The intramuscular fat content was obtained in triplicates via the Soxhlet extraction method using petroleum ether as solvent and determined gravimetrically after evaporating the extracting solvent (AOAC 2000).

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. Fibre 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 *et al.* (2000). The muscle fibre and fat cell traits in individual skeletal muscles were analyzed using an image analysis system equipped with Jenaval microscope (Carl Zeiss, Jena, Germany), Altra20 CCD camera (OSIS, Münster, Germany) and CELL^D image analysis software (OSIS, Münster, Germany). Adipocyte size was measured using the interactive measurement module. Where available, 200 to 300 adipocytes were randomly selected and measured, after following the contour using the interpolating polygon function. In muscle sections containing less adipocytes, all available cells were measured. Muscle fibre traits were measured with a special muscle fibre measurement module (MAS, Freiburg, Germany) of the same system as described in detail by Albrecht *et al.* (2011). A minimum of 300 muscle fibres per animal in randomly selected muscle fibre primary bundles, usually 4 to 6 fields of one section, were measured and classified.

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) and the StdSens RNA Assay of an Experion Automated Electrophoresis System (BioRad Laboratories, Munich, Germany). The RQI numbers of all samples were between 7.7 and 9.4. 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 45 s at 70 °C. The sequences of specific bovine primers used were as follows: RPS18 (GenBank accession No: NM_001033614; product size: 218 bp) forward: 5'-CTT AAA CAG ACA GAA GGA CGT GAA-3', reverse: 5'-CCA CAC ATT ATT TCT TCT TG GACA-3' (Tib Molbiol, Berlin, Germany); DCN (GenBank acc. No: NM_173906; product size: 218 bp) forward: 5'-AAC TCT TTT GCT TGG GCT GA-3', reverse: 5'-CCA GAA GCC TCA TCT TCC AG-3' (Sigma-Aldrich, Munich, Germany); MSTN (GenBank acc. No: NM_001001525; product size: 191 bp) forward: 5'-GTG TTG CAG AAC TGG CTC AA-3', reverse: 5'-TCA TCA CAA TCA AGC CCA AA-3' (Sigma-Aldrich, Munich, Germany). The specificity of amplification was determined by melting curve analysis and agarose gel electrophoresis. The cDNA structure was checked by sequencing. The reported sequences matched exactly to those published in GenBank. 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 mRNA abundance was calculated as pg/µg total RNA, using the known concentration of standard oligonucleotides and amplification efficiency displayed by the iCycler. The values were normalized to RPS18 mRNA.

Western blotting

Total protein was extracted from LD and ST muscles using CelLytic MT lyses 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 10×10 mini 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 MSTN propeptide (mouse anti human, ab37254, Abcam, Cambridge, UK), MSTN (rabbit anti human, AB3239, Millipore, Schwalbach, Germany), or DCN (rabbit anti human, sc-22753, Santa Cruz Biotechnology, Heidelberg, Germany) at 4 °C overnight. Cross reactivity with the respective bovine proteins were expected due to sequence homologies between synthetic peptides used for antibody generation and bovine sequences. 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 Femto, PIERCE, Rockford, USA) and a Chemocam HR-16 imager (INTAS, Göttingen, Germany). The intensity of specific bands was quantified using LabImage 1D Electrophoresis Software (Kapelan Bio-Imaging, Leipzig, Germany). Two independent blots were analyzed for each sample, whereby protein of both muscles of one animal was on the same blot. For comparability, individual band volumes were normalized dividing them by the average band volume of the blot.

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 (as used for Western blots), or DCN (mouse monoclonal, H00001634-M01, Abnova, Taipei, Taiwan), 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 labelled with Alexa Fluor 488 (Molecular Probes, Eugene, USA). In MSTN-DCN double labelling experiments, MSTN was detected by an Alexa Fluor 594 labelled 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 33 258 (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 antibodies 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 colour camera (OSIS, Münster, Germany).

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 immunohistochemistry). 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-labelled proteins were visualized using a transmission electron microscope Libra 120 (Zeiss, Oberkochen, Germany).

Statistical analysis

Statistical analysis was performed using the SAS statistical software version 9.2 (SAS Inst. Inc. 2008). 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.

Results and discussion

The study was conducted to investigate MSTN and DCN expression in muscles showing different muscle fibre size and profile, different meat quality properties, and intramuscular fat deposition. Meat quality and muscle structure data (Table 1) showed the expected clear difference between LD and ST muscles. Values of brightness, water holding capacity, and

shear force were lower ($P \leq 0.02$) and intramuscular fat content was higher ($P < 0.01$) in LD. Based on myofibrillar ATPase activity, ST displayed a larger proportion ($P < 0.01$) of fast fibres and a reduced proportion ($P < 0.01$) of slow fibres compared with LD (Table 1). The muscle fibre area was larger ($P \leq 0.02$) for all three types in ST. The LD had therefore a higher ($P < 0.01$) muscle fibre density than ST. Intramuscular fat cell size was not different ($P = 0.39$) between muscles. Nevertheless, the samples varied sufficiently to enable the detection of possible associations between these traits and MSTN and DCN expression.

Table 1
Meat quality and muscle structure traits of two muscles in 18 months old bulls

Trait	*Longissimus* muscle	*Semitendinosus* muscle	SE	*P*
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 fibre cross sectional area, μm^2				
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 fibre type profile, %				
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 fibre number per cm^2	36 299	22 602	1 983	<0.001
Fat cell diameter, µm	66.2	62.6	3.4	0.459

The mRNA abundance of DCN and MSTN (Figure 1) was not significantly different between the muscles ($P \geq 0.12$). A fibre type specific MSTN expression was mentioned in a review by Kobolák & Gócza (2002). Carlson *et al.* (1999) found higher MSTN expression in fast muscles than in slow type muscles and a correlation between the level of MSTN mRNA and the percentage of MyHC IIb in muscle. The difference in fibre type composition between LD and ST in our study was not accompanied by different MSTN mRNA levels. Posttranscriptional modifications and protein interactions may play a greater role. The used antibodies were able to discriminate between the mature form of MSTN and its propeptide. By means of Western blotting, the propeptide was detected at ~23 kDa, whereas the mature MSTN migrated to ~26 kDa. This was also described by McPherron *et al.* (1997) and Sharma *et al.* (1999), suggesting a homodimerization. The relative quantification revealed higher amounts of MSTN propeptide and DCN in LD ($P = 0.07$ and $P < 0.01$, respectively) compared to ST (Figure 2). The DCN antibody detected two bands, one at ~40 kDa, which corresponds to the theoretical size of the DCN core protein (http://www.uniprot.org/uniprot/P21793), and a second band around 60 kDa, which is caused by an undefined number of glycosaminoglycan chains (Wight *et al.* 1991). A protein band of similar size was also described by Eggen *et al.* (1994) for bovine muscle.

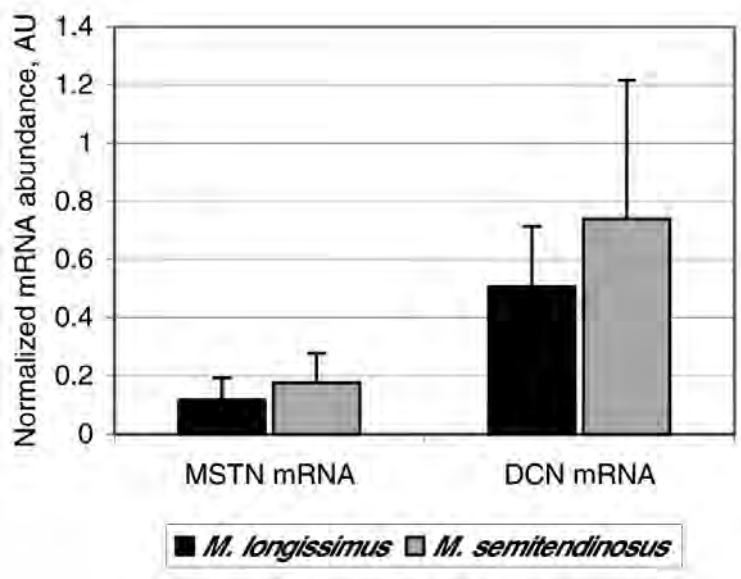

Figure 1
Abundance of myostatin (MSTN) and decorin (DCN) mRNA in two muscles of 18 months old bulls, normalized to RPS18 mRNA (AU - arbitrary units)

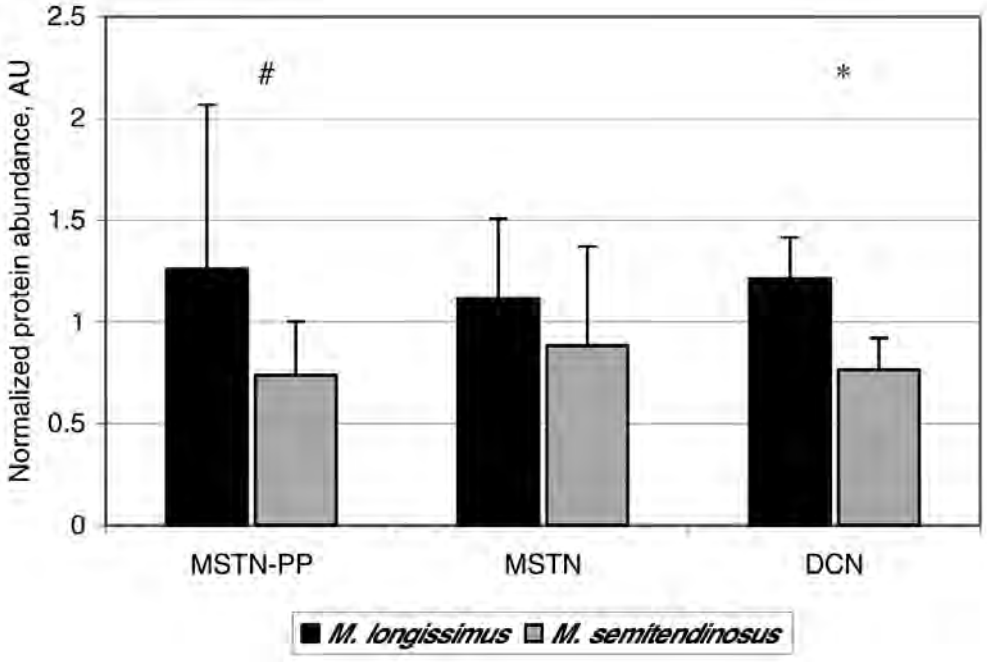

Figure 2
Normalized protein abundance (arbitrary units - AU) of Myostatin (MSTN) propeptide, mature MSTN, and decorin (DCN) in *M. longissimus* and *M. semitendinosus* (# $P<0.1$, * $P<0.05$)

Decorin can affect morphogenesis of the intramuscular connective tissue that supports muscle fibres, which is important for the tenderness of meat (McCormick 1999). An increased DCN level could affect the formation of collagen fibres and therefore negatively influence meat quality. However at 18 months of age, the LD had lower shear force values despite the higher DCN protein abundance. Thus, a direct association between protein abundance and meat quality traits could not be detected.

It is well known that overexpression of MSTN elicits a significant decrease in muscle mass, muscle fibre cross-sectional area, and muscle protein content (Durieux *et al.* 2007). On the other hand, the MSTN-null genotype produces »double muscling« in mice (McPherron *et al.* 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 fibres, with varying intensities, but was never seen outside the muscle fibres (data not shown). The mature form of MSTN was detected in slow myofibres and in the intermyocellular space. For DCN and the mature form of MSTN, immunohistochemical and electron microscopic studies showed a distinct distribution of the proteins and a partial colocalization (Figure 3, 4). Both proteins were often detected in close proximity to slow muscle fibres in the intermyocellular space (Figure 3) or in connective tissue in the neighbourhood of developing adipocytes. The colocalization of MSTN and DCN was supported by electron microscopic findings (Figure 4).

This is the first report of a colocalization in bovine skeletal muscle and may be indicative for an interaction. However, colocalization 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 fibres 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 fibre properties in LD and ST. Further investigations are necessary to elucidate the role of MSTN and DCN in muscle development and composition of cattle. The first demonstration of colocalization of MSTN and DCN in bovine skeletal muscle provides evidence for a similar mechanism of MSTN regulation as previously described for the rat.

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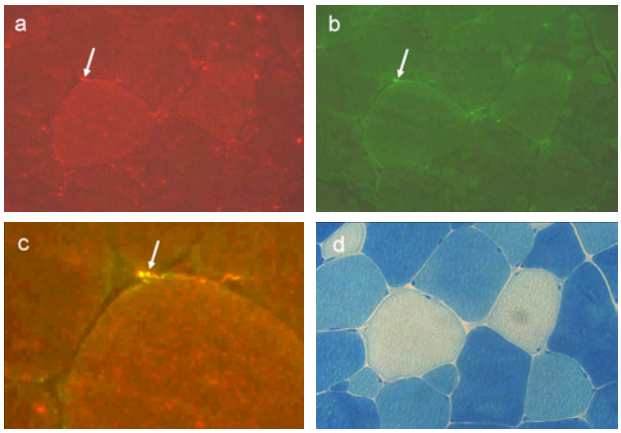

Figure 3
Immunohistochemical detection of MSTN (a: rabbit anti MSTN and Alexa Fluor 594 goat anti rabbit IgG) and DCN (b: mouse anti DCN and Alexa Fluor 488 rabbit anti mouse IgG) in muscle cross sections and the respective, magnified overlay (c) of double labeled section, and fibre typing in a serial section (d). Arrows show colocalization of MSTN and DCN, indicated by yellow color.

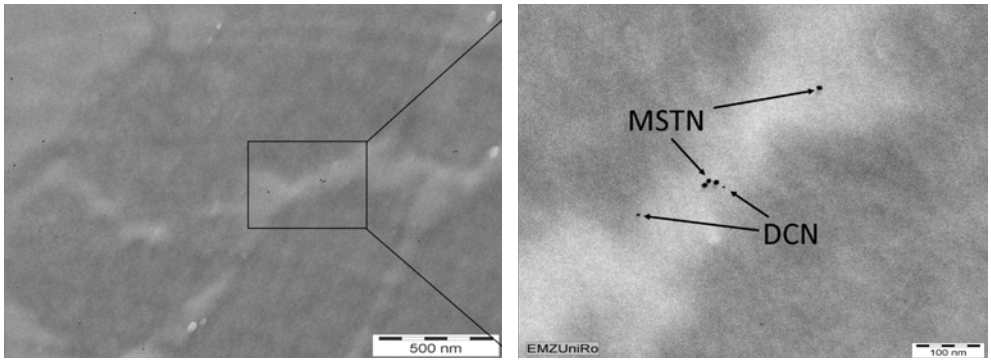

Figure 4
Immune electron microscopic detection of MSTN (rabbit anti MSTN, 10 nm colloidal gold labelled anti rabbit IgG) and DCN (mouse anti DCN, 5 nm colloidal gold labelled anti mouse IgG)

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