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Localization and abundance of early markers of fat cell differentiation in the skeletal muscle of cattle during growth — Are DLK1-positive cells the origin of marbling flecks?

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ABSTRACT

Localization and abundance of early markers of adipogenic differentiation were investigated in bovine muscle tissue to verify their association with marbling development. Bovine skeletal muscle samples were used for immunohistochemical localization and measurement of mRNA and protein abundance of delta-like homolog 1 (DLK1) and CCAAT/enhancer-binding protein beta (CEBPB). The localization of DLK1-positive cells confirmed the position of small clusters of adipocytes which can be considered as the origin of marbling flecks. The results indicated higher DLK1 expression in the less marbled muscle of Holstein (HS) steers ($P < 0.05$) and a trend to higher CEBPB expression in Japanese Black (JB) steers ($P < 0.1$) at slaughter. The number of DLK1-positive cells and fat content were negatively correlated. The lower expression of DLK1 together with higher CEBPB abundance during fattening in JB may have contributed to the development of more adipocytes in the skeletal muscle of JB.
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1. Introduction

Intramuscular adipose tissue or marbling is appreciated for its positive effects on palatability and tenderness of meat (Platter et al., 2005). The capability to develop intramuscular adipose tissue varies widely between breeds and also within breeds even under similar feeding conditions (Gotoh et al., 2009). During growth and fattening of cattle, the number and size of marbling flecks increase which is caused by the increasing number and size of adipocytes which are embedded in the connective tissue between muscle fiber bundles (Albrecht, Teuscher, Ender, & Wegner, 2006; Cianzio, Topel, Whitehurst, Beitz, & Self, 1985; Hood & Allen, 1973; Yang, Albrecht, Ender, Zhao, & Wegner, 2006). These adipocytes derive from progenitor cells which are able to proliferate and start to incorporate lipids upon stimulation (Gregoire, Smas, & Sul, 1998; Hausman et al., 2009; May et al., 1994; Rosen & Spiegelman, 2000). Terminal differentiation of an adipocyte needs the concerted action of peroxisome proliferator-activated receptor γ2 (PPARG2) and CCAAT/enhancer-binding proteins (CEBPs) to turn on lipid synthesis and other adipocyte-specific programs (Fernyhough, Okine, Hausman, Vierck, & Dodson, 2007; Hausman et al., 2009; Rosen & Spiegelman, 2000). Several genes are transiently expressed during differentiation of preadipocytes to mature adipocytes serving as markers for different stages of adipogenesis. Among them are proteins stabilizing the preadipocyte stage like delta-like 1 homolog (DLK1; also known as preadipocyte factor 1, PREF1), transcription factors of the CEBP family and PPARG2, fatty acid binding protein 4 (FABP4) and several adipokines which are produced and secreted by mature adipocytes.

Understanding cellular events leading to the development of intramuscular adipocytes and to individual differences in fat deposition can help to optimize production of high quality beef. In our earlier study (Albrecht et al., 2011), we investigated the cellularity of different adipose tissue depots in association with late markers of adipogenic differentiation and revealed not only the expected up-regulation with increasing fat deposition during growth, but also breed differences under similar feeding conditions. Our current investigation focused on early markers of adipogenic differentiation like DLK1 and CEBPB which were proposed as possible markers for the adipogenic potential in young animals by Pickworth et al. (2011).

The transmembrane protein DLK1 is involved in the differentiation of several cell types including adipocytes (Sul, 2009). Cell culture models demonstrated high expression in preadipocytes and complete disappearance during differentiation to adipocytes (Garcés, Ruiz-Hidalgo, Bonvini, Goldstein, & Laborda, 1999; Wang, Kim, Kim, & Sul, 2006). Thus, cells expressing DLK1 represent the very early stage of adipocyte development, before lipids are incorporated. Subsequently, CEBPB expression occurs and is accompanied by lipid incorporation and lipid droplet formation. This stage precedes the terminal differentiation of the adipocyte (Gregoire et al., 1998). Both genes, among others, were

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investigated on mRNA level by Wang et al. (2009) during growth and fattening of 2 crossbreeds of cattle and found differences between crossbreeds in young and older animals. We extended the investigation to protein expression and used a new approach for its quantification. Preadipocytes expressing DLK1 were detected using immunohistochemistry of muscle cross sections from biopsy samples and counted. We hypothesized that these DLK1-positive cells are the origins of marbling flecks in bovine muscle tissue.

If DLK1-positive cells represent preadipocytes waiting for a stimulus to start the adipogenic differentiation program, their localization and gene expression in muscle should be related to marbling development. We expected that DLK1 expression decreases during life of cattle when fat deposition increases and cattle with increased intramuscular fat (IMF) deposition like JB have lower overall DLK1 expression. However, CEBPB is expected to increase transiently during adipogenesis and would indicate adipogenic differentiation activity if up-regulated. Our objective was to clarify the localization and abundance of DLK1 and CEBPB in the skeletal muscle of cattle at different developmental stages. We investigated the muscle tissue of a fetus, neonatal calves, and adult cattle by immunohistochemistry, western blot analysis and RT-qPCR. Additionally, mRNA expression in different adipose tissues was investigated for comparison.

2. Materials and methods

2.1. Animals and sampling

Six Japanese Black (JB) and 6 Holstein (HS) steers were kept under equal conditions from 10 to 26 months of age as described by Albrecht et al. (2011). The animals were cared for and slaughtered according to the Guidelines for Animal Experiments in the Faculty of Agriculture of Kyushu University and to the laws of the Japanese Government (Law No. 105, Notification No. 6). Steers were raised in a pen with group feeding using the standard feeding system for the production of marbled beef (Gotoh et al., 2009). They were fed twice daily a high energy diet starting at 10 months of age until slaughter at 26 months of age. All steers had permanent access to water and to mineral salt blocks. Consumed feed of the groups was recorded daily.

Steers were weighed monthly and biopsy samples of subcutaneous fat (SCF) and *longissimus thoracis* (LT) were taken from the left side at the 12th to 13th rib region at 10, 14, 18, and 22 months of age by the shot biopsy technique (Bellmann, Wegner, Teuscher, Schneider, & Ender, 2004; Schöberlein, 1989; Wegner & Schöberlein, 1984). Samples were divided into SCF and 3 pieces of LT. Subcutaneous fat was placed in RNA later (Applied Biosystems, Tokyo, Japan) and frozen at −70 °C. Muscle samples were mounted on cork with Tissue Tek (tissue freezing medium, Sakura Finetechnical, Tokyo, Japan) and snap frozen in liquid nitrogen and stored for further processing, either for RNA extraction or for histology.

Samples of LT (between the 12th and 13th ribs) and IMF, intermuscular fat (INTER), SCF, perirenal fat (PERI), and visceral fat (VISC) were taken within 45 min after exsanguination and placed either in Tissue Tek (sample size ~5 × 5 × 5 mm) and snap frozen in liquid nitrogen or in RNA later (1 ml per sample of 100 mg) for 2 h and stored at −70 °C.

Additional muscle samples were taken from 4 German Holstein calves, slaughtered at day 4 of life, from an experiment described by Steinhoff-Wagner et al. (2011). Furthermore, muscle sections of a Holstein fetus at 6th month of gestation were used from the experiment described by Albrecht, Lembcke, Wegner, and Maak (2013).

2.2. RNA isolation and RT-qPCR

Muscle and fat samples taken during biopsies and/or at slaughter were used for RNA extraction with TRIzol reagent (Invitrogen GmbH, Karlsruhe, Germany) or the RNeasy Lipid Tissue Mini Kit (QIAGEN, Hilden, Germany), respectively, according to manufacturer's instructions. Details of RNA extraction, cDNA synthesis and qPCR were described elsewhere (Albrecht et al., 2011). Briefly, 1-μl aliquots of each RT reaction (1/20 of total) were primed, in each 10 μl PCR, using an iQ-SYBR Green Supermix (BioRad Laboratories GmbH, Munich, Germany) and gene-specific oligonucleotides (final concentration of 0.2 μM). The following specific primers were used: for DLK1 (GenBank accession No. NM_174037; product size: 200 bp) forward: 5′-GGCTTC ATGGACAAGACCTG-3′, reverse: 5′-TTGTAGCGCAGATTGGACAC-3′ and for CEBPB (GenBank accession No. NM_176788; product size 154 bp) forward: 5′-ACAGCGACGAGTACAAGATCC-3′, reverse: 5′-GACAGTTGCT CCACCTTCTTCT-3′ (TIB MOLBIOL, Berlin, Germany). PCR was performed in 40 cycles after an initial denaturation step (94 °C for 3 min) with 10 s at 94 °C, 30 s at 60 °C and 45 s at 70 °C. The specificity of amplification was determined by melting curve analysis and agarose gel electrophoresis. The oligonucleotide structure was checked by sequencing. The reported sequences matched exactly to those published in GenBank. Quantification of ribosomal protein S18 (RPS18) mRNA (GenBank accession No. NM_001033614; product size: 218 bp; primer forward: 5′-CTTAAACAGACAGAAGG ACGTGAA-3′, reverse: 5′-CCACACATTATTTCTT CTTGGACA-3′, TIB MOLBIOL, Berlin, Germany) was used for normalization. Each cDNA was quantified in duplicate. For comparison of breeds and biopsy time points, data were calculated as relative expression values and for breed comparisons within tissues, the ΔΔ-Ct method was used (Pfaffl, 2001).

2.3. Immunohistochemistry

Samples of LT were cryosectioned (10 μm thick) using a Leica CM3050 S (Leica, Bensheim, Germany) cryostat microtome, fixed in 4% paraformaldehyde for 15 min, washed 3 times in PBS, and permeabilized in PBS containing 0.1% TritonX100. Unspecific bindings of the secondary antibody were blocked using 10% goat serum in PBS-TritonX100 (PBS-T) for 15 min. Sections were incubated with primary antibody against either DLK1 (ab21682, ABCAM, Cambridge, UK) or CEBPB (sc-150, Santa Cruz Biotechnology, Heidelberg, Germany), each diluted 1:200 in PBS-T containing 2% goat serum, for 1 h at room temperature in a humidity chamber. After washing 3 times with PBS-T, sections were incubated for 45 min in the dark with a goat anti-rabbit IgG secondary antibody labeled with Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA). Nuclei were counterstained with 1 μg/ml Hoechst 33258 (Sigma-Aldrich, Munich, Germany) for 5 min, after washing sections 3 times with PBS-T. Slides were covered using a MobiGLOW mounting medium (MoBiTec, Göttingen, Germany) and appropriate cover-slips. Negative controls were incubated in PBS-T containing 2% goat serum instead of primary antibody. No unspecific bindings of the secondary antibody were detected.

Immunofluorescence was visualized with a Nikon Microphot SA fluorescence microscope (Nikon Instruments Europe B.V., The Netherlands) and an image analysis system equipped with CELL^F image analysis software and a CCD-12 high resolution color camera (OSIS, Münster, Germany). DLK1-positive cells were counted in 25 randomly selected images in neonatal calves covering a total area of >1.6 mm^2 and for other cattle 26 to 63 images covering a total area of >6.7 mm^2 per animal and time point. These values were used to calculate the number of cells per mm^2. The ratio between number of CEBPB positive nuclei and total number of nuclei was determined in 2 randomly selected images per animal and time point covering an area of 0.52 mm^2.

2.4. Protein extraction and western blotting

Total protein was extracted using CelLytic MT lyses reagent (Sigma-Aldrich, Munich, Germany) with protease inhibitor according to the manufacturer′s instructions. Protein extract, 40 μg, was mixed with loading buffer and denatured by boiling for 5 min before loading on a 12.5% SDS-PAGE Criterion Precast gel (BioRad Laboratories GmbH,

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