EXPRESSION OF THYROID HORMONE-RESPONSIVE PROTEIN (THRSP) IS RELATED TO INTRAMUSCULAR FAT IN A F_2-CROSS BETWEEN CHAROLAIS AND HOLSTEIN

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Abstract – Intramuscular fat (IMF) is a major determinant of beef quality but the underlying genetic mechanisms for its accumulation in skeletal muscle are only partially understood. The study aimed at identification of candidate genes for IMF deposition in cattle. Global gene expression was analyzed in *M. longissimus* of 2 x 10 bulls of a Charolais x Holstein F_2 population differing largely in IMF content. Among the differently expressed genes 4 were verified by quantitative PCR and revealed significant correlations to IMF and further marbling traits. Most of the candidate genes are known to be expressed predominantly in adipocytes. This made it likely that the observed differential expression resulted from higher IMF content of the muscle. In contrast, thyroid hormone-responsive protein (THRSP) is expressed in muscle as well as in fat cells. Thus, the observed 6-fold increase of expression in highly marbled animals may result from genuine expression differences and makes it a promising candidate for further investigations.

I. INTRODUCTION

Identification of genes influencing intramuscular fat (IMF) deposition in cattle is of interest since IMF is a major determinant of beef palatability and tenderness (1). To this end, several studies compared global gene expression profiles between (2, 3) or within (4) breeds and crosses with divergent marbling traits. Comparisons between breeds are more likely to identify a larger number of differentially expressed genes. However, expression differences related to the target traits must be dissected from those which are specific for a breed but not related to the trait of interest (5). On the other hand, phenotypic differences in IMF are often small within a breed thus exacerbating the identification of differently expressed genes. In contrast, our study employed bulls from a F_2-population derived from a Charolais x Holstein cross. The F_2 offspring is characterized by a high variability in IMF content and a well-defined genetic background.

The aim of this study was to identify candidate genes for IMF content in *M. longissimus* by comparative expression profiling. Furthermore, the relationships between expression of candidate genes and histological traits of *M. longissimus* and of the embedded IMF cells were elucidated.

II. MATERIALS AND METHODS

Animals and phenotypes

Each 10 bulls with either high or low IMF ("High", "Low") were selected from a F_2 population derived from Charolais and Holstein (6). Group selection considered equal appearance of sires in both groups. Bulls were kept under standardized conditions and slaughtered at an age of 18 months. Details of sampling and phenotyping were described elsewhere (7).

Analysis of gene expression

Total RNA was isolated from *M. longissimus* of the bulls at slaughter. Gene expression patterns were assessed using the GeneChip® Bovine Genome Array (Affymetrix, Santa Clara, USA). Between groups comparisons for single genes were done with Fisher's LSD test. Regulated pathways were identified with Ingenuity Pathway Analyses (IPA) software (Ingenuity Systems, Redwood City, USA).
Expression differences for selected genes were verified with RT-qPCR (iCycler MyiQ 2, BioRad, Munich, Germany) in duplicates (Table 1). The qPCR protocol included an initial denaturation step (95 °C for 3 min) followed by 45 cycles (95 °C for 10 s, 60 °C for 30 s, 70 °C for 45 s) and a final melting curve analysis. Crossing point values were determined automatically by iQ5 Software (Bio-Rad). The amplification efficiency E was calculated from a standard curve derived from six serial dilutions.

Table 1: Primers for RT-qPCR

Gene symbol	Primer (forward/reverse, 5'-3')
THRSP	GAGATGGAAGAGGCTGAGGA
	CAGGGTAAGATGGGTGAGGA
CIDEC	CCGTATTCATGGTCCTCCAC
	TGCCATAGAGAGTTGCCTTC
INSIG1	AGTCACCTTGGAGAGCCACA
	ACGGTCAAATGTCCACCAGA
ACLY	CAAGAAGGCAGACCAGAAGG
	CTGGGCGGTACAGCTTAGAG
ZFP423	GAGGAGAGGAATGAGGACGA
	TCCTTACTGGAGGGAGACGA
FNDC5	GGTAAGCTGGGATGTCTTGG
	CTGACCCTGGATGGATATGG
TOP2B	AAGAAAACAGCACCGAAAGG
	GAGGTCTGAGGGGAAGAGGT
B2M	CAGCTGCTGCAAGGATGG
	ATTTCAATCTGGGGTGGATG

Expression values were normalized to beta-2-microglobulin (B2M) and topoisomerase II beta (TOP2B). Expression differences between the two cattle groups (High vs. Low IMF) were analyzed using the REST algorithm (REST 2009, Qiagen, Hilden, Germany).

Statistical analyses

Phenotypic traits were analyzed with ANOVA (Statistica, StatSoft, Hamburg, Germany) considering group and sire as fixed effects. Between-breed comparisons for single genes on the microarray were done with Fisher's LSD test.

III. RESULTS AND DISCUSSION

Phenotypes

As expected, both groups of bulls selected for divergent IMF content in *M. longissimus* differed significantly in fatness traits despite comparable carcass weights. All fat depots were larger in the "High" group.

Meat quality traits were not significantly different between the groups (Table 2).

Table 2: Carcass and meat quality traits (LSM ± S.E.)

Trait	Low IMF	High IMF
Cold carcass weight (kg)	408.1 ± 8.1	389.7 ± 8.1
Visceral fat (kg)	34.2 ± 2.4^{A}	56.7 ± 2.4^{B}
Fat (% of CCW)	12.8 ± 1.0^{A}	21.7 ± 1.0^{B}
Protein (% of CCW)	15.1 ± 0.2^{a}	13.3 ± 0.2^{b}
LM area (cm²)	106.6 ± 4.0^{a}	92.5 ± 4.0^{b}
IMF content (LM; %)	1.9 ± 0.6^{A}	7.0 ± 0.6^{B}
pH (LM, 24 h p. m.)	5.51 ± 0.07	5.44 ± 0.07
Shear force (LM, 14 d p. m., kp)	11.6 ± 0.2	10.3 ± 0.2

a, b: $p<0.05$; A, B: $p<0.001$; CCW: cold carcass weight; LM: *Musculus longissimus*

Tenderness of *M. longissimus* was slightly but not significantly improved in "High IMF".

Muscle structure was not different between the groups, whereas marbling traits and fat cell size differed significantly (Table 3). The mean area of muscle fibers was not different between the groups. In contrast, bulls with high IMF content were characterized by a higher mean fat cell diameter which accounts at least in part for the larger fat area percentage in *M. longissimus*. The fat cells in the "High" group were connected to larger marbling spots as measured by the parameter "largest fat area in LM".

Table 3: Muscle fiber and marbling traits in *M. longissimus* (LSM ± S.E.)

Trait	Low IMF	High IMF
Fiber area (µm²/fiber)	2722 ± 166	3085 ± 157
Fiber number (n/cm²)	37553 ± 1891	33292 ± 1794
Fat area (%)	3.1 ± 0.8^{A}	9.6 ± 0.8^{B}
Largest area (mm²)	99 ± 42^{a}	243 ± 42^{b}
Cell diameter (µm)	81.8 ± 4.0^{A}	100.6 ± 4.0^{B}

a, b: $p<0.05$; A, B: $p<0.001$

Analysis of gene expression in M. longissimus

Analysis of microarray expression data in both groups revealed a total of 178 differentially expressed genes (DEGs; $p < 0.05$) out of 12,138 probe sets with present calls in at least 10 out of 20 animals. Thirteen genes fulfilled additional criteria (I) log expression > 6, (II) present in all 10 bulls of the group "High IMF", and (III) expression difference ≥ 1.3. All 13 genes were significantly up-regulated in the "High IMF" group. Among them, 6 genes belonged to gene sets defined by De Jager et al. (4). SCD (stearoyl-CoA desaturase) and INSIG1 (insulin induced gene 1) are members of the gene set for fatty acid synthesis whereas FABP4 (fatty acid binding protein 4, adipocyte), THRSP (thyroid hormone-responsive protein) and CIDEC (cell death-inducing DFFA-like effector c) belong to the gene set for triacylglyceride synthesis and storage. ACLY (ATP citrate lyase) belongs to the gene set of PPARgamma-signaling. Further genes assigned to above gene sets were increased in the "High IMF" group of our study but did not reach significance level. Next we selected 4 genes (THRSP, CIDEC, INSIG1 and ACLY) for verification of differential expression by RT-qPCR. Additionally, two putative, functional candidate genes for fatness traits not represented on the microarray were analyzed.

FNDC5 (fibronectin type III domain-containing protein 5) was recently proposed as related to muscle mass and obesity in human and mice (8, 9) and ZNF423 (zinc finger protein 423) was identified as potent promoter of adipogenesis in cattle (10).

Table 4: Validation of differentially expressed genes by RT-qPCR

Gene symbol	Fold change "High" vs. "Low"	
	Microarray	RT-qPCR
THRSP	6.06	6.34
CIDEC	3.22	4.31
INSIG1	1.78	2.07
ACLY	1.58	$1.55^{p=0.08}$
ZNF423	n/a	0.91
FNDC5	n/a	0.92

The results of RT-qPCR confirmed largely the expression differences revealed by microarray. Expression of THRSP, CIDEC and INSIG1 was significantly increased in the "High" group. The fold-changes were in the same magnitude like observed in the microarray analysis. The differential expression of ACLY however, was confirmed only as trend although the fold changes corresponded well. Both functional candidates, FNDC5 and ZNF423, were not differentially expressed between both groups (Table 4).

Ingenuity pathway analysis (IPA) of differentially expressed genes revealed significant enrichment only of genes related to lipid metabolism (Fig. 1).

This result conforms to the assignment of the candidate genes to the gene sets proposed by De Jager et al. (4). The concordance of results from both studies indicate that the genes identified here represent true candidates for marbling traits independent of the cattle population investigated. Further genes with a trend to differential expression in both groups of cattle could not be assigned to pathways or functional groups by IPA. However, a trend to increased expression was observed for genes related to cytoskeletal organization and cell expansion (PAK3 - p21 protein [Cdc42/Rac]-activated kinase 3; CAMTA1 - calmodulin binding transcription activator 1, MYLK - myosin light chain kinase; S100A10 - S100 calcium binding protein A10) in the "High IMF" group. This indicates a relationship between increased expression of genes of these functional classes and altered cellularity of IMF in high-marbled cattle.

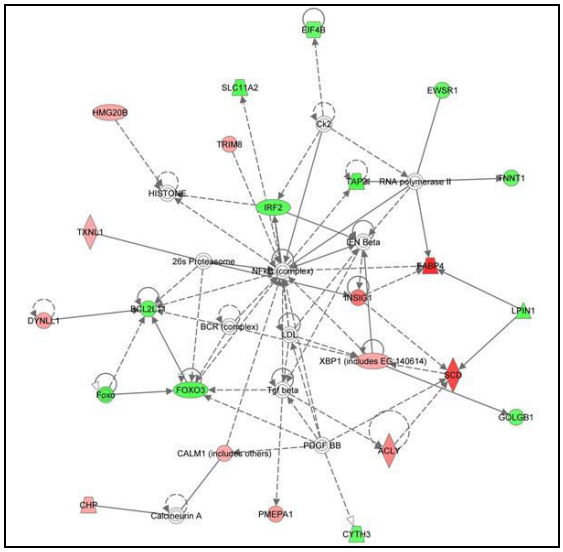

Fig. 1. Differentially expressed genes in pathway "lipid metabolism" (red: up-regulated; green: down-regulated)

Correlation analysis between individual gene expression levels of the investigated candidates and IMF revealed significant relationships for THRSP (r = 0.51), CIDEC (r = 0.56), INSIG1 (r = 0.48), and ACLY (r = 0.48). These correlation coefficients are higher than those reported by De Jager et al. (4) in a larger group (n = 48) for these genes (r = 0.14 – 0.30). Since IMF content is closely related to the fat cell parameters measured in our experiment, similar correlations exist between gene expression levels and these traits (r = 0.44 – 0.52, $p < 0.05$).

The bovine candidate genes were then analyzed on existing expression data in human tissues (GeneCards, 11). Comparison of gene expression levels between human skeletal muscle and adipocytes revealed three groups of genes. SCD and FABP4 are expressed predominantly in adipocytes (ratio adipocytes vs. skeletal muscle = 50 – 55 - fold). Thus higher expression values for these genes are likely to be caused by increased adipocyte number in skeletal muscle tissue rather than by increased expression in single adipocytes. A second group of genes (CIDEC, INSIG1, ACLY) are characterized by a 13 – 20 – fold higher expression in adipocytes than in muscle. Again, a higher number of adipocytes in muscle of bulls with increased expression of these genes may account for a significant part of the group differences. In contrast, THRSP is only 2-fold higher expressed in adipocytes compared to skeletal muscle. This makes it

likely, that the observed 6-fold increase in gene expression is not caused by higher adipocyte number to a large amount.
The genomic interval on bovine chromosome 29 where THRSP is located was recently identified as QTL for marbling (12). Furthermore, data from different cattle populations indicate that an increased expression of THRSP at different ontogenetic stages is related to marbling in cattle (13, 14).

IV. CONCLUSION

Most differently expressed genes, identified in a sample of this experimental F_2 cross, were predominantly expressed in adipocytes. Consequently, expression differences were likely to originate from increased adipocyte numbers in the muscle samples. In contrast, THRSP is expressed in muscle and fat cells similarly. The 6-fold increase of expression in the "High IMF" group qualifies THRSP as candidate gene for increased IMF deposition in bovine *M. longissimus*. Characterization of its physiological role in IMF deposition may shed light on this process.

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