Expression Profiling of Muscle Reveals Transcripts Differentially Expressed in Muscle That Affect Water-Holding Capacity of Pork

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To identify biological processes as well as molecular markers for drip loss, a parameter for water holding capacity of meat, the M. longissimus dorsi transcriptomes of six divergent sib pairs were analyzed using Affymetrix Porcine Genome Array. Functional categories of differentially regulated transcripts were determined by single-gene analysis and gene set analysis. The transcripts being up-regulated at high drip loss belong to groups of genes functionally categorized as genes of membrane proteins, signal transduction, cell communication, response to stimulus, and cytoskeleton. Among genes down-regulated with high drip loss, functional groups of oxidoreductase activity, lipid metabolism, and electron transport were identified. Differential regulation of the abundance of transcripts of these biological networks in live muscle affect mortem biochemical processes of meat maturation. Knowledge of this functional link is indicative for the identification of candidate genes for improvement of meat quality.

KEYWORDS: Gene expression; microarray; gene set analysis; muscle; pig; drip loss

INTRODUCTION

Variability in meat quality traits such as water holding capacity (WHC) is a major concern for both the meat industry and consumers. Meat quality largely depends on stressors, slaughter, and storage conditions, which are relevant at ante and post mortem, and on properties of muscle expressed during life, which are marked by muscle structural and metabolic features (*1, 2*). Loss of water and soluble constituents occurs during storage of meat at different levels. It is principally caused by shrinking myofibrils because of changes in pH and temperature post mortem (*3*). Rapid pH decline while muscle temperature is still high causes denaturation of many proteins, including those involved in binding water. Genetically determined muscle properties and environmental effects influence the WHC and the rate change of pH post mortem, that are still not completely understand (*4, 5*). Functional genomics provide new opportunities for determining the molecular processes underlying phenotypic differences (*6, 7*). The complexity of the relationship

between physiological characteristics of the live muscle and meat quality is probably the reason why so few attempts have been made to improve meat quality by monitoring the physiological prerequisites of the muscle at the time of slaughter. Transcriptome profiles of M. longissimus dorsi of animals providing meat with high and low WHC offers insight into the biological processes in the muscle and the maturing meat and their influence on meat quality. Therefore, in this study, M. longissimus dorsi expression profiles of six discordant sib pairs selected from 572 F2 animals of a cross of Duroc and Pietrain were analyzed.

MATERIALS AND METHODS

Animals and Tissue Samples. M. longissimus dorsi samples were taken from animals of the F2 generation of a cross of the commercial breeds Duroc and Pietrain, known to differ in meat and carcass properties. Out of 572 F2 animals of the DUPI population, six discordant sib pairs were selected from the tails of the distribution of the trait drip loss. The discordant sib pair design was applied to increase the probability of detecting differential expression due to differences in particular genes affecting the trait of interest, rather than due to the overall genetic background or bias arising from family effects (*6, 8–10*). Whereas the average WHC was 1.92 ± 0.94% drip (mean ± standard deviation), among the 572 DUPI, the two extreme groups of six discordant sib pairs had 4.14 ± 0.77 vs 0.9 ± 0.77% drip (mean ±

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standard deviation) ($p < 0.0001$). The number of animals was chosen as to provide a compromise between necessary phenotypic differentiation between the sibs, which limits the number of suitable sib pairs within the population, and adequate representation of biologic variation among the extremes. The animals used in this study were free of the Halothane gene mutation (RYR1; C1843T), ensured by DNA testing prior to the experiment. Animals were slaughtered at 180 days of age, and tissues samples were taken immediately and snap-frozen. Ranking of animals with regard to WHC measured as percent drip loss was done on the basis of residuals of this phenotype after preadjustment by analysis of variance taking into account the carcass weight and slaughter date.

WHC Measured As Percent Drip Loss. Drip loss was scored on the basis of the bag method using a size-standardized sample from the M. longissimus dorsi that was collected at 24 h post mortem. The samples were weighed, suspended in a plastic bag, held at 4 °C and reweighed 48 h later (*11, 12*). Drip loss was calculated as a percentage of lost weight based on the starting weight of a sample.

Whole Genome Expression Profiling. Total RNA was isolated from M. longissimus dorsi tissue samples taken immediately after slaughter using TRI reagent (Sigma, Taufkirchen, Germany). After DNaseI treatment, the RNA was cleaned up using the RNeasy Kit (Qiagen, Hilden, Germany). The quantity of RNA was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop, Peqlab, Germany), and the integrity was checked by running 1 μg of RNA on 1% agarose gel. Muscle expression patterns were assessed using in total 12 Affymetrix GeneChip porcine Genome Arrays (Affymetrix, U.K.), which contain 23 937 probe sets that interrogate ~23 256 transcripts from 20,201 S. scrofa genes. Preparation of antisense biotinylated RNA targets from 5 μg of total RNA, hybridization, and scanning on a GeneChip scanner 3000 were performed according to Affymetrix protocols. The quality of hybridization was assessed in all samples following the manufacturer's recommendations. Data were analyzed with Affymetrix GCOS 1.1.1 software using global scaling to a target signal of 500. Data were then imported into Arrays Assist software (Stratagene Europe, Amsterdam, The Netherlands) for subsequent analysis. The data were processed with MAS5.0 to generate cell intensity files (present or absent) (*13*). Quantitative expression levels of the present transcripts were estimated using PLIER (Probe Logarithmic Intensity Error) (*14*) for normalization. The microarrays data related to all samples were deposited in the Gene Expression Omnibus (GEO) (*15*) public repository (GEO accession number: GSE11193: GSM257696, GSM257715, GSM257716, GSM257720, GSM257722, GSM257760, GSM257697, GSM257708, GSM257717, GSM257719, GSM257759, GSM257761).

Data Analyses, Statistics and Functional Annotation. Paired *t* tests, with differences considered significant at $p < 0.05$, were performed among the samples of the two groups with six biological replicates per group. Significance analysis of microarrays (SAM) (*16*) was applied to identify genes with highly reproducible expression changes. SAM estimates differential expression from the ratio of the differences of expression levels at the different states as compared to the standard deviation obtained from the repeated measurements for that gene (*17*). A gene is considered significantly changed if it exceeds an adjustable threshold. SAM calculates a FDR, which represents the median percentage of genes that are incorrectly identified as significant (*17*). On the basis of these statistical analyses, a list of genes showing significant trait-associated transcript abundance was established independent of the direction of regulation between the two groups of animals with high and low WHC. These genes were assorted and clustered to pathways and functional categories annotated by KEGG (*18*), and Gene Ontology database (GO) (*19*) using the DAVID bioinformatic resources (*20*). Once the relevance of any functional pathway or category was indicated by this analysis, gene sets were defined and acquired from GO. These gene sets were used to perform gene set enrichment analyses as implemented in SAM, SAM-GS (*21*).

Quantitative Real-Time RT-PCR (qPCR). Differential expression data of selected genes obtained from porcine oligonucleotide microarray were validated by using real-time RT-PCR carried out in a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) using LightCycler DNA Master SYBR Green I (Roche). The reaction mixture

Table 1. Primers for Quantitative Real-Time RT-PCR

probe set ID	gene	primer sequence 5'—3'
Ssc.309.1.S1_at	VTN	GTGTCCTGGACCCCAATTAC GCTGCTGCTGGAACACATAC
Ssc.1894.1.S1_at	AMBP	CCGGAGGACTCCATCTTTAC GCAGGAATCTTCTTTCTTGCT
Ssc.7090.1.A1_at	SERPINA1	AAGACACCGTATTTGCTCTGG AGCTTGTCACAGTGGTGGAG
Ssc.955.1.S1_at	CYP2C	CGGAATACTTCGTGCCTTTC ACCGAAATGGGTTTGGTCTC
Ssc.30334.1.A1_at	CYP3A	TGCATTCGATGGATGATTAGA CACTTTGGCCACCACAATAAC
Ssc.16645.1.S1_at	AHNAK	TGTCACTGGCTCACCAGAAG GTCGCTGAAGGAATTTGAGC
Ssc.9365.5.S1_at	IGF2	CGTCTACGCAAGGCCAAC AAGGCCAAGAAGACGAGCA
Ssc.25503.1.S1_at	UNKNOWN	AGTCTGGTCTTACTTGCTTGCT CCAGAATCTCTGGGCTTGG
Ssc.507.1.A1_at	TYROBP	ATCTGGTGCTGACCCTCCT CTGTGTGTTGAGGTCGCTGT
Ssc.16678.1.S1_at	ZYX	CCCAGGGATAAAGTGAGCAG GTGCAGGCTTGGTATTGGAC

consisted of cDNA, 5 μM up-stream and down-stream primers, and LightCycler DNA Master SYBR Green I (1X). The template was amplified by 45 cycles of 95 °C for 15 s of denaturation, 60 °C for 10 s of annealing, and 72 °C for 15 s for extension, preceded by initial denaturation of 95 °C for 10 min as a universal thermal cycling parameter. On the basis of the analysis of melting curves of the PCR products a high temperature, the fluorescence acquisition point was estimated and included in the amplification cycle program. For all assays, a standard curve was generated by amplifying serial dilutions of specific PCR product. Normalization of variation in PCR efficiency and initial RNA/cDNA input was performed by using the *RPL*32 gene as the internal standard. The sequences of primers for qPCR are shown in **Table 1**. The same 12 individual samples of muscle mRNA used for microarray analysis were used in dublicate. qPCR data from the discordant sib pairs were analyzed using paired *t* tests, and differences were considered significant at $p < 0.05$ (SAS version 8.02; SAS Institute, Cary, NC).

RESULTS

As described previously, WHC depends on many factors, so ranking of animals with regard to drip loss was done on the basis of the residual of this phenotype after correction for the covariable carcass weight and the effect of the slaughter date. The sib pair design of extremes for the drip loss phenotype was used to reduce possible bias due to the genetic background. The two groups with high and low drip loss phenotype consisting of six discordant sib pairs showed drip loss that different by more than 3%, corresponding to more than 3 standard deviations of the trait.

From each M. longissimus dorsi RNA sample of these animals, 23,256 expression measurements were obtained using the Affymetrix GeneChip porcine genome array. On the basis of BLAST comparison of EnsEMBL human cDNA and genomic sequences and the Affymetrix porcine target sequence, which were extended with porcine sequence information of the Pig Gene Index (Institute for Genome Research, TIGR), 19,675 of 24,123 transcripts on the Affymetrix GeneChip porcine Genome Arrays, representing 11,265 unique genes, were annotated (*22*). This source of annotation list was used in this study. After processing the Affymetrix CEL files with MAS5, where a "present call" was assigned, this preselected data set was further analyzed with the more sophisticated hybrid algorithm PLIER (*14, 23*). The quality check of arrays after normalization by displaying
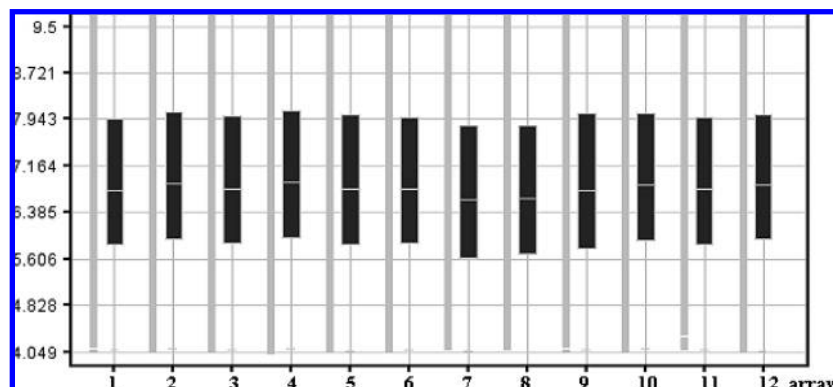
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Figure 1. Boxplots displaying the average normalized $\log_2$ gene expression intensity value (*y* axis) of all 12 Affymetrix Porcine microarrays after MAS5 and PLIER as well as variance stabilization. The box plot results indicated adequate normalization with small variation across the arrays.

Table 2. GO Categories of Genes with up Regulation with High Drip Loss

		genes in category		EASE
		number	proportion, %	score
Functional Group 1; Median: 1.00×10^{-4}				
GOTERM_CC_ALL	membrane	198	34.26	1.71×10^{-6}
GOTERM_CC_ALL	integral to membrane	142	24.57	1.00×10^{-4}
GOTERM_CC_ALL	intrinsic to membrane	142	24.57	1.15×10^{-4}
Functional Group 2; Median: 7.72×10^{-5}				
GOTERM_BP_ALL	signal transduction	118	20.42	3.01×10^{-5}
GOTERM_BP_ALL	cell communication	124	21.45	7.73×10^{-5}
GOTERM_BP_ALL	intracellular signaling cascade	50	8.65	4.75×10^{-2}
Functional Group 3; Median: 0.0025				
GOTERM_BP_ALL	antigen processing	7	1.21	3.01×10^{-4}
GOTERM_MF_ALL	MHC class I receptor activity	5	0.87	6.57×10^{-3}
Functional Group 4; Median: 0.0135				
GOTERM_MF_ALL	transmembrane receptor activity	34	5.88	1.73×10^{-4}
GOTERM_BP_ALL	G-protein coupled receptor protein signaling pathway	19	3.29	1.62×10^{-2}
Functional Group 5; Median: 0.0181				
GOTERM_BP_ALL	immune response	38	6.57	8.93×10^{-5}
GOTERM_BP_ALL	defense response	39	6.75	2.01×10^{-4}
GOTERM_BP_ALL	response to biotic stimulus	40	6.92	4.40×10^{-4}
GOTERM_BP_ALL	response to external stimulus	23	3.98	2.54×10^{-2}
GOTERM_BP_ALL	response to stress	42	7.27	1.40×10^{-1}
Functional Group 6; Median: 0.0362				
GOTERM_CC_ALL	cytoskeleton	41	7.09	3.57×10^{-2}
GOTERM_CC_ALL	nonmembrane-bound organelle	69	11.94	3.62×10^{-2}
GOTERM_CC_ALL	intracellular nonmembrane-bound organelle	69	11.94	3.62×10^{-2}

box plots confirmed adequate normalization (**Figure 1**). After calculation using MAS5 and PLIER probe set algorithms, a total of 12,966 probe sets were used for further analysis.

Expression Profile between High and Low Drip Loss. Comparative expression profiling by hybridization of the Affymetrix GeneChip porcine genome arrays revealed 789 differential expressions of transcripts between high and low WHC group at $p < 0.05$. Significance analysis of microarrays (SAM) revealed a corresponding false discovery rate of about 35%. Of 789 transcripts, 166 showed down-regulation in the high drip loss group (Supporting Information Table 1). Only 23 of this down-regulated transcript showed a fold change higher than 1.5. The expression level of 34 transcripts was found to be 1.5-fold higher in the high drip loss group.

To identify functionally related gene sets that are differentially perturbed in high and low drip loss, two methods were applied: over representation analysis using Fisher's exact test and significance analysis of microarray for gene sets (SAM-GS). Statistically, significantly overrepresented GO terms associated with genes up- and down-regulated in the high drip loss group are shown in **Tables 2** and **3**. Six

Table 3. GO Categories of Gene with Down-Regulation with High Drip Loss

		genes in category		EASE
		number	proportion, %	score
Functional Group 1; Median: 1.00×10^{-4}				
GOTERM_MF_ALL	oxidoreductase activity	16	10.26	0.005
GOTERM_BP_ALL	electron transport	9	5.77	0.035
GOTERM_BP_ALL	generation of precursor metabolites and energy	10	6.41	0.214
Functional Group 2; Median: 0.0341				
GOTERM_BP_ALL	alcohol metabolism	8	5.13	0.011
GOTERM_BP_ALL	cholesterol metabolism	4	2.56	0.028
GOTERM_BP_ALL	steroid metabolism	5	3.21	0.029
GOTERM_BP_ALL	lipid metabolism	9	5.77	0.187
GOTERM_BP_ALL	cellular lipid metabolism	7	4.49	0.297

functional groups were found to be significant at the median of EASE score of 0.05 for the positively trait associated genes. The transcripts up-regulated with high drip loss belong to groups of genes functionally categorized as genes of membrane proteins and transmembrane receptor activity,

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Table 4. Gene Sets Significantly Enriched with Genes Being Either Up- or Down-Regulated in Samples with High Drip Loss vs Low Drip Loss As Revealed by SAM-GS ($p < 0.0001$; FDR < 0.0001)

gene set name	description	origin of genes sets
	Gene Sets of Up-Regulated Genes at High Drip Loss	
functional group 1	integrin signaling: integrins are transmembrane receptors binding to ligands in the extracellular matrix	MSigDB
functional group 2	cancer-related genes involved in the cell signaling	MSigDB
functional group 3	proteins associated with membranes	GO
functional group 4	signal transducer activity	GO
functional group 5	response to stimulus	GO
functioanl group 6	cytoskeleton	GO
	Gene Sets of Down-Regulated Genes at High Drip Loss	
functional group 7	genes involved in fatty acid metabolism	MSigDB
functional group 8	ceramide is a lipid signaling molecule that can activate proliferative or apoptotic pathways	MSigDB
functional group 9	genes involved in tyrosine metabolism	MSigDB
functional group 10	oxidoreductase activity	GO
functional group 11	metabolism	GO
functional group 12	response to stimulus	GO
functional group 13	calcium ion binding	GO
functional group 14	electron transport	GO

Table 5. The Fold Changes (FC) of Expression Levels between High and Low Drip Loss from qPCR as Compared to Microarray Experiments

probe set ID	gene name	qPCR			microarrays		
		FC	p value	regulate	FC	p value	regulate
Ssc.309.1.S1_at	VTN	3.45	0.036	down	2.86	0.045	down
Ssc.1894.1.S1_at	AMBP	4.05	0.042	down	2.79	0.057	down
Ssc.7090.1.A1_at	SERPINA1	3.92	0.042	down	2.70	0.055	down
Ssc.25503.1.S1_at	UNKNOWN	2.01	0.009	down	1.51	0.021	down
Ssc.955.1.S1_at	CYP2C	3.49	0.004	down	2.76	0.037	down
Ssc.30334.1.A1_at	CYP3A	4.42	0.001	down	1.69	0.019	down
Ssc.16645.1.S1_at	AHNAK	1.65	0.006	up	1.40	0.047	up
Ssc.9365.5.S1_at	IGF2	1.33	0.096	up	1.40	0.009	up
Ssc.16678.1.S1_at	ZYX	1.325	0.085	up	1.35	0.007	up
Ssc.507.1.A1_at	TYROBP	1.83	0.056	up	1.71	0.050	up

Table 6. Comparison of Log 2 of Relative Transcript Abundance Obtained by qPCR and Microarray Analyses of 10 Genes and Coefficient of Correlation between qPCR and Microarray Results

probe set ID	gene	qPCR	microarray	coefficient of correlation	p value
Ssc.309.1.S1_at	VTN	7.88	7.63	0.99	<0.0001
Ssc.1894.1.S1_at	AMBP	4.12	6.95	0.99	<0.0001
Ssc.7090.1.A1_at	SERPINA1	6.46	7.14	0.97	<0.0001
Ssc.955.1.S1_at	CYP2C	10.76	6.44	0.70	0.0118
Ssc.30334.1.A1_at	CYP3A	8.45	5.36	0.85	0.0004
Ssc.16645.1.S1_at	AHNAK	14.62	11.05	0.80	0.0018
Ssc.9365.5.S1_at	IGF2	9.07	6.25	0.73	0.0073
Ssc.25503.1.S1_at	unknown	9.31	7.54	0.98	<0.0001
Ssc.507.1.A1_at	TYROBP	6.90	6.82	0.75	0.0050
Ssc.16678.1.S1_at	ZYX	10.56	7.36	0.76	0.0044

signal transduction, antigen processing, and immune response as well as those of the cytoskeleton. Among the genes down-regulated with high drip loss, categories of the functional groups of oxidoreductase activity and lipid metabolism were identified (**Table 3**).

Gene set analysis evaluates the expression of members of biological pathways or an a priori defined gene set rather than that of individual genes. The gene set was obtained from a GO category, KEGG pathways, and the Molecular Signature Database (MSigDB) (*24*). On the basis of all 789 genes differentially expressed between the high and low WHC group in the study, a total of 40 gene sets were created by assigning the genes to functional groups according to GO or KEGG pathways. These gene sets were supplemented by 1600 gene sets obtained from MSigDB that consist of functional groups reasonable for our study on muscle and meat properties but which also cover other functional groups with highly specific impact on the original studies, mostly in human cancer science. Only meaningfully significant gene sets with FDR < 0.0001 are shown in **Table 4**. Gene sets of integrin signaling and cell signal obtained from MSigDB as well as those of membrane, signal transducer activity, response to stimulus, and cytoskeleton assembled here were enriched in the high drip loss group. In the low drip loss group, eight gene sets were found to be enriched with fatty acid metabolism, ceramide pathways, tyrosine metabolism, oxidoreductase activity, metabolism, defense response, calcium ion binding, and electron transport.

Quantitative Real-Time RT-PCR Validation of Microarrays. qPCR was used to confirm differential expression as indicated by microarray expression patterns. Several differential expressed genes between high and low drip loss were selected for validation of the microarrays results. Six genes were selected for qPCR that were down-regulated [vitronectin (VTN); AMBP protein (AMBP); serpin peptidase inhibitor,

clade A (α-1-antiproteinase, antitrypsin); member 1 (SER-PINA1); cytochrome P450, family 2, subfamily C (CYP2C); cytochrome P450, family 3, subfamily A (CYP3A); and a nonannotated locus (unknown)], and four genes were chosen that were up-regulated as drip loss increases [TYRO protein tyrosine kinase-binding protein (TYROBP), AHNAK nucleoprotein (AHNAK), insulin-like growth factor 2 (IGF2), and zyxin (ZYX)]. The expression levels of all genes obtained by qPCR were normalized to ribosomal protein L32 (RPL32). The fold change between the groups designated high and low detected by qPCR was in most cases slightly higher than by microarray experiments (**Table 5**). The *p* values of the differences of transcript abundance between the high and low WHC groups ranged from 0.009 to 0.096 (**Table 6**). The correlations of qPCR and microarrays calculated for the log 2 of the ratio of transcript levels obtained in the high vs the low drip loss group varied between 0.70 and 0.99 with high statistical significance.

DISCUSSION

Here, we report on transcript profiling for getting more insight into biological processes involved in the occurrence of extreme drip loss phenotypes. Microarray technology is a powerful tool that permits measurement of the expression of many thousands of genes simultaneously to gain a better physiological understanding (*6, 7, 25*). There are a number of studies of expression profiles of muscle during fetal muscle development that have implications for muscle structural properties and, thus, also for meat quality traits (*26–28*). Here is the first report about pigs concerning comparative expression profiling of phenotypical divergent individuals in WHC that aims to elucidate the biological processes taking place in live muscle (immediately after slaughter) and affecting properties of meat (72 h post mortem). Ten genes were selected for evaluation of trait

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associated expression by qPCR. All of them showed corresponding fold changes of expression between the extreme phenotypes by microarray analysis and by qPCR, except IGF2 and ZYX, which did not reveal significant expression differences by qPCR but by microarray analysis only. The correlation between qPCR data and Affymetrix microarray data was high as compared to previous studies with other long oligonucleotide microarrays (*6, 29*).

Comparative Expression Profiling of Muscle Tissue with High and Low Drip Loss. Drip loss is a quantitative trait with low heritability, so many environmental factors play a significant role (*30, 31*). In this study, nongenetic effects were accounted for by performing analysis of variance, including systematic impacts on drip, such as slaughter day and weight of carcass, and subsequent use of residuals estimated for the phenotypes when ranking the animals and selecting suitable discordant sib pairs. The analysis of divergent sib pairs selected from a high number of animals on the basis of preadjusted phenotypes ensured display of the best contrasts on the level of genetic control of the target phenotype. On one hand, the high number of genes that are addressed in microarray experiments enabled us to identify genes with trait-dependent expression. These genes could further be assigned and clustered into groups according to their common functions, and hypotheses on the physiological pathways that are implicative for the expression of the phenotype could be derived. On the other hand, however, due to the very high number of genes (each with different dependencies on intrinsic and extrinsic factors) that are addressed in microarray experiments, the identification of differential expressed genes between the two groups of animals with high and low drip loss is often still tainted by high false discovery rates. In the microarray analysis, no individual gene meets the threshold for statistical significance after correcting for the testing of multiple hypotheses. This is because the relevant biological differences between high and low drip are modest relative to the noise inherent to microarray expression profiling. However, validation of microarray data by qPCR revealed a high correspondence of the analyses and confirmed differential expression for 8 out of 10 genes tested. Thus, single-gene analysis provided lists of genes with trait-dependent expression (either up- or down-regulated at acquiescent statistical settings) suitable for building of a hypothesis on the physiological background and underlying pathways and genes of differential trait expression. Too stringent thresholds when detecting differentially expressed genes might hamper bioinformatics pathway analysis and biological interpretation of the data in terms of generating hypotheses (*32*). Therefore we intended to first identify regulated genes due to trait variation by single-gene analysis, to subsequently suggest relevant functional networks of genes, then to set up gene sets that are finally used in gene set enrichment analysis. Gene set enrichment analysis (GSEA) was developed to overcome the challenges of microarray analysis related to high numbers of simultaneous tests (*33*). Significance analysis of microarray for gene sets, which has overcome the limitations of GSEA was selected to analyze the gene set method (*21, 34*).

Single Gene Analysis Using Over-Representation Analysis. Comparative gene expression profiles of M. longissimus dorsi samples with high and low drip loss were evaluated by single-gene analysis. This analysis revealed lists of genes with trait-dependent expression at $p \leq 0.05$ that were used for functional annotation to get hints on relevant functional networks and to derive new or support existing hypotheses on the physiology of the trait.

Many studies have shown that degradation of the cytoskeleton and other structural proteins plays an important role in drip loss

at post mortem (*35–38*). Correspondingly, in this study, transcripts in the functional categories of membrane proteins, cytoskeleton, and other structural proteins were found to be enriched among the up-regulated genes in the high drip loss phenotype. This implies that up-regulation of such transcripts in live muscle also plays an important role in final meat quality. Some examples of genes in these categories will be discussed. Titin is a giant filamentous elastic protein that spans from the Z-disk to M-band regions of the sarcomere (*39*). Titin also contains binding sites for muscle-associated proteins, so it serves as an adhesion template for the assembly of the contractile machinery in muscle cells. The titin gene locus was shown to be associated with drip loss and color and other meat quality traits (*1, 37, 40*). Physical training studies shown that the up-regulation of titin expression after exercise may be an adaptative mechanism to increase muscle elasticity and stability in response to the physical burden in the repair process following damage or involvement in continuing adaptation during endurance training (*41, 42*). Here in this study, it was found that up-regulation of titin expression in muscle is associated with high drip loss in meat ($p = 0.022$, Supporting Information Table 1). It may be evident that high expression of such marker molecules is due to repair processes taking place due to the physical impact on muscle that finally leads to lower meat quality.

Phenotypic variation among individuals may be due to variation in adaptability to exogenous stressors. This is in line with the finding that biological processes that were up-regulated with high drip loss were also those covering the response to biotic and abiotic stimuli. The negative impact of preslaughter stress on meat quality traits, including drip loss, has been established for a long time (*43, 44*). The transcripts of genes involved in the maintenance of homeostasis might be the potential markers of the changes in muscle cells relevant for meat quality. Furthermore, high levels of titin transcripts in live muscle may also contribute to an increased source of muscle structural proteins that are shrinking during meat maturation and, thus, increasing drip loss. This may also be valid for AHNAK. AHNAK, a 700 kDa protein, is expressed in a variety of cells and has been implicated in different cell-type-specific functions (*45–47*). In muscle cells, AHNAK is localized to the sarcoplemma membrane and T-tubules (*48*). Recently, the carboxyl-terminal AHNAK domain was identify to link the Ca^{2+} channels to the actin-based cytoskeleton (*49*) and exerts a stabilizing effect on muscle contractility via its interaction with the actin of thin filaments (*47*). Microarray analysis and qPCR showed that AHNAK was up-regulated in the high drip loss group. The overexpression of this gene in the muscle state influences the final state of the meat quality; however, the biological process is still unknown. Further studies are needed to address this process.

The change of biochemical and energy metabolism during early post mortem plays an important role in the final meat quality (*50*). Restricted oxidative capacities results in the development of intramuscular acidosis post mortem and impaired meat quality (*51*). This view is supported by the finding of down-regulation of transcripts of the functional group of oxidoreductase activity, electron transport, and generation of precursor metabolites and energy, as well as lipid metabolism in the high drip loss group of animals. Here, we have showed that reduced transcriptional activity of genes involved in the oxidative capacity of skeletal muscle is associated with a high drip loss.

CYPs are primarily membrane-associated proteins that are located either in the inner membrane of mitochondria or in the

endoplasmic reticulum of cells. Three of the CYP genes, which belong to the CYP2C and the CYP3A families, were found significantly down-regulated in high drip with more than 1.5 factors in microarray and qPCR analysis. This family of genes belongs to the GO category of electron transport as well as oxidoreductase activity. CYP3A and CYP2C are members of the cytochrome P450 mixed-function oxidase system and catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids, and other lipids. Most CYPs are predominantly found in the liver, but they are also present in other organs and tissues of the body, where they may play an important role in metabolism. Cytochrome P450 is associated with skeletal muscle sarcoplasmic reticulum (*52*). Induction of cytochrome P450 and the consequent formation of reactive intermediates in the metabolism of some compounds result in the activation of calcium-release channels (*53*). Genes of the GO category of lipid metabolism were also enriched in the low drip loss group. This finding corresponds to the negative phenotypic correlation that exists between the carcass traits related to fatness (back fat, fat area, as well as fat-to-meat ratio) and drip loss in DUPI population (−0.19, −0.27, −0.31, respectively). High to moderate negative (−0.70, −0.42) genetic correlations between intramuscular fat and drip loss and cooking loss were reported in Duroc by Suzuki et al. (*54*).

Significance Analysis of Microarray for Gene Sets. On the basis of the results of the single-gene analysis indicating the relevance of particular pathways for the trait drip, gene sets were derived and complemented by selected gene sets covering related pathways of the molecular signaling database in order to perform SAM-GS. The functional groups of membrane, signal transducer activity, response to stimulus, and cytoskeleton, which showed significant enrichment in the high drip group as revealed by the single-gene analysis, were also found by SAM-GS; however, with higher statistical confidence. In addition, other functional categories were identified from the gene sets selected from the MSigDB; namely, integrin signaling and cell signaling that were associated with high drip (FDR < 0.0001). Furthermore, five additional pathways were found to be negatively associated with drip (ceramide pathways, tyrosine metabolism, metabolism, defense response, calcium ion binding) by SAM-GS (FDR < 0.0001). Interestingly, SAM-GS revealed that genes of pathways representing response to stimuli are activated in either the high or low drip group, indicating that any extreme deviation from the populations mean is partly due to distortion of homeostasis due to insufficient adaptation to exogenous effects. In fact, the two major extreme deviations of meat quality, DFD, dark firm dry on the one hand, and PSE, pale soft exudative, on the other hand, occur as a result of insufficient response to stimuli affecting muscle cell metabolism. Interestingly, most of the genes found either up- or down-regulated at fold changes higher than 1.5 belong to the functional group of response to stimulus. When using single-gene analysis at commonly used thresholds of fold change at 1.5 or higher, only these genes would have been identified, and a considerable proportion of information would have been mimicked. Many of the genes with a modest difference between high and low drip loss belong to metabolic processes or muscle structure. This functional network with modest regulation due to the trait can by identified by gene set analysis with high confidence (*21, 33*).

We have demonstrated here that differentially expressed transcript profiles of M. longissimus dorsi offer an insight into the biological processes in the live muscle that affect the process of meat maturation and, finally, meat quality. Knowledge of this functional link will shed light on the genes responsible for individual variation in meat quality and thus be promising for the development of new DNA-based breeding tools for improved meat quality. The gene set analysis method provides improved statistical confidence in identification of trait-dependent regulated pathways and genes, especially at modest levels of difference.

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