Tissue-specific mRNA expression patterns reveal a coordinated metabolic response associated with genetic selection for milk production in cows

R. Weikard, T. Goldammer, R. M. Brunner, and C. Kuehn

Research Unit Molecular Biology, Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany

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CATTLE EVOLVED THROUGH THE slow process of natural selection until human domestication, which rapidly accelerated the development of specific, phenotypically and genetically different breeds selected for traits desirable to humans, primarily for milk and meat production. The general mechanisms at the endocrine and autocrine level controlling partitioning of absorbed nutrients between body tissues and compartments are well known (3, 5, 12, 18, 67), but the genetic and molecular determinants that modulate how ruminants transform nutrients preferentially into body tissues or milk are not completely understood.

The onset of lactation in cows is accompanied by increased nutrient requirements for milk synthesis, for which the glucose supply to the mammary gland is a metabolic priority. The metabolism of the cow has to adapt to lactation-associated challenges and requires major adjustments in liver and peripheral tissues, which also result in the mobilization of endogenous body reserves (5, 14, 32, 67). A variety of transcriptomic and proteomic single-tissue studies have been performed to elucidate the metabolic adaptation associated with lactation in dairy cows with a focus on different stages of lactation or metabolic and environmental conditions (2, 9–11, 36, 38, 39, 42, 45, 46, 48).

To investigate the genetic and physiological background underlying the phenotypic variation of nutrient partitioning and the mechanisms associated with genetic selection for meat and milk production in cattle, a unique F2 resource cross population was established from Charolais (CH) and German Holstein (GH) founder breeds (40). Although the CH and GH founder breeds, which represent beef and dairy type cattle breeds, are of comparable body weight and size, they differ substantially in their muscle growth and milk performance traits (6, 26, 27, 57, 68, 77). Previous studies revealed that heifers of the founder breeds and F2 cows of the Charolais × German Holstein (CH×GH) cross population showed remarkable differences in milk yield and hormone levels (22, 23, 57). The aim of the present study was to elucidate tissue-specific molecular mechanisms affected by metabolic modulation due to genetically determined lactation performance in segregating F2 offspring of this unique CH×GH resource population with a combined genetic dairy and beef background (CH×GH) compared with purebred dairy cows (GH) that had been selected for high milk production performance. We hypothesized that genetic background and phenotype for milk production affect the molecular mechanisms on gene expression level and the coordinated gene expression patterns associated with metabolic adaptation processes.

Therefore, we investigated mRNA expression levels of genes included in pathways of glucose and energy metabolism in tissues crucial for metabolic adaptation during lactation. Target genes for quantitative real-time PCR (qRT-PCR) were selected due to their key functional role in ruminant energy metabolism pathways with a specific focus on glucose metabolism. The selected genes are involved in different biochemical pathways including gluconeogenesis, glyceroconeogenesis, propionate metabolism, tricarboxylic acid cycle, insulin binding, and signaling, as well as transcriptional regulation of gluconeogenesis and mitochondrial biogenesis (Table 1). These pathways are potential targets of modulation by genetic selection for milk production. Tissues selected for our study included the mammary gland as the target tissue for milk synthesis, the liver as the most active metabolic organ during lactation, which is critical in nutrient signaling and regulation of feed intake, and the skeletal muscle, which accounts for most of the body mass of the cow and contributes to mobilization of endogenous body reserves to preserve glucose and amino acid supply for mam-

Address for reprint requests and other correspondence: R. Weikard, Research Unit Molecular Biology, Leibniz Institute for Farm Animal Biology (FBN), 18196 Dummerstorf, Germany (e-mail: weikard@fbn-dummerstorf.de).
mammary milk production and physiological maintenance processes.

MATERIALS AND METHODS

Animals and phenotypic data. The study involved 30 lactating cows from the F2 generation of a CH×GH cross population [SEGFAM, (40)] and purebred GH. Environmental conditions, feeding, and housing were identical for all cows. The CH×GH and GH cows were at a similar stage of lactation, housed and fed as described elsewhere (22), and milked twice daily. At 42 days in milk, the GH cows had a total milk yield (days 1–42: MY-42d) in the range from 1,062 to 1,442 kg, and milked twice daily. At 42 days in milk, the GH cows had a total milk yield (days 1–42: MY-42d) in the range from 1,062 to 1,442 kg, whereas for the F2 CH×GH cows, MY-42d varied between 209 and 1,158 kg. On average, high milk yield (MY) levels comparable to high milk performance, MY-7d in the range from 10 to 20 kg/day, (Table 2). Each group consisted of 10 animals. Individual MY was recorded daily, and milk composition was determined weekly. Energy-corrected milk (ECM-7d) was calculated according to Kirchgessner: (37)

$$ECM - 7d = \frac{0.37 \cdot F\% + 0.21 \cdot P\% + 0.95}{3.1} \cdot MY - 7d,$$

where F% is milk fat percentage, and P% is milk protein percentage (as determined by routine milk recording). At slaughter, the cows were ~100 days in milk. Tissue samples from liver, skeletal muscle (M. longissimus dorsi), and mammary gland (parenchymal tissue) were collected immediately 30 min after slaughter. The tissues were

<table>
<thead>
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<th>Symbol</th>
<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Function</th>
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<td>Phosphoenolpyruvate carboxykinase 1 cytosolic isoform</td>
<td>NM_174737</td>
<td>Glucose homeostasis, regulation of hepatic gluconeogenesis, glyceroneogenesis</td>
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<tr>
<td>G6PC</td>
<td>Glucose 6 phosphate subunit</td>
<td>NM_001076124</td>
<td>Glucose homeostasis, regulatory function in gluconeogenesis and glycogenolysis</td>
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<td>Glucose homeostasis, involved in gluconeogenesis from glucogenic amino acids, lipogenesis, insulin secretion and gluconeogenesis</td>
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<tr>
<td>FBP1</td>
<td>Fructose 1,6 bisphosphatase 1</td>
<td>NM_001034447</td>
<td>Glucose homeostasis, regulation of gluconeogenesis</td>
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<td>FBP2</td>
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<td>Insulin receptor</td>
<td>XM_590552</td>
<td>Insulin binding, stimulation of glucose uptake, regulation of glycolysis, glucose homeostasis</td>
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<td>NM_177945</td>
<td>Regulation of transcription, energy metabolism, mitochondrial biogenesis</td>
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</table>

Table 1. Target genes investigated by qRT-PCR and their physiological function

Table 2. Milk performance traits of the experimental cow groups

<table>
<thead>
<tr>
<th>Trait</th>
<th>Group</th>
<th>Trait Data</th>
<th>Analysis of Variance P Value</th>
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<tbody>
<tr>
<td>Mean milk yield-7d</td>
<td>GH</td>
<td>32.68 ± 2.78</td>
<td>&lt;0.0001</td>
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<tr>
<td>(kg/d), MY-7d</td>
<td>CH×GH-M</td>
<td>16.75 ± 5.41</td>
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<tr>
<td></td>
<td>CH×GH-L</td>
<td>3.21 ± 3.29</td>
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<tr>
<td>Mean energy corrected</td>
<td>GH</td>
<td>35.34 ± 5.85</td>
<td>&lt;0.0001</td>
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<td>milk yield-7d</td>
<td>CH×GH-M</td>
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<td>(kg/d), ECM-7d</td>
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<td>Total milk yield-42d</td>
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<td>(kg), MY-42d</td>
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<td></td>
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<td>Fat yield-7d</td>
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<td>(kg/d)</td>
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<td>CH×GH-L</td>
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<tr>
<td>Fat/protein ratio</td>
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<td></td>
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Cows were divided into 3 experimental groups according to their mean milk yield during 7 days (MY-7d) before the end of the experiment (slaughter). GH, high milk performance, MY-7d >30 kg/d; CH×GH-M, medium milk performance, MY-7d between 10 and 20 kg/d; L, low milk performance, CH×GH-MY-7d <10 kg/d; MY-42d, total milk yield of cows from 1–42 days in milk.
cut in small pieces, snap-frozen in liquid nitrogen, and stored at −80°C until use.

The experimental procedures were carried out according to the animal care guidelines with respect to welfare and health of the animals and were approved by the relevant authorities of the State Department of Agriculture, Food Security and Fisheries, Mecklenburg-Western Pomerania, Germany.

RNA preparation and quantitative RT-PCR. Total RNA was extracted from liver and mammary gland using the Nucleospin Extract II kit (Macherey & Nagel, Düren, Germany) according to the manufacturer’s instructions. In general, prior to RNA extraction, tissue samples (30 mg) were transferred into lysis tubes prefilled with 1.4 mm ceramic beads and the respective lysis buffer/reagent and homogenized for 2 × 15 s at 5,000 rpm using the Precellys-24 tissue homogenizer (peQLab, Erlangen, Germany). For skeletal muscle tissue, an initial lysis/prepurification step using the TRIzol reagent (Invitrogen, Darmstadt, Germany) was carried out. Genomic DNA was carefully eliminated from RNA preparations by repeated on-column digestion using twice the concentration of RNase-free DNase I (Invitrogen, Darmstadt, Germany) was carried out. Genomic DNA was purified from liver and mammary gland using the Nucleospin Extract II kit (Macherey & Nagel), and pooled. The purified cDNA pool was finally diluted with one volume of DNase/RNase-free water.

Gene-specific intron-spanning primers for qRT-PCR were designed using the OLGKO Primer Analysis Software (MedProbe, Oslo, Norway). The primers used in qRT-PCR were selected in intron-overlapping exons located near the 3’ terminus of the respective gene. Primer sequences (Table 3) were analyzed for secondary structure formation, G/C content, primer-dimer formation, hairpin formation, and PCR reaction compatibility as implemented in the OLGKO primer analysis software. The specificity of qRT-PCR primers was checked by BLASTing against the *Bos taurus* reference transcriptome (RefSeq mRNA database at http://blast.ncbi.nlm.nih.gov/ and http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome).

Table 3. Primer sequences and PCR conditions of genes analyzed using qRT-PCR

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<th>Primers</th>
<th>Primer Sequences (5’-3’)</th>
<th>Amplicon Size, bp</th>
<th>Annealing Temperature, °C</th>
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The first 2 primers (For and Rev) in a column represent the forward and reverse primers used for qRT-PCR; the third primer (RT) is a gene-specific primer used for reverse transcription.

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Primers were synthesized and purified by reverse-phase cartridge chromatography (Sigma-Aldrich Chemie, Steinheim, Germany). Amplified cDNA fragments were purified using the NucleoSpin Extract kit II (Macherey & Nagel) and verified by sequencing.

Quantitative real-time PCR was performed in duplicate on a LightCycler 2.0 Real-Time PCR System (Roche Applied Science, Mannheim, Germany) with the LightCycler FastStart DNA MasterPLUS SYBR Green I system (Roche Applied Science) and gene-specific primers (Table 3). The qRT-PCR analyses were performed in a final assay volume of 10 μl containing 2 μl 5× SYBR Green Hot start mix PCR buffer, 0.4 μM gene-specific primers, and 5 μl cDNA aliquot equivalent to 50 ng of total RNA input in first-strand cDNA synthesis. The cycling conditions consisted of a single preincubation step for 10 min at 95°C followed by 40 cycles comprising 15 s at 95°C for denaturation, 5 s at an annealing temperature specific for each gene (Table 3), and 10–25 s (depending on the amplicon length of the analyzed gene according to the manufacturer’s instructions) at 72°C for elongation. Acquisition was performed for 5 s at the appropriate gene-specific temperature (Table 3). Melting curve analyses were carried out following quantification to ensure the specificity of the qRT-PCR reaction. After quantification, the qRT-PCR products were electrophoresed on 2% agarose gels to monitor the expected size of PCR products and confirm melting curve analysis results. The analysis of the raw data was performed with the LightCycler Software 3.5 by employing the second derivative method (Roche Applied Science).

The efficiency of each qRT-PCR assay was evaluated by standard curves based on serial dilutions of a respective standard cDNA specific to each gene (range: 10⁻⁴⁻¹⁰⁷ copies). The respective standard dilution series were freshly prepared for each qRT PCR experiment. Testing of the suitability of each pair of designed gene-specific primers and optimization of specific qRT PCR assays has revealed PCR efficiencies >90%, indicating that the conditions of these assays were appropriate for subsequent mRNA expression analyses. However, in the following experiments of our study, the efficiencies were lower on average (as indicated in Table 3). The cDNA standards used for qRT-PCR were generated by amplifying the respective transcript fragment of each gene from liver cDNA, purification with the NucleoSpin Extract kit II (Macherey & Nagel), cloning into the pDrive plasmid using the QiaGenPCR cloning kit (Qiagen, Hilden, Germany), and verification by sequencing. Plasmid DNA standards used for qRT-PCR were linearized by NotI digestion and purified using the NucleoSpin Extract kit II.

Reference genes and statistical analysis. Statistical analysis of mRNA abundance was performed using the qbasePLUS program (30), available from Biogazelle, Zwijnaarde, Belgium. Gene expression levels were defined as nonexpressed, when the Crossing point (Cp) values of the targeted gene exceeded 35. The mRNA expression levels were defined as nonexpressed, when the Crossing point (Cp) values of the targeted gene exceeded 35. The mRNA expression levels of the analyzed genes between experimental animal groups. Variance analyses for a fixed effect of the experimental group on mRNA expression levels within tissues were carried out by applying the Proc GLM procedure of the SAS Statistics package (SAS Institute, Cary, NC). Pairwise differences in normalized mRNA expression levels of the analyzed genes between experimental animal groups within tissues (GH vs. CH vs. GH-M vs. CH vs. GH-L and CH vs. GH) were determined by the paired t-test. Results were considered as statistically significant at P < 0.05.

Spearman rank correlations between mRNA expression levels of the respective genes and milk performance traits were calculated using the Proc CORR procedure as implemented in the SAS Statistics package. Correlations exceeding a threshold of P < 0.01 were considered.

RESULTS AND DISCUSSION

High-lactating dairy cows have undergone intensive genetic selection for milk performance, which has pushed the prioritization of energy for milk performance in lactating dairy cows and consequent metabolic adaptations to support lactation (15, 32, 40, 68). The aim of the present study was to contribute to a better understanding of the transcriptional regulation of genes included in glucose and energy metabolism of lactating cows of beef and dairy type in the liver, mammary gland, and skeletal muscle.

To address the genetic and physiological background underlying the phenotypic variation in milk performance traits, we took advantage of a beef-dairy CH×GH resource population. The F₂ design of this unique cross population focused on disrupting the disequilibrium of unlinked traits, and therefore, the segregating heterogeneous F₂ individuals will allow the investigation of distinct metabolic types compared with purebred lines (40). An unexpected observation in the F₂ cows of
the CH×GH resource population was that their MY was generally on a low level compared with high-lactating cows from GH or other dairy breeds (22, 23) and more similar to milk production of CH cows (34, 57, 65). In addition, Hammon et al. (22, 23) reported that changes in basal glucose and insulin concentrations before and during lactation differed in the F2 cows of the CH×GH resource population from those in Holstein dairy cows and were similar to those found in beef cows (28, 64) and in dairy cows with low genetic merit for milk production (20). Furthermore, the F2 CH×GH cows showed a relatively high ratio of hot carcass weight to empty body weight (>60%, (23)]. Specifically, those F2 CH×GH cows characterized by particularly low milk production also displayed a higher carcass weight and muscle cross section of M. longissimus dorsi (21). These characteristics indicate that the genetic dairy background is suppressed in the female F2 offspring of the beef × dairy cross population and suggest a beef-like phenotype for the F2 CH×GH cows.

In the present study, we used the lactating F2 cows of the CH×GH cross population with a combined genetic dairy and beef background but a beef phenotype (CH×GH cows) to compare their tissue-specific expression profiles of relevant metabolic genes with those from purebred high-lactating dairy cows (GH cows), all of which were at a similar stage of lactation and kept under identical environmental conditions. A unique feature of this study is the parallel investigation of quantitative changes of expression levels of the selected genes in three metabolically important tissues originating from the same animal in response to challenges associated with divergent milk performance. The results of the study showed that the mRNA expression levels of genes encoding key mediators of glucose and energy metabolism display different gene expression patterns across the three tissues between cow groups with divergent milk performance (Table 4, Fig. 1).

Liver: different transcriptional regulation of gluconeogenic genes relative to divergent milk production and energy status. The comparison of gene expression patterns in liver tissue between cows of divergent milk production performance revealed that the most pronounced group effects were detected for PCCA (P = 0.0001) and PCK1 (P = 0.0008) transcripts (Table 4). Pairwise comparison of high-lactating GH with CH×GH-L cows representing the group with the lowest milk production performance showed significant differences in mRNA expression levels for PCCA and INSR transcripts. Hepatic mRNA expression levels of PCCA, FBP1, PCK1, and PPARGC1A were significantly different between GH and CH×GH-M cow groups. PCK1 was the only gene differently expressed between CH×GH-M and CH×GH-L cows (Fig. 1).

The most prominent difference in hepatic mRNA expression levels of GH cows and CH×GH cows was found for PCCA, which was markedly higher in the GH cows than in both CH×GH cow groups (Fig. 1), suggesting transcriptional reg-

<table>
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<tr>
<th>Gene</th>
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<th>Analysis of Variance in Tissues (P Value)</th>
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<td></td>
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Experimental groups are characterized according to milk yield during 7d (MY-7d) at the end of the experiment (slaughter): GH, high milk performance; CH×GH-M, medium milk performance; CH×GH-L, low milk performance. Variance analyses were performed by testing for a fixed effect of the experimental group on mRNA expression levels within tissues (Proc GLM, SAS Statistics). N.A., not analyzed.
ulation of PCCA in response to challenges associated with high milk production performance or dairy phenotype. Functionally, the PCCA enzyme catalyzes the conversion of propionyl-CoA into (S)-methylmalonyl CoA, which is the final product of odd-chain or methyl-branched fatty acid metabolism and used for the formation of gluconeogenic intermediates refilling the TCA cycle. Propionyl-CoA is of great importance as a glucose precursor, particularly in ruminants due to the large amount of propionate that is generated by nutrient fermentation in the rumen (75). During lactation, propionate is the primary substrate for hepatic gluconeogenesis, accounting for 50–60% of total glucose supply in dairy cattle (44). Our results indicate an affected propionate metabolism and a differently modulated energy production via TCA cycle in the liver in high-lactating GH cows. However, the divergent hepatic PCCA mRNA expression in association with milk production is not co-regulated in parallel with other gluconeogenic genes. In the high-lactating GH cows, the intermediate oxaloacetate generated by the

Fig. 1. Tissue-specific mRNA expression levels of analyzed genes in liver (A), mammary gland (B), and skeletal muscle (C) of lactating dairy and beef-type cows differing in milk performance. Relative target gene expression levels in tissues (arbitrary units) are depicted on the left y-axis. Expression data represent the mean values and standard deviations in respective experimental cow groups. Values for energy-corrected milk yield [EMC-7d (kg/day), red bars] are depicted on the right y-axis. Experimental groups are characterized according to milk yield during the last 7 days before the end of the experiment (slaughter): GH, high milk performance; CH×GH-M, medium milk performance; CH×GH-L, low milk performance. Different letters indicate significant differences in mean gene expression levels between experimental groups compared within tissues: GH and CH×GH-M, respectively, vs. CH×GH-L and GH vs. CH×GH-M (t-test, P < 0.05).
TCA cycle seems not to be completely directed toward gluconeogenesis because we found lower PCK1, FBP1, and PPARGC1A mRNA expression levels in the liver of these cows compared with CH×GH cows. The particularly lower transcript abundance of these genes in GH cows compared with CH×GH-M cows points to an impaired hepatic gluconeogenesis in the GH cows. In contrast, hepatic gene expression analysis between both CH×GH cow groups with combined genetic dairy and beef background revealed a trend for concomitantly higher hepatic mRNA expression levels of PCK1, FBP1, G6PC, PC, and PPARGC1A transcripts in CH×GH-M cows in contrast to the CH×GH-L cows (Fig. 1). This trend agrees with the generally accepted regulatory mechanism of activated lactation-associated hepatic gluconeogenesis to cope with higher glucose demands.

Functionally, the differential PCK1 expression pattern adjusted to the lactation-dependent metabolic situation or genetic merit for milk production performance seems to make sense because an excessively elevated PCK1 gene expression and most likely higher PCK1 enzyme activity in cows with a high milk production would require large amounts of oxaloacetate from the TCA cycle, which could result in impaired acetyl-CoA oxidation and, consequently, elevated ketone synthesis. A dramatic downregulation of PCK1 gene expression has also been found in periparturient lactating cows afflicted with ketosis, and a low PCK1 enzyme activity was reported in dairy cows with fatty liver (45, 53).

Noticeably, the mRNA expression levels of PC and PCK1 did not display the same pattern between the three cow groups. We observed a trend for elevated PC mRNA expression levels in the livers of GH (P = 0.06) and CH×GH-M (P = 0.1) cows relative to the CH×GH-L cows (Fig. 1). This could be explained by the complex regulatory role of PC in carbohydrate and lipid metabolism at a biochemical branch-point between gluconeogenesis and TCA cycle. A discrepancy between the regulation of mRNA expression levels of PC and PCK1 genes was also observed in livers of cows at calving, the onset of lactation, and during feed restriction (19, 29, 69). The hepatic mRNA expression of PC of the GH and CH×GH-L cow groups was similar to the pattern of PCCA, PCCB, and INSR expression (Fig. 1), which might indicate a possible coordinated transcriptional regulation for these genes in response to the different genetic potential for milk performance. Reduced PCCA and PC gene expression and enzyme activity levels have been observed in biotin deficiency in human and mice (63, 71). PC and PCCA/B enzymes require biotin as cofactor, which has stimulatory effects on genes whose action favors hypoglycemia, like insulin and INSR (17). This link may offer a potential explanation for a putative co-regulation of PCCA/B, PC, and INSR genes. In our study, the hepatic INSR mRNA abundance had a significantly lower level in CH×GH-L cows compared with GH cows, which is in contrast to results from a recent report investigating dairy genotype effects on the expression of genes critical to energy homeostasis and appetite regulation in duodenal and hepatic tissue from Holstein Friesian and Jersey cows and their F1 hybrids. Therein, hepatic INSR gene expression levels did not vary due to dairy genotype in a very recent study (2). However, the phenotype differences in milk production did not reach a magnitude similar to our study, which may be the reason for the different results.

In the liver, the expression patterns of genes acting in the gluconeogenesis pathway were strongly correlated (P < 0.001) with each other (except for PC) and with the upstream acting transcription factor coactivator PPARGC1A (Fig. 2A) that is known to coordinate the expression of proteins controlling the regulation of several metabolic pathways in response to met-
abolizing challenges. PPARGC1A has been shown to play a pivotal role in the regulation of cellular energy metabolism, such as hepatic gluconeogenesis with response to fasting and food deprivation in other species (59, 61), and to be a key gene in mitochondrial oxidative phosphorylation metabolism (43, 51, 58). In our previous studies, polymorphisms in the bovine PPARGC1A gene were found to be associated with milk performance traits in GH populations (73, 74). In the present study, hepatic PPARGC1A mRNA abundance showed correlations to transcripts of genes included in the gluconeogenesis pathway and INSR, but not to MY.

The highest numbers of significant correlations ($P < 0.001$) between hepatic mRNA expression levels of single genes were found for $G6PC$, $PC$, and PPARGC1A, whereas PCCB and FBP2 transcripts did not show any correlations to other genes (Fig. 2A). Significant positive correlations to milk performance were only found for hepatic PCCA mRNA expression levels ($P < 0.001$, Fig. 2A), indicating that higher hepatic PCCA mRNA expression levels were linked to higher milk performance of the cows.

Mammary gland: different transcriptional regulation of PCK2, PC, PCCA, INSR, and PPARGC1A genes in response to divergent milk performance. In mammary gland, we found highly divergent mRNA expression levels between experimental cow groups for PCK2, PPARGC1A ($P < 0.0001$), and PC ($P = 0.0003$) as well as for PCCA, INSR ($P = 0.0006$), and FBP2 ($P = 0.0002$), as shown in Table 4. In pair-wise comparison of GH with CH×GH-L cows, significant differences in mRNA expression levels were observed for PCK2, PC, PCCA, and INSR transcripts (Fig. 1). These genes were also significantly differently expressed between GH and CH×GH-M cows in addition to FBP1, FBP2, and PPARGC1A transcripts. Mammary mRNA expression levels of PC, FBP1, FBP2, and PPARGC1A transcripts varied substantially between CH×GH-M and CH×GH-L cows (Fig. 1).

The most pronounced difference in mammary transcript patterns was observed for PCK2, which showed profoundly larger mRNA expression in the GH cows compared with the CH×GH cow groups. The PCK2 enzyme located in the mitochondrial matrix is also involved in gluconeogenesis, like the cytosolic isoform PCK1, with the difference that lactate serves as substrate (24, 72). In contrast to PCK1, the tissue-specific functional role of PCK2 has not yet been investigated in detail. Stark et al. (66) proposed that PCK2 is involved in the catastrophic recycling of citric acid cycle anions during glucose-stimulated release of insulin from rodent pancreatic cells. Like PCK1, PCK2 was also postulated as a candidate gene for diabetes and obesity in humans (1, 4, 35, 47, 55, 66).

The mammary tissue is composed of several cell types, including epithelial cells and adipocytes, both lacking $G6PC$ expression, which argues against glucose production via gluconeogenesis in this tissue. Based on the similar structure and catalytic properties of both PCK isozymes, it can be postulated that PCK2 might also have a function in gluconeogenesis of lipogenic tissues similar to that of PCK1 in adipose tissue during fasting or compromised feed intake (25, 60) and in adipocytes and epithelial cells of mammary tissue during lactation (31). However, a gluconeogenic role has not yet been demonstrated for PCK2. Earlier studies in guinea pigs showed that during transition to lactation, PCK2 enzyme activity was substantially higher in the mammary gland compared with PCK1 activity (35), suggesting that during this period, PCK2 may play the more important role in this tissue. This hypothesis is supported by results obtained in humans and in our study. Gene expression analysis of the human milk fat globule transcriptome revealed that $PCK2$ but not $PCK1$ was expressed (47). We found clearly different mammary $PCK2$ mRNA transcript levels in the GH cows compared with the CH×GH cows, whereas $PCK1$ mRNA expression showed no lactation-associated differences. Therefore, we assume that in high-lactating cows, PCK2 may contribute to the formation of milk triglycerides via gluconeogenesis in mammary cells. However, further investigations are required to confirm our hypothesis.

A second relevant result of our study in mammary tissue is the significantly different variation of $PC$ mRNA abundance between all three cow groups, with the highest value in CH×GH-M cows (Fig. 1). However, the $PC$ mRNA expression pattern differs clearly from that of PCK2, indicating that the expression levels of these genes are not interconnected proportionally to milk production. Compared with the CH×GH-L cows, the reduced mammary $PC$ mRNA expression levels in the GH cows were accompanied by lower transcript abundance of INSR and PCCA, whereas in the CH×GH-M cows, PPARGC1A, FBP1, and FBP2 displayed substantially elevated transcript levels compared with the CH×GH-L cows. The prominently higher $PPARGC1A$ mRNA expression levels in the CH×GH-M cows compared with CH×GH-L and GH cows (Fig. 1) may reflect a divergent regulatory function of PPARGC1A in the mammary gland of the CH×GH cows with the dairy × beef genetic background. PPARGC1A mRNA expression was found to be increased in mammary explants in response to insulin (50) and after the onset of lactation and appeared to independently regulate the expression of several lipogenic genes (10).

Differences observed in mammary INSR mRNA abundance between GH cows and both CH×GH cow groups (Fig. 1) suggest the relevance of insulin signaling for milk production. The main functional activity of INSR is to induce insulin-mediated glucose uptake, which failed to be affected by insulin in the mammary gland of lactating cows (41). This result is supported by the lack of or very low expression of insulin-responsive glucose-transporters in this tissue (76), confirming that insulin-responsive glucose uptake should be excluded in the mammary gland. However, insulin has been found to regulate milk production via direct effects on blood flow and amino acid flux in the mammary gland of lactating goats (8). Recently, it has been reported that insulin- and mTOR-related signaling play a regulatory role for milk protein synthesis in the mammary gland of cows during lactation (50). These results are in agreement with those of Bionaz and Loor (11), who observed an upregulation of INSR mRNA expression level after the onset of lactation in the mammary gland. Their results documenting processes of initiation of lactation seem to be controversial with the higher INSR expression levels of the low-lactating cows in our study; however, there is no clear interpretation for the inconsistency due to the different experimental design.

The considerably lower PCCA expression in the mammary gland of GH cows in our study (Fig. 1) would support a reduced catabolism of branched-chain amino acids, odd-numbered chain length fatty acids, or cholesterol and other metabolites under conditions of higher lactation in this tissue with a
positive effect on the availability of these amino acids for incorporation into milk protein (16). Bionaz and Loor (11) found that the gene expression of transporters for branched-chain amino acids is very high in the mammary gland during lactation, favoring the flux of the respective amino acids toward utilization in milk protein synthesis.

The analysis of correlative relationships between mRNA expression levels of the analyzed genes in the mammary gland showed PPARG1A and PC transcripts as most closely correlated to mRNA abundance of other genes analyzed in our study (Fig. 2B) and highlighted their relevance in this tissue. The PC mRNA expression levels revealed correlations to all genes (except for both PCK isoforms), suggesting a particular regulatory role for PC. Strong correlations \((P < 0.001)\) between PPARG1A, PC, FBP1, and FBP2 mRNA expression levels were noticeable, whereas PCK1 did not show any correlations to other genes in the mammary gland. Negative correlations of INSR and PC mRNA abundance to milk production traits suggest a reciprocal relationship between expression of these genes and milk performance. In contrast, the strong positive correlations between PCK2 mRNA expression and milk performance parameters suggest a potential molecular link between expression of this gene in the mammary tissue and milk production performance.

**Skeletal muscle: modulation of FBP2, PCCA/B, PCK2, INSR, and PPARGC1A transcription according to milk production performance.** Analysis of gene expression differences between GH and CH×GH cow groups in skeletal muscle revealed the largest contrast in mRNA expression levels of FBP2, PCCB \((P < 0.0001)\), and PCCA \((P = 0.0005)\) transcripts. Furthermore, mRNA abundances of PPARG1A \((P = 0.0058)\), PCK2 \((P = 0.029)\), and INSR \((P = 0.0028)\) were also significantly affected by group (Table 4). Pairwise comparison of GH with CH×GH-L cows revealed significant differences in mRNA expression levels for all genes but PCK1, PC, and FBP1 transcripts. The transcript levels of FBP2, PCCA, PCCB, INSR, and PPARC1A were significantly different between GH and CH×GH-M cows, whereas PCK2 was the only gene with different expression between CH×GH-M and CH×GH-L cows (Fig. 1).

Out of those genes, the most evident differences in mRNA expression levels in skeletal muscle were detected for FBP2. Its transcript levels were highest in GH cows, pointing to a role in improving sensitivity in metabolic control. FBP2 enzyme activity has been shown to correlate with uptake and incorporation of lactate into glycogen (49) and to be generally lower in red than in white muscles across species (56). In cattle, expression levels of the FBP2 gene have been found to vary between different muscle types and to be larger in skeletal muscle with a fast-glycolytic fiber structure (52). Based on our and literature results, we conclude that higher FBP2 mRNA expression levels in skeletal muscle of GH cows could reflect activation of metabolic processes (glycolysis, storage, and sequestering of glycogen) to meet the metabolic demands of the cows under conditions of high lactation. Skeletal muscle glycolysis and glycogen metabolism have been found to contribute to the metabolic adaptation of dairy cows during early lactation (39).

Our results in skeletal muscle showed that larger FBP2 mRNA abundance in GH cows relative to CH×GH cows was accompanied by larger PPARG1A mRNA expression (Fig. 1), which may suggest a simultaneously activated mitochondrial biogenesis in muscle tissue of high-lactating cows to promote adaptation of the organism by mobilizing endogenous body reserves in response to metabolic challenges related to higher milk production. Higher PPARG1A levels, similar to those that can be induced by physiological stimuli, were found to alter intramuscular lipids and improved fatty acid oxidation, insulin signaling, and insulin-stimulated glucose transport (7). Further investigations are necessary to delineate in detail if FBP2 and PPARG1A gene expression is coordinately regulated in skeletal muscle of highly lactating cows, or if the similar gene expression pattern of these genes is due to independently regulated pathways.

In contrast to the elevated FBP2 and PPARG1A transcript levels in skeletal muscle of GH cows, the transcript levels of INSR, PCCA, PCCB, and PCK2 were substantially lower in these cows compared with the CH×GH cow groups (Fig. 1). The markedly lower PCCA and PCCB expression levels in skeletal muscle of high lactating GH cows may be associated with its functional role in the catabolism of branched-chain amino acids, as has been discussed above for mammary gland, and may presumably improve the availability of these amino acids for incorporation into muscle protein or, more likely, provide them for milk protein synthesis in the mammary gland. Impaired INSR expression in skeletal muscle of GH cows was found to be consistent with low plasma insulin concentrations and higher insulin resistance, which were associated with selection for high MY (13, 33, 62).

In skeletal muscle (Fig. 2C), the correlation networks of the analyzed genes indicate the highest numbers of significant \((P < 0.001)\) correlations of mRNA expression levels for INSR, PCCB, and PCK2. Transcriptional levels of PC and PCK1 revealed no correlation to expression levels of other genes, confirming that gluconeogenesis does not play a role in skeletal muscle tissue. The FBP2 transcript levels exhibited a strong correlation to PPARG1A mRNA expression levels and were profoundly positively correlated to milk performance traits, whereas the PPARG1A mRNA abundance revealed a correlation to milk production traits with a lower significance threshold. In addition, there are several genes (INSR, PCCA, PCK2, and PCCB) in skeletal muscle with mRNA expression levels negatively correlated to milk performance traits.

Liver, mammary gland, and skeletal muscle showed different variability of adaptive performance in response to different genetic background and phenotype for milk production. Altogether, the correlative relationships between mRNA expression levels and milk performance parameters found in our study indicate that in addition to the expected high impact of the intrinsically, metabolically very active liver tissue, the mam-
primary gland and the skeletal muscle also appear to play an important role in the adaptive regulatory processes and metabolic changes associated with the genetic selection for higher milk production. The results of our cross-tissue study demonstrated that the metabolic adjustment relative to the genetic potential for milk performance is clearly reflected at the molecular level; the transcription of the selected functional candidate genes is linked to the interrelated pathways of gluconeogenesis, glyceroneogenesis, propionate metabolism, tricarboxylic acid cycle, insulin binding and signaling, as well as transcriptional regulation of gluconeogenesis and mitochondrial biogenesis.

In particular, the PCCA and PPARGC1A genes should be highlighted because they displayed significantly altered mRNA abundance across all three cow groups and in all three tissues, pointing to key gene functions in the metabolic adaptation relative to milk performance. When one focuses on characteristics of the purebred dairy-type GH cows compared with the cows from the CH×GH cross population, it is particularly noticeable that alterations of PCCA and INSR transcript levels showed a concordant pattern in all three tissues, indicating a potential metabolic connection between these genes across tissues in cows with the higher lactation potential. Furthermore, our results point to yet unknown gene regulatory, adaptive mechanisms in highly lactating dairy cows to accommodate the metabolic milk performance-linked challenges, e.g., upregulation of PCK2 transcripts in the mammary gland and of FBP2 transcripts in skeletal muscle of GH cows.

Compared with the cows with the lowest milk performance (CH×GH-L), in the cows with a higher genetic merit for milk performance (GH and CH×GH-M), we observed a concordant unidirectional modulation of hepatic transcript levels for PCK, G6PC, and INSR. This would suggest that these genes may have a general function associated with hepatic regulatory adaptation processes for milk production. In contrast, hepatic PCK1, FBP1, and PPARGC1A mRNA expression levels were found to vary in a different, nonuniformly directed pattern in GH and CH×GH-M cows. Compared with CH×GH-L cows, the mRNA abundance of these genes was higher in CH×GH-M cows but lower in GH cows, indicating a divergently regulated function of these genes due to different levels of milk production.

In skeletal muscle and mammary gland, concordant changes of gene expression levels were not observed in the GH and CH×GH-M cows relative to CH×GH-L cows (except for PCK2 in skeletal muscle). This would suggest that, in these tissues, contrasts due to the genetic selection for milk performance or different genetic phenotype were divergently reflected by discordant changes of gene expression levels in the GH and CH×GH-M cows groups, as for PCCA, PPARGC1A, INSR, and FBP2.

Several genes in liver (PCK1) and mammary gland (PPARGC1A, FBP1, and FBP2) showed significantly elevated transcript levels in CH×GH-M cows compared with CH×GH-L cows but displayed no different transcript levels between GH and CH×GH-L cows.

The divergent tissue-specific expression patterns of the CH×GH cross population with a combined dairy and beef genetic background compared with the dairy GH cows may reflect the result of alternative dairy vs. beef selection and can be used to define specific molecular fingerprints for these populations.

Conclusions

The results presented in this study provide evidence for the hypothesis that the transcriptional gene expression levels in the liver, mammary gland, and skeletal muscle of lactating cows are modulated due to the genetic background and phenotype for milk performance. New insights are revealed about coordinated gene expression patterns in tissues involved in the metabolic response to lactation-associated challenges and genetic selection for milk performance and about the regulatory functions of genes included in the interrelated pathways of gluconeogenesis, glyceroneogenesis, propionate metabolism, tricarboxylic acid cycle, insulin binding/signaling, and transcriptional regulation of gluconeogenesis and mitochondrial biogenesis. Specifically, we highlight genes that showed modulated mRNA abundance between all three tissues and all cow groups, such as PCCA and PPARGC1A, suggesting key gene functions in the metabolic adaptation relative to milk performance. Furthermore, we detected genes with yet unknown functions in dairy and beef type cows, such as for PCK2 and FBP2, that displayed strongly elevated transcripts levels in the mammary gland or in skeletal muscle of dairy type cows selected for high milk production.

The results of this study also indicate that further investigation is needed to elucidate and clearly delineate the adaptive responses of tissue-specific and intertissue gene expression networks in more detail, including additional tissues (e.g., adipose and gastrointestinal compartments) and advanced molecular techniques like unbiased global gene expression approaches (e.g., microarray analyses or next-generation sequencing via RNAseq). Furthermore, it has to be considered, that there is also evidence for numerous posttranscriptional and particularly, posttranslational regulatory steps and protein-protein interaction processes, which may play important roles to adjust for metabolic short-term regulation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: R.W. conception and design of research; R.W., T.G., and R.M.B. performed experiments; R.W. and C.K. analyzed data; R.W. interpreted results of experiments; R.W. prepared figures; R.W. and C.K. drafted manuscript; R.W., T.G., R.M.B., and C.K. edited and revised manuscript; R.W., T.G., R.M.B., and C.K. approved final version of manuscript.

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