The bovine PPARGC1A gene: molecular characterization and association of an SNP with variation of milk fat synthesis

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The bovine PPARGC1A gene: molecular characterization and association of an SNP with variation of milk fat synthesis. The bovine PPARGC1A gene (PPARGC1A) as a plausible positional and functional candidate gene for a previously described QTL for milk fat yield on BTA6 because of its chromosomal position and its key role in energy, fat, and glucose metabolism. To analyze the role of the bovine PPARGC1A gene in regulation of milk fat synthesis in dairy cattle, we determined its cDNA sequence, genomic organization, chromosomal localization, and expression pattern. The bovine PPARGC1A gene is organized in 13 exons comprising 6,261 bp and is expressed at different levels in a large number of tissues. Bovine PPARGC1A cDNA and protein sequences showed substantial similarity (92–95%) to its respective orthologs from human, rat, and mouse. Screening for polymorphisms in the coding sequence, exon/intron boundaries, 5’- and 3’-untranslated regions, and promoter region of the PPARGC1A gene in sires with a different genotype at the QTL for milk fat yield as well as in a multibreed panel revealed a total of 11 polymorphic loci. A significant association between an SNP in intron 9 of the PPARGC1A gene and milk fat yield was observed in a major dairy cattle population, indicating that the PPARGC1A gene could be involved in genetic variation underlying the QTL for milk fat synthesis on BTA6.

quantitative trait locus; positional and functional candidate gene; single nucleotide polymorphisms; association study; cattle; BTA6

THE DISSECTION of quantitative trait loci (QTL) with impact on complex, economically important traits in livestock and the identification of the underlying genetic variation will help to gain insight into the metabolic pathways and associated genes involved. Concurrently, this will provide the molecular means for a successful marker-assisted selection in livestock populations and may contribute to comparative investigation of basic physiological mechanisms of genetic variation related to similar metabolic pathways and functions of linked and interacting genes in humans and other species. Recently, strategies of comparative positional and functional candidate cloning have been used to identify the molecular background of QTL affecting milk yield and milk composition in dairy cattle and muscle growth in pigs, respectively (e.g., see Refs. 3, 18, 51, 56). A number of independent studies in different populations reported QTL for milk production traits on bovine chromosome 6 (BTA6) (e.g., see Refs. 1, 4, 13, 25, 35, 43, 52). Particularly, there is evidence from several of these studies for at least two QTL for milk production traits on this chromosome. In our previous mapping study in German Holstein cattle, we found a QTL with effects on milk fat and protein yield in an interval comprising 46 cM (ILSTS090–ILSTS097) in the middle part of BTA6 (25). Applying multivariate QTL mapping analysis, we confirmed the QTL and localized the suggested QTL position to a chromosomal segment of 8 cM (12). In Norwegian Dairy cattle, Olsen et al. (36) mapped the position of a highly significant QTL affecting milk fat and protein percentage to a 7.5-cM interval between markers BMS2508 and FBN12. From multiple QTL-multitrait analysis, Olsen et al. (36) had also indication for a second QTL interval downstream of the first interval with effects on milk fat yield. This interval is in agreement with our previous results and narrows down the QTL to 3 cM between markers BM143 and BMS690.

To define positional candidate genes underlying the QTL, we had constructed a high-resolution comparative radiation hybrid (RH) map for BTA6 containing the intervals harboring previously confirmed QTL on this chromosome (54). Among the genes that have been mapped comparatively from the human orthologous chromosomal region of HSA4–BTA6 (Fig. 1), peroxysome proliferator-activated receptor-γ coactivator-1α (PPARGC1A, also known as PGC1α) and cholecystokinin A receptor (CCKAR), both genes suggested to be implicated in the development of obesity according to the obesity gene map (45), stand out as strong positional and functional candidates underlying the QTL effect on milk fat synthesis.

In our study we focused on PPARGC1A as the most plausible comparative functional candidate gene affecting milk fat yield. Whereas CCKAR mediates cholecystokinin action mainly involved in gallbladder contraction, pancreatic enzyme secretion (50), and neuromodulation of feeding behavior (e.g., see Refs. 31, 41), PPARGC1A has a key function in activating a variety of nuclear hormone receptors and transcription factors regulating energy homeostasis. Moreover, PPARGC1A has been shown to mediate the expression of genes involved in adaptive thermogenesis, oxidative metabolism, adipogenesis, and gluconeogenesis (reviewed in Refs. 24, 40). The human PPARGC1A gene was mapped to a chromosomal region that was linked to fasting serum insulin concentrations (39) and...
several obesity-related parameters (e.g., see Refs. 38, 44, 47).
Genetic variations in the human PPARGC1A gene were found to be associated with insulin resistance, susceptibility to type II diabetes, lipodystrophy, and indicators for obesity (e.g., see Refs. 7, 8, 19, 23, 32). As recorded from the integrated obesity gene map (45), a number of QTL for obesity-associated traits have been localized in mouse and pig cross-breeding experiments on equivalent syntenic chromosomal regions, Mmu5 and SSC8, respectively, overlapping the same interval to which PPARGC1A has been mapped. Thus, comparative genomics suggests genetic evidence for a conserved chromosomal region with impact on fat synthesis.

The potential role of PPARGC1A in mammary gland metabolism, however, has been investigated neither in humans nor in mice nor in ruminants. Because of its dynamic and critical role in the regulation of programs linked to energy homeostasis and its ability to coordinate the process of metabolic adaptation in liver, fat tissue, and muscle, PPARGC1A may be regarded as a possible general mediator of the metabolic demands that accompany the onset and progression of lactation in dairy cows. Lactational performance in high-yielding dairy cows has its limits in metabolic processes due to dramatic alternations in glucose and fat metabolism at the onset of lactation. During lactation availability of glucose, depending on a state of continuous hepatic gluconeogenesis, is a limiting factor for milk production. The importance of hepatic gluconeogenesis is especially highlighted by the fact that, in genetically superior lactating dairy cows, glucose production must increase up to sevenfold to satisfy the requirements of the mammary gland compared with their nonlactating counterparts (2). In the mammary gland, ~60–70% of glucose is used for lactose synthesis, and ~20–30% of glucose goes through the pentose phosphate

Fig. 1. Alignment of the gene order on the high-resolution radiation hybrid (RH) map of the targeted bovine chromosome 6 (BTA6) region with the gene sequence map of the orthologous Homo sapiens chromosome 4 (HSA4) region. Left: targeted region of BTA6 taken from the 12,000-rad RH map (54). Right: selected HSA4 region from the human gene sequence map (human draft NCBI MapView, build 34/3). Peroxysome proliferator-activated receptor-γ coactivator-1α gene (PPARGC1A) is highlighted by a light-gray box. Direct gene anchors connecting both maps upstream and downstream from PPARGC1A are given in hatched boxes. The areas marked in dark gray on the HSA4 gene sequence map contain genes displayed in the boxes beside, whereas the open areas are not covered with known genes.

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shunt to generate NADPH2 that is used as reducing equivalents in milk fatty acid synthesis (reviewed in Refs. 2, 26). Regarding this latter fact, glucose is also limiting for the fatty acid synthesis pathway during lactation. Given the critical role of PPARGC1A in several aspects of glucose, fat, and energy metabolism and its ability to coordinate the process of metabolic adaptation in liver, fat tissue, and muscle of humans and mice, it is conceivable that the metabolic adaptive processes modulated during lactation in dairy cattle might be coordinated by PPARGC1A. Extending this idea of the functional relevance of PPARGC1A in conjunction with the fact that the respective bovine gene is mapped to a chromosomal region on BTA6 linked to a QTL for milk fat synthesis, we hypothesized that variability in the PPARGC1A gene, as a candidate gene, could be associated with the QTL effect. This study reports the molecular description of the bovine PPARGC1A gene, the results of a comprehensive screening for sequence polymorphisms, and the identification of an intron polymorphism that is reproducibly associated with milk fat synthesis in a major dairy cattle breed.

MATERIALS AND METHODS

Molecular Characterization of the PPARGC1A Gene

Bacterial artificial chromosome screening, mapping, and sequencing. Bacterial artificial chromosome (BAC) clones harboring the bovine PPARGC1A gene were identified by PCR screening of DNA superpools from a bovine BAC library (57), available at the Resource Center, Berlin, Germany (http://www.rzpd.de). The procedures of blood collecting from cows were performed in the course of standard clinical care, with respect to the welfare and health of the animals, by a qualified veterinarian with experience and practice in the treatment of cattle. Primers were derived from exon 1 of the human PPARGC1A gene (AF106698: forward 5’-ATGAGTGTGTCGTCGTC-3’ and reverse 5’-TCATGTTCACTCATGAGG-3’) and a bovine expressed sequence tag (EST) (AF213835: forward 5’-CTGCTGATTTGATGGAGGAC-3’ and reverse 5’-GGCTGATGTACTGCAC-3’) highly homologous to a human EST (DS5928) from the 3’-UTR of the gene. Specificity of the identified BACs (BBI_750O11357, BBI_750I03315) was verified by direct sequencing with primers used for BAC library screening. To determine the sequence of the bovine PPARGC1A gene, BACs were directly sequenced using vector primers and gene-specific primers derived from bovine ESTs (BE665709, AV594535, BB76129, BF076255, BE752704, BM445563, BM086947, BB48938, AF213835) that were identified by sequencing the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) using BLASTN with the human PPARGC1A cDNA sequence (AF106698). The chromosomal location of the BACs harboring the bovine PPARGC1A gene was determined by fluorescence in situ hybridization according to Brummer et al. (5).

Rapid amplification of cDNA ends. To obtain the 5’-end of the cDNA, rapid amplification of cDNA ends (RACE)-PCR was performed by use of the GeneRacer kit (Invitrogen, Karlsruhe, Germany), following the manufacturer’s instructions, based on RNA ligase-mediated and oligo-capping RACE methods. Expression analysis. The RT-PCR assay included total RNA extracted from nine bovine tissues (liver, small intestine, kidney, thyroid gland, mammary gland, pituitary gland, skeletal muscle, and subcutaneous and intestinal fat) originating from a lactating Holstein cow. The RNA was prepared using the RNeasy mini kit (Qiagen, Hilden, Germany) and the TRizol procedure (Invitrogen, Karlsruhe, Germany). The cDNAs were synthesized using the Superscript preamplification system for first-strand cDNA synthesis (Invitrogen) according to the manufacturer’s instructions. The investigation of a tissue-specific PPARGC1A gene expression pattern by PCR was done with primers spanning the exons 4–5 (forward 5’-AAGAGCCCTTTACTGACACCC-3’ and reverse 5’-ATGGTGTTGTCTGGAGTTG-3’; 318 bp) and the exons 8–13 (forward 5’-CAGTGGAATTTTCCAAACCGG-3’ and reverse 5’-GGGAAAATTTCCAAATGAGC-3’; 1,337 bp). As a control, expression of gyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed in all tissues (forward 5’-TACATGGTCTACATGTTCCAGATG-3’ and reverse 5’-CAGTCCTCGGGGGAGTATG-3’; 440 bp).

Screening for polymorphisms. On the basis of our sequence of the bovine PPARGC1A gene (GenBank accession no. AY321517), all exons of the gene including their flanking regions, the complete introns 6, 9, and 11 (GenBank accession nos. AY547552, AY547554, and AY547555), and a part of the promoter comprising a region of 1 kb upstream from the transcription start (GenBank accession no. AY547550) were comparatively sequenced. Additionally, the screening for polymorphisms was extended to the internal and end sequences of BAC clones (BBI_750I07162, BBI_750A19302) close to BACs containing the PPARGC1A gene. These BACs were identified by screening with a sequence (FBNS10; GenBank accession no. AY398242) derived from a library specific to the targeted BTA6 region (53). Position of the BAC clones on BTA6 has been inferred from comparative alignment of BAC end sequences onto the human orthologous chromosomal region of Homo sapiens chromosome 4 (HSA4) and RH mapping of the chromosome region-specific sequence FBNS10 used for BAC screening (unpublished data).

Comparative sequencing to screen for polymorphisms was applied to seven samples. On the one hand, genomic DNAs from specific individuals were analyzed: one Holstein bull was most likely heterozygous Qq at the QTL for milk fat yield on BTA6 (25), while another Holstein bull was most likely homozygous at the QTL. In addition, one animal originating from a Holstein × Charolais cross and the corresponding region of the identified BAC clone derived from a Jersey male were analyzed as reference sequences. On the other hand, we also performed a DNA pool sequencing approach to obtain a comprehensive list of polymorphisms within and adjacent to the PPARGC1A locus. Three DNA pools, each containing equimolar amounts of genomic DNA from a total of 60 individuals, were included in the analysis. Two unibred DNA pools included DNA from 40 female animals representative for the German Holstein cattle population (20 samples in each pool). The multibreed DNA pool consisted of male animals representing 16 different European taurine breeds (Galloway, Red Pied Cattle, Red Angus, German Angus, Angler, Welsh Black, Dairy Shimmel, Brown Swiss, Uckermaerker, Jersey, Limousin, Highland, Charolais, Belgian Blue, Salers, Hereford) and one indicine breed (Dwarf zebu). Polymorphisms detected by the pool sequencing approach were verified by amplifying and sequencing DNA from single individuals contained in the corresponding pool.

Generally, PCR products amplified from genomic DNA were directly sequenced using Big Dye Terminator Cycle sequencing reaction on an ABI 310 Automated sequencer (PE Applied Biosystems, Foster City, CA). Primers used for PCR amplification and sequencing of the PPARGC1A locus were derived from exon-flanking intronic regions as well as from the 5’- and 3’-untranslated regions. Primer sequences are available as supplemental online information (Supplementary Tables S1 and S2; available at the Physiological Genomics web site) or from the authors upon request. Usually, gene fragments were PCR amplified and sequenced using identical primers. In case of longer PCR products or difficult sequence regions (e.g., A stretch), as for exons 8, 9–10, and 11–12 and the 3’-UTR, additional primers were used for sequencing. Primers from the adjacent BAC clones were derived from BAC ends (BAC end sequences (BES);

*The Supplemental Material for this article (Supplemental Tables S1 and S2) is available online at http://physiolgenomics.physiology.org/cgi/content/full/00103.2004/D1C1.*
GenBank accession nos. AY838273–AY838276| by direct sequencing and from within the BAC inserts by exon trapping (unpublished results; GenBank accession nos. AY838277, AY839825). Sequence data were analyzed with the use of the Phred/Phrap/Polyped/Consed software package (9, 10, 16, 33). Coding of polymorphic loci was done according to the current recommendations for the description of sequence variants (http://www.hgvs.org/mutnomen| or http://www.genomic.unimelb.edu.au/mdi/mutnomen/) and based on the deposited bovine reference cDNA sequence (AY321517), where +1 corresponds to the adenine of the translation codon ATG.

Genotyping tests. The single nucleotide polymorphisms (SNPs) in exon 8 (c.1209T>C), exon 9 (c.1847C>T), intron 9 (c.1892+19T>C), and the 3’-UTR (c.3359A>C, c.5314C>T) of the PPARGC1A gene, as well as an SNP in the sequence derived from the neighboring BAC BBI_750f07162, were genotyped by PCR-restriction fragment length polymorphism methods employing restriction site-generating PCR with one of the primers containing a nucleotide mismatch, which enables the use of restriction enzymes for discriminating sequence variations. The length variation in the promoter region of the PPARGC1A (c.−298−301delCTT) was detected by conventional microsatellite analysis (25). Primers, restriction enzymes selected (MBI Fermentas, St. Leon-Rot, Germany), and fragment sizes are given in Table 1. The detection of allelic variation at the SNP sites was performed with a fluorescence-labeled primer on an Automated ALF sequencer (Pharmacia Biotech, Uppsala, Sweden), based on the electrophoretic pattern of the restriction enzyme-treated PCR products.

Statistical Analysis

Animals. Genomic DNA from a panel including 54 female animals representative of the German Holstein cattle population was used to determine the allele frequencies of the PPARGC1A sequence polymorphisms. In addition, a set of 26 male animals from 17 different European taurine breeds (German Holstein, Galloway, Red Pied Cattle, Red Angus, Angler, Welsh Black, Dairy Simmental, Brown Swiss, Uckermärker, Jersey, Limousin, Highland, Charolais, Belgian Blue, German Angus, Salers, Hereford), African zebu (Dwarf zebu), and Tibetan yak (Poephagus mutus) was used to analyze the detected polymorphisms.

The data set for the association study consisted of DNA samples from 434 artificial insemination (AI) bulls from the German Holstein population originating from different sire groups. All bulls were born between 1990 and 1996. The average paternal half-sib family size was 54.3 sons (1–157 sons/family). The basic structure of the pedigree material was a granddaughter design (55). The dams of the AI bulls represent the top of the Holstein population regarding milk production traits, because <0.05% of the total Holstein cow population in Germany is selected for production of AI bulls.

Markers. In the PPARGC1A gene, the only identified polymorphisms for which the Qq bull at the QTTL for milk fat yield was heterozygous were those in intron 9 (c.1892+19T>C) and the 3’-UTR (c.3359A>C, c.5314C>T). Consequently, both SNPs were genotyped in the 434 bulls according to the protocol given above to assess an association of PPARGC1A SNPs on milk yield and milk fat synthesis. To evaluate the results received from these SNPs, five additional polymorphic positions within and adjacent to the PPARGC1A gene were included: c.−298−301delCTT, c.1209T>C, c.1847C>T, c.5314C>T, and BES_J07162 250G>A. To account for the effects of the diacylglycerol acyltransferase (DGAT1) locus previously shown to exhibit a major gene effect on milk production traits, notably milk fat synthesis (explaining 64 or 21% of genetic variance for milk fat percentage or milk fat yield, respectively; Ref. 19), all individuals were genotyped for the nonconservative DGAT1 K232A polymorphism according to Winter et al. (56).

Genotypes and haplotypes. All genotypes were checked for Mendelian inheritance and double recombinants within a previously described marker data set on BTA6 (12). The most likely paternally and maternally inherited alleles for the PPARGC1A polymorphisms of the sons were estimated by a Markov Chain Monte Carlo (MCMC) algorithm using the SIMWALK2 program haplotype option (46). Frequencies of the maternally transmitted alleles and haplotypes of sons were obtained by allele/haplotype counting.

Phenotypic data. To investigate associations between PPARGC1A polymorphisms and milk fat synthesis, estimated breeding values (EBVs) of the bulls for milk yield, milk fat yield, and milk fat percentage were included as phenotypic values corrected for environmental effects. EBVs were calculated by a multilactation test day animal model from lactation data of daughters of the sons. The whole data set comprised 202,501 performance-tested daughters, with an average number of daughters per bull of 466.6. The reliability of the calculated EBVs was very high, with an average of 92.4%. The EBVs

Table 1. PCR and PCR-RFLP tests used for genotyping SNPs detected in the bovine PPARGC1A gene and the adjacent BAC end sequence

<table>
<thead>
<tr>
<th>SNP</th>
<th>Primer Sequences</th>
<th>Amplicon Size, bp</th>
<th>Restriction Enzyme</th>
<th>RE Size, bp/ Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARGC1A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.−298−301delCTT</td>
<td>F: *GGTGGGTTACAGACTGCG</td>
<td>210/206</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: AAAAGTAGGCTGGGTGTTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1209T&gt;C</td>
<td>F: TTAGTATGCAAAGGAGATCCA</td>
<td>123</td>
<td>Pagl</td>
<td>103, 20/</td>
</tr>
<tr>
<td></td>
<td>R: *CGGTCTCTTCCAGGGAACAC</td>
<td></td>
<td></td>
<td>c.1209T</td>
</tr>
<tr>
<td>c.1847C&gt;T</td>
<td>F: *CAGAATTCCGAGGGACATCTC</td>
<td>165</td>
<td>Bgll</td>
<td>149, 16/</td>
</tr>
<tr>
<td></td>
<td>R: TTGACGTCGAGGCGGACAGG</td>
<td></td>
<td></td>
<td>c.1847C</td>
</tr>
<tr>
<td>c.1892+19T&gt;C</td>
<td>F: *CTGGTACTCCTCGAGGTGTC</td>
<td>178</td>
<td>BsuRI</td>
<td>158, 20/</td>
</tr>
<tr>
<td></td>
<td>R: TTTGACGACGTAGGACAG</td>
<td></td>
<td></td>
<td>c.1892+19C</td>
</tr>
<tr>
<td>c.3359A&gt;C</td>
<td>F: TTAAAGAGGGATCTAGT</td>
<td>339</td>
<td>Xml</td>
<td>319, 20/</td>
</tr>
<tr>
<td></td>
<td>R: GGTCCCCATGAAATGAAC</td>
<td></td>
<td></td>
<td>c.3359C</td>
</tr>
<tr>
<td>c.5314C&gt;T</td>
<td>F: CTTGAAATATTGCTGAAATC</td>
<td>191</td>
<td>HpyF10VI</td>
<td>171, 20/</td>
</tr>
<tr>
<td></td>
<td>R: *AATTGGCTTCCAGGTGATG</td>
<td></td>
<td></td>
<td>c.5314C</td>
</tr>
<tr>
<td>BES</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BES_J07162 250G&gt;A</td>
<td>F: *CACAGAGTTGGACAGCAGTTATG</td>
<td>220</td>
<td>Pdml</td>
<td>200, 20/</td>
</tr>
<tr>
<td></td>
<td>R: AATCTGGAAGACCTGAAATC</td>
<td></td>
<td></td>
<td>250A</td>
</tr>
</tbody>
</table>

Primers are given as forward (F) and reverse (R) in 5’−3’ direction. Underlined nucleotides mark nucleotide mismatches used in the selected restriction enzymes for discriminating sequence variations. RE size, size of fragments at the indicated allele after digestion of the PCR product using the respective restriction enzyme; SNP, single nucleotide polymorphism; RFLP, restriction fragment length polymorphism; PPARGC1A, peroxysome proliferator-activated receptor-γ coactivator-1α gene; BAC, bacterial artificial chromosome; BES, BAC end sequence. *Fluorescence-labeled primers.
for milk yield and milk fat yield were taken directly from the national breeding value evaluation for the Holstein breed in February 2001 (VIT, Verden, Germany), while EBVs for milk fat percentage were calculated as described by Thaller et al. (48). For the association studies, EBVs were deregressed by dividing each estimated breeding value by the square of its reliability

\[
\text{Dereg}_i = \left(1r_i^2\right)\text{REBV}_i
\]

where Dereg, is the deregressed estimated breeding value of son \(i\), REBV, is the relative estimated breeding value of son \(i\), and \(r_i^2\) is the reliability of the REBV of son \(i\).

To test the hypothesis that the polymorphisms c.1892+19T>C and c.3359A>C in the PPARGC1A gene are associated with the variation in milk fat synthesis, an association analysis between both SNPs and milk yield, milk fat yield, and milk fat percentage using general linear model procedures (SAS procedure GLM; SAS Institute, Cary, NC) was performed

\[
y_{ij} = \mu + g_{si} + \text{dgat1} + \text{ppargc1a} + e_{ij}
\]

where \(y_{ij}\) is the trait value of son \(j\) within grandsire \(i\), \(\mu\) is the overall mean, \(g_{si}\) is the fixed effect of grandsire \(i\), \(\text{dgat1}\) is the fixed effect of the DGAT1 genotype, \(\text{ppargc1a}\) is the fixed effect of the c.1892+19T>C genotype or c.3359A>C PPARGC1A genotype, respectively, and \(e_{ij}\) is the random residual effect. The weight of each observation was proportional to one over the \(\text{var}(e_{ij})\)

\[
\text{var}(e_{ij}) = \frac{1}{4}h^2 + \frac{1}{4n_{ij}}r_p^2
\]

where \(h^2\) is the heritability and \(r_p^2\) is the phenotypic variance of the trait investigated, respectively; \(n_{ij}\) is the number of daughters of son \(j\) within sire \(i\) included for the calculation of the EBV of son \(j\).

This analysis indicated that the c.1892+19T>C PPARGC1A genotype is associated with milk fat yield. Because this association at the c.1892+19T>C position might have reflected the effect of another polymorphism within or adjacent to the PPARGC1A gene, we consequently extended the genotype association analysis to five additional polymorphisms (c.-298_-301delCTTT, c.1209T>C, c.1847C>T, c.301delCTTT, and BES_J07162 250G>A) to evaluate, if other additional loci within or adjacent to the PPARGC1A gene also displayed a trait association. However, any genotypic associations within our data set had to be treated with caution, because it included sons from only a limited number of sires. Therefore, an association study using the genotypes of sons might be influenced strongly by the son’s paternally inherited chromosomes, possibly carrying a causal allele at a locus linked to the PPARGC1A gene.

Because of these limitations of the genotype association study, we performed a comprehensive analysis of the maternally transmitted alleles and haplotypes at all loci investigated. The extent of pairwise linkage disequilibrium (LD) between allelic loci within and adjacent to the PPARGC1A gene was calculated as \(D'\) and \(r^2\) according to Lewontin (28) and Hill and Robertson (20), respectively, while the significance of the LD was determined independently by \(X^2\) analysis. An additional phenotypic association study was performed considering only the effect of the maternally inherited PPARGC1A alleles and tested if the assumed association could be confirmed in the pool of bull dam alleles. Applying the following model, we tested whether the maternally inherited PPARGC1A alleles were associated with milk fat synthesis in our data set

\[
y_{ij} = \mu + g_{si} + \text{DGAT1} + \text{PPARGC1Am} + e_{ij}
\]

where \(y_{ij}\) is the trait value of son \(j\) within grandsire \(i\), \(\mu\) is the overall mean; \(g_{si}\) is the fixed effect of grandsire \(i\); \(\text{DGAT1}\) is the fixed effect of the DGAT1 K232A genotype; \(\text{PPARGC1Am}\) is the fixed effect of the maternally inherited PPARGC1A alleles c.1892+19T>C and c.3359A>C or alleles c.-298_-301delCTTT, c.1209T>C, c.1847C>T, c.301delCTTT, and BES_J07162 250G>A, respectively; and \(e_{ij}\) is the random residual effect.

Finally, we calculated the effects of the maternally inherited haplotypes either spanning all loci investigated (BES_J07162 250G>A, c.-298_-301delCTTT, c.1209T>C, c.1847C>T, c.301delCTTT, and BES_J07162 250G>A) or comprising alleles of adjacent loci (BES_J07162 250G>A, c.-298_-301delCTTT, c.1209T>C, c.1847C>T, c.301delCTTT, and BES_J07162 250G>A) using a model analogous to the single allele effects.

RESULTS

Bovine PPARGC1A Gene: Genomic Organization, Comparison with Related Sequences, and Gene Expression

To facilitate sequence analysis of the bovine PPARGC1A gene, we screened a bovine BAC library (57) using primers from exon 1 of the human PPARGC1A gene (AF106698) and a bovine EST (AF213835) highly homologous to a human EST (D59328) from the 3’ UTR of the gene. We identified two overlapping BAC clones, BBI_750011357 and BBI_75003315, carrying parts of the bovine PPARGC1A genes, and allocated a physical mapping region on chromosome BTA10.
gene. Fluorescence in situ hybridization of these BACs confirmed the localization of the gene on BTA6 by mapping the bovine PPARGC1A gene to the region BTA6q17–q19 (Fig. 2). This is in agreement with the previous mapping of the bovine EST (AF213835) on our high-resolution RH map (54). To identify further bovine PPARGC1A sequences, we screened available sequence databases with the human PPARGC1A cDNA sequence and discovered nine bovine ESTs covering different parts of the human sequence that are partially the exons 3, 7, 8, and 10 and the 3′-UTR and completely the exons 4, 5, 6, and 9 of the human PPARGC1A cDNA sequence. Remaining gaps not represented by bovine ESTs were closed by direct sequencing of the BAC clones by primer walking and using primers derived from the human cDNA sequence. The sequences of the bovine ESTs and from direct BAC sequencing were aligned to the human PPARGC1A cDNA sequence. On this basis, we constructed a consensus sequence for the bovine PPARGC1A cDNA (AY321517). Long-range RT-PCR was done to verify the existence of the assembled predicted cDNA transcripts. Amplification of overlapping transcripts spanning exon 1 to 8 (c.69 to c.1309, 1,378 bp), 2 exon 8 to 13 (c.1156 to c.2492, 1,337 bp), and 3 exon 13 with two fragments (c.2296 to c.5298, 3,003 bp; and c.4471 to c.6155, 1,685 bp) confirmed the predicted cDNA assembly (data not shown). In total, the determined cDNA sequence spans 6,261 bp. As summarized in Fig. 3, the bovine PPARGC1A gene is divided into 13 exons ranging from 46 to 1,982/3,884 bp. The transcription start site of the bovine PPARGC1A gene was determined by 5′-RACE experiments on mRNA from bovine liver. Exon 1 contains the translation start site that is predicted by the programs HMMGene and Genscan (http://genius.embl.de). Thus the bovine PPARGC1A gene is transcribed into an mRNA containing 90 bp of 5′-UTR sequence and 2,391 bp of sequence coding for a protein of 797 amino acids. Two consensus AATAAA polyadenylation signals suggest 3′-UTR sequences comprising 1,878 and 3,779 bp, respectively, downstream of the translational stop codon. The exon-intron boundaries of the bovine PPARGC1A gene were predicted by aligning the bovine and human cDNA sequences with the known structure of the human PPARGC1A gene (Table 2). We found that all exons of the bovine PPARGC1A gene followed the AG-GT rule for splice acceptor and donor sites. The exon-intron organization of the gene is perfectly conserved between the human and the bovine gene. Intron sizes were precisely determined for all introns except introns 2, 7, 10, and 12 (Table 2; GenBank accession nos. AY547551–AY547555, AY839821–AY839823). The complete size of the bovine genomic PPARGC1A sequence was

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Table 2. Exon-intron boundaries of the bovine PPARGC1A gene (AY321517)

<table>
<thead>
<tr>
<th>5′-Splice Donor</th>
<th>Intron No.</th>
<th>Size, bp</th>
<th>3′-Splice Acceptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>...ATCGAGtgagctggagctgg</td>
<td>1</td>
<td>5,357</td>
<td>eccccgtcctcgcagTGTGCT...</td>
</tr>
<tr>
<td>...TTTGGAtgagacgacccctt</td>
<td>2</td>
<td>ND</td>
<td>ttactgtgctgtagAAGATA...</td>
</tr>
<tr>
<td>...TCTCTAgtagacccctgcttct</td>
<td>3</td>
<td>1,826</td>
<td>tggatatgtgctgtagCTTA...</td>
</tr>
<tr>
<td>...GTAAAGtgaatgtaaatgtag</td>
<td>4</td>
<td>859</td>
<td>tgcctgtttagtgtagACCGG...</td>
</tr>
<tr>
<td>...TACAAGtgagccgacctg</td>
<td>5</td>
<td>3,884</td>
<td>tttaacccttcttgtagCAAC...</td>
</tr>
<tr>
<td>...ACCAAAtgagatgtagtag</td>
<td>6</td>
<td>109</td>
<td>tgcctggagctgtagACCC...</td>
</tr>
<tr>
<td>...GCGAAGtgaacctggcatcttt</td>
<td>7</td>
<td>ND</td>
<td>tggattagctgtagCTATCA...</td>
</tr>
<tr>
<td>...TCTCAAggacagtgtgccttca</td>
<td>8</td>
<td>563</td>
<td>ttgatccttcttgtagACCC...</td>
</tr>
<tr>
<td>...GCCCAAgtagtgatgcgagt</td>
<td>9</td>
<td>151</td>
<td>agttatgtagtcttgtagCTATG...</td>
</tr>
<tr>
<td>...GCAATTgtgagacgacgctggag</td>
<td>10</td>
<td>ND</td>
<td>cactcttcggctgccacGAGG...</td>
</tr>
<tr>
<td>...TGATGGtgaagtctctctcag</td>
<td>11</td>
<td>331</td>
<td>cttggctgtgtagtgAGAAC...</td>
</tr>
<tr>
<td>...ACCTAggttagcttttactg</td>
<td>12</td>
<td>ND</td>
<td>ttggtgtctgtagtCTGCAA...</td>
</tr>
</tbody>
</table>

Exon sequences are shown in uppercase letters, and intron sequences are shown in lowercase letters. Conserved GT-AG exon-intron junctions are marked in boldface type. ND, intron size not determined.
not determined, because we expected large introns spanning up to 50 kb, as reported for the human orthologous gene that comprises a total length of ~99 kb.

Alignment of the bovine PPARGC1A cDNA with corresponding coding sequences from other mammalian species showed similarity of 94% for humans and 91.1% for mice and rats. On the protein level, the bovine amino acid sequence revealed 94.9, 92.3, and 91.7% identity with human, mouse, and rat orthologs, respectively, which is demonstrated in Fig. 4.

The expression of the bovine PPARGC1A gene was analyzed using RT-PCR within a set of nine different tissues of a lactating Holstein Friesian cow, using primers spanning exons 4–5 and 8–13. The results shown in Fig. 5, A and B, revealed expression of the PPARGC1A gene in all analyzed tissues including the mammary gland. Predominant expression was observed in liver, kidney, thyroid gland, and pituitary gland, whereas in intestinal fat, PPARGC1A was expressed at a lower, but clearly detectable, level.

Identification of Polymorphisms in the Bovine PPARGC1A Gene

To test the hypothesis that a PPARGC1A gene variant is associated with the QTL effect, we subsequently comparatively sequenced the PPARGC1A gene locus to identify sequence polymorphisms in samples of animals differing in their genetic background. In our previous linkage studies in the German Holstein breed, we had identified sires that were most likely heterozygous at the QTL locus. Sires that are heterozygous at the QTL should also be heterozygous at a nucleotide polymorphism with putative causal effect in the underlying candidate gene. Consequently, we included individual genomic DNA from two Holstein sires (one of them is highly probable heterozygous (Qq) at the QTL locus, and the other is most likely homozygous (QQ or qq) at the QTL) and as references a bull from a beef/dairy cattle cross (Holstein × Charolais) and the identified BAC clone (derived from of a Jersey male) in our sequencing investigation. Furthermore, striving for a comprehensive detection of polymorphic sites within and around the PPARGC1A locus, we extended the screening for polymorphisms to DNA pool

Fig. 4. Comparison of sequence homology between bovine, human, mouse, and rat amino acid sequences of PPARGC1A. Amino acid sequences were derived from the following reference sequences: AY321517 (bovine), AF106698 (human), AF049330 (mouse), and AB025764 (rat). White letters indicate different amino acids compared with the bovine sequence.

Fig. 5. Electrophoretic expression pattern of the bovine PPARGC1A gene in different tissues of a lactating Holstein cow, using primers spanning exons 4–5 and 8–13. The results shown in Fig. 5, A and B, revealed expression of the PPARGC1A gene in all analyzed tissues including the mammary gland. Predominant expression was observed in liver, kidney, thyroid gland, and pituitary gland, whereas in intestinal fat, PPARGC1A was expressed at a lower, but clearly detectable, level.
sequencing. These DNA pools comprised two unibreed panels containing cows representing the German Holstein population and one multibreed panel involving bulls from a variety of taurine and one indicine breeds.

Those regions of the gene most likely affecting gene function were analyzed by comparative sequencing of the bovine PPARGC1A gene, namely, the coding sequence, the exon/intron boundaries, the 5′- and 3′-untranslated regions, and ~1 kb upstream from the transcription start (GenBank accession no. AY547550). In addition, the smaller introns 6, 9, and 11 were screened.

Furthermore, screening for sequence variation was extended to end sequences and internal sequences of two BAC clones that were positioned nearby the BACs carrying the PPARGC1A gene.

In total, we found 11 polymorphic sites within the bovine PPARGC1A gene (Fig. 3) scanning the 7 DNA samples described above. A conservative SNP was detected in exon 8 at nucleotide position 1209 (c.1209T>C), causing no amino acid substitution at position 403 of the PPARGC1A protein. A nonconservative SNP was identified in exon 9 at nucleotide position c.1847C>T encoding a proline-to-leucine substitution in the amino acid sequence in codon 616 (Pro616Leu). Two additional SNPs, based on a T-to-C nucleotide polymorphism, were found in intron 1 (c.49–9T>C) and intron 9 (c.1892+19T>C) of the gene. In addition, in the 3′-UTR three SNPs were identified: an A-to-C substitution (c.3359A>C), a variation based on a G-to-C replacement (c.4223G>C), and a C-to-T exchange (c.5314C>T). Furthermore, sequence analysis of the 3′-UTR of the bovine PPARGC1A gene revealed a microsatellite motif (c.2660–301delCTTT). The second polymorphism at position c.–644_–645delAA represents a length variation within a stretch of A nucleotides, and the third one is based on a G>A substitution at position c.–920.

We did not find any sequence variations in the FBNS10 sequence and internal BAC sequences but in one of the BAC end sequences. The BES of BBI_750J07162 carries a polymorphic site, a G>A variant, at position 250.

**Association Study of PPARGC1A Polymorphisms**

To test the hypothesis that the polymorphisms within the PPARGC1A gene would contribute to the variation of milk fat synthesis, we performed an association analysis between PPARGC1A gene variants and milk yield, milk fat yield, and milk fat percentage. In analyzing the genotypes at the identified polymorphic sites, we found that the Holstein sire highly probably heterozygous Qq at the QTL is heterozygous at two SNP sites, in intron 9 (c.1892+19T>C) and in the 3′-UTR (c.3359A>C). In contrast, the second Holstein sire, which is most likely homozygous at the QTL, revealed heterozygous genotypes at the SNP sites in exon 8 (c.1209T>C) and the 3′-UTR (c.3359A>C, c.4223G>C, and c.5314C>T). Allele frequencies of the polymorphic loci within and adjacent to the PPARGC1A gene included in the further association analyses were determined in a control panel representative for the German Holstein population (Fig. 6). In contrast to the SNPs PPARGC1A c.3359A>C and BES_J07162 250G>A, which had allele frequencies close to 0.5, one of the alleles of the other sequence variants was observed predominantly. In the Holstein control panel, we detected no homozygous animals for the rare alleles of the sequence variants c.1209T>C, c.1847C>T, c.1892+19T>C, c.5314C>T, and c.–298_–301delCTTT. The genotype distributions of the seven polymorphisms analyzed were in Hardy-Weinberg equilibrium within the Holstein control panel (data not shown). For maternally inherited alleles in a large data set of bulls originating from cows highly

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![Fig. 6](https://physiolgenomics.physiology.org/ by 10.220.33.6 on June 20, 2017)

**Fig. 6.** Frequencies of the alternative alleles at diallelic polymorphic loci within and around the bovine PPARGC1A gene: comparison between a Holstein control population and maternally inherited alleles from a Holstein dam population highly selected for milk production traits. At bottom, in shaded boxes: C, control population; S, maternally inherited alleles from a dam population highly selected for milk production traits. *P < 0.05 and ***P < 0.001.
selected for milk production traits, highly significant differences in the allele frequencies compared with the control population were observed for the SNP in intron 9 (c.1892+19T>C), but no or only marginal differences for the other polymorphic loci were observed (Fig. 6). Allele c.1892+19T from the SNP c.1892+19T>C had a significantly higher frequency in the population of alleles inherited from dams highly selected for milk production traits compared with the control population.

A total of 32 maternally inherited haplotype patterns comprising the alleles of all 7 loci investigated (BES_J07162 250G>A; c.1892+19T/C; c.1892+19T>C - c.3359A/C and c.3359A/C; c.5314C>T) were observed, with 10 of them detected in at least 10 individuals and comprising 84.6% of all maternally inherited haplotypes. Calculation of \( D^2 \) and \( r^2 \) values. Significance of pairwise LD (C). C: pairwise \( X^2 \) values. For calculation of \( D^2 \) and \( r^2 \), see MATERIALS AND METHODS. I, BES_J07162 250G>A; II, c.1892+19T>C; III, c.1209T>C; IV, c.1847C>T; V, c.1892+19T>C; VI, c.3359A>C; VII, c.5314C>T

Table 3. Association of milk production traits with PPARGC1A c.1892+19T>C and PPARGC1A c.3359A>C genotypes in German Holstein bulls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>MY, kg</th>
<th>MFP, %</th>
<th>MY, kg</th>
<th>MFP, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARGC1A c.1892+19T&gt;C Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-T (n = 5)</td>
<td>887.45 (±255.30)</td>
<td>0.0022 (±0.1096)</td>
<td>34.00 (±10.46)</td>
<td>0.0020 (±0.1096)</td>
</tr>
<tr>
<td>T-C (n = 132)</td>
<td>642.23 (±88.39)</td>
<td>-0.0071 (±0.0379)</td>
<td>22.11 (±3.62)</td>
<td>-0.0062 (±0.0369)</td>
</tr>
<tr>
<td>C-C (n = 297)</td>
<td>534.39 (±86.00)</td>
<td>-0.0062 (±0.0369)</td>
<td>17.02 (±3.53)</td>
<td>-0.0062 (±0.0369)</td>
</tr>
<tr>
<td>( P ) value</td>
<td>0.0840</td>
<td>0.8897</td>
<td>0.0361</td>
<td>0.2540</td>
</tr>
</tbody>
</table>

| PPARGC1A c.3359A>C Genotype |            |           |            |           |
| T-T (n = 65)       | 686.82 (±104.76) | -0.0035 (±0.0448) | 25.04 (±4.29)   | 0.0020 (±0.1096) |
| T-C (n = 251)     | 575.69 (±84.61)   | -0.0064 (±0.0362) | 18.71 (±3.47)   | -0.0020 (±0.1096) |
| C-C (n = 118)     | 525.63 (±95.07)   | -0.0058 (±0.0407) | 16.77 (±3.90)   | -0.0020 (±0.1096) |
| \( P \) value     | 0.2020       | 0.7961     | 0.0762      |           |

Values are given as least square means (±SE) of estimated breeding values (EBV) for milk production traits; \( n \) = no. of animals. MY, milk yield; MFP, milk fat percentage; MY, milk fat yield.
With respect to the QTL for milk fat yield on BTA6, we present here the PPARGC1A encoding gene as a potential regulator of fat synthesis in dairy cattle. On the one hand, PPARGC1A has been identified as a positional candidate gene according to its chromosomal localization linked to the QTL for milk fat yield on BTA6 and located within homologous chromosomal regions affecting obesity-related traits in other species. In addition to its position within a QTL region for milk fat yield, PPARGC1A qualified also as a functional candidate gene because of its significant role in coordinated regulation of energy partitioning and homeostasis, gluconeogenesis, and fat synthesis in response to endogenous physiological and environmental stimuli.

We have isolated the bovine PPARGC1A gene, determined its cDNA sequence, and detected its expression in all samples of our multitissue panel, including tissues contributing to the regulation of energy homeostasis and milk production in cattle (e.g., liver, mammary gland). In addition to the identification and characterization of the bovine PPARGC1A cDNA and flanking genomic regions, we report a systematic screening for variation in the coding sequence, the exon-intron boundaries, three introns, the 3' and 5' untranslated regions, and ~1,000 bp immediately 5'-upstream of the gene, as well as in sequences from BACs close to the PPARGC1A gene.

In a sample consisting of a total of 64 animals with different QTL genotype and from a large variety of breeds, we identified a total of 11 DNA sequence polymorphisms within nearly 7,900 bp of the PPARGC1A gene corresponding to one polymorphic nucleotide per 718 bp. In the sequence region analyzed around the PPARGC1A locus comprising ~2 kb from closely adjacent BACs, we detected one additional SNP.

The detection of only one nonsynonymous SNP in the coding region of PPARGC1A is in agreement with our results from comparison of human, mouse, rat, and bovine cDNA and amino acid sequences demonstrating that the PPARGC1A gene is highly conserved across species.

We proposed the hypothesis that the polymorphic sites found within the bovine PPARGC1A candidate gene might be associated with the QTL effect for milk fat synthesis on BTA6. Any causal polymorphism should then cosegregate with the assumed genotype at the QTL. The grandsire, which was previously shown to be heterozygous Qq at the QTL for milk fat yield in the region of the PPARGC1A gene, showed a heterozygous genotype exclusively for the SNPs in intron 9 (c.1892+19T>C) and in the 3'-UTR (c.3359A>C) while homozygous at all SNPs in the coding region and all other polymorphic positions within or adjacent to the gene.

In our association study, we found indication supporting a trait relationship of the sequence variation c.1892+19T>C in intron 9 of the bovine PPARGC1A gene with milk fat yield.

1) We found an association of the genotypes at the c.1892+19T>C SNP in a large data set of paternal half-sib bull families that showed a significantly increased milk fat yield of individuals with genotypes 1892+19T/1892+19T and 1892+19T/1892+19C.

2) This association was confirmed for the maternally inherited alleles of this data set, with allele 1892+19C being associated with decreased milk fat yield.

3) Neither the comprehensive haplotypes of the whole genomic region nor pairwise-adjacent haplotypes as determined from the maternally transmitted alleles showed an association with milk fat yield similar or more significant than the maternally transmitted c.1892+19T>C SNP alleles individually.

4) The allele frequency of the favorable allele regarding milk fat yield was significantly increased in the maternally inherited alleles from dams highly selected for milk production traits compared with the allele frequency in a control population.
The tendency of association between SNP c.3359A>C and milk fat yield may be due to the detected LD between the c.1892+19T>C and the c.3359A>C locus. Regarding the history of the polymorphism in intron 9, the c.1892+19T variant is an ancient sequence variant, because it was observed in many taurine breeds as well as in African zebu (Dwarf zebu) and Tibetan yak (Poephagus mutus).

Previous studies in humans emphasized the significance of a comprehensive characterization of the intragenic LD within candidate genes (e.g., Refs. 30, 49), because frequently intragenic haplotype blocks within candidate genes are observed, which would impede dissection of the effects of the single polymorphisms. Although a direct conclusion from humans to cattle may be hampered by the observation that the distribution of LD across the genome differs substantially between humans and bovines (11), similar intragenic haplotype blocks would also affect gene association studies in cattle. However, our analysis of the LD within and adjacent to the PPARGC1A gene did not reveal indication on a haplotype block including the c.1892+19T>C locus associated with milk fat yield.

Recently, there is an increasing indication that variation in complex traits results from noncoding regulatory variants more frequently than from coding sequence polymorphisms (reviewed, e.g., in Refs. 15, 37). Although regulatory elements have been localized predominantly in the 5′-flanking regions of genes, there is a growing number of examples for intronic and 3′-untranslated sequence motifs that appear to play a significant role in regulating the expression level of a gene or in defining its tissue-specific expression pattern (e.g., Refs. 7, 14, 15, 17, 27, 37, 42, 51). Albeit the ability to identify and evaluate functionally important polymorphisms in noncoding regions is still poorly developed, there is a recent prominent example in farm animal research supporting the view that variation in regulatory regions is important for controlling phenotypic variation of complex traits. Van Laere et al. (51) showed that a QTL affecting muscle growth, fat deposition, and heart size in pigs is caused by a nucleotide substitution in intron 3 of the insulin-like growth factor-2 gene.

PPARGC1A plays a critical role in several aspects of glucose, fat, and energy metabolism and coordinates the process of metabolic adaptation in liver, fat tissue, and muscle. Thus it seems conceivable that a variation in the noncoding region of the gene with potential effects on pre-mRNA processing, splicing efficiency, or mRNA level might affect the response on the increased glucose demand of the lactating mammary gland during the high-lactation state. Particularly in high-yielding dairy cows, this physiological adaptation requires major adjustments in glucose production and utilization in liver, adipose tissue, skeletal muscle, and other tissues.

However, it has to be excluded that the observed trait association of the c.1892+19T>C variant is due to an LD with a yet-undetected functional polymorphism in close proximity to PPARGC1A. As outlined in a comprehensive evaluation of association studies in humans (e.g., Ref. 21), independent replication of association studies is necessary before final conclusions on a genetic association should be drawn. Thus future investigations will have to replicate the association study of PPARGC1A SNPs presented here for German Holsteins. In addition to this breed, belonging to the world-wide dominating dairy breed of Holsteins, other populations have to be studied independently. These investigations may also integrate the exploration of gene-gene and gene-environment interactions as requested from genetic association studies in humans (e.g., Refs. 21, 29).

It remains to be demonstrated, finally, whether the nucleotide substitution in intron 9 of PPARGC1A is causal for the observed QTL for milk fat yield on BTA6. Further functional and physiological research elucidating the impact of the PPARGC1A gene on bovine fat synthesis, including information from comparative physiology and genomics (34), will help in understanding the complex interplaying processes and pathways linked to lipid, glucose, and energy metabolism and possibly the genetic basis of variation in obesity-related phenotypes across species.

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