Phenotypic population screen identifies a new mutation in bovine DGAT1 responsible for unsaturated milk fat

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Selective breeding has strongly reduced the genetic diversity in livestock species, and contemporary breeding practices exclude potentially beneficial rare genetic variation from the future gene pool. Here we test whether important traits arising by new mutations can be identified and rescued in highly selected populations. We screened milks from 2.5 million cows to identify an exceptional individual which produced milk with reduced saturated fat content, and improved unsaturated and omega-3 fatty acid concentrations. The milk traits were transmitted dominantly to her offspring, and genetic mapping and genome sequencing revealed a new mutation in a previously unknown splice enhancer of the DGAT1 gene. Homozygous carriers show features of human diarrheal disorders, and may be useful for the development of therapeutic strategies. Our study demonstrates that high-throughput phenotypic screening can uncover rich genetic diversity even in inbred populations, and introduces a novel strategy to develop novel milks with improved nutritional properties.

Livestock selection for the improved production of milk, meat, skin, and fiber has influenced the evolution of human society and animal breeds1–2. Traditional selection based on ancestral performance is currently being supplanted by “genomic selection” based principally on the summation of genome-wide marker or haplotype associations with ancestral performance parameters3. While both industrial processes have provided considerable genetic gain, application of either methodology excludes rare alleles or new mutations with significant phenotypic impact from the future gene pool.

Haldane4 estimated the mutation rate for hemophilia in humans to be approximately $10^{-5}$, and subsequent studies have confirmed the rate of new mutations per gene to range from $10^{-7}$ to $10^{-4}$ per generation5–7. Due to the comparable sizes of human and cow genomes8,9, we hypothesized that if novel mutations were to arise in the bovine at a comparable rate, rare alleles for extreme phenotypes should be detectable provided that the sample size is above one million and an appropriate screening protocol for the rare trait is available. We also hypothesized that variations in these genetically and biochemically complex components could be proxies for individually refined milk compositions, including variations in individual milk fatty acids.

We have tested our hypothesis using a large and well-characterized bovine population. Our aim was to discover new alleles that are responsible for milks with extremely low or high milk fat or protein content.

Results

We screened the lactation records of 2.5 million cows and identified 29 individuals with milks deviating by more than 3.5 standard deviations (SD), over repeated milk tests and lactations, from the breed- and age-adjusted means for protein or fat content. Of these, Holstein-Friesian cow ‘363’ produced milk with average fat content of $2.81\% \pm 0.10\%$ (mean $\pm$ SD), 3.9 SD below the breed mean of 4.52%. Milk protein concentration of cow 363 was 1 SD lower than the population group mean, while mean seasonal milk production of 6223 $\pm$ 925 L was 3.0 SD above the breed average. At selection, cow 363 was 8 years old and had completed...
five lactations, indicating normal fertility and survival rate. Information provided by her breeder and lifetime owner confirmed normal history and health status, and the cow showed no abnormalities on inspection (Fig. 1b). The 74,618 paternal half-sisters of cow 363, farmed throughout New Zealand, produced milks with an average fat content of 4.4 ± 0.7%. Milks from cow 363’s deceased dam and her maternal half-sister contained 3.98 ± 0.15% and 5.60 ± 0.3% fat, respectively.

Figure 1 | An outlier cow with a heritable mutation responsible for low milk fat content. (a) Pedigree of cow 363 showing segregation of the low milk fat phenotype. Founder cow 363 is indicated by the large filled circle. Filled symbols represent affected females or carrier males, open symbols represent wild-type individuals. Numbers inside symbols indicate average milk fat content in percent, determined during the first lactations of the founder’s filial generations, or during the lactation lifespan for all other animals. For offspring from bull sons, the milk fat averages of their phenotypic daughter groups are stated together with the number of animals in each group. Hash symbols indicate unavailable DNA samples, and the asterisks denote offspring generated by embryo transfer. (b) Founder cow 363 during the dry phase at 13 years of age. (c) Segregation of milk fatty acid composition in the F1 generation. Milk contents of saturated (SFA, blue), monounsaturated (MUFA, red), and polyunsaturated fatty acids (PUFA, black) for three individual mutant and wild-type daughters of cow 363, and for three unrelated, breed-matched control cows in the same herd, are indicated by open symbols. Means are indicated by bold horizontal bars, and P values (two-tailed Student’s t-test) for fatty acid groups differences are stated between genotype groups. Differences between wild-type daughters and control cows were not significant (P_{SFA}=0.96, P_{MUFA}=0.96, P_{PUFA}=0.93).
Detailed milk composition analysis revealed a substantial improvement in the ratio of saturated:unsaturated milk fat. Saturated fat content was reduced by 3–4 SD, while mono- and polyunsaturated fatty acids were 2–3 SD above the New Zealand average (Supplementary Table 1). In contrast, content and composition of casein and whey proteins were within one SD of the national breed average (Supplementary Table 2). The extreme milk fat content and milk fat composition phenotypes remained unchanged after cow 363 was relocated from her home farm to our research farm, indicating a negligible influence of environmental factors.

To assess the genetic basis of the extreme milk fat phenotypes, we generated four additional sons and seven daughters of cow 363 (Fig. 1a). Three daughters produced milks with an average fat content of 2.62% ± 0.09%, and fatty acid compositions similar to cow 363; in contrast, average fat content (4.14% ± 0.37%) and fatty acid composition of milks from her other four daughters were similar to unrelated control cows (Fig. 1c, Supplementary Table 3). When unrelated Holstein-Friesian dams were bred with semen from the five sons of cow 363, 18 of the 50 daughters sired by three of the sons produced milks with fat content similar to that of founder cow 363. Milks from their paternal half-sisters, and from the 42 daughters of the other two bull sons of cow 363, were similar to that of the breed average (Fig. 1a). Similar to the founder, no other phenotypes were apparent in her offspring. Taken together, these results suggested that a heterozygous genetic variation with a dominant effect was responsible for the milk fat phenotypes of cow 363.

Genotyping of 56,000 single-nucleotide markers in the pedigree of cow 363 and genome-wide association analysis identified a single locus associated at a genome-wide level of significance [p-value ≤ 1.27 × 10⁻¹⁰] (Fig. 2). This locus harbors the DGAT1 gene encoding diacylglycerol O-acyltransferase 1, which catalyzes the terminal reaction in triglyceride synthesis (DGAT1). Sequencing of the DGAT1 locus of cow 363 revealed a heterozygous, non-synonymous A>C transition in exon 16 (Fig. 3a). This variation was present in all females producing milks with less than 3% fat and in the three bulls producing offspring with reduced milk fat content but was absent in all other offspring of cow 363, her sire, and her grand sires. Moreover, the variant was absent in 185 bulls that have sired the majority of the New Zealand dairy cows. As historical records showed that the milk fat phenotype of cow 363’s deceased dam was typical of the breed, the mutation has likely occurred de novo. Cow 363 was homozygous for the common Ala 232 variant of DGAT1.

Quantitative PCR amplification polymerase and cDNA sequencing revealed that the mutation disrupts splicing and causes exon 16 skipping. In liver and mammary gland biopsies from heterozygous animals, transcripts lacking the 63-nucleotide exon 16 were as abundant as full-length transcripts (Fig. 3b). In contrast, only low levels of full-length transcripts were detectable in liver biopsies from homozygous mutants (Fig. 3b,c). Analysis of the wild-type sequence of exon 16 identified the putative consensus exonic splicing enhancer motif 5'-ATGATG overlapping the mutation (Fig. 3a).

To assess the effect of the mutation on enzyme activity, we expressed wild-type and mutant DGAT1 cDNAs in a baker’s yeast strain lacking endogenous diacylglycerol acyltransferase activity. In contrast to the full-length variants, the mutant enzyme lacking the 21 amino acids encoded by exon 16 was unable to transfer oleic acid from CoA to diacylglycerol (Fig. 4).

These results show that a novel single nucleotide substitution in an exonic splicing enhancer of the DGAT1 gene induces exon 16 skipping and results in enzymatically inactive diacylglycerol O-acyltransferase 1. The mutation also reveals a link between triglyceride synthesis capacity and milk fat saturation, and provides an important clue to the role of DGAT1 in milk fat saturation.

Cows heterozygous for the new mutation thrived in a pastoral environment, and displayed normal growth, survival, and fertility rates. In contrast, homozygous calves exhibited scurrying (non-bloody watery diarrhea), slow growth, sensitivity to dietary fats, reduced levels of serum cholesterol, free fatty acids and triglycerides, and high levels of serum cholesterol, free fatty acids and triglycerides.

Figure 2 | Association mapping of the mutation responsible for low milk fat content. Genome-wide association of milk fat content. The chromosomal position of SNP markers (x axis) is plotted against -log10 GWAS P-value (y axis). The threshold for genome-wide significance (P≤1.27×10⁻¹⁰) is indicated by a horizontal line. Markers on chromosome 14 showing significant association with milk fat content are identified.
flattened intestinal microvilli. Scouring severity and incidence was reduced on iso-energetic low-fat diets, and growth rates improved by regular parenteral administration of essential lipids. These phenotypes overlap in part with the single reported case of loss of function, and whether the pleiotropic effects of over the longer lifespan of cows any novel effects of be screened to identify new and rare mutations responsible for extreme traits, and that these mutations can be employed to establish new lines, together with unrelated control cows.

Animal procedures. Animals were managed under standard New Zealand seasonal farming routines on mixed ryegrass/white clover pasture and milked twice daily. Adult females were artificially inseminated for calving in July and August. Experimental samples were collected from September to February before cows were dried off in April or May. Lactating females from the founder pedigree were farmed under standard New Zealand seasonal routines on mixed ryegrass/white clover pasture and milked twice daily. Adult females were artificially inseminated for calving in July and August. Experimental samples were collected from September to February before cows were dried off in April or May. Lactating females from the founder pedigree were farmed together with unrelated control cows.

In vitro fertilization and embryo implantation into surrogate dams was performed according to routine industry protocols (Artificial Breeding Services, Hamilton, New Zealand). Liver and mammary gland tissues were collected post milking by needle biopsy, or at slaughter. Samples were snap-frozen in liquid nitrogen and stored at −86 °C until use. Animals were monitored after biopsy as described. Procedures were approved by the AgResearch Ruakura Animal Ethics Committee.

Milk composition analysis. Milk samples were collected during routine morning and evening milking and combined into a representative daily sample. Milk fat and protein content was determined by FTIR. Fatty acid composition was determined by gas chromatography.

Methods

Population screen. New Zealand’s National Herd Testing database records milk volume, protein and fat content, and somatic cell count determined by Fourier transformed infrared spectrometry (FTIR, http://www.foss.com) for 70–80% of dairy cows 3–4 times per season. We retrieved statistical outlier cows with low or high milk fat percentage, high milk protein percentage, or high milk protein : fat content ratio in New Zealand’s National Herd Testing database records milk volume, protein and fat content, and somatic cell count determined by Fourier transformed infrared spectrometry (FTIR, http://www.foss.com) for 70–80% of dairy cows 3–4 times per season. We retrieved statistical outlier cows with low or high milk fat percentage, high milk protein percentage, or high milk protein : fat content ratio in multiple herd tests over at least two lactation seasons. Candidates were compared to herd mates to exclude cows with extreme milks due to environmental factors, and we biased selection towards de novo mutations by excluding candidates with paternal half-sibs or maternal ancestors showing similar, albeit less extreme phenotypes.

Candidates were inspected for gross phenotypic abnormalities, and their owners interviewed to reveal possible environmental contributions to the extreme phenotypes. The owners were informed of the aims and potential contribution of their animal to the program before the cows were purchased.
Figure 4 | The mutation abolishes the diacylglycerol acyltransferase activity of Dgat1. Thin-layer chromatography (a) and quantification by densitometry (b) of triglycerides produced by recombinant Dgat1 wild-type (Lys232 and Ala232) and mutant (Ala232–E16) enzymes, compared to vector-only control (pYES2). Extracts were adjusted to contain equivalent levels of recombinant DGAT1 mRNA or yeast TDH1 mRNA (for pYES2). Panel a shows results from a typical experiment, while data in panel b show mean ± s.e.m. of triacylglyceride levels produced by at least seven extracts prepared from at least two independent yeast transformations for each plasmid. P-values determined by two-tailed Student’s t-test are indicated. As previously reported15, the full-length Ala232 variant showed slightly lower activity than the ancestral Lys232 enzyme.

Genotyping of the 8078A>C mutation. Animals from the 363 pedigrees, 185 sires frequently used in the New Zealand dairy population, and 80 sires and 1595 cows representing a crossbred herd were genotyped for the mutation by the Australian Genome Research Facility using a custom-designed iPLEX Gold assay (SEQUENOM) with primers detailed in supplementary file 2.

Identification of splicing regulatory motifs. The effect of the 8078A>C substitution was analyzed against a set of functionally validated hexamer motifs that are statistically overrepresented in exons31,32 by querying the RESCUE-ESE web server (http://genes.mit.edu/burgelab/rescue-es/) with wild-type and mutant bovine DGAT1 sequences.

Analysis of DGAT1 mRNA splicing. Total RNA was prepared from liver samples using RNeasy columns (QIAGEN) and on-column DNase treatment, and reverse transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen). Presence of DGAT1 exon 16 was determined by gel electrophoresis and sequencing of the PCR products obtained with primer pairs to exon 15 and 16, or to exons 15 and 17. Exon 16 skipping was quantified by qPCR on a Lightcycler 480 using the Lightcycler 480 Probes Master System and universal probes (Roche). Briefly, exon16-containing transcripts were PCR-amplified using primers to exons 15 and 16 with fluorescent probe #44, and normalized to DGAT1 transcripts quantified using primers to DGAT1 exons 4 and 5 with probe #98. Detailed parameters are provided in the Supplementary MIQE File. Statistical significance of expression differences was determined using Student’s t-test (two-sided, unequal variance).

Yeast expression and diacylglycerol acyltransferase assay. Yeast expression vectors encoding wild-type (Lys232 and Ala232) or mutant (Ala232–E16) DGAT1 were constructed by cloning bovine cDNA into pYES2 (Invitrogen). Plasmids and vectors were transformed into tricylglyceride-negative strain H12464, and transgene expression was induced using galactose as described (pYES2 Manual, Invitrogen). Twenty OD600 nm of culture was harvested, and 1 OD600 nm was retained for mRNA extraction. Cells were sedimented, washed with water, and disrupted by vigorous vortexing in 50 μL of 10 mM Tris-HCl (pH 7.9), 10 mM MgCl2, 1 mM EDTA, 5% glycerol, 1 mM DTT, 0.3 M ammonium sulfate, Complete protease inhibitor cocktail (Roche), 0.8 mM Pefabloc SC (Roche), and 600 mg of glass beads (diameter 450–600 μm, Sigma-Aldrich). The cell lysate was recovered in 500 μL disruption buffer and cleared by centrifugation at 12000 g-1 for 10 min. Protein concentration was determined using the DC protein assay (Bio-Rad).

Diacylglycerol acyltransferase activity in 50 μg of clarified yeast lysate was determined as described11. TLC plates were exposed to a phosphor imaging screen and scanned (Pharos FX+, Bio-Rad). DGAT1 cDNA was quantified by qPCR using primers to exons 4 and 5 (see above), while endogenous TDH1 transcripts were quantified using fluorescent probe #92 and primers detailed in supplementary file 2. DGAT1 expression levels were computed as the ratio of mean quantification cycle (Cq) for the bovine DGAT1 reaction to the mean Cq for the yeast TDH1 reaction. Statistical significance of gene expression differences was determined using Student’s t-test (two-sided, unequal variance).

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Author contributions
R.G.S. conceived the project. K.L., S.D.B. and R.G.S. designed and directed experiments, and analyzed results. A.A.-U., B.H., A.K.H.M., R.J.S. and R.G.S. performed the population screen and Outlier selection. A.A.-U. managed and bred the animal pedigree. Y.v.d.D. and A.K.H.M. analyzed milk fat composition. K.L., H.W., A.A.-U., A.B., L.F.A. and N.L.T. contributed to genotyping. E.M.B. and S.R.B. contributed to DGAT1 sequencing, bioinformatics analysis, cDNA cloning, and expression plasmid construction. P.R. and W.T.J. advised on enzyme function assay. H.W. performed enzyme assays and qPCR reactions. G.V. and S.R.D. provided advice on cow management and animal physiology. K.L., H.W., A.A.-U., A.B., N.L.T. and G.V. performed animal biopsies. K.L., S.D.B., R.J.S. and R.G.S. wrote the first draft of the manuscript, and all authors contributed to and read the final version.

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