

Targeted microRNA expression in dairy cattle directs production of β -lactoglobulin-free, high-casein milk

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Edited by R. Michael Roberts, University of Missouri, Columbia, MO, and approved August 28, 2012 (received for review June 22, 2012)

Milk from dairy cows contains the protein β -lactoglobulin (BLG), which is not present in human milk. As it is a major milk allergen, we wished to decrease BLG levels in milk by RNAi. In vitro screening of 10 microRNAs (miRNAs), either individually or in tandem combinations, identified several that achieved as much as a 98% knockdown of BLG. One tandem construct was expressed in the mammary gland of an ovine BLG-expressing mouse model, resulting in 96% knockdown of ovine BLG in milk. Following this in vivo validation, we produced a transgenic calf, engineered to express these tandem miRNAs. Analysis of hormonally induced milk from this calf demonstrated absence of BLG and a concurrent increase of all casein milk proteins. The findings demonstrate miRNA-mediated depletion of an allergenic milk protein in cattle and validate targeted miRNA expression as an effective strategy to alter milk composition and other livestock traits.

nuclear transfer | transgenic cattle

Milk is the primary source of nutrition for young in mammals and contains all the essential nutrients for mammalian growth. In humans, the incidence of breastfeeding is decreasing and cows' milk has become a substitute for human milk. The protein composition of cows' milk is, however, different from that of human milk, and this may cause allergic reactions. In developed countries, 2% to 3% of infants are allergic to cows' milk proteins in the first year of life (1). The milk whey protein β -lactoglobulin (BLG) is produced in cows and other ruminants but is not found in human milk, so it is not surprising that it constitutes a major milk allergen (2). It naturally occurs in a number of genetic variants, and the most prevalent bovine variants are known as BLG A and BLG B (3). BLG has been associated with the transport of small hydrophobic molecules (4), linked to hypocholesterolemia (5) and has been ascribed antioxidant properties (6), but its exact biological role remains unknown. BLG may primarily constitute another source of protein for the young, and it is generally believed that it is dispensable from milk without greatly altering its properties.

To reduce the allergenic potential of milk, hypoallergenic formulas based on enzymatically hydrolyzed whey proteins have been developed by the dairy industry. However, hydrolysis is not a perfect solution because the resulting peptides can still possess residual antigenicity, it may cause milk to taste bitter as a result of the exposure of hydrophobic amino acids (7, 8), and the processing technique is costly. Alternatives to postharvest processing include more direct approaches to reduce BLG, such as knockdown by RNAi or KO of the BLG gene (*LGB*) by using homologous recombination (9). Gene KOs in livestock species have proven to be notoriously difficult to achieve (10), and, despite being an attractive target, no BLG KO animals have yet been reported to our knowledge. Applying zinc-finger nuclease technology, Yu et al. recently demonstrated successful biallelic modification of the *LGB* gene in bovine cells (11). The targeted modifications of the one live *LGB* mutant calf consisted of small in-frame deletions and did not create the *LGB* KO alleles required for the production of BLG-free milk.

In comparison with a KO strategy, the use of RNAi is a less complex approach to down-regulate BLG and reduce the

allergenicity of cows' milk (9). Moreover, RNAi could allow fine-tuning of BLG expression, which may be advantageous if some BLG is required for normal milk physiology. Artificial RNAi molecules that enable the knockdown of target transcripts, either by mRNA degradation or a block of translation, have been used in different forms such as siRNAs, shRNAs, or miRNAs (12, 13).

A recent in vitro study demonstrated the effectiveness of several shRNAs and miRNAs directed against the porcine variant of BLG (14). We used artificial miRNAs based on the murine miRNA-155 to knock down BLG. miRNAs can be driven by Pol II promoters, which enable spatiotemporally restricted expression and greatly limit off-target effects that may arise from constitutive miRNA expression. Indeed, constitutive expression of BLG-specific RNAi constructs negatively affects primary cell growth, indicating that abundant interfering RNAs aimed at BLG may be toxic (14). When the same RNAi constructs were controlled by a lactation-specific promoter, they showed no adverse effects and were compatible with early pig development (14). However, the feasibility of reducing BLG in milk by an RNAi-mediated approach remains untested.

Here, we present data on the in vitro screening for efficient BLG-specific miRNAs. Considering the substantial costs involved in generating transgenic cows, the most effective miRNAs were tested in a mouse BLG knockdown model engineered for the mammary gland-specific expression of miRNAs and BLG, which rodents do not normally express. The in vivo validated miRNAs were used to generate a transgenic calf whose milk contained no detectable BLG and more than twice the amount of casein milk proteins.

Results

BLG Knockdown in Cultured Cells. The whey protein BLG is highly conserved between sheep and cattle. This suggests that miRNAs can be designed that possess knockdown activity against BLG variants of both species. Because dairy cattle will be the ultimate target of this approach, we have used the common bovine variant BLG B for designing effective miRNAs against ovine and bovine BLG. The designs suggested by the RNAi designer tool were screened against the mouse, human, and bovine genomes to exclude miRNAs with strong homology to any off-target sequences. We then selected 10 of the most highly ranked miRNAs targeting homologous sequences within the coding regions of ovine and bovine BLG, avoiding the signal peptide encoding sequence and regions with multiple mismatches between the ovine and bovine

Author contributions: A.J., S.W., and G.L. designed research; A.J., S.W., J.M., and D.N.W. performed research; A.J., S.W., and G.L. analyzed data; and A.J., S.W., and G.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1210057109/-DCSupplemental.

sequences (Fig. 1). By using these design criteria, we expected to identify miRNAs that are effective against ovine and bovine BLG.

BLG expression is limited to mammary tissue during lactation and is difficult to emulate in cultured cells. This made it necessary to first engineer a cell culture system for BLG expression to evaluate the 10 BLG-specific miRNAs for their knockdown potential. Thus, COS-7 cells were cotransfected with an expression plasmid for BLG and a plasmid bicistronically encoding GFP and one of the BLG-specific miRNAs. Knockdown of BLG was determined by Western blot analysis (Fig. 2). BLG expression for cells cotransfected with ovine BLG and a scrambled control miRNA was set as 0% knockdown. To account for differences in transfection efficiency, BLG expression was normalized against GFP expression encoded on the miRNA construct. Most miRNAs down-regulated ovine BLG expression, with the exception of miRNA 7 ($-46 \pm 49\%$) and miRNA 10 ($-9 \pm 45\%$), which appeared to have no or possibly a slight enhancing effect. The knockdown activities of the effective miRNAs varied and were classified as low ($12 \pm 19\%$ for miRNA 1), medium ($33 \pm 14\%$ for miRNA 3 and $31 \pm 16\%$ for miRNA 5), and high ($94 \pm 2\%$ for miRNA 2, $78 \pm 9\%$ for miRNA 4, $93 \pm 1\%$ for miRNA 6, $66 \pm 7\%$ for miRNA 8, and $83 \pm 10\%$ for miRNA 9).

By using an equivalent cotransfection approach, replacing ovine BLG with a bovine BLG expression plasmid, the 10 miRNAs were also analyzed for their potential to knock down bovine BLG. Compared with ovine BLG, a greater number of the miRNAs showed high knockdown activity. miRNA 1 ($81 \pm 9\%$), miRNA 2 ($92 \pm 7\%$), miRNA 3 ($78 \pm 17\%$), miRNA 4 ($94 \pm 2\%$), miRNA 5 ($80 \pm 13\%$), miRNA 6 ($97 \pm 3\%$), miRNA 8 ($78 \pm 7\%$), and miRNA 10 ($70 \pm 28\%$) reduced bovine BLG levels by 70% or more. Thus, some of the miRNAs (1, 3, 5, 10) were more specific for bovine BLG and less active against ovine BLG. miRNA 9 showed the reversed specificity and was more active against ovine than bovine BLG, whereas miRNA 7 slightly enhanced bovine BLG expression ($-72 \pm 42\%$) and was the only miRNA that had no knockdown activity against either target.

Cumulative action of multiple miRNAs targeting different regions of the same transcript often yields a higher knockdown

than the individual miRNAs on their own. We therefore tested tandem miRNA constructs and compared their activity vs. that of their respective single miRNAs. We generated three tandem constructs with different combinations of three miRNAs that showed high knockdown efficiency for ovine and bovine BLG (miRNAs 2, 4, and 6). All tandem constructs showed high BLG knockdown activity in the range of 90% to 98% with very little difference between ovine and bovine BLG (Fig. S1). Consistent with the selection of single miRNAs possessing high knockdown efficiency against ovine and bovine BLG, we observed no significant differences between the *in vitro* activity of single and tandem miRNA constructs (Fig. S1).

Having established that miRNAs can effectively knock down ovine and bovine BLG *in vitro*, we selected miRNA 6–4, one of the most efficient tandem constructs, for subsequent *in vivo* evaluation.

BLG Knockdown in Mice. To limit miRNA expression to the lactating mammary gland, the constitutive promoter used *in vitro* was replaced with a murine whey acidic protein (WAP) promoter. In addition, the GFP expression cassette was deleted to avoid any effects the reporter gene may have on endogenous or transgene function in transgenic animals. WAP-miRNA 6–4 transgene fragments were coinjected into zygotes with chicken β -globin insulator sequences to minimize the likelihood of position effects exerted by the chromosomal context on the expression of the miRNAs. Four founders that carried the miRNA 6–4 transgene were produced, but only one transmitted the transgene through its germline and was used for all subsequent experiments.

To determine the effect of the tandem miRNA construct on transcript levels and the production of BLG in milk, we crossed miRNA 6–4 mice with an existing transgenic mouse line (15) expressing ovine BLG under the control of a minimal ovine BLG promoter. Single and double transgenic offspring were identified by PCR, and females were bred to induce a natural lactation. Real-time PCR quantification of the BLG transcript levels in lactating mammary gland tissue revealed that expression of the miRNA construct had a strong effect on the level of the ovine BLG mRNA. Relative to the housekeeping gene for GAPDH,

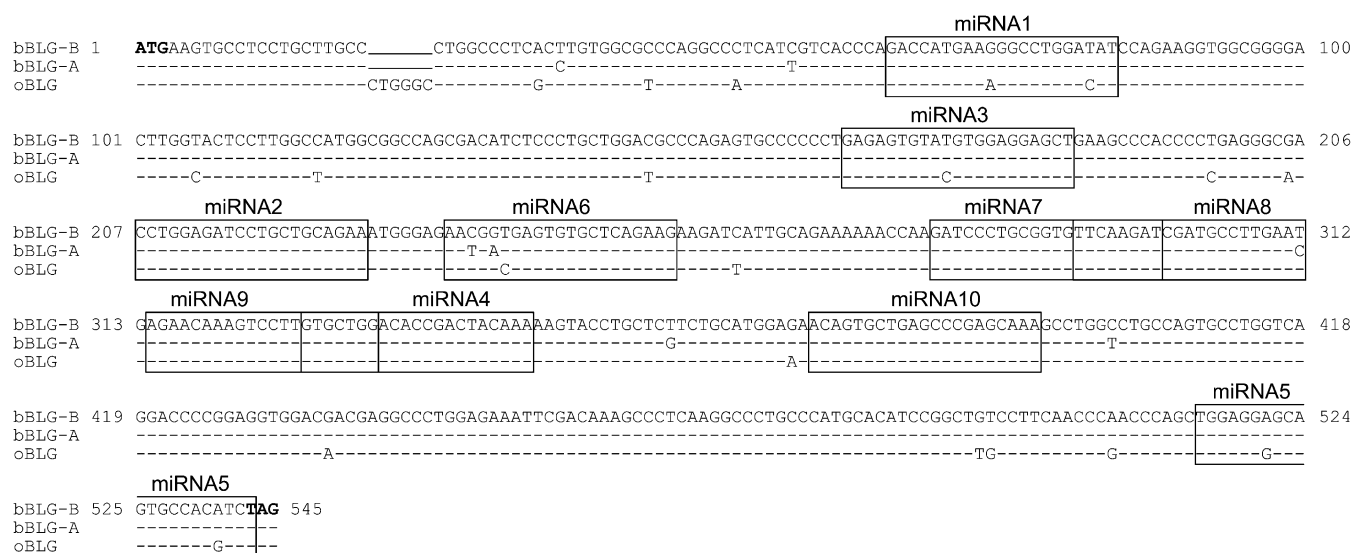


Fig. 1. Position of miRNAs targeting the BLG mRNA. Alignment of the mRNA sequences for the two bovine BLG isoforms B (bBLG-B) and A (bBLG-A) and ovine BLG (oBLG). The sequence for bBLG-B (GenBank accession no. NM_173929) is shown with position 1 referring to the first base of the start codon (bold) and position 545 the last base of the stop codon (bold). For the other two sequences, only the sequence differences to bBLG-B are indicated and conserved bases are depicted as dashes. The underline represents a gap in the alignment of the bBLG sequences where the oBLG sequence has a 6-bp insertion. Boxes mark the position of the miRNA sequences suggested by the Invitrogen RNAi designer tool for the bBLG target sequence. Please note that sequences for miRNA 7 and miRNA 8 and miRNA 9 and miRNA 4 partially overlap.

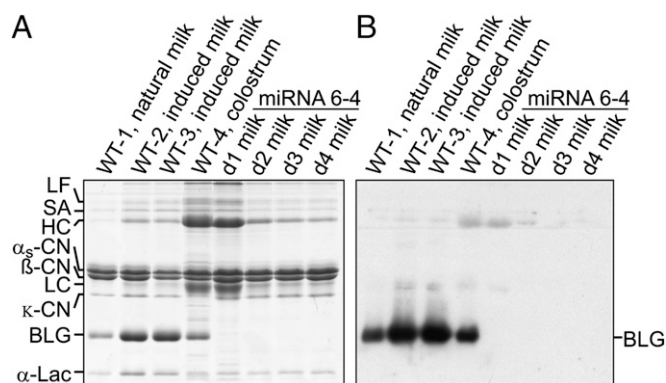


Fig. 4. miRNA-mediated depletion of BLG in bovine milk. Levels of BLG in the milk of a transgenic calf expressing miRNA 6–4 were assessed by (A) Coomassie blue staining and (B) Western analysis following SDS/PAGE separation of milk samples. Equal amounts of total milk protein were loaded onto each lane of the gel. α -Lac, α -lactoglobulin; α_s -CN, α_s -casein; β -CN, β -casein; HC, IgG heavy chain; κ -CN, κ -casein; LC, IgG light chain; LF, lactoferrin; miRNA 6–4, induced milk samples produced by the transgenic miRNA 6–4 calf at four consecutive days (days 1–4); WT 1–4, four different WT cow samples of natural and induced milk and colostrum; SA, serum albumin. Positions of main milk proteins are indicated.

induced WT milks to 0.30 for the transgenic calf samples. Although the concentration of whey protein α -lactalbumin was also more than twofold higher, the strong increase of all caseins and lack of BLG decreased the whey-to-casein ratio from an average of 21:79 for induced WT samples to a 4:96 average ratio for the transgenic calf samples.

Discussion

BLG has been suspected as a major milk allergen for decades, but its biological function(s) and impact on milk composition or nutritional and processing properties remain elusive. This is primarily because of the lack of a mouse model. Rodents do not express the protein, and the development of a mouse KO model—as demonstrated for all other major milk proteins (17–21)—is therefore not feasible. Second, KOs in ruminants are much more difficult to achieve than in mice, and, although a first attempt at knocking out BLG in cattle was made recently, the one resulting animal does not exhibit a BLG KO phenotype (11). RNAi knockdown technology based on a more basic but versatile overexpression strategy is an attractive alternative, as it was shown in mice to be capable of producing phenotypes similar to a KO (22).

Most of the 10 candidate miRNAs we tested in vitro were effective against ovine and bovine BLG, which recapitulates the successful design of effective RNAi molecules against porcine BLG in a previous study (14). Although a few of our miRNAs showed some target specificity for either of the two tested forms of BLG, this was not correlated with sequence homology between the miRNA and target. Indeed, miRNA 6, with the most numbers of mismatches for the target sequence, was one of the best-performing miRNAs. Most likely, these 3' mismatches (one and two for ovine and bovine, respectively) do not impede knockdown because they are outside the “seed region,” which determines the specificity of the target recognition in translational repression (23).

To date, only a few studies have applied RNAi to modify important traits in livestock animals, with relatively mixed success. Knockdown of the prion protein (PrP) to make cattle resistant to the prion disease bovine spongiform encephalopathy only resulted in knockdown of the PrP encoding mRNA and had no significant effect at the protein level of PrP (24). A similar study in goats proved to be more successful, and demonstrated the successful reduction of PrP levels, at least in fetal brain (25). Five calves were produced in an attempt to increase muscle mass in cattle by knocking down myostatin. However, the calves showed no correlation between the expression levels of the interfering RNA and myostatin mRNA and, in only one of the calves were the myostatin mRNA levels reduced (26). These studies included GFP reporters and expressed a single interfering RNA from strong constitutive promoters. By contrast, we avoided a GFP reporter, relying instead on a tightly spatiotemporally regulated promoter and a tandem array of two miRNAs. This excludes potential interference from the reporter gene, minimizing off-target effects from uncontrolled expression, while targeting two separate regions of the BLG mRNA. Expression of a tandem miRNA construct achieved strong knockdown of ovine BLG in our mouse model and knocked down BLG expression in cattle to levels that could no longer be detected by protein analysis. Although this difference in the degree of knockdown might be a reflection of the strong overexpression of ovine BLG in the transgenic mice, it nevertheless demonstrates that the miRNAs are highly effective against different forms of BLG and are likely to be effective against other natural variants of bovine BLG.

mRNA degradation and the repression of translation have been implicated in the mechanism of miRNA-mediated gene silencing. However, the intricate interconnectivity between these two processes has made it difficult so far to fully understand the molecular mechanism underpinning miRNA-mediated silencing (12, 27). Analysis of BLG transcript levels in our mouse model demonstrated that strong mRNA decay is involved in the observed

Table 1. Milk composition of induced milk from transgenic and control cows

Cow	Milk	Casein, mg/g					Whey, mg/g				
		α_{s1} -CN	α_{s2} -CN	β -CN	κ -CN	Total	α -Lac	BLG-A	BLG-B	Total	Whey:CN ratio
miRNA 6–4	Induced, day 1	28.0	6.3	24.5	39.3	98.2	3.9	0.0	0.0	3.9	4:96
miRNA 6–4	Induced, day 2	32.1	7.1	29.0	28.7	96.8	3.5	0.0	0.0	3.5	3:96
miRNA 6–4	Induced, day 3	36.0	9.1	33.2	28.3	106.6	4.3	0.0	0.0	4.3	4:96
miRNA 6–4	Induced, day 4	43.7	11.0	42.7	31.2	128.6	5.3	0.0	0.0	5.3	4:96
WT-1	Natural, day 69	11.4	5.8	16.1	6.4	39.6	1.5	5.7	0.6	7.8	16:84
WT-2	Induced, day 5	11.2	6.2	13.6	7.8	38.8	1.5	7.6	0.7	9.8	20:80
WT-3	Induced, day 5	9.4	5.8	10.0	7.3	32.5	1.2	7.3	0.9	9.4	22:78
WT-4	Colostrum, day 1	15.9	6.1	13.5	12.5	48.1	1.7	10.1	4.0	15.7	25:75
SEM*	—	0.41	0.51	0.31	0.36	1.27	0.09	0.12	0.11	0.12	0.43

α_{s1} -CN, α_{s1} -casein; β -CN, β -casein; CN, casein.

*Values represent SEM of duplicates, averaged over all samples of a particular milk protein. BLG samples with a value of 0 were excluded from the calculation.

knockdown of BLG. Still, repression of translation may be the initial trigger for the mRNA instability, or, alternatively, may further contribute and enhance the knockdown.

In contrast to our mouse model in which we did not observe any obvious phenotypic anomalies, a phenotype was observed in the transgenic calf, which was born without a tail. Taillessness in cattle is a rare congenital abnormality (28), and has been previously reported as a phenotype in a transgenic calf produced from a microinjected, cloned embryo (29). A similar tail phenotype was observed in a cloned mouse and was shown to be caused by a mutation in this individual donor cell that was below the detection limit for chromosomal abnormalities and unmasked by cloning (30). Thus, it appears unlikely that the tailless phenotype of the calf is correlated with the transgene insertion, but rather with an unrelated mutation present in the donor cell used for NT. Further characterization of this transgenic cattle line will be required to clarify the cause for the taillessness in the miRNA 6–4 calf.

The intended aim of expressing the miRNA 6–4 construct has been to direct production of milk depleted of BLG, which was achieved in transgenic mice and cattle. A more detailed analysis of the milk produced by the transgenic calf demonstrated that this change in BLG also affected the levels of all other milk proteins, revealing an intricate balance of milk proteins synthesis. The increase in all other milk proteins as a result of BLG depletion is consistent with previous observations of a decrease in endogenous milk proteins in transgenic mouse models overexpressing BLG (31). Moreover, it is known that low BLG levels are associated with high casein levels in cows' milk (32, 33). Astonishingly, the BLG knockdown approach greatly surpasses the efforts to increase β -casein (>200% vs. 20%) and κ -casein (~400% vs. ~200%) in cows' milk through overexpression of these two milk proteins (16).

Induced-lactation milk has higher levels of BLG than natural lactation milk (34) (Fig. 4A), which is advantageous for a vigorous assessment of BLG knockdown and further strengthens the finding of the high efficiency for the knockdown of BLG. In addition, an induced lactation is a reliable predictor of major changes, such as the observed increase in casein that can be expected in a natural lactation (35, 36). Thus, the BLG-depleted milk not only has potentially hypoallergenic properties, its high total casein content, particularly high κ -casein correlated with smaller casein micelle sizes, should provide for increased calcium levels and high cheese yields (37, 38). Ultimately, natural-lactation milk will be required to confirm the changes to the milk composition and fully assess the functional properties of this milk.

In conclusion, our results demonstrate that targeted expression of miRNAs is an approach that is capable of abolishing a protein's production in livestock. Thus, it validates miRNAs as an efficient tool to change livestock traits. Importantly, this can be achieved with animals possessing a hemizygous transgenic locus. Considering long gestation times and potentially large herd sizes, this offers a clear advantage for disseminating such a trait over KO technology that is dependent on homozygous KO animals to achieve a similar phenotype. The production of dairy cows with a comprehensive knockdown of BLG, will allow direct testing of the longstanding conjecture that milk with greatly reduced BLG level will be less allergenic than normal cows' milk. Furthermore, the BLG knockdown cattle line will provide the opportunity to fully investigate the impact of the associated milk composition changes on nutritional and processing properties and will provide new insights into the still obscure biological function of BLG.

Materials and Methods

The applied methods are briefly summarized here, and a more detailed description is provided in *SI Materials and Methods*.

Animal Studies. All animal studies were undertaken in compliance with New Zealand laws, and were approved by the Environmental Protection Agency of New Zealand and the Ruakura Animal Ethics Committee.

DNA Constructs. In COS-7 cells, expression of artificial miRNAs was based on the BLOCK-iT Pol II miR RNAi system (Invitrogen) using the pcDNA6.2-GW/EmGFP-miR vector. BLG was constitutively expressed from cDNAs [bovine BLG A (GenBank accession no. X14712) and ovine BLG B (GenBank accession no. NM_001009366)]. In mice, miRNA expression was driven by the WAP promoter without a GFP reporter. For expression of the miRNAs in cattle, tandem chicken β -globin insulators were inserted upstream of the WAP promoter.

Southern Blot. Genomic DNA from cultured bovine fetal fibroblasts was extracted with a DNeasy kit (Qiagen) and isolated from blood of the transgenic calf using the Illustra DNA extraction kit BACC 2 kit (GE Healthcare). EcoRV/HindIII restricted genomic DNA was hybridized with a 0.7-kb XhoI fragment comprising the proximal WAP promoter and entire miRNAs 6 and 4 that was labeled by using the Gene Images AlkPhos Direct Labeling and Detection System (Amersham).

COS-7 Cells: Transfection and BLG Knockdown Analysis. COS-7 cells were transiently cotransfected with miRNA and BLG expression constructs at a molar ratio of 1:1 by using Lipofectamine 2000 (Invitrogen). Total cell lysates were prepared in the presence of protein inhibitors 48 h after transfection. Ovine and bovine BLG and GFP were detected on Western blots with rabbit anti-bovine BLG and rabbit anti-GFP primary antibodies, respectively, in combination with goat anti-rabbit-HRP conjugate as secondary antibody.

Generation of Transgenic miRNA Mice. The miRNA transgene fragment was microinjected with tandem chicken β -globin insulators (39, 40) into the pronuclei of C57/BL6 mouse zygotes and embryos transferred into pseudo-pregnant recipients according to standard protocols (41).

Real-Time PCR Analysis of BLG mRNA Levels. Total RNA was isolated from lactating mammary tissue of ovine BLG and ovine BLG/miR6-4 mice and converted into cDNA. Transcript copies of ovine BLG were calculated relative to those of the housekeeping gene GAPDH following amplification of respective gene-specific sequences in triplicate by real-time PCR (42). Results are presented as mean $\pm$ SD.

Knockdown Analysis of BLG Levels in Mouse Milk. Milk was collected manually into capillary tubes by gentle massage of teats following oxytocin administration. Milk proteins were separated by SDS gel electrophoresis and quantified following Coomassie blue staining. Western blots of milk proteins were performed as described for COS-7 cell extracts.

Generation of Transgenic miRNA Cattle. Bovine fetal fibroblasts (16) homozygous for the BLG variant A were cotransfected at a molar ratio of 5:1 (Lipofectamine 2000; Invitrogen) with tandem chicken β -globin insulators and the miRNA 6–4 transgene, including a puromycin selection cassette. Stable cell lines were isolated following puromycin selection. Zona-free NT with two confirmed cell clones (312/3 and 313/4) was performed essentially as described (43). Embryos were nonsurgically transferred singularly to synchronized recipient cows. One live transgenic calf was delivered by Caesarian section on day 255 of gestation after being diagnosed with hydroallantois.

Induction of Lactation and Analysis of Induced Bovine Milk. Lactation of the transgenic calf was hormonally induced at the age of 7 mo, essentially as described (44), except that hormones were injected s.c. WT samples of colostrum (provided by Jessica van Leeuwen, AgResearch, Hamilton, New Zealand), milk generated by hormonal induction (35), and natural milk were derived from cows of similar breed. The Western analysis was performed as described for mouse milk with a detection limit of 0.04 μ g for bovine BLG. The concentrations of the main milk proteins were determined by reversed-phase HPLC against purified milk protein standards with a detection limit for BLG-A and BLG-B of 0.14 μ g and 0.08 μ g, respectively (45).

ACKNOWLEDGMENTS. We thank R. Martinus for his support as university doctorate supervisor for A.J.; R. Broadhurst for pronuclear microinjection; members of the reproductive technologies group for in vitro bovine embryo production and transfers; Ali Cullum and animal facility staff for inducing lactation and dedicated animal husbandry; H. Henderson and P. Hunt for assistance with statistical analyses and the artwork, respectively; and R. Thresher and B. Oback for critical reading of the manuscript. This work was funded by the Ministry of Science and Innovation and AgResearch.

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Supporting Information

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SI Materials and Methods

Generation of DNA Constructs. To express artificial microRNAs (miRNAs), we used a BLOCK-iT Pol II miR RNAi system (Invitrogen). The miRNA vectors are based upon the structure of murine miRNA-155 (1), which is highly conserved in bovine (92% identity) and has been optimized in the stem loop and internal loop structures to enhance knockdown efficiency (BLOCK-iT Pol II miR RNAi Expression Vector Kits user manual; Invitrogen). Annealed miRNA duplexes were inserted into the linearized miRNA expression vector pcDNA6.2-GW/EmGFP-miR according to the manufacturer's protocol (Invitrogen). Individual miRNAs were chained up into tandem constructs based on the provider's cloning strategy. For expression of β -lactoglobulin (BLG) in COS-7 cells, cDNAs for bovine BLG (isoform A; GenBank accession no. X14712) and ovine BLG (isoform B; GenBank accession no. NM_001009366) were inserted into the pcDNA5/FRT expression vector (Invitrogen). For expression of the miRNAs in mice, the CMV promoter and GFP expression cassette were excised from the pcDNA6.2-GW/EmGFP-miR6-4 vector used for the *in vitro* study and replaced with the 2.5-kb whey acidic protein (WAP) promoter (2) to target expression of the miRNAs to the mammary gland. For expression of the miRNAs in cattle, tandem chicken β -globin insulators were inserted upstream of the WAP promoter. All miRNA and BLG constructs were validated by DNA sequence analysis.

Southern Blot. Genomic DNA from cultured bovine fetal fibroblasts was extracted with a DNeasy kit (Qiagen) and isolated from blood of the transgenic calf by using the Illustra DNA extraction kit BACC 2 kit (GE Healthcare). Equal amounts of genomic DNA (1.85 μ g) were digested with EcoRV/HindIII and analyzed by Southern blotting. Transgene copies were detected with a 0.7-kb XhoI hybridization probe comprising the proximal WAP promoter and entire miRNAs 6 and 4, which was labeled and visualized using the Gene Images AlkPhos Direct Labeling and Detection System (Amersham). Equal loading was confirmed following the detection of the endogenous single copy gene for β -casein with a 1.2-kb HindIII/EcoRV hybridization probe, and transgene copy numbers were determined by signal quantification using densitometry (GS-800; Bio-Rad) in comparison with controls spiked with known amounts of transgene copies.

COS-7 Cells: Transfection and BLG Knockdown Analysis. For transient transfections, 2×10^5 COS-7 cells were cultured in a 24-well tissue culture plate in DMEM-F12 medium (Gibco) supplemented with 10% (vol/vol) FBS. Cells were cultured for 12 to 16 h before transfection and transfected at ~70% to 80% confluence. The plasmid DNAs of miRNA and BLG expression constructs were mixed at a molar ratio of 1:1 and cotransfected into cells by using Lipofectamine 2000 (Invitrogen). After 4 to 6 h of transfection, the medium was replaced with fresh medium. Cells were harvested 48 h after transfection, resuspended in lysis buffer (20 mM Tris, pH 7.5, 5 mM EDTA, 5 mM EGTA, 1% triton, 1 mM PMSF) containing protease inhibitor mixture (1 μ L per million cells; Roche) and sonicated (Misonix) for complete lysis. The protein concentration of the cleared lysates was measured by BCA protein assay kit (Pierce). SDS/PAGE was performed with 10% Bis-Tris precast polyacrylamide gels by using a NOVEX gel apparatus (Invitrogen). Proteins were transferred to a nitrocellulose membrane using an iBlot gel transfer device (Invitrogen). Ovine and bovine BLG and GFP were detected with rabbit anti-bovine BLG (1:10,000; Bethyl Laboratories) and

rabbit anti-GFP (1:2,000; Invitrogen) primary antibodies, respectively, in combination with goat anti rabbit-HRP conjugate (1:20,000; Sigma) as secondary antibody and visualized by enhanced chemiluminescence (Amersham). Each experiment analyzed by Western blot was conducted in triplicate. The intensity of BLG and control GFP signal was measured by Quantity One software (Bio-Rad). The expression level of BLG was normalized for GFP expression to account for variability of transfections. BLG knockdown by miRNAs was calculated relative to the negative control with scrambled miRNA, which was set as 0% BLG knockdown.

Generation of Transgenic miRNA Mice. A 2.8-kb transgene fragment comprising the WAP promoter and miRNAs 6 and 4 was excised from the vector without the pA sequence by SpeI-KspI restriction and subsequently gel-purified. The miRNA transgene fragment was microinjected together with a 2.4-kb NotI-XhoI fragment containing tandem chicken β -globin insulators (3, 4) into the pronuclei of C57/BL6 mouse zygotes. Resulting two-cell embryos were transferred into pseudopregnant recipients according to standard protocols (5). PCR genotyping to identify founder animals was performed on DNA from tail tip biopsy specimens with the following primers and cycle conditions: forward primer, GAGGTAGTGAGTCGACCA GTG; reverse primer, GAGGTAGTGAGTCGACCA GTG; 35 cycles of 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C, and an initial denaturing step (5 min at 95 °C) and final extension step (7 min at 72 °C) at the beginning and end of the program, respectively.

Isolation of RNA from Mammary Tissue, cDNA Synthesis, and Real-Time PCR. Total RNA was isolated from lactating mammary tissue of heterozygous ovine BLG and heterozygous ovine BLG/miRNA 6-4 mice with TRIzol (Invitrogen). cDNA was prepared from 1 μ g RNA using the SuperScript III first-strand synthesis system (Invitrogen) according to the manufacturer's instructions. Real-time PCR amplifications (95° for 3 min followed by 40 cycles of 95° at 10 s, 62° at 25 s) for ovine BLG (primers, AGATCCTGCTGCAGAAATGG and AGGCATCGATCTTGAACACC) and murine GAPDH (primers, TGCACCACCAACTGCTTAG and GATGCAGGGATGATGTTT) were performed in triplicate on a Corbett Rotor-Gene 6000 cycler. Transcript copies of ovine BLG were calculated relative to those of the housekeeping gene GAPDH according to described methodology (6) and are presented as mean $\pm$ SD.

Knockdown Analysis of BLG Levels in Mouse Milk. The lactating transgenic miRNA-BLG mice were separated from their litters for 4 h before milking. Mice were then anesthetized by i.p. injection with ketamine, and lactation was stimulated by intramuscular injection of 0.5 mL oxytocin (200 IU/mL). Milking was started 2 to 3 min after oxytocin administration, and milk was collected manually by gentle massage of teats into capillary tubes. A total of 10 μ g defatted milk proteins were separated by SDS gel electrophoresis and stained with Coomassie blue R 350. Signal intensities of milk proteins were measured by Quantity One software (Bio-Rad). The expression level of BLG was normalized to an endogenous mouse milk protein (α -casein) and miRNA-mediated knockdown calculated relative to the BLG levels observed in milk of transgenic mice expressing BLG but lacking the miRNA transgene (0% knockdown). Western blots of milk proteins were performed as described earlier for COS-7 cell extracts.

Generation of Transgenic miRNA Cattle. Bovine fetal fibroblasts (BFFs) (7) homozygous for the BLG variant A were co-transfected at a molar ratio of 5:1 (Lipofectamine 2000; Invitrogen) with a 5.6-kb MluI/FspI fragment comprising tandem chicken β -globin insulators, the WAP promoter linked to the tandem array of miRNAs 6 and 4 including a thymidine kinase pA signal sequence, and a 1.7 kb EcoRI/NotI fragment comprising a puromycin selection cassette. Stable cell lines were isolated following puromycin selection (1 μ g/mL) and miRNA 6–4 transgene insertion was determined by PCR (forward primer, CACACTGTGCTCATCACTTTG; reverse primer, CTCTTGCTTGCTCCTCTGAGC; one precycle step, 5 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C; final step, 5 min at 72 °C). Positive cell clones were further verified for normal chromosome numbers as previously described (8). Zona-free nuclear transfer (NT) with two confirmed cell clones (312/3 and 313/4) was performed essentially as described (9) using proliferating (G1) or serum deprived (G0) donor nuclei. For the 312/3 cell line, the development of NT reconstructs was compared with those produced following a double cytoplasm NT procedure, whereby, within 1 h of successful somatic cell fusion, a second cytoplasm was fused to effectively double the cytoplasmic volume in the reconstructed embryo. In vitro development was assessed on day 7 after fusion and grade 1/2 quality embryos

(10) were nonsurgically transferred singularly to synchronized recipient cows. Fetal development was monitored by regular ultrasonography and rectal palpation throughout gestation. One live transgenic calf was delivered by caesarian section on day 255 of gestation after being diagnosed with hydroallantois.

Induction of Lactation and Analysis of Induced Bovine Milk. Lactation of the transgenic calf was hormonally induced at the age of 7 mo essentially as described (11), except that hormones were injected s.c.. The calf was milked for 5 d and then dried off. Induced milk from two 10-mo-old WT cows with similar genetics according to the method described by Ball et al. (12) served as controls for induced milk. The WT sample of natural milk was from peak lactation (day 69). Colostrum from a cow of similar breed was provided by Jessica van Leeuwen. The Western analysis was performed as described for mouse milk with a detection limit of 0.04 μ g for bovine BLG. The concentrations of the main milk proteins were determined from skim milk samples by reversed-phase HPLC against purified milk protein standards with detection limits for BLG-A and BLG-B of 0.14 μ g and 0.08 μ g, respectively (13). In the absence of a commercially available standard for α_{S2} -casein, the concentration for α_{S2} -casein was quantified with the standard for α_{S1} -casein, which has a similar primary sequence.

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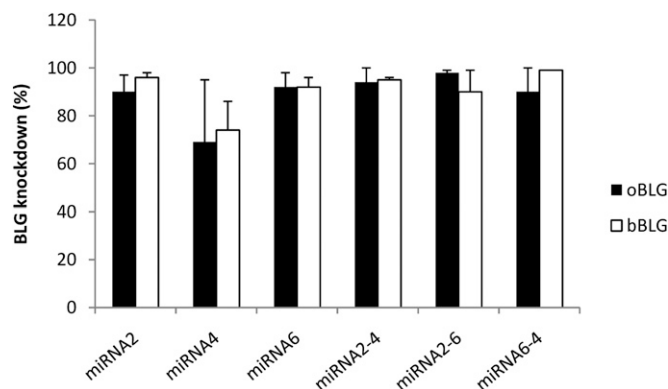


Fig. S1. In vitro BLG knockdown activity of single and tandem miRNAs. Knockdown of ovine and bovine BLG as determined by quantification of Western blot results from three independent experiments. Signals were normalized to GFP expression and knockdown expressed relative to the control sample transfected with a scrambled, nonspecific miRNA, which was defined as 0% BLG knockdown. Error bars denote SEM.

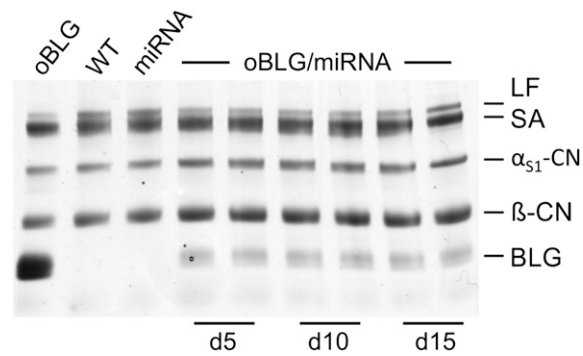


Fig. S2. Persistence of ovine BLG knockdown throughout lactation. Coomassie blue staining of equal amounts of milk proteins from milk samples of an ovine BLG expressing mouse (oBLG), WT mouse, an miRNA 6–4–expressing mouse (miRNA), and samples from two littermates of double transgenic mice expressing ovine BLG and miRNA 6–4 (oBLG/miRNA) obtained on day 5, day 10, and day 15 of lactation. Positions of the main milk proteins are indicated. α_{s1} -CN, α_1 -casein; β -CN, β -casein; LF, lactoferrin; SA, serum albumin.

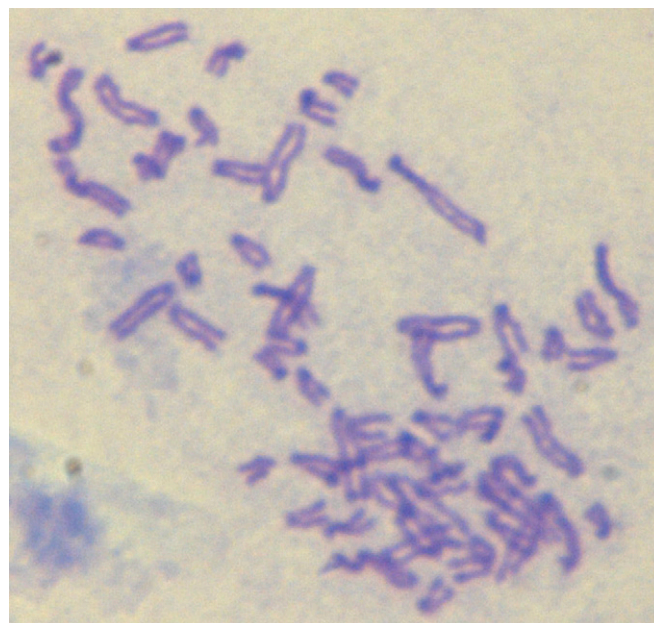


Fig. S3. Metaphase chromosome spread of cell clone 312/3. Metaphase chromosomes from a single 312/3 cell visualized by Giemsa staining.

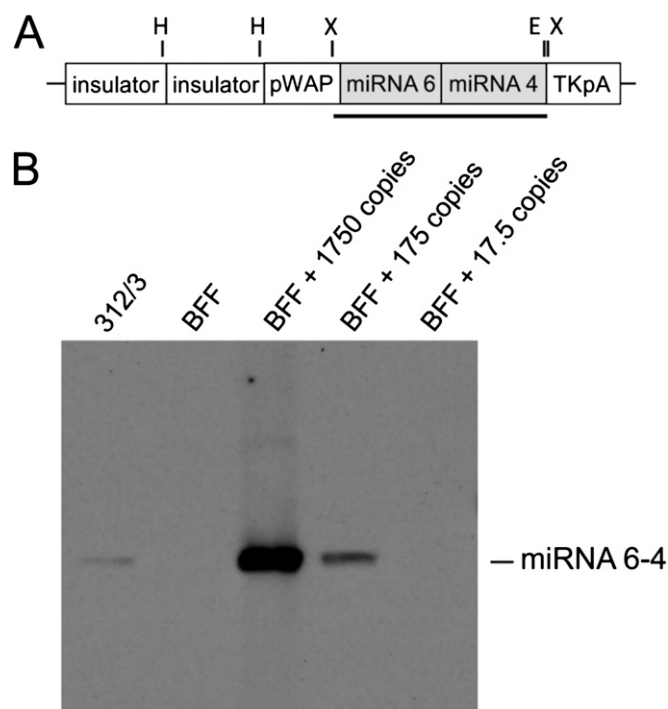


Fig. S4. Southern blot analysis of transgene copies for the transgenic calf derived from cell clone 312/3. (A) Schematic representation of the integrated transgene with indicative positions of restriction sites for HindIII ("H") and EcoRV ("E") which were used to digest genomic DNA. An XhoI ("X") fragment spanning the 3' sequence of the WAP promoter and miRNAs 6 and 4 was used as hybridization probe (black bar). (B) Southern blot results for HindIII/EcoRV restricted genomic DNA from the transgenic calf derived from cell line 312/3, nontransfected BFFs, and BFFs spiked with a transgene containing plasmid equivalent to the indicated transgene copy numbers. Insulator, chicken β -globin insulator element; pWAP, murine promoter; TK pA, thymidine kinase poly(A) sequence.

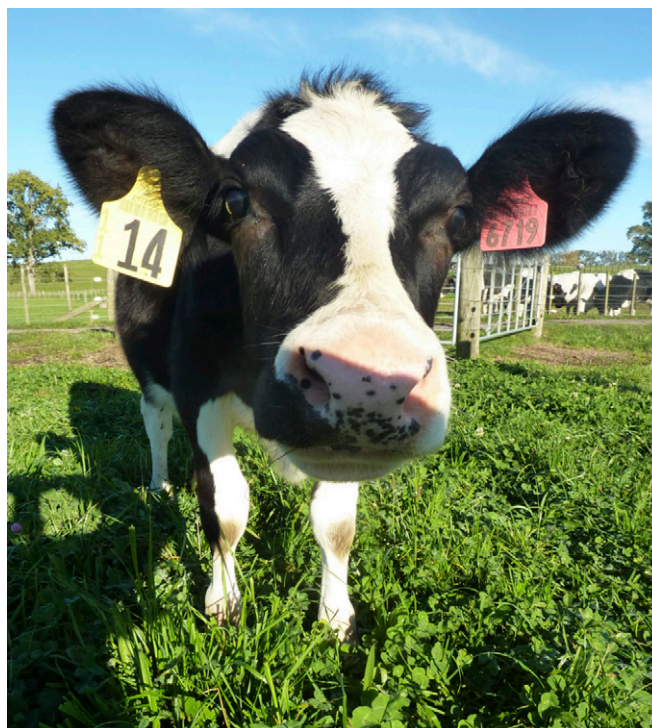


Fig. S5. Transgenic calf at 7 mo of age, shortly after completion of the induced lactation trial.

DCNT, double cytoplasm NT.

*Transferable quality (grade 1/2) late morulae and blastocyst-stage embryos on day 7. Values with different superscript letters differ significantly ($P < 0.05$) by Fisher exact test in 2×2 tables.

†Grade 1–3 late morulae and blastocyst-stage embryos on day 7. Values with different superscript letters differ significantly ($P < 0.05$) by Fisher exact test in 2×2 tables.

‡Pregnancy initiation as indicated by the presence of trophoblast and allantoic fluid, with or without a fetal mass observed by ultrasonography at the time of the first scan (between days 34 and 44 of gestation).

§Number of fetuses detected with heartbeats following ultrasonography.

¶One fetus recovered on day 65 of gestation to derive a rejuvenated cell line.

||One elective abortion on day 140 of gestation to terminate a hydrops pregnancy.

**Two spontaneous abortions; one between day 63 and day 77 and the other on day 117.

††Calf delivered by Caesarian section on day 255 of gestation after being diagnosed with hydroallantois.