AGRICULTURAL SCIENCES


The authors wish to note the following: “The article and Supporting Information (SI) have incorrect designations for the tandem micro RNA constructs that were used. In the article and SI, the constructs were designated as miRNA 2–4, miRNA 2–6, and miRNA 6–4, whereas they should have been designated miRNA 6–4, miRNA 3–3, and miRNA 3–4, respectively.”

“We introduce the tandem microRNA ‘miRNA 6–4’ in the Results section. We then repeatedly mentioned ‘miRNA 6–4’ in the Results and Discussion sections. Accordingly, it has been used in the legend for Fig. 3, Fig. 4 and its legend, and in Table 1. We have now discovered that the designation ‘miRNA 6–4’ is incorrect and that it should have been labeled ‘miRNA 3–4.’”

“In Fig. S1, we show results for in vitro BLG knockdown for tandem constructs miRNA 2–4, miRNA 2–6, and miRNA 6–4. These tandem constructs should have instead been designated as miRNA 6–4, miRNA 3–3, and miRNA 3–4, respectively. In addition, miRNA 6–4 and miRNAs 6 and 4 are indicated in SI Materials and Methods as the tandem micro RNA construct that was used and the two functional components thereof, respectively. They are also mentioned in the legends for Fig. S2 and Fig. S4 and shown in Fig. S1 and Fig. S4. In each case, the description should have been referring to miRNA 3–4 or miRNAs 3 and 4.”

Appearing below are: Fig. 3 and its corrected legend; corrected Fig. 4 and its corrected legend; corrected Table 1; corrected Fig. S1 and its legend; Fig. S2 and its corrected legend; and corrected Fig. S4 and its corrected legend. The SI has been corrected online. The contents of the main article have not been updated online and remain as originally published.

Fig. 3. miRNA-mediated knockdown of ovine BLG in transgenic mouse milk. Levels of BLG in the milk of transgenic mice were assessed by (A) Coomassie blue staining and (B) Western analysis following SDS/PAGE separation of mouse milk samples. An equal amount of total milk protein was loaded onto each lane of the gel. WT mouse milk was used as negative control. αs1-CN, αs1-casein; β-CN, β-casein; LF, lactoferrin; miRNA, milk from an miRNA 3–4-expressing mouse (negative control); oBLG, milk from ovine BLG-expressing mouse (positive control); oBLG/miRNA, milk obtained on day 5 and day 10 of lactation from a double transgenic mouse expressing ovine BLG and miRNA 3–4; SA, serum albumin. Positions of main milk proteins are indicated.
miRNA-mediated depletion of BLG in bovine milk. Levels of BLG in the milk of a transgenic calf expressing miRNA 3–4 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; αs-CN, αs-casein; β-CN, β-casein; HC, IgG heavy chain; κ-CN, κ-casein; LC, IgG light chain; LF, lactoferrin; miRNA 3–4, induced milk samples produced by the transgenic miRNA 3–4 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.

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

<table>
<thead>
<tr>
<th>Cow</th>
<th>Milk</th>
<th>αs1-CN</th>
<th>αs2-CN</th>
<th>β-CN</th>
<th>κ-CN</th>
<th>Total</th>
<th>α-Lac</th>
<th>BLG-A</th>
<th>BLG-B</th>
<th>Total</th>
<th>Whey:CN ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>miRNA 3–4</td>
<td>Induced, day 1</td>
<td>28.0</td>
<td>6.3</td>
<td>24.5</td>
<td>39.3</td>
<td>98.2</td>
<td>3.9</td>
<td>0.0</td>
<td>0.0</td>
<td>3.9</td>
<td>4:96</td>
</tr>
<tr>
<td>miRNA 3–4</td>
<td>Induced, day 2</td>
<td>32.1</td>
<td>7.1</td>
<td>36.6</td>
<td>28.7</td>
<td>96.8</td>
<td>3.5</td>
<td>0.0</td>
<td>0.0</td>
<td>3.5</td>
<td>3:96</td>
</tr>
<tr>
<td>miRNA 3–4</td>
<td>Induced, day 3</td>
<td>36.0</td>
<td>9.1</td>
<td>33.2</td>
<td>28.3</td>
<td>106.6</td>
<td>4.3</td>
<td>0.0</td>
<td>0.0</td>
<td>4.3</td>
<td>4:96</td>
</tr>
<tr>
<td>miRNA 3–4</td>
<td>Induced, day 4</td>
<td>43.7</td>
<td>11.0</td>
<td>42.7</td>
<td>31.2</td>
<td>128.6</td>
<td>5.3</td>
<td>0.0</td>
<td>0.0</td>
<td>5.3</td>
<td>4:96</td>
</tr>
<tr>
<td>WT-1</td>
<td>Natural, day 69</td>
<td>11.4</td>
<td>5.8</td>
<td>16.1</td>
<td>6.4</td>
<td>39.6</td>
<td>1.5</td>
<td>5.7</td>
<td>0.6</td>
<td>14.0</td>
<td>16:84</td>
</tr>
<tr>
<td>WT-2</td>
<td>Induced, day 5</td>
<td>11.2</td>
<td>6.2</td>
<td>13.6</td>
<td>7.8</td>
<td>38.8</td>
<td>1.5</td>
<td>7.6</td>
<td>0.7</td>
<td>18.1</td>
<td>20:80</td>
</tr>
<tr>
<td>WT-3</td>
<td>Induced, day 5</td>
<td>9.4</td>
<td>5.8</td>
<td>10.0</td>
<td>7.3</td>
<td>32.5</td>
<td>1.8</td>
<td>7.3</td>
<td>0.9</td>
<td>17.6</td>
<td>22:78</td>
</tr>
<tr>
<td>WT-4</td>
<td>Colostrum, day 1</td>
<td>15.9</td>
<td>6.1</td>
<td>13.5</td>
<td>12.5</td>
<td>48.1</td>
<td>1.7</td>
<td>10.1</td>
<td>4.0</td>
<td>29.8</td>
<td>25:75</td>
</tr>
<tr>
<td>SEM*</td>
<td></td>
<td>0.41</td>
<td>0.51</td>
<td>0.31</td>
<td>0.36</td>
<td>1.27</td>
<td>0.09</td>
<td>0.12</td>
<td>0.11</td>
<td>0.12</td>
<td>0.43</td>
</tr>
</tbody>
</table>

αs1-CN, αs-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.

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 3–4–expressing mouse (miRNA), and samples from two littermates of double transgenic mice expressing ovine BLG and miRNA 3–4 (oBLG/miRNA) obtained on day 5, day 10, and day 15 of lactation. Positions of the main milk proteins are indicated. αs1-CN, αs1-casein; β-CN, β-casein; LF, lactoferrin; SA, serum albumin.

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 3 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.
Targeted microRNA expression in dairy cattle directs production of β-lactoglobulin-free, high-casein milk

Anower Jabeđ,1,2, Stefan Wagner,2,3, Judi McCracken,4, David N. Wells5, and Goetz Laible6,3

1AgResearch, Hamilton 3240, New Zealand; and 2Department of Biological Sciences, University of Waikato, Hamilton 3240, New Zealand

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

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 hypcholesterolemia (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 BLG.

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.

1Present address: Department of Pharmacology and Clinical Pharmacology, University of Auckland, Auckland 1142, New Zealand.

2A.J. and S.W. contributed equally to this work.

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

www.pnas.org/cgi/doi/10.1073/pnas.1210057109
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 was 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 ± 49%) and miRNA 10 (−9 ± 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 ± 19% for miRNA 1), medium (33 ± 14% for miRNA 3 and 31 ± 16% for miRNA 5), and high (94 ± 2% for miRNA 2, 78 ± 9% for miRNA 4, 93 ± 1% for miRNA 6, 66 ± 7% for miRNA 8, and 83 ± 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 ± 9%), miRNA 2 (92 ± 7%), miRNA 3 (78 ± 17%), miRNA 4 (94 ± 2%), miRNA 5 (80 ± 13%), miRNA 6 (97 ± 3%), miRNA 7 (78 ± 7%), and miRNA 10 (70 ± 26%) 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 ± 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 cojected 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,
two transgenic cell lines were determined from Southern blot analyses to be approximately 30 for 312/3 (Fig. S4) and more than 200 for 313/4.

Initial attempts to generate live calves from cell line 313/4 were unsuccessful. NT with cell line 312/3 and the transfer of 57 cloned embryos to recipients resulted in five pregnancies at approximately day 65 of gestation (Table S1). Because most of the available 312/3 cells were used for NT, one of the pregnancies was terminated to recover the fetus and derive rejuvenated cells to preserve the unique genetics and provide future NT options. Of the remaining four pregnancies, one resulted in the production of a live female calf (Fig. S5). Unexpectedly, the miRNA 6–4 calf was born without a tail.

To obtain milk and evaluate miRNA-mediated knockdown of BLG expression, we hormonally induced the transgenic calf into lactation. Analysis of the milk samples by SDS-gel electrophoresis and Coomassie blue staining revealed that none of the milk samples from the miRNA 6–4 calf contained detectable levels of BLG. By contrast, BLG was readily detectable as a major milk protein in all WT controls, including colostrum and natural and induced milk samples, with the latter showing the highest levels of BLG (Fig. 4). A more sensitive analysis of BLG levels by Western blot confirmed the highly effective knockdown, as all milk samples from the transgenic calf were devoid of any detectable BLG (Fig. 4B). To further quantify BLG levels and determine any effects the knockdown of BLG may have on milk protein composition, the milk samples were quantified for all major milk proteins by HPLC. Consistent with the Coomassie blue staining and Western results, HPLC analysis could not detect any BLG in the transgenic calf milk samples and confirmed comprehensive knockdown of BLG (Table 1). The absence of BLG had a strong, compensatory effect on the levels of other milk proteins. In comparison with natural and induced WT samples, all other major milk proteins were greatly increased in the milk produced by the transgenic calf, in particular the caseins (α- and β-casein more than twofold and κ-casein approximately fourfold). This had a strong effect on the κ-casein–to–total casein ratio, which increased from an average of 0.21 for the

ovine BLG transcript levels in BLG mice were $1,460 \pm 290$ and $0.34 \pm 0.02$ in BLG/miRNA 6–4 mice. Thus, the level of the mRNA for BLG is down-regulated by more than four orders of magnitude ($4,294$ fold) inBLG mice expressing miRNA 6–4 compared with BLG mice without the miRNA transgene.

Next, we analyzed the milk proteins by SDS/PAGE and Western blotting and directly compared milk from WT mice, double transgenic mice, and those expressing either transgene (Fig. 3). As expected, BLG is absent in the milk of WT mice and transgenic mice containing only the miRNA 6–4 transgene, but is the most highly expressed milk protein in the milk produced by the transgenic ovine BLG mice. Double transgenic mice with transgenes for ovine BLG and miRNA 6–4, however, show a $96 \pm 2\%$ reduction in BLG expression level compared with milk from transgenic mice expressing BLG but no miRNAs. We also addressed the question whether the miRNA-induced knockdown will persist as lactation progresses. Milk was collected 5, 10, and 15 d after the onset of lactation and was analyzed for BLG levels (Fig. 3 and Fig. S2). Knockdown of ovine BLG induced by miRNA 6–4 remained at the same high level, which indicates persistent expression of the miRNAs and knockdown activity against BLG. Thus, the results demonstrate the utility of the miRNA approach to reduce the level of BLG in milk.

**BLG Knockdown in Cattle.** We next attempted to knock down BLG in cattle. The donor cell line (bovine fetal fibroblasts), previously validated for nuclear transfer (NT) (16), was transfected with an expression cassette for miRNA 6–4. Two expanded cell clones, 312/3 and 313/4, were karyotyped and found to have normal chromosome numbers (Fig. S3). Transgene copy numbers for the

![Fig. 2.](image-url) In vitro knockdown of BLG in COS-7 cells. Representative Western blot analysis (with indicative sizes) of cell extracts from COS-7 cells cotransfected with ovine BLG (oBLG; A) or bovine BLG (bBLG, B) expression plasmids and GFP-miRNA (miRNA 1–10) expression plasmids or a GFP-scrambled miRNA control (miRNA ctrl). Knockdown of ovine and bovine BLG was determined by quantification of Western blot results from three independent experiments (C). Signals were normalized to GFP expression and knockdown expressed relative to the control sample transfected with a scrambled, nonspecific miRNA (miRNA ctrl), which was defined as 0% BLG knockdown. NTC, nontransfected cell. Error bars denote SEM.

![Fig. 3.](image-url) miRNA-mediated knockdown of ovine BLG in transgenic mouse milk. Levels of BLG in the milk of transgenic mice were assessed by (A) Coomassie blue staining and (B) Western analysis following SDS/PAGE separation of mouse milk samples. An equal amount of total milk protein was loaded onto each lane of the gel. WT mouse milk was used as negative control. αs1-CN, αs1-casein; β-CN, β-casein; LF, lactoferrin; miRNA, milk from an miRNA 6–4-expressing mouse (negative control); oBLG/miRNA, milk from ovine BLG-expressing mouse (positive control); oBLG/miRNA, milk obtained on day 5 and day 10 of lactation from a double transgenic mouse expressing ovine BLG and miRNA 6–4; 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 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.

miRNA 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

<table>
<thead>
<tr>
<th>Cow</th>
<th>Milk</th>
<th>αs1-CN</th>
<th>αs2-CN</th>
<th>β-CN</th>
<th>κ-CN</th>
<th>Total</th>
<th>α-Lac</th>
<th>BLG-A</th>
<th>BLG-B</th>
<th>Total</th>
<th>Whey:CN ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>miRNA 6–4</td>
<td>Induced, day 1</td>
<td>28.0</td>
<td>6.3</td>
<td>24.5</td>
<td>39.3</td>
<td>98.2</td>
<td>3.9</td>
<td>0.0</td>
<td>0.0</td>
<td>3.9</td>
<td>4:96</td>
</tr>
<tr>
<td></td>
<td>Induced, day 2</td>
<td>32.1</td>
<td>7.1</td>
<td>29.0</td>
<td>28.7</td>
<td>96.8</td>
<td>3.5</td>
<td>0.0</td>
<td>0.0</td>
<td>3.5</td>
<td>3:96</td>
</tr>
<tr>
<td></td>
<td>Induced, day 3</td>
<td>36.0</td>
<td>9.1</td>
<td>33.2</td>
<td>28.3</td>
<td>106.6</td>
<td>4.3</td>
<td>0.0</td>
<td>0.0</td>
<td>4.3</td>
<td>4:96</td>
</tr>
<tr>
<td></td>
<td>Induced, day 4</td>
<td>43.7</td>
<td>11.0</td>
<td>42.7</td>
<td>31.2</td>
<td>128.6</td>
<td>5.3</td>
<td>0.0</td>
<td>0.0</td>
<td>5.3</td>
<td>4:96</td>
</tr>
<tr>
<td>WT-1</td>
<td>Natural, day 69</td>
<td>11.4</td>
<td>5.8</td>
<td>16.1</td>
<td>6.4</td>
<td>39.6</td>
<td>1.5</td>
<td>5.7</td>
<td>0.6</td>
<td>7.8</td>
<td>16:84</td>
</tr>
<tr>
<td>WT-2</td>
<td>Induced, day 5</td>
<td>11.2</td>
<td>6.2</td>
<td>13.6</td>
<td>7.8</td>
<td>38.8</td>
<td>1.5</td>
<td>7.6</td>
<td>0.7</td>
<td>9.8</td>
<td>20:80</td>
</tr>
<tr>
<td>WT-3</td>
<td>Induced, day 5</td>
<td>9.4</td>
<td>5.8</td>
<td>10.0</td>
<td>7.3</td>
<td>32.5</td>
<td>1.2</td>
<td>7.3</td>
<td>0.9</td>
<td>9.4</td>
<td>22:78</td>
</tr>
<tr>
<td>WT-4</td>
<td>Colostrum, day 1</td>
<td>15.9</td>
<td>6.1</td>
<td>13.5</td>
<td>12.5</td>
<td>48.1</td>
<td>1.7</td>
<td>10.1</td>
<td>4.0</td>
<td>15.7</td>
<td>25:75</td>
</tr>
<tr>
<td>SEM*</td>
<td></td>
<td>0.41</td>
<td>0.51</td>
<td>0.31</td>
<td>0.36</td>
<td>1.27</td>
<td>0.09</td>
<td>0.12</td>
<td>0.11</td>
<td>0.12</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Supershadows, 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 miRNA 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. 4.4), 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. Impotantly, 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 Iillustra 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 used for protein measurements. For detection of ovine and bovine BLG and GFP, Western blots were performed 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 coinjected with tandem chicken β-globin insulators (39, 40) into the pronuclei of C57BL6 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 ± 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 (3123 and 3134) were performed essentially as described (43). Embryos were nonsurgical 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 Jeroen Leeuwen, AgBea van Hamilton, New Zealand, milk generated by hormonal induction (35), and natural milk were derived from cows of similar breeds. The Western analysis was performed as described for mouse milk with a detection limit of 0.04 μg 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 μg and 0.08 μg, respectively (45).


