

# 1 Genome-wide profiling of microRNAs 2 and prediction of mRNA targets in 17 3 bovine tissues

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## 14 **Keywords**

15 known and novel microRNAs, miRNA target sites, differential expression, whole genome sequence

16 variants, lactating dairy cow, FAANG

## 17 **Abstract**

18 MicroRNAs regulate many eukaryotic biological processes in a temporal- and spatial-specific  
19 manner. Yet in cattle it is not fully known which microRNAs are expressed in each tissue, which  
20 genes they regulate, or which sites a given microRNA bind to within messenger RNAs. An improved  
21 annotation of tissue-specific microRNA network may in the future assist with the identification of  
22 causal variants affecting complex traits. Here, we report findings from analysing short RNA sequence  
23 from 17 tissues from a single lactating dairy cow. Using miRDeep2, we identified 699 expressed  
24 mature microRNA sequences. Using TargetScan, known (60%) and novel (40%) microRNAs were  
25 predicted to interact with 780,481 sites in bovine messenger RNAs homologous with human.  
26 Putative interactions between microRNA families and targets were significantly enriched for  
27 interactions from previous experimental and computational identification. Characterizing features of  
28 microRNAs and targets, we showed that (1) mature microRNAs derived from different arms of the  
29 same precursor targeted different genes in different tissues; (2) miRNA target sites preferentially  
30 occurred within gene regions marked with active histone modification; (3) variants within  
31 microRNAs and targets had lower allele frequencies than variants across the genome, as identified  
32 from 65 million whole genome sequence variants; (4) no significant correlation was found between  
33 the abundance of microRNAs and messenger RNAs differentially expressed in the same tissue; (5)  
34 microRNAs and target sites weren't significantly associated with allelic imbalance of gene targets.  
35 This study contributes to the goals of Functional Annotation of Animal Genomes consortium to  
36 improve the annotation of genomes of domestic animals.

## 37 **Introduction**

38 Micro RNAs (miRNAs) are a class of single-stranded, short-length (typically 19-24nt), endogenous,  
39 non-coding RNAs (ncRNAs) that are involved in almost all biological processes, including  
40 development, differentiation, immunity, reproduction and longevity (Kloosterman and Plasterk  
41 2006; Hasuwa et al. 2013; Renthal et al. 2013; Li and Belmonte 2015; Mehta and Baltimore 2016;  
42 Wang et al. 2016a; Cowled et al. 2017; Ioannidis and Donadeu 2017; Bartel 2018). Close to three  
43 decades of miRNA studies have revealed that miRNAs have broadly conserved biogenesis and  
44 conserved target sites at the three prime untranslated regions (3'UTRs) of the messenger RNAs  
45 (mRNAs) in eukaryotes (Bartel 2018). Mutations in miRNA target sites have been linked to numerous  
46 complex phenotypes in humans, livestock and plants (Mallory et al. 2004; Abelson et al. 2005; Clop  
47 et al. 2006; Kloosterman and Plasterk 2006; Leung and Sharp 2010). For example, in humans a single  
48 base deletion in the binding sites of *hsa-miR-189* within the 3'UTR of the *SLITRK1* mRNA was shown  
49 to be associated with Tourette's syndrome (Abelson et al. 2005). In Texel sheep, a single-base-  
50 nucleotide substitution had created a binding site for the *miR-1/miR-206* family within the 3'UTR of  
51 the *GDF8* mRNA, leading to translational repression of the *GDF8* transcript and 'double muscling'  
52 (Clop et al. 2006). In Arabidopsis, the *GGU* (Gly) to *GAU* (Asp) single-base-nucleotide substitution  
53 within the *PHABULOSA* mRNA, which was associated with the development of stunted leaves, was  
54 within the binding site of the *miR165/166* family. When a synonymous single-base-nucleotide  
55 substitution *GGA* (Gly) was introduced, which didn't change the protein sequence but disrupted the  
56 miRNA binding site, the same stunted leaf phenotype was observed. This confirmed the disruption  
57 of miRNA binding site, not the change of *PHABULOSA* protein sequence, had caused the stunted  
58 leaf development (Mallory et al. 2004).

59 In cattle the spatial- and temporal- interactions between miRNAs and mRNAs are not yet fully  
60 understood or characterised. An improved annotation of tissue-specific miRNA interaction networks  
61 will provide opportunities to better examine the effects of mutations in miRNA genes and target

62 sites in the bovine genome, particularly in the era of large collections of whole genome sequence  
63 (WGS) variants.

64 In animals, the miRNA gene is transcribed by RNA polymerase II in the nucleus to produce primary  
65 miRNA before being processed into precursor miRNA that consists of a hairpin structure (Lee et al.  
66 2004; Morlando et al. 2008; Pawlicki and Steitz 2008; Nojima et al. 2015). The precursor miRNA is  
67 exported to cytosol where the stem loop of the hairpin is cleaved to release the duplex. The duplex  
68 consists of two RNA strands that are partially reverse-complementarily matched. One strand will  
69 become the dominant functional product (i.e. the mature miRNA) and be incorporated into the  
70 Argonaute protein as part of the RNA-induced silencing complex to direct post-transcriptional  
71 repression of mRNA targets. The other strand will become passenger sequence (i.e. the star miRNA)  
72 which is usually discarded and degraded (Bartel 2018). In cytosol, the mature miRNA is mostly  
73 known to bind to the 3'UTR of target mRNA to inhibit translation (Bartel 2018). A contiguous 6-  
74 nucleotide Watson-Crick base pairing is required between the mRNA target sequence and the  
75 miRNA seed sequence, which is the 2<sup>nd</sup>-7<sup>th</sup> nucleotides at the 5'-end of the mature miRNA. When a  
76 target-seed match is extended to 1<sup>st</sup> or 8<sup>th</sup> nucleotide or both nucleotides, the repression effect is  
77 higher. Other features including sequence context at the 3'UTRs and open reading frame (ORF) have  
78 been shown to affect the repression efficacy (Grimson et al. 2007; Friedman et al. 2009; Garcia et al.  
79 2011; Agarwal et al. 2015). Apart from targeting the 3'UTR, miRNAs have also been shown to target  
80 the coding regions (CDS), 5'UTRs and introns in the mRNA (Schnall-Levin et al. 2010; Meng et al.  
81 2013; Li et al. 2016).

82 Experimental and computational methods have been developed to identify miRNA genes and target  
83 sites (Krek et al. 2005; Licatalosi et al. 2008; Chi et al. 2009; Betel et al. 2010; Hafner et al. 2010;  
84 Zisoulis et al. 2010; Friedländer et al. 2012; Reczko et al. 2012; Helwak et al. 2013; Grosswendt et al.  
85 2014; Agarwal et al. 2015; Paicu et al. 2017). Particularly with recent advances in high-throughput  
86 sequencing, more lowly-abundant miRNAs, which are less conserved than the highly-abundant

87 miRNAs, have been detected with unprecedented sensitivity (Friedländer et al. 2012; Londin et al.  
88 2015). This has already led to the discovery of many novel miRNAs in humans and other mammals (Li  
89 et al. 2011; Londin et al. 2015; Penso-Dolfin et al. 2016; Wake et al. 2016; Ji et al. 2017; Wong et al.  
90 2017). In bovine, profiling expressed miRNAs using high-throughput sequencing has been performed  
91 in a number of tissues and developmental stages (Gu et al. 2007; Jin et al. 2009; Zhixiong et al.  
92 2014). High-throughput sequencing of RNA isolated by crosslinking and immunoprecipitation (HITS-  
93 Seq, also known as CLIP-Seq) was recently used to identify the mRNA sequences that were bound by  
94 the miRNA-directed Argonaute protein in bovine kidney cells on a genome-wide scale (Scheel et al.  
95 2017). However, because miRNAs have well-defined temporal- and spatial- expression patterns (Guo  
96 et al. 2014), we reasoned that many more miRNAs and their target sites are present in cattle and can  
97 be identified through analysing additional samples representing more diverse tissue types.

98 In this study, we present a genome-wide identification, quantification and differential expression of  
99 known and novel miRNAs in 17 tissues from a single lactating dairy cow. We predicted the target  
100 sites of all expressed miRNAs in bovine mRNA transcripts and demonstrated an enrichment of  
101 putative interactions between miRNA families and cognate targets in interactions from previous  
102 experimental and computational identification. We summarized features of expressed miRNAs and  
103 target sites. We showed a preferential localisation of miRNA target sites at active bovine enhancer  
104 regions. Using 65 million WGS variants from the 1000 Bull Genomes Project (Daetwyler et al. 2014)  
105 (*Bos Taurus* Run 6), we found rare sequence variants were significantly enriched within miRNA genes  
106 and target sites compared with the entire genome. But we didn't find the expression levels of  
107 mature miRNAs significantly associated with allelic imbalance within miRNA target sites, nor did we  
108 find heterozygous sites within miRNA target sites significantly contributed to allelic imbalance within  
109 exons of the same target genes. Our study provides an updated annotation of bovine miRNA-mRNA  
110 regulatory networks that in the future may aid in the identification of causal variants affecting  
111 complex traits.

## 112 **Results**

### 113 **Samples, RNA sequencing and alignment**

114 The miRNA profiles were generated by sequencing short RNA libraries prepared in duplicate from  
115 adrenal gland, black skin, brain caudal lobe, brain cerebellum, heart, intestinal lymph node, kidney,  
116 leg muscle (semimembranosus), liver, lung, mammary gland, ovary, spleen, thymus, thyroid, tongue,  
117 and white skin, which were collected from a first-lactation dairy cow as part of another study  
118 (Chamberlain et al. 2015). Over 800 million paired-end reads, 5.8-17.8 million reads per library, were  
119 generated (Supplemental Table S1). Two trimmers, cutadapt (Martin 2011) in combination with  
120 sickle (Joshi and Fass 2011) and trimmomatic (Bolger et al. 2014), were used to compare the efficacy  
121 of trimming. Although no adapter contamination was detected by FastQC (Andrews 2010) in any  
122 trimmed reads, a much larger number of reads were retained by cutadapt and sickle (83.25-95.88%)  
123 than trimmomatic (14.72-60.51%) (Supplemental Table S1).

124 In order to assess whether adapter residues remained in trimmed libraries, and to identify the best  
125 aligner for short RNA sequence reads, reads output from both trimmers were respectively mapped  
126 with five aligners: BWA (Li and Durbin 2009), bowtie (Langmead 2010), bowtie2 (Langmead and  
127 Salzberg 2012), STAR (Dobin et al. 2013) and HISAT2 (Kim et al. 2015). Across all aligners, sequence  
128 reads that were trimmed by the single-end trimmer (i.e. cutadapt and sickle) were aligned to bovine  
129 reference genome with much larger gaps than those that were trimmed by the paired-end trimmer  
130 (i.e. trimmomatic). Since trimmomatic has the advantage over cutadapt and sickle to utilise paired-  
131 end information to identify adapter residues, this observation indicated that adapter residues  
132 remained in the single-end trimmed reads even though FastQC (Andrews 2010) could not detect  
133 them. Across all libraries, only when short RNA sequence reads were trimmed by trimmomatic and  
134 aligned to bovine reference genome using bowtie2, the averaged inferred insert size matched with  
135 the insert size from experimental design (10-50nt; Table 1). Bowtie2 was also one of the best two  
136 aligners in returning more paired and mapped reads, more exact matched reads, more inward

137 oriented pairs and less low mapping quality reads (Supplemental Table S1). Read depths varied  
138 between tissues as expected. Tongue had the largest proportion of mapped and paired reads  
139 (98.19%), and black skin had the lowest ratio (90.45%; Supplemental Table S1). All subsequent  
140 analyses were progressed with reads processed by trimmomatic and bowtie2.

141 Table 1 Here

## 142 **Identification of expressed known and novel miRNAs**

143 A total of 13,807 candidate precursor miRNAs genomic coordinates along with their cognate mature  
144 and star miRNA sequences from 34 libraries (17 tissues × 2 technical replicates) were identified by  
145 miRDeep2 (Friedländer et al. 2012) (Supplemental Table S2). Of those 7,862 passed filtering (false  
146 discovery rate ≤0.1 for novel miRNAs), which included 655 unique precursor sequences (44-149nt)  
147 that were expressed from 657 genomic coordinates, 699 unique mature miRNA sequences (18-25nt)  
148 from 722 genomic coordinates, and 736 unique star miRNA sequences (13-28nt) from 748 genomic  
149 coordinates. The inconsistency between the number of unique sequences (precursor, mature or  
150 star) and the number of unique genomic locations were due to identical miRNA sequences  
151 expressing from >1 distinct genomic location. For example, a novel precursor sequence was  
152 expressed from multiple genomic coordinates including location 1: *chr25:32389474-32389547(+)*,  
153 location 2: *chr25:32411299-32411372(+)* and location 3: *chr25:32395491-32395564(+)*. All three  
154 locations were expressed in ovary; whereas in adrenal gland only location 1 and 2 were expressed. In  
155 all three locations, many mature reads (read count: 544-2521) were observed from each tissue. In  
156 ovary, a small amount of loop and star miRNAs were observed. Because the loop and star miRNAs  
157 degrade quickly, the detection of these sequences along with the high levels of mature sequences  
158 increased the confidence of a true detection (Friedländer et al. 2012) (accuracy: 73-79%). Other  
159 examples of identical known miRNA sequences (e.g. *bta-miR-7-5p*) or novel miRNA sequences (e.g.  
160 *ACUAUACAACCUACUACCUCA*) that were expressed from different genomic locations (within the

161 same chromosome or between chromosomes) in different tissues are also observed (Supplemental  
162 Table S2).

163 Sixty percent of the miRNAs identified were nearly identical to bovine, goat or sheep miRNAs that  
164 were annotated in miRBase (Kozomara and Griffiths-Jones 2014) (version 22), and the remaining  
165 40% were novel (Supplemental Table S2). miRDeep2 (Friedländer et al. 2008; Friedländer et al. 2012)  
166 classified known and novel miRNAs by comparing our expressed precursor miRNA sequence with the  
167 precursor miRNA sequences in miRBase (Kozomara and Griffiths-Jones 2014) (version 22). If an  
168 expressed mature miRNA has never been reported to miRBase but is excised from a precursor  
169 miRNA in miRBase, the mature miRNA sequence is still classified as 'known' (e.g. *bta-miR-26c-5p*;  
170 Supplemental Table S2). Because such cases were rare, in this study we used the miRDeep2  
171 classification of known and novel miRNAs.

172 Known miRNAs were more abundant than novel miRNAs (Figure 1A). Close to 48.19% of the novel  
173 mature miRNAs overlapped with the antisense strand of known miRNAs, and these 'known-  
174 antisense' novel miRNAs were more abundant than the rest of the novel miRNAs (Figure 1B). Known  
175 and novel mature miRNAs were more abundant in brain cerebellum than all other tissues (Figure  
176 1C). Most mature miRNAs were expressed in either only one tissue (where >67% were novel) or all  
177 17 tissues (where >98% were known; Figure 1D). Across all tissues, most miRNAs were expressed in  
178 clusters within 3,000nt of each other (Figure 2). Many known and novel mature miRNAs were  
179 identified on chromosome 21, X and 19 (Figure 1E). No known nor novel miRNAs were identified on  
180 the bovine mitochondrial chromosome, even though miRNAs have previously been identified in the  
181 mitochondria of liver cells from humans, mice and rats (Kren et al. 2009). This could be because  
182 mitochondria have a unique population of miRNAs, and required the isolation of mitochondria from  
183 tissue samples to enable RNAs to be extracted from mitochondria as opposed from total cellular  
184 content (Kren et al. 2009).

185 Figure 1 and Figure 2 here



186 Close to 27% of the expressed mature miRNAs, which were known to miRBase (version 22) and were  
187 detected in our datasets, were not included in the General Feature Format (GFF) of the UMD3.1.1  
188 reference genome annotation (e.g. *miR-654-5p*, *miR-6715-3p*). A small proportion of the expressed  
189 mature miRNAs (<1.5%), which had nearly identical sequences to annotated miRNAs in the GFF and  
190 miRBase (one or two nucleotides differences), were also identified, and those miRNAs were  
191 expressed from genomic regions that were not annotated in the GFF file. For example, *bta-miR-29d-*  
192 *3p* (*UAGCACCAUUUGAAAUCGAUUA*) is annotated on *chr16:77562591-77562612(+)* in GFF and this  
193 sequence is identical to that annotated in miRBase (version 22). In our dataset, 13 tissues (adrenal  
194 gland, black skin, brain caudal lobe, brain cerebellum, heart, kidney, lung, intestinal lymph node,  
195 mammary, ovary, thyroid, tongue and white skin) that had the mature miRNA  
196 (*uagcaccuuugaaaucgguua*) expressed were all from *chr16:77478646-77478667(+)*. Because *bta-*  
197 *miR-29d-3p* and *uagcaccuuugaaaucgguua* only differed in the 19<sup>th</sup> nucleotide, miRDeep2  
198 annotated *uagcaccuuugaaaucgguua* as *miR-29d-3p*. Overall, apart from the miRNAs that were  
199 already annotated in UMD3.1.1, expressed known and novel miRNAs were also found within the  
200 coding regions (CDS) of other annotated genes, 5'- or 3'- untranslated regions (UTRs), introns of  
201 annotated genes or non-coding RNAs, intergenic regions, and in genomic regions that crossed over  
202 from one of these features to another feature (Figure 1F), consistent with previous observations that  
203 miRNAs could arise throughout the genome (Melamed et al. 2013; Londin et al. 2015).

204 We traced the strand of the precursor that each mature and star miRNA were derived from,  
205 following the call of the miRNA community to provide a more precise nomenclature (Kozomara and  
206 Griffiths-Jones 2014; Budak et al. 2016) (Supplemental Table S2). We found that 83% of precursors  
207 had only one mature miRNA. We also observed some miRNAs had both arms highly co-expressed  
208 (read counts > 1000) in a tissue in both technical replicates, although most of those co-expressed  
209 duplexes were in lower abundance (read counts < 100). For example, *bta-mir-140* and *bta-mir-145*  
210 had both arms dominant in kidney (read counts: 1,088 to 13,635). *Bta-mir-361* had both arms  
211 dominant in adrenal gland, brain caudal lobe, brain cerebellum, heart, kidney, liver, ovary, spleen,

212 thyroid and tongue (read counts: 223 to 1704). Some miRNAs changed the dominant arms across  
213 tissues. For example, *bta-miR-195-5p* was preferred in both technical replicates of black skin, lung,  
214 ovary, spleen and white skin (read counts: 230 to 3235), whereas *bta-miR-195-3p* was preferred in  
215 leg muscle, mammary gland and thyroid (read counts: 30 to 190). *Bta-miR-335-5p* was preferred in  
216 both technical replicates of thyroid (read count: 349 to 379), whereas *bta-miR-335-3p* was preferred  
217 in brain caudal lobe, heart, kidney, lung, ovary, spleen, tongue and white skin (read count: 24 to  
218 464).

### 219 **Identification of differentially expressed mature miRNAs**

220 Differential expression analysis was performed to identify mature miRNAs that were more often  
221 expressed (up-regulated) or less often expressed (down-regulated) in a tissue than the mean  
222 expression across all other tissues (Supplemental Table S3). Significant differentially expressed (after  
223 corrected for multiple testing P-value <0.01 and absolute fold change >2) mature miRNAs were  
224 observed in all tissues except in black skin (Table 2; Table 2Figure 3Figure 3Figure 3Figure 3Figure  
225 3A). Known miRNAs with high tissue-specificity showed significant differential expression in our  
226 analysis, such as *miR-375* in adrenal gland (Ludwig et al. 2016; Gai et al. 2017), *miR-219* in brain  
227 caudal lobe and brain cerebellum (Ludwig et al. 2016), and *miR-122* in liver (Jopling 2012; Szabo and  
228 Bala 2013; Modepalli et al. 2014; Wang et al. 2016a). No novel miRNAs were found to be up-  
229 regulated in any tissues, but some novel miRNAs were down-regulated in adrenal gland, brain  
230 cerebellum, lung, ovary, spleen and thyroid. Tissues with similar biological function were grouped  
231 into clusters based on the full normalised read count matrix for all miRNAs (Figure 3B). The  
232 clustering profile of miRNAs was similar to that of mRNAs from the same tissues of the same cow  
233 shown previously (Chamberlain et al. 2015). For example, brain tissues and skin tissues were  
234 clustered together, respectively. However, some tissues were no longer grouped into the same  
235 cluster as they were in the mRNA profile. For example, leg muscle was separated from the muscle  
236 group formed by tongue and heart.

237 Table 2 and Figure 3 here

## 238 **Prediction of miRNA seed match sites**

239 A miRNA seed match site is a segment of mRNA sequence that perfectly reverse-complements the  
240 miRNA seed sequence, which is the 2<sup>nd</sup> to 7<sup>th</sup> nucleotide at the 5'-end of the mature miRNA  
241 sequence. To identify miRNA seed match sites within bovine mRNA transcripts for our expressed  
242 mature miRNAs, all clean (Materials and Methods) mature miRNA sequences were provided as input  
243 to TargetScan (Agarwal et al. 2015) (version 7.2). This consisted of 434 known and 265 novel mature  
244 miRNA sequences, which were expressed in at least one of the 17 bovine tissues. Based on the  
245 identical extended seed sequence (i.e. 2<sup>nd</sup>-8<sup>th</sup> nucleotides at the 5'-end of mature miRNA sequence),  
246 all mature miRNAs were grouped into 600 miRNA families. Examining members within each miRNA  
247 family, we found that 455 families were formed by grouping known and novel expressed miRNAs  
248 with miRNAs in miRBase (Kozomara and Griffiths-Jones 2014) (version 22), 125 families were formed  
249 by a single expressed novel miRNA that was not grouped with any miRBase miRNAs, and the  
250 remaining 20 families were formed by a single known expressed miRNA that was also not grouped  
251 with any miRBase miRNAs. Most of these “ungrouped” known miRNAs were recently discovered, as  
252 the name of these miRNAs had large numbers (e.g. *bta-miR-11995-3p*) as opposed to small numbers  
253 (e.g. *bta-miR-1*), noting miRNAs are mostly named in chronological order of their discovery  
254 (Kozomara and Griffiths-Jones 2014).

255 TargetScan (Agarwal et al. 2015) (version 7.2) returned information including the genomic position of  
256 each seed match site of a miRNA family in bovine reference genome bostau7 that was orthologous  
257 to the 3'UTR of mRNA transcripts in human reference genome (UCSC ID) hg19. Although TargetScan  
258 only predicted miRNA seed match sites in the 3'UTRs, we searched through all genes in bostau6 for  
259 seed match sites, because miRNAs were found to be reverse-complements to the seed match  
260 sequences in the promoter sequence, 5'UTR and open reading frames (Lewis et al. 2005; Stark et al.  
261 2007; Place et al. 2008; Schnall-Levin et al. 2010; Cheng et al. 2015; Xiao et al. 2016).

262 Our prediction using TargetScan returned a total of 6,411,460 records of “mRNA target: seed match  
263 site: miRNA family” from bostau7. Among those, 879,240 records were recovered (Materials and  
264 Methods) on the 31 standard chromosomes (1-29, X and M) from bostau6. This included a total of  
265 780,481 putative seed match sites from 18,196 mRNA transcripts from 16,381 genes on the 31  
266 chromosomes. Although TargetScan returned putative seed match sites on bostau7 that was at least  
267 6-8nt long, because only the seed match sites were searched on bostau6, each putative seed match  
268 site on bostau6 was 6nt. A miRNA family was predicted to target 4 to 18,448 sites (on average 1,306)  
269 from 4 to 7,591 mRNA transcripts (on average 1,292) from 4 to 6,767 genes (on average 1,154). A  
270 target gene was predicted to have 1 to 199 miRNA seed match sites (on average 48). Given the  
271 many-to-many relationship among miRNA family and mRNA, a total of 692,685 putative interactions  
272 between miRNA families and cognate gene targets were returned from our prediction on bostau6,  
273 which resulted in 3,484,895 putative interactions between expressed miRNAs and cognate gene  
274 targets. Genomic coordinates of all predicted seed match sites of our expressed miRNAs are  
275 reported (Supplemental Table S4).

276 Acetylated lysine 27 on histone H3 (H3K27ac) and tri-methylation of lysine 4 on histone H3  
277 (H3K4me3) regions have been identified by chromatin immunoprecipitation followed by high-  
278 throughput sequencing (ChIP-Seq) experiments to identify active enhancer regions and active  
279 promoter regions (Creyghton et al. 2010; spicuglia and Vanhille 2012). H3K27ac and H3K4me3 ChIP-  
280 Seq marks from bovine liver tissue were mostly found at intergenic and intronic regions, and were  
281 also found at 5'UTRs, 3'UTRs and CDS (Wang et al. 2017). In the nucleus, miRNAs were shown to  
282 bind to the promoter sequence through the same 6nt miRNA seed match site to increase  
283 transcription of the gene that the promoter regulates (Place et al. 2008; Zhang et al. 2014; Cheng et  
284 al. 2015; Xiao et al. 2016). This inspired us to examine within genes, whether DNA sequences that  
285 were marked by bovine H3K27ac or H3K4me3 signals (Villar et al. 2015; Zhao et al. 2015) were more  
286 or less often also putative miRNA seed match sites.

287 To answer this question, a contingency table was constructed to show the number of nucleotides  
288 within gene regions in UMD3.1 (Ensembl release 91) that were (1) inside both a histone modification  
289 mark and putative miRNA target sites, (2) inside a histone modification mark but outside putative  
290 miRNA target sites, (3) outside a histone modification mark but inside putative miRNA target sites,  
291 and (4) outside both a histone medication mark and putative miRNA target sites (Table 3). Because  
292 putative miRNA seed match sites were DNA-strand specific, where a histone modification mark  
293 existed we assumed both strands affected. Using data from the contingency table, a Chi-Square test  
294 showed that within genes, the proportion of miRNA seed match sites that were also within the  
295 histone modification mark regions was significantly higher than the proportion of non-miRNA seed  
296 match sites that were within the same histone mark regions (P-value <0.00001; Table 3), indicating  
297 that miRNA seed match sites preferentially occurred within gene regions undergoing active histone  
298 modifications.

299 Table 3Error! Reference source not found. Here

### 300 **Confirmation of putative interactions between miRNAs and targets**

301 Putative interactions between miRNA families and cognate targets were confirmed by interactions  
302 identified from previous publications through experimental and computational procedures (Table 4).  
303 Messenger RNA (mRNA) sequences bound with miRNAs by the AGO protein in bovine kidney cells  
304 were identified through CLIP-Seq (Scheel et al. 2017). After converting genomic coordinates of those  
305 mRNA sequences from (UCSC ID) bostau7 to bostau6, 208,688 interactions from 224 expressed  
306 miRNAs and 200,459 cognate target sequences remained. We identified 297,802 putative  
307 interactions from 238 miRNA families and 15,999 seed match sites in our lactating cow's kidney  
308 tissue. Of those, 11,885 putative interactions from 163 miRNA families and 9,282 seed match sites  
309 overlapped with those from the Scheel *et al.* set (Table 4). Other miRNA interaction datasets from  
310 public domains did not provide any cell/tissue information, and therefore all our putative  
311 interactions were counted and compared. We found 3,173 putative interactions between miRNA

312 families and target gene names overlapped with that in miRTarBase (Chou et al. 2018), 229,473  
313 putative interactions between miRNA families and target mRNA transcript IDs overlapped with that  
314 in miRWalk (Dweep and Gretz 2015), and 182,685 putative interactions between miRNA families and  
315 target gene names overlapped with that in TargetScan (Agarwal et al. 2015) (version 7) (Table 4).

316 Table 4 Here

317 We investigated the relationship between miRNA transcription and target mRNA transcription.  
318 mRNA transcripts that were differentially expressed in a tissue compared with the mean expression  
319 in all other tissues (after corrected for multiple testing P-value <0.01 and absolute fold change >2)  
320 from the same cow as used in this study and previously identified (Chamberlain et al. 2015) were  
321 utilised. Combining those results with differentially expressed miRNAs that were identified in this  
322 study, we found that 84,585 tissue-specific putative interactions between miRNA families and target  
323 genes had both a miRNA in the miRNA family and the gene target differentially expressed in the  
324 same tissue from the same cow. Of those 84,585 interactions, which consisted of 15 tissues, 2,333  
325 target gene IDs and 473 miRNA families, 233 putative interactions from all tissues that were  
326 confirmed by miRTarBase (Chou et al. 2018) and 8 putative interactions in kidney that were  
327 confirmed by CLIP-Seq (Scheel et al. 2017) had both the miRNA and gene differentially expressed in  
328 the same tissue, and one putative interaction overlapped the miRTarBase, CLIP-Seq and differential  
329 expression sets. KEGG and GO analyses on differentially expressed mRNAs were previously  
330 performed (Chamberlain et al. 2015). Combining functional terms from KEGG and GO, we found that  
331 mRNAs that were co-differentially expressed with cognate miRNA within the same tissue were most-  
332 frequently related to metabolism or metabolic processes, immune or inflammatory responses,  
333 signalling pathways, cell activation and differentiations, blood coagulation and transportations of  
334 inorganic and organic molecules (Chamberlain et al. 2015).

335 To investigate whether there was a general direction between miRNA transcription and target mRNA  
336 transcription, a least square analysis was performed to test the association between the log2 fold

337 change of miRNA transcription from this study and the log<sub>2</sub> fold change of mRNA transcription from  
338 Chamberlain *et al.* for miRNA and target mRNAs that were both significantly differentially-expressed  
339 (after correction for multiple testing P-value <0.01 and absolute fold change >2) in the same tissue of  
340 the same cow. The number of records for the fold change of expression between miRNAs and target  
341 mRNAs across tissues varied from 62 to 95,586. We found no significant (P-value < 10<sup>-8</sup>) association  
342 between the fold change of miRNA transcription and target mRNA transcription in any tissues (Table  
343 5). Note that although leg muscle and lung appeared to be significant, manual examination of the  
344 data did not support a true correlation. These results indicated that in general if miRNAs were up-  
345 regulated in a tissue, their target mRNAs could be up- or down-regulated in the same tissue.

346 Table 5 Here

347 Mature miRNAs that were derived from different arms of the same precursor were found to target  
348 different genes (Supplemental Table S5 **Error! Reference source not found.**). *Bta-miR-140-5p*, *bta-*  
349 *miR-140-3p*, *bta-miR-145-5p*, *bta-miR-145-3p*, *bta-miR-195-5p*, *bta-miR-195-3p*, *bta-miR-335-5p*,  
350 *bta-miR-335-3p*, *bta-miR-361-5p* and *bta-miR-361-3p* were respectively the dominant product in at  
351 least one tissue from this lactating cow (Supplemental Table S2). These mature miRNAs were  
352 predicted to target 1431, 1712, 770, 897, 5868, 521, 1437, 3415, 411 and 1932 bovine mRNA genes,  
353 respectively. Among those, 24, 16, 11, 3, 1432, 3, 192, 2, 11 and 10 target genes were confirmed by  
354 experimental procedures that were published in either miRTarBase (Chou et al. 2018) (all tissues  
355 were considered) or CLIP-Seq (Scheel et al. 2017) (only kidney was considered) **Error! Reference**  
356 **source not found..Error! Reference source not found.**

### 357 **Enrichment analysis**

358 A permutation test was performed to examine whether putative interactions between the miRNA  
359 families and cognate targets were significantly enriched for interactions identified from  
360 experimental and computational procedures in previous publications (Table 6). We found that  
361 putative interactions were statistically significantly enriched for previously-identified interactions

362 from all confirmation datasets. Compared with 10,000 random interactions, putative interactions  
363 that were confirmed by miRTarBase (Chou et al. 2018) were 1.61-fold higher, CLIP-Seq (Scheel et al.  
364 2017) were 1.19-fold higher, miRWalk (Dweep and Gretz 2015) were 1.11-fold higher, TargetScan  
365 (Agarwal et al. 2015) were 1.40-fold higher, and by the differential expression of mRNAs  
366 (Chamberlain et al. 2015) and miRNAs from the same tissue of the same cow were 1.10-fold higher  
367 (Table 6).

368 Table 6 Here

### 369 **Polymorphisms within miRNA genes and targets**

370 To investigate the possibility that polymorphism within miRNA genes and target sites might be  
371 detrimental for fitness and therefore could be a target for natural selection, we compared both the  
372 rate of polymorphism and the allele frequency distributions within miRNA genes and targets with  
373 that across the genome.

374 Firstly, we defined the polymorphic rate as the proportion of a genomic feature that were  
375 polymorphic sites. We used whole genome sequence (WGS) variants from the 1000 Bull Genomes  
376 Project (Daetwyler et al. 2014) (*Bos Taurus* Run 6) that were filtered based on quality metrics as  
377 described (Daetwyler et al. 2017). The filtered variants (including single-base-nucleotide  
378 substitutions, insertions and deletions) consisted of sequence variants with high confidence, which  
379 have been shown to improve the quality of variant sets judged by the concordance of sequence and  
380 SNP chip genotypes at overlapping positions as well as the rate of opposing homozygotes found in  
381 parent-offspring pairs (Daetwyler et al. 2017). We found that polymorphic rates were lower within  
382 miRNA genes (precursor, mature and star) and miRNA targets (target genes and seed match sites)  
383 than the polymorphic rate averaged across the genome

384 Table 7

385 Table 7 (Table 7)



386 Table 7

387 Table 7). For mRNA genes that were targeted by our expressed miRNAs, their gene regions (including  
388 introns) had a higher polymorphic rate than 3'UTRs. miRNA seed match sites from *in-silico* prediction  
389 and miRNA target sequences from previous experimental identification (Scheel et al. 2017) had even  
390 lower polymorphic rates than 3'UTRs. Compared with genes that were targeted by expressed  
391 miRNAs (1.556%), miRNA genes (0.710%) were much more depleted for polymorphic sites. Known  
392 expressed miRNAs had a lower polymorphic rate than novel expressed miRNAs. Mature miRNAs had  
393 the lowest polymorphic rate among all genomic features examined. Surprisingly, although star  
394 miRNAs were partially reverse-complementary with mature miRNAs, the star sequence had a higher  
395 polymorphic rate than the mature sequence (Table 7  
396 Table 7).

397 Table 7

398 Table 7 Here

399 All alleles in the variant call file, including each alternative allele at the same polymorphic site, were  
400 calculated for their frequencies in the 2,333 animals from the 1000 Bull Genomes Project (Daetwyler  
401 et al. 2014) (*Bos Taurus* Run 6). To examine whether miRNA genes and target sites had more variants  
402 with extreme frequencies than the entire genome, which could be evidence of selection at these  
403 genomic features, we used raw WGS variants (single-base-nucleotide substitutions only). Raw WGS  
404 variants contained all variants called, so rare variants were not filtered out. We found that variants  
405 with extreme allele frequencies were more likely within mature, star and precursor miRNAs,  
406 followed by miRNA target sequences from experimental identification (Scheel et al. 2017) and  
407 putative miRNA seed match sites within target genes from computational prediction, compared to  
408 the entire genome (Table 8; Figure 4Figure 4Figure 4).

409 Table 8Error! Reference source not found. and Figure 4Figure 4 Here

410 Insertions and deletions (INDELs) were categorised by the number of positional shifts,  
411  $n \in (-\infty, +\infty)$ , between the alternative allele and reference allele at the same locus. To examine  
412 whether INDELs with any  $n$ -th positional shift were more enriched within miRNA genes and target  
413 sites than the entire genome, we used the raw INDELs (unfiltered) from 1000 Bull Genomes Project  
414 (Daetwyler et al. 2014) (*Bos Taurus* Run 6). We found that most INDELs only shifted a small number  
415 of positions (Figure 5), which was consistent with previous research in humans that INDELs with a  
416 small number of positional shifts were more frequent than INDELs with a large number of positional  
417 shifts, even though those studies did not use WGS variants (Mills et al. 2006; Saunders et al. 2007;  
418 Bhattacharya et al. 2012; Gong et al. 2012). Compared with the entire genome and mRNA genes that  
419 were targeted by our expressed miRNAs, miRNA genes were highly depleted for INDELs of all  
420 lengths. Interestingly, both miRNA seed match sites from our computational prediction and miRNA  
421 target sequences from experimental identification (Scheel et al. 2017) had a higher density of INDELs  
422 than the entire genome and target mRNA genes (Figure 5). INDELs with a single base pair positional  
423 shift and even number of positional shifts were more dominant in putative miRNA seed match sites.  
424 Deletions were more frequently observed in experimentally identified miRNA target sequences of  
425 Scheel *et al.* (2017) than insertions.

426 Figure 5 Here

## 427 **Examination of associations among miRNAs, miRNA target regions and** 428 **allele-specific expression of target genes**

429 Allele-specific expression (ASE) or allelic imbalance is the biased expression from one parental allele  
430 for a gene. In 18 tissues from this cow, the degree of allelic imbalance at a locus was previously  
431 reported as a Chi-Square value (Chamberlain et al. 2015). To examine whether allelic imbalance was  
432 associated with the miRNAs and miRNA target sites, we defined ASE score as the square root of this  
433 Chi-square value, and putative miRNA target sequence as the 56nt mRNA sequence surrounding the  
434 miRNA seed match site (Materials and Methods).

435 Firstly, we examined across tissues, whether allelic imbalance at each heterozygous site within  
436 putative miRNA target sequence was associated with the expression level of cognate mature miRNA.  
437 A dataset with 176,478 records was constructed, which consisted of 682 mature miRNAs and 17,626  
438 heterozygous sites within 19,119 cognate putative target sequences in 6,704 target genes across 17  
439 tissues. Results of analysis of variance (ANOVA) showed that the expression level of miRNA did not  
440 have a significant effect on the allelic imbalance within its putative miRNA target sequence (P-value  
441 = 0.367).

442 Secondly, we examined across genes, whether zygosity within putative miRNA target sequences was  
443 associated with allelic imbalance within exons of miRNA target genes. A dataset with 8,406,848  
444 records was constructed, which consisted of 191,866 WGS variants from 1000 Bull Genomes Project  
445 (Daetwyler et al. 2014) (*Bos Taurus* Run 5) within 158,466 putative miRNA target sequences from  
446 7,072 miRNA gene targets, and ASE scores from 22,238 heterozygous sites within exons of miRNA  
447 target genes. Results from linear regression showed that zygosity within putative miRNA target  
448 sequences had an effect on allelic imbalance within exons of target genes. Also, with an addition of a  
449 heterozygous site within a putative miRNA target sequence, we could expect the mean allelic  
450 imbalance within exons of miRNA target genes to increase 0.0389 (P-value  $\leq 10^{-8}$ ).

451 A permutation test was performed to assess whether the effect of zygosity within putative miRNA  
452 target sequences was significant compared with the effect of zygosity within genes that were not  
453 putative miRNA target sequence nor exons (termed the 'null regions'). Results showed that all  
454 effects from the null regions were positive. Additionally, the effect of zygosity within putative miRNA  
455 target sequences, 0.038937, was only larger than 845 out of 10,000 effects from the null regions.  
456 The observed standard error of the effect within putative miRNA target sequences, 0.002797, was  
457 bigger than 9,998 out of 10,000 standard errors from null regions. This showed that an increment of  
458 polymorphic sites anywhere within a gene increased allelic imbalance in exons, and zygosity within  
459 putative miRNA target sequences were not more significantly associated with allelic imbalance at

460 exons of target genes than zygosity within the gene but outside putative miRNA target sequence and  
461 exons.

## 462 **Discussion**

463 Improving the annotation of functional elements (or regulatory elements) in the bovine genome will  
464 help to better understand the effects of sequence variants across the genome, to fill the gap  
465 between genotype and phenotype, and to contribute to the application of molecular phenotype to  
466 predict complex phenotypes (Andersson et al. 2015). A functional element is denoted as a discrete  
467 genomic segment that encodes a defined product (e.g. protein, mRNA or miRNA) or a reproducible  
468 biochemical signature (e.g. protein binding sites or miRNA seed match sites) (ENCODE Project  
469 Consortium 2012). In this study, we improve the annotation of tissue-specific miRNAs that were  
470 expressed in a single lactating dairy cow. We provide a list of putative seed match sites for all  
471 expressed mature miRNAs and propose possible functions of those miRNA-led interactions.

472 Most of our findings on expressed miRNAs aligned with previous studies in human and other  
473 animals. First, we confirmed the expression of many widely-reported, tissue-specific and highly-  
474 expressed miRNAs (Supplemental Table S2), such as myotubes-related *miR-1* and *miR-133b* (Lim et  
475 al. 2005; Zhao et al. 2005) in heart, leg muscle and tongue as well as neuron-specific *miR-9* (Wang et  
476 al. 2012) in brain caudal lobe and brain cerebellum. Second, a large proportion of miRNAs identified  
477 in this study were novel, and overall had a lower expression level but a higher tissue-specificity than  
478 known miRNAs (Figure 1) (Friedländer et al. 2012; Londin et al. 2015; Huang et al. 2017). Novel  
479 miRNAs tended to be expressed from the antisense strand of known miRNAs (Figure 1), which were  
480 suggested to have functional importance in different stages of development (Bender 2008; Stark et  
481 al. 2008; Tyler et al. 2008). Finally, most expressed miRNAs were close to one another on the DNA  
482 strand, forming into discrete “blocks” or “clusters” (Figure 2). miRNA clusters were previously  
483 defined as a set of two or more miRNAs that are transcribed in the same orientation and are not

484 separated by a transcription unit or a miRNA in the opposite orientation (Altuvia et al. 2005). miRNA  
485 clusters are known to play important roles controlling various cellular process in the human genome  
486 (Yu et al. 2006; Griffiths-Jones et al. 2008; Lai and Vera 2013; Marco et al. 2013). Constituents of the  
487 same miRNA cluster were suggested to be expressed from the same transcription unit and evolve to  
488 coordinate regulation of functionally related genes (Marco et al. 2013; Wang et al. 2016b). Overall,  
489 our results suggested that bovine miRNAs were more extensive than currently represented by public  
490 repositories, and we have characterised a significant number of novel tissue-specific miRNAs.

491 We observed cases of identical miRNA sequences being expressed at different magnitudes from  
492 different genomic locations in different tissues. This tissue-specificity of miRNA expression could be  
493 controlled by super-enhancers (SEs) and strong insulator signals, e.g. topological association  
494 domains (TADs) and the DNA binding sequence patterns of the CCCTC protein (CTCF binding  
495 motifs) (Bouvy-Livrand et al. 2017; Suzuki et al. 2017). In mouse embryonic stem cells (mESCs),  
496 active enhancers that were bound by transcription factors Oct4, Sox2 and Nanog (OSN) (Whyte et  
497 al. 2013), and active promoters that were marked by H3K4me3 (Marson et al. 2008), were identified  
498 through ChIP-Seq. Interactions between promoters and enhancers in mESCs that were initialised by  
499 cohesin were identified by chromatin interaction analysis with paired-end tag sequencing (CHIA-PET)  
500 (Downen et al. 2014). These data from mESCs showed that miRNA genes that were transcribed from  
501 genomic regions close to one another on a chromosome in mESCs were spatially close to a small  
502 subset of super-enhancers specific to mESCs (Suzuki et al. 2017). Super-enhancers are enhancers  
503 close to one another on the same DNA strand (typically  $\leq 30\text{Kb}$ ), which have been shown to control  
504 cellular identity (Khan and Zhang 2016). When CRISPR/Cas9 deletion was performed on each of the  
505 constituents of the miRNAs-associated super-enhancers, substantial but different levels of decrease  
506 in the *de novo* production of mature miRNAs were observed (Suzuki et al. 2017). Additionally, strong  
507 insulator signals from TAD boundaries and CTCF binding sites were observed between the  
508 transcription start sites (TSSs) of miRNAs close to one another on the chromosome (Bouvy-Livrand  
509 et al. 2017). Those insulator elements separated the TSSs of miRNAs into two groups: miRNAs that

510 were on the same side of the insulator elements as the super-enhancer expressed a large number of  
511 transcripts, whereas miRNAs that were on a different side of the insulator elements from the super-  
512 enhancer expressed fewer transcripts (Bouvy-Liivrand et al. 2017). These results indicated that the  
513 observation of identical miRNA sequences from different locations being expressed at different  
514 magnitudes in different bovine tissues could be because those miRNAs were controlled by different  
515 enhancers (or different constituents of the same super-enhancer), and/or because those miRNAs  
516 were separated by insulator elements, leading to only a subset of miRNAs accessible to enhancer  
517 regulatory control.

518 To predict the seed match sites of all expressed miRNAs on bovine mRNA transcripts, we firstly  
519 grouped expressed mature miRNAs into miRNA families based on their extended seed sequence (2<sup>nd</sup>  
520 to 8<sup>th</sup> nucleotides at the 5'-end of mature miRNA sequence). We found that 52% of novel mature  
521 miRNAs were grouped into the same miRNA families with mammalian miRNAs in miRBase  
522 (Kozomara and Griffiths-Jones 2014), indicating that these novel miRNAs had a similar function as  
523 known mammalian miRNAs in the same family. The remaining 48% of novel mature miRNAs did not  
524 group with any known miRNAs in miRBase. Along with those newly-discovered-known miRNAs that  
525 could not be grouped with any other miRNAs, the function of these miRNAs from recent discoveries  
526 could be inferred from the function of cognate targets that we predicted (Supplemental Table S4).

527 The 600 miRNA families that were formed from our expressed miRNAs were predicted to target  
528 780,481 seed match sites on bovine mRNAs homologous to human. Because a putative seed match  
529 site could be shared between 1 and 3 miRNA families, there were 879,240 putative interactions  
530 between miRNA families and their mRNA targets (Supplemental Table S4). We demonstrated an  
531 enrichment of these putative interactions in interactions identified previously (Table 6). The fold  
532 change of enrichment, which compared the number of confirmed actual interactions with the  
533 average number of confirmed random interactions, were lower than 1.7 across all confirmation sets  
534 (i.e. miRTarBase, CLIP-Seq, miRWalk and TargetScan). This was because a miRNA family could

535 interact with 4 to 6,767 putative target genes, which caused an incompletely shuffled dataset that  
536 had more permuted interactions identical to actual interactions and led to a lower level of fold  
537 change of enrichment.

538 *In-silico* identification of seed match sites on bostau6 was challenging because firstly, the prediction  
539 relied on the extended seed sequence within the mature miRNA sequence. miRDeep2 named miRNA  
540 sequences by comparing the precursor miRNA sequence from RNA-Seq data with that from miRBase  
541 (Friedländer et al. 2012). Mature miRNA sequences that were identified through this procedure  
542 could have the same name as that in miRBase even though the mature miRNA sequence, or even the  
543 seed sequence, differed by a few nucleotides from that in miRBase. This gave rise to the different  
544 putative seed match sites between miRNA and mRNA that were identified by TargetScan 7 (Agarwal  
545 et al. 2015) and by our study. Secondly, whole genome sequence (WGS) alignment from 100  
546 vertebrates were required to filter false positive putative miRNA seed match sites (Lewis et al. 2005;  
547 Agarwal et al. 2015). Currently only mRNAs orthologous to humans were included in the WGS  
548 alignment, which was constructed from old versions of reference genomes including hg19 and  
549 bostau7 (UCSC IDs) (Agarwal et al. 2015). As a result, miRNA seed match sites on bovine-specific  
550 mRNAs or on newly annotated mRNAs could not be identified using this method. Many miRNA seed  
551 match sites in bostau7 were not found in bostau6 because the human genes could not be identified  
552 through orthologs in bovine or because sequence compositions of the same gene were different  
553 between bostau7 and bostau6.

554 Confirmation of interacting miRNA sequences and mRNA sequences was also challenging because  
555 most public repositories only provided miRNA name and cognate target gene names. This made it  
556 difficult to determine (1) the strand of the DNA duplex where the mature miRNA was derived from,  
557 (2) the target gene names, because some were missing, and neither gene IDs or transcript IDs were  
558 provided, and (3) the target mRNA sequences, particularly when multiple target sequences were on  
559 the same target gene. To overcome these challenges, future studies should use CLIP-Seq to directly

560 identify the interacting miRNA and target RNA target sequences. This will expand the repository of  
561 miRNA targets to all RNA transcripts. This will also validate the novel miRNAs, putative seed match  
562 sites and putative interactions that were identified in this study.

563 The choice of which strand of the duplex becomes the mature miRNA depends on the orientation of  
564 the duplex as well as the sequence context of the strand, but the mechanism is not fully known yet  
565 (Bartel 2018). Although most miRNAs only have one dominant strand consistent across samples,  
566 some miRNAs had both strands as the dominant products, while other miRNAs had different  
567 preferences for the strand between tissues, species or developmental stages (Griffiths-Jones et al.  
568 2011; Choo et al. 2014). This observation has been called ‘arm switching’ or ‘arm selection’  
569 (Griffiths-Jones et al. 2011). Examination of arm switching requires a large number of samples, and  
570 therefore in previous studies arm switching was more often examined across species (Griffiths-Jones  
571 et al. 2011; Kozomara and Griffiths-Jones 2011). With high throughput sequencing, arm switching  
572 has increasingly been shown between tissues or between cells (Kuchenbauer et al. 2011; Li et al.  
573 2012; Gong et al. 2014; Kuo et al. 2015; Tsai et al. 2016; Lin et al. 2018), and was suggested to  
574 provide a fundamental mechanism to evolve the function of a miRNA locus and target gene network  
575 (Griffiths-Jones et al. 2011). In this study, we showed that cattle also had different arm usages in  
576 different tissues (Supplemental Table S2), and mature miRNAs that were produced from different  
577 strands of the same precursor miRNA led to different confirmed gene targets (Supplemental Table  
578 S4), similar to the different arm usages observed in humans, sheep, insects and rice (Marco et al.  
579 2010; Griffiths-Jones et al. 2011; Bortoluzzi et al. 2012; Li et al. 2012; Hu et al. 2014; Kuo et al. 2015;  
580 Laganà et al. 2015; Tsai et al. 2016; Lin et al. 2018). Variants that control arm preference were  
581 suggested to be within the primary miRNA but outside the duplex (Griffiths-Jones et al. 2011). To  
582 identify the complete primary miRNA sequences, one could use nascent RNA assays such as CAGE  
583 (Kodzius et al. 2006) or PRO-Seq (Kwak et al. 2013) to identify the transcription start sites of RNA  
584 transcripts, use polyadenylated RNA termini assays such as 3P-seq (Jan et al. 2010) or TAIL-Seq  
585 (Chang et al. 2014) to identify the transcription termination sites, and use long-read RNA sequencing



586 methods (Tilgner et al. 2015) to define the exact transcript in genomic regions with large repeats or  
587 large gene families.

588 Using 65 million WGS variants from the 1000 Bull Genomes Project (Daetwyler et al. 2014) (*Bos*  
589 *Taurus* Run 6), we showed that compared with the entire genome and mRNA transcripts, miRNA  
590 genes were depleted for common variants (Table 7

591 Table 7), but were enriched for sequence variants with extreme allele frequencies (Table 8**Error!**  
592 **Reference source not found.**; Figure 4; Figure 5), supporting the hypothesis that miRNA genes are  
593 under strong natural selection. We also showed that miRNA target sites were more depleted for  
594 filtered variants than the entire genome but were less depleted than miRNA genes (Table 7

595 Table 7). Additionally, miRNA target sites were more enriched for variants with extreme frequencies  
596 than the entire genome but were less enriched than miRNA genes (Table 8**Error! Reference source**  
597 **not found.**; Figure 4). These results indicate that miRNA target sites were under weaker natural  
598 selection than miRNA genes.

599 The stronger selection of miRNA genes and weaker selection of miRNA target sites could be a  
600 mechanism to retain the specific role that miRNA plays in the cellular processes yet also to  
601 contribute to the variation of phenotypes. To retain the specific functional role, miRNA genes are  
602 highly conserved in evolution, particularly at the 5' end of the mature miRNAs (Lee et al. 1993;  
603 Reinhart et al. 2000; Bartel 2018). Mutations in miRNA genes were expected to rewire the miRNA  
604 targeting network, affect many cellular processes and cause deleterious consequences to the animal.  
605 To compensate these strong effects, miRNAs expanded their members forming a miRNA family. If  
606 one miRNA gene was mutated, other members in the same family could be expressed to target the  
607 same transcript through the same seed target site (Agarwal et al. 2015; Bartel 2018). Alternatively,  
608 miRNAs from other families could target the same transcript through a different seed target site  
609 because there were often more than one target sites on a transcript (Supplemental Table S4). A

610 mechanism for miRNAs to contribute to variation of phenotypes could be to mutate miRNA seed  
611 match sites and target sequences more frequently such as through insertions and deletions (INDELs)  
612 (Figure 5). Mutations in miRNA target sequences abolish or decrease the repression effects of  
613 cognate mature miRNAs. It has been suggested that each miRNA target site contributes a modest  
614 repression effect in most cases (with multiple target sites in the same 3'UTR adding up to much  
615 more substantial repression) (Baek et al. 2008; Selbach et al. 2008). By mutating miRNA target  
616 sequences, miRNAs could provide a finer tuning of the protein profile. Previous studies on the  
617 functions of miRNAs proposed that occasionally the collective function of a group of miRNAs could  
618 be enough to either trigger or sharpen a developmental transition, but more generally, miRNAs were  
619 expected to produce a much more complex topology of gene expression in the nucleus, with more  
620 optimal levels of protein synthesis in the cytoplasm of each cell of each tissue (Bartel 2018).

621 miRNAs have been found to have functional applications in cattle. *Bta-miR-103-2*, *bta-miR-150* and  
622 *bta-miR-181b-2* were shown to be up-regulated during heat events in Frieswal (*Bos Taurus* x *Bos*  
623 *Indicus*) crossbred dairy cattle (Sengar et al. 2018). Tissue collection for this cow was conducted in  
624 the early spring of Victoria, Australia when the weather was mild (Chamberlain et al. 2015), and  
625 these miRNAs were lowly expressed in this *Bos Taurus* dairy cow's skin tissues (mature miRNA read  
626 count: 4-74). Whereas *bta-miR-142*, which has been shown to be down-regulated during heat event  
627 (Sengar et al. 2018), was moderately expressed in skin tissues (mature miRNA read count: 248-317).  
628 This provides the possibility of using these miRNAs as biomarkers for heat tolerant cattle.

629 The *DGAT1* gene is known to have a large effect on milk yield and composition in dairy cattle (Grisart  
630 et al. 2004).

631 In another study, a genomic region around *TFCP2* was associated with fertility in both Australian and  
632 Irish dairy cattle (Moore et al. 2016). We found 4 single-base-nucleotide substitutions, 0 insertion  
633 and 0 deletion from WGS variants from 1000 Bull Genomes Project (Daetwyler et al. 2014) (*Bos*  
634 *Taurus* Run 6) within the seed match sites within the *TFCP2* mRNA transcript (Table 9). The seed

635 match site that had two WGS variants, *chr5:28806521-28806526(+)*, was predicted to be targeted by  
636 the *bta-miR-11995-5p* miRNA family. *TFCP2* is known to be targeted by *bta-miR-660-5p* (Chou et al.  
637 2018), but in our data, there was no variant within the only putative seed match site,  
638 *chr5:73427696-73427701(+)*, for the *bta-miR-660-5p* family within the *TFCP2* mRNA transcript  
639 region. Both *TFCP2* and *bta-miR-660-5p* were expressed in all 17 tissues from this cow, whereas *bta-*  
640 *miR-11995-5p* was only detected in brain caudal lobe and brain cerebellum. Our prediction showed  
641 that the *bta-miR-11995-5p* miRNA family would have a higher repression effect than the *bta-miR-*  
642 *660-5p* miRNA family (Table 9), suggesting that in brain caudal lobe and brain cerebellum tissues,  
643 *bta-miR-11995-5p* was likely to bind to the *TFCP2* mRNA transcript to repress *TFCP2* translation.

644 Similarly, gene *CD40* encodes the protein CD40 which is a receptor on antigen-presenting cells of  
645 the immune system and is essential for mediating a broad variety of immune and inflammatory  
646 responses including mastitis in dairy cattle (Lutzow et al. 2008). *CD40* is known to be regulated by  
647 *bta-miR-145-5p* (Chou et al. 2018). Our prediction showed that apart from *bta-miR-145-5p*, *CD40*  
648 could also be regulated by several other known or novel miRNAs, including the *miR-378g/miR-6637-*  
649 *3p/miR-7482-5p/GCUGGGCUGCGUCGGCGCUCGGA* family which had a more effective repression site  
650 than *bta-miR-145-5p* (Supplemental Table S4), indicating that these miRNAs are likely to target *CD40*  
651 if they are expressed. We found 6 single-base-nucleotide substitutions, 0 insertion and 0 deletion  
652 from WGS variants from 1000 Bull Genomes Project (Daetwyler et al. 2014) (*Bos Taurus* Run 6)  
653 within the seed match sites within the *CD40* mRNA transcript region (Table 9). Although none of  
654 those seed match sites that had WGS variants had a higher repression effect than *miR-bta-145-5p*,  
655 an A-to-G substitution mutation at position *chr13:75569617* within the seed match site  
656 *chr13:75569617-75569622(+)* was interesting, because in most cases the allele that formed the seed  
657 match sequence had a frequency of over 99% (Table 9), but at *chr13:75569617*, the 'A' allele that  
658 formed the seed match sequence had a frequency of 46.13% (Table 9), indicating that variant  
659 *chr13:75569617* is an old mutation, and animals from the 1000 Bull Genomes Project have adapted  
660 to and favoured the alternative allele 'G'.

661 Table 9 Here

662 Given both miRNAs and mRNAs are transcribed by RNA polymerase II in the nucleus, transcription of  
663 miRNAs and that of their target mRNAs was speculated to be correlated. Our results didn't appear to  
664 support this speculation, as we detected no general association between miRNAs and their putative  
665 mRNA targets that were differentially expressed from the same tissue from the same cow (Table 5).  
666 We also did not observe significant association between the expression of mature miRNAs and the  
667 allele-specific expression of putative miRNA target sequences from the same tissue. Neither did we  
668 find the heterozygous sites within putative miRNA target sequences more significantly associated  
669 with allelic imbalance of exons within the same target genes than the heterozygous sites within the  
670 gene regions other than exons and putative miRNA target sequences. Additionally, of a total of  
671 902,172 putative pairs of miRNA and target mRNA, we found that 26 putative pairs had the miRNA  
672 gene overlapped the target mRNA gene on the same DNA strand. Another 26 putative pairs had the  
673 miRNA overlapped the target mRNA gene region but on the antisense strand. There were 32,466  
674 putative pairs had the miRNA transcribed from the same chromosome as the target mRNA but  
675 outside the target mRNA region. And the remaining 869,609 pairs had the miRNA transcribed from a  
676 different chromosome from the target mRNA. Overall our observations suggested that miRNAs and  
677 their putative target mRNAs were more likely to scatter at different transcriptional units within the  
678 same nucleus (Iborra et al. 1996; Osborne et al. 2004) and therefore showed different transcription  
679 rates. Since miRNAs are known to repress the translation of target mRNAs, instead of comparing  
680 transcriptional levels between miRNAs and target mRNAs, it will be interesting for future studies to  
681 investigate whether the transcription levels of miRNAs are associated with the translation levels of  
682 target proteins, and whether quantitative trait loci (QTLs) for the translation levels of individual  
683 protein are enriched within miRNAs or miRNA target sequences.

684 In conclusion, our study has profiled expressed miRNAs and predicted their mRNA targets in 17  
685 bovine tissues from a single lactating dairy cow. We have demonstrated that although miRNAs and

686 seed match sites are depleted for common variants compared with entire genome, they are  
687 enriched for rarer variants, providing evidence for miRNA genes and target sequences under natural  
688 selection. Additionally, through switching the dominant miRNA sequence, a conserved precursor  
689 miRNA can regulate different target mRNAs in different bovine tissues, potentially contributing to  
690 the specialised function of each tissue. We also bring a closer connection between miRNAs and  
691 enhancer RNAs by demonstrating that miRNA seed match sites are significantly enriched at active  
692 enhancer regions. Contributing to the goal of the Functional Annotation of Animal Genomes  
693 (FAANG) consortium, our results help to complete the catalogue of tissue-specific interaction  
694 network between miRNAs and mRNA targets, which in the future may assist in better assessing the  
695 combined effects of genes and their regulators on dairy traits.

## 696 **Materials and Methods**

697 All methods are summarized in a flow diagram provided in Supplemental Figure S1.

### 698 **Samples, RNA sequencing and alignment**

699 Seventeen tissues from a dairy cow were collected in the early spring (8 September 2010) at  
700 Ellinbank, Victoria, Australia as part of another study (Chamberlain et al. 2015). The cow was 25  
701 months old and 65 days into her first lactation, non-pregnant, and was euthanised following an  
702 incurable injury.

703 Small (10-50 nucleotides) RNA transcripts in the cell nucleus and cytosol were isolated from 50mg of  
704 ground frozen tissue using the mirVana miRNA isolation kit (Ambion) in duplicate as per the  
705 manufacturer's instructions.

706 Libraries were prepared for sequencing using the NEBNext Multiplex Small RNA Library Prep kit (New  
707 England Biolabs Inc) according to manufacturer's instructions with the following modification.  
708 Following PCR 6ul of each library were pooled and purified with the Qiaquick PCR purification kit  
709 (Qiagen) as per the manufacturer's instructions. A second elution was performed for a total eluate of

710 60ul. 45ul of purified library pool was run with 6X loading buffer on a 3% TBE gel in 2 lanes alongside  
711 5ul of Quick Load pBR322 DNA MspI Digest. The gel was run at 70 volts for 2 hours. Short RNA  
712 libraries were cut from the gel at 130-170bp with an expected insert size of 10-50nt. DNA was  
713 extracted from gel slices using the Qiagen QIAEX II gel extraction kit (Qiagen) as per the  
714 manufacturer's instructions and eluted in 40ul Tris.

715 Libraries were sequenced on HiSeq3000 (Illumina) in a paired-end 50 cycle run and FASTQ files were  
716 generated with bcl2fastq2 conversion software. Raw sequence quality was assessed using FastQC  
717 (Andrews 2010) (version 0.10.1).

718 Illumina adapter sequences and poor-quality bases were trimmed from raw RNA sequence reads.  
719 Two trimming pipelines were used and compared. The first was cutadapt (Martin 2011) (version 1.9)  
720 and sickle (Joshi and Fass 2011) (version 1.33) following the micro RNA-Seq Data Processing Pipeline  
721 from Encyclopedia of DNA Elements (ENCODE) consortium. Raw RNA sequence reads were trimmed  
722 from the 3'-end when  $\geq 5$  consecutive base pairs matched with the adapter sequences. Trimmed  
723 sequence reads were discarded if final read length was  $< 18$ nt or had low quality score (read error  
724 rate  $> 0.1$ ). The second, trimmomatic (version 3.6) (Bolger et al. 2014), trimmed raw RNA sequence  
725 reads from the 3'-end when  $\leq 2$  nucleotides matched with the adapter sequence during the standard  
726 'seed and extend' approach (Li and Homer 2010) and  $\geq 5$  consecutive nucleotides matched with the  
727 whole adapter sequence. To trim partial read-through adapter sequences, initial-trimmed RNA  
728 sequence reads were trimmed from the 3'-end when  $> 10$  consecutive nucleotides matched with part  
729 of the adapter sequence. Nucleotides at both ends were further trimmed off if read quality was  $< 15$ .  
730 The remaining reads were trimmed from the 3'-end if the averaged read quality among 4  
731 consecutive nucleotides were  $< 15$ . Trimmed reads were retained if final read length was  $\geq 18$ nt.  
732 Trimmed sequence quality for all trimmers was also assessed using FastQC (Andrews 2010) (version  
733 0.10.1).

734 Trimmed paired sequence reads were aligned to bovine reference genome UMD3.1.1. (bostau8)  
735 using BWA (Li and Durbin 2009) backtrack algorithm (aln) (version 0.7.17), bowtie (Langmead 2010)  
736 (version 1.2.2), bowtie2 (Langmead and Salzberg 2012) (version 2.3.4.1), STAR (Dobin et al. 2013)  
737 (version 2.5) and HISAT2 (Kim et al. 2015) (version 2.1.0) respectively. Default settings were applied  
738 to all aligners, except that the maximum insert size was 50nt in BWA, the overhang was 49  
739 (maximum sequence length minus 1) (Dobin et al. 2013) in the first step of the two-step STAR  
740 alignment, and both mapped and unmapped reads were required to return in the output BAM file  
741 for all aligners. Alignment statistics were generated using SAMtools (Li et al. 2009) (version 1.6). Only  
742 mapped and paired reads with mapping quality over 20 were retained for analysis.

### 743 **Identification of expressed known and novel miRNAs**

744 Canonical and non-canonical micro RNAs (miRNAs) that were expressed in 17 bovine tissues were  
745 identified using miRDeep2 (Friedländer et al. 2012) (version 2.0.0.8). Aligned paired RNA sequence  
746 reads with mapping quality  $\geq 20$  were provided as input to the second module of miRDeep2  
747 (Quantifier; default settings) and processed through to the last module (miRDeep2; default settings).  
748 The first module (Mapper) was excluded because it required raw single-end RNA sequence data as  
749 input, and the built-in trimmer did not take partial adapter read-through into account. Technical  
750 replicates were provided as input to the miRDeep2 pipeline. Also provided were the complete  
751 collection of precursor and mature miRNA sequences from bovine and bovine-related species from  
752 miRBase (Kozomara and Griffiths-Jones 2014) (version 22), where goat and sheep were chosen as  
753 the bovine-related species. A surge of miRNAs from deep sequencing had been added to the  
754 complete collection of miRBase (version 22), but the high confidence set from miRBase (version 22)  
755 had not been updated at the time this study was conducted and therefore was not used. Consistent  
756 with the case study in the miRDeep2 paper (Friedländer et al. 2012), candidate miRNAs that  
757 resembled other small RNAs such as tRNA and rRNA were removed. The miRDeep2 algorithm assigns  
758 each novel precursor miRNA a log-odds score, which indicates the probability that the sequence is a

759 true miRNA precursor instead of a background hairpin (Friedländer et al. 2008; Friedländer et al.  
760 2012). For each analysis, the lowest miRDeep2 score cut-off that yielded a signal-to-noise ratio of  
761 10:1 or higher was used to select the candidate miRNAs for further analysis. The actual threshold for  
762 each library is listed in “filters for each library” tab of Supplemental Table S2.

763 miRDeep2 returned the genomic coordinates of precursor miRNAs and the DNA sequence aligning to  
764 cognate mature and star miRNAs (Friedländer et al. 2012). The genomic coordinates of mature and  
765 star miRNAs were identified by searching respective sequences within the cognate precursor. ‘-5p’  
766 and ‘-3p’ ends were identified by comparing the relative genomic positions of mature and star  
767 miRNAs that were derived from the same precursor. Due to variation among sequence libraries, a  
768 stack of sequence reads aligning to the same known precursor, mature or star miRNA varied in  
769 length (e.g. Figure 2) (Friedländer et al. 2012). In those cases, a consensus sequence was derived  
770 from the stack of reads, and the genomic coordinates were updated accordingly. The consensus  
771 sequence was selected as follows:

- 772 (1) If the stack of reads were classified as known and fell into a miRNA region annotated in  
773 UMD3.1.1 (GCF\_000003055.6), the consensus sequence was the sequence in UMD3.1.1  
774 annotation.
- 775 (2) If the stack of reads fell outside the annotated miRNA regions in UMD3.1.1, the consensus  
776 sequence was the sequence with the highest miRDeep2 score (Friedländer et al. 2012).  
777 miRDeep2 scores a miRNA by considering the hairpin folding, the mature, star and loop  
778 sequence from the hairpin, and the proportion of nucleotides in the mature miRNA passing  
779 the RNA folding threshold from the randfold software (Bonnet et al. 2004; Lorenz et al.  
780 2011) (Github: eb00/randfold).
- 781 (3) If multiple sequences reached the same highest miRDeep2 score, the consensus sequence  
782 was the sequence with the highest frequency across all libraries.



783 (4) If multiple sequences reached the same highest frequency, the consensus sequence was the  
784 longest sequence.

785 (5) If multiple sequences reached the same longest length, the consensus sequence was the  
786 collapsed sequence from the stack (this only applies to mature and star sequences).

787 The consensus miRNA genomic coordinates and miRNA sequences were considered as “clean”  
788 miRNAs and were retained for analysis. Note, the observation that a stack of reads aligning to the  
789 same known precursor, mature or star miRNA varied in length in this lactating dairy cow, is similar to  
790 the miRNA isoforms that were detected in healthy humans, where depending on gender, population  
791 and race, precursor miRNAs gave rise to many isoforms that typically differed in either 5’ or 3’  
792 termini or both, and the most abundant isoform was frequently annotated as the canonical  
793 sequence in miRBase (Telonis et al. 2015; Magee et al. 2018). However, miRNA isoform was out of  
794 the scope of this study, and our method to obtain consensus sequence didn’t consider miRNA  
795 isoforms.

## 796 **Identification of differentially expressed mature miRNAs**

797 Mature miRNA read counts from miRDeep2 were used to produce a tissue-by-miRNA read count  
798 matrix (17 tissues × 610 miRNAs). The final read count of a miRNA was the averaged read count  
799 between technical replicates. miRNAs with <10 reads across all tissues were removed from the  
800 analysis. DESeq2 (version 1.18.1) (Love et al. 2014) with default shrinkage estimator was used to  
801 identify mature miRNAs that were more or less often expressed in a tissue than the average of all  
802 other tissues. A miRNA was considered differentially expressed if the miRNA was found expressed by  
803 miRDeep2, and after adjustment for multiple testing (Benjamini-Hochberg correction) the P-value  
804 was <0.01, and the expression of miRNA in the tissue was >2 fold different to the averaged  
805 expression across all other tissues.

## 806 **Prediction of miRNA seed match sites**

807 TargetScan suite (Lewis et al. 2005; Friedman et al. 2009; Agarwal et al. 2015) (version 7.2) was used  
808 to predict the seed match sites of expressed miRNAs. All input files and Perl scripts from TargetScan  
809 7 website (downloaded on 19 June 2018) were used in our analysis except the miRNA seed file,  
810 miRNA sequence file and the UTR profile file were prepared as per instruction from TargetScan 7  
811 (Agarwal et al. 2015).

812 The miRNA seed file was table of grouped miRNA names (i.e. miRNA family), seed sequence, and  
813 grouped animal IDs from miRBase that contributed constituents of the miRNA family. To construct  
814 the miRNA file, all cleaned mature miRNA sequences from 17 bovine tissues, and the mature miRNA  
815 sequences of ten vertebrates from miRBase (Kozomara and Griffiths-Jones 2014) (version 22), were  
816 grouped into miRNA families based on the identity of extended seed sequence (2<sup>nd</sup>-8<sup>th</sup> nucleotides  
817 from 5'-end of mature miRNA). The ten vertebrates from miRBase were the same as those in  
818 TargetScan 7 predictions, which were mouse, rat, opossum, western clawed frog, chicken, rhesus  
819 macaque, chimpanzee, human, dog and cattle.

820 The miRNA sequence file was a table of grouped miRNA names (i.e. miRNA family), animal ID from  
821 miRBase that contributed constituents of the miRNA family, miRBase ID of a constituent of the  
822 miRNA family and the mature miRNA sequence. miRNA family ID, miRBase species ID, miRBase ID  
823 and mature miRNA sequence were sorted according to the miRNA file.

824 The UTR profile file was a table of transcript ID or gene ID or gene name from human reference  
825 genome hg19 (UCSC ID), animal ID from miRBase that contributed the 3'UTR sequence for whole  
826 genome sequence (WGS) alignment, and the aligned 3'UTR sequence. As per instruction from  
827 TargetScan 7 (Agarwal et al. 2015), the UTR profile was constructed from WGS alignment of 84  
828 vertebrate species.

829 TargetScan 7 did not directly output the genomic coordinates of putative miRNA seed match sites on  
830 to UMD3.1 (NCBI) or bostau6 (UCSC), but instead provided the positions of the seed match sites on  
831 the UTR profile. To identify the genomic coordinates of putative miRNA seed match sites on bostau6  
832 chromosome 1-29, X and mitochondria, the Ensembl transcript IDs on bostau6 that were orthologs  
833 to hg19 (UCSC ID) were identified using the getLDS function from R Bioconductor biomaRt package  
834 (version 2.34.2). Then the genomic coordinate of each seed match site on bostau6 was identified  
835 through local pairwise alignment between the miRNA seed sequence and the bostau6 gene  
836 sequence, using the pairwiseAlignment function (parameter: type='local') from R Bioconductor  
837 Biostrings package (version 2.46.0).

### 838 **Confirmation of putative interactions between miRNAs and targets**

839 High-throughput sequencing of RNAs isolated by crosslinking immunoprecipitation (HITS-CLIP, also  
840 known as CLIP-Seq) was used to identify miRNA and mRNA sequences that were bound by the  
841 Argonaute (AGO) protein in bovine kidney cells (Scheel et al. 2017). The data was a table (S3) listing  
842 target gene name, genomic coordinate of target sequence (i.e. chromosome, start, end and strand),  
843 target sequence and cognate miRNA name. The miRNA target sequences were aligned to bovine  
844 reference genome Btau4.6.1 (NCBI) or bostau7 (UCSC). We converted their genomic coordinates to  
845 UMD3.1 (NCBI) or bostau6 (UCSC) using the UCSC liftOver tool (Kuhn et al. 2013).

846 To confirm putative interactions between bovine miRNA families and their cognate seed match sites  
847 using the Scheel *et al.* set, we identified putative miRNA seed match sites for miRNAs that were  
848 expressed in kidney tissues and also overlapped with miRNA target sequences in the Scheel *et al.*  
849 set. Then for each putative seed match site, we examined whether the miRNA name in the Scheel *et*  
850 *al.* set could be found in the miRNA family in the prediction set. If the miRNA name in the Scheel *et*  
851 *al.* set was found, the putative interaction between the miRNA seed match sites and cognate miRNA  
852 was considered “confirmed”.

853 miRTarBase (Chou et al. 2018) (version 7.0) is a public database that used natural language  
854 processing techniques to collect experimentally identified “miRNA and cognate gene target”  
855 interactions in animals from published literature. The animals were *Bos Taurus*, *Caenorhabditis*  
856 *elegans*, *Canis familiaris*, *Drosophila melanogaster*, *Danio rerio*, *Gallus Gallus*, *Homo sapiens*, *Mus*  
857 *musculus*, *Rattus norvegicus*, *Sus scrofa*, *Xenopus tropicalis* and *Ovis aries*. The experimental  
858 identification techniques included but not limited to qRT-PCR, Luciferase reporter assay, western  
859 blot, microarray and CLIP-Seq. The data consisted of a table listing miRTarBase ID, miRNA name,  
860 species, target gene name, experimental technique and PubMed ID. Gene names from species other  
861 than bovine were converted to orthologous bovine gene names using the getLDS function from R  
862 Bioconductor biomaRt package (version 2.34.2). Any records with target gene name as NA were  
863 removed from analysis.

864 TargetScan (Agarwal et al. 2015) (version 7.0) published putative miRNA seed match sites from  
865 human reference genome GRCh37 (NCBI) or hg19 (UCSC) for all miRNAs in miRBase (version 21). The  
866 data consisted of a table listing the genomic coordinate of seed match site, gene target and cognate  
867 miRNA family. Human target gene names were converted to orthologous bovine gene names using  
868 the getLDS function from R Bioconductor biomaRt package (version 2.34.2). Genomic coordinates of  
869 human miRNA seed match sites were converted to bovine reference genome bostau6 using the  
870 UCSC liftOver tool (Kuhn et al. 2013). Any records with target gene name as NA were removed from  
871 analysis.

872 miRWalk (Dweep and Gretz 2015) (version 3.0) is a public database for computationally-predicted  
873 interactions between miRNAs and cognate gene targets from human, mouse, rat, cow and dog using  
874 a random-forest-based approach. We downloaded the cow putative interactions set. The data  
875 consisted of a table listing miRNA name, target transcript Ensembl ID and target gene name.

876 To confirm putative interactions between miRNA families and cognate targets using miRTarBase,  
877 TargetScan and miRWalk, target gene names in miRTarBase and TargetScan and target transcript IDs

878 in miRWalk (Dweep and Gretz 2015) were matched exactly with that in the our predicted miRNA  
879 targets. Then for each record, the cognate miRNA name in the miRTarBase, TargetScan or miRWalk  
880 was searched within the miRNA family in our prediction result. If the miRNA name was found, the  
881 putative interaction between the miRNA family and cognate targets was considered “confirmed”.

882 Messenger RNAs, which were either up- or down-regulated in one tissue compared with the mean  
883 expression in the other 17 tissues from the same lactating cow as used in this study were identified  
884 (Chamberlain et al. 2015). The tissue that was not included in our study was white blood cells. The  
885 data consisted of a table listing the Ensembl gene ID, gene name, gene genomic coordinates, the  
886 tissue where the gene was differentially expressed, fold change (all >2-fold change) and P-value after  
887 corrected for multiple testing (all <0.01). Also provided were summaries of the GO terms and KEGG  
888 pathways of those differentially expressed genes.

889 The differentially expressed genes (Chamberlain et al. 2015) were combined with the differentially  
890 expressed miRNAs that were identified in this study to create a differential expression set. To  
891 identify miRNAs and cognate putative gene targets that were differentially expressed in the same  
892 tissue of the same cow, differentially expressed gene IDs were matched exactly with putative target  
893 gene IDs. Then the tissues where the genes were differentially expressed in were matched exactly  
894 with the tissues where the miRNAs were differentially expressed. For each putative interaction  
895 between the miRNA and the mRNA within tissue, if the miRNA name in the differential expression  
896 set was found in the miRNA family in the prediction set, then the miRNA and cognate gene target  
897 were considered as differentially expressed in the same tissue of the same cow.

## 898 **Enrichment analysis**

899 A permutation test was performed to examine whether putative interactions between the miRNA  
900 families and cognate targets (named as the ‘prediction set’) overlapped with interactions from  
901 previous experimental and computational identifications (named as the ‘confirmation set’) more  
902 often than by chance. Data from CLIP-Seq (Scheel et al. 2017), miRTarBase (Chou et al. 2018),

903 TargetScan (Agarwal et al. 2015) and miWalk (Dweep and Gretz 2015). The actual number of  
904 overlapping interactions between the prediction set and the confirmation set, which was denoted as  
905  $n$ , was the same as was described in the previous section (Confirmation of putative interactions  
906 between miRNAs and targets). To create a random interaction set, the list of miRNA families in the  
907 prediction set was shuffled randomly and then combined with the list of predicted targets. The  
908 number of overlapping interactions between the random set and the confirmation set, which was  
909 denoted as  $m$ , was then calculated. This shuffling and counting procedure were repeated 10,000  
910 times. The ranking position of  $n$  within the distribution of 10,000  $m$  values, denoted as  $R$ , was  
911 determined, and a P-value to test the significance of the ranking was computed. If  $n$  was larger than  
912 all 10,000  $m$  values, the P-value was set to  $< 0.0001$  and otherwise it was  $\frac{R}{10001}$ . The fold change of  
913 enrichment was defined as the ratio of the actual number of validated pairs to the average number  
914 of validated pairs in 10,000 random sampling.

## 915 **Polymorphisms in miRNA genes and targets**

916 A genomic feature is a predefined genomic range, which could be miRNAs that were expressed in  
917 one or more bovine tissues, miRNA targets that were identified through experimental or  
918 computational procedures, or the entire bovine genome (genome-wide). Raw (65,195,092) and  
919 filtered (Daetwyler et al. 2017) (44,678,426) WGS variants from 1000 Bull Genomes Project  
920 (Daetwyler et al. 2014) (*Bos Taurus* Run 6) from 2,333 individuals were utilised. Raw and filtered  
921 variants within a genomic feature were examined in the following three ways:

922 (1) The polymorphic rate in a genomic feature,  $y_1$ , was defined as follows:

$$y_1 = \frac{x_1}{w}$$

923 Where  $x_1$  was the total number of polymorphic sites (including single-base-nucleotide  
924 substitutions, insertions and deletions) within the genomic feature, and  $w$  was the total width of  
925 the same genomic feature. Here, the map file for the filtered WGS variants in TXT format was  
926 used to calculate  $x_1$ .

927 (2) The density of rarer variants with an allele frequency less than  $\alpha$  in a genomic feature,  $y_2$ , was  
928 defined as follows:

$$y_2 = \frac{x_2}{m}$$

929 Where  $x_2$  was the number of variants (single-base-nucleotide substitutions only) with an allele  
930 frequency less than  $\alpha$  within the genomic feature, and  $m$  was the total number of variants  
931 (single-base-nucleotide substitutions only) within the same genomic feature. Here, the variant  
932 call file for all raw sequence variants in VCF format was provided as input to the vcftools (version  
933 0.1.15) to calculate the allele frequency for each variant, and custom R scripts were used to  
934 calculate  $x_2$  and  $m$ . If >1 alternative allele at a locus was detected, the frequency of each  
935 alternative allele was calculated.

936 (3) INDELS were categorised by the number of positional shifts,  $n \in (-\infty, +\infty)$ , between the  
937 alternative allele and reference allele at the same locus, following a classification system similar  
938 to what was previously used (Mills et al. 2006; Bhattacharya et al. 2012). The density of INDELS  
939 with an  $n$ -th positional shift in a genomic feature,  $y_3$ , was defined as follows:

$$y_3 = \frac{x_3}{w \times k}$$

940 Where  $x_3$  was the number of variants (INDELS only) with an  $n$ -th positional shift comparing the  
941 alternative allele with the reference allele among all the  $k$  animals from the 1000 Bull Genomes  
942 Project (Daetwyler et al. 2014) (*Bos Taurus*, Run 6) and  $w$  was the total width of the same  
943 genomic feature. Here, the variant call file for all raw sequence variants in VCF format was  
944 provided as input to the vcftools (version 0.1.15) to count the number of alleles (INDELS only) at  
945 each locus, and custom R scripts were used to calculate  $x_3$ ,  $w$  and  $k$ . If >1 alternative allele at a  
946 locus was detected, each alternative allele was calculated.

947 **Examination of associations among miRNAs, miRNA target regions and**  
948 **allele-specific expression of target genes**

949 RNA sequence (RNA-Seq) data was previously used to assess the degree of allelic imbalance across  
950 18 tissues from the same cow (Chamberlain et al. 2015). The degree of allelic imbalance was  
951 estimated as a Chi-Square value at each heterozygous site that was identified from the 1000 Bull  
952 Genomes Project (Daetwyler et al. 2014) (*Bos Taurus* Run 5). Although only results within exons  
953 were published, allelic imbalance had been calculated for heterozygous sites within all genes that  
954 were annotated in UMD3.1 (Ensembl release 75). We obtained this ASE data (Chamberlain et al.  
955 2015), and defined ASE scores as the square root of Chi-Square values from their ASE analysis.

956 Very few variants were located within putative miRNA seed match sites because putative miRNA  
957 seed match sites were short (6nt) and highly conserved (perfectly reverse-complementary to miRNA  
958 seed sequences). Because sequence contexts around miRNA seed match sites were shown to affect  
959 miRNAs binding to target mRNA sequences (Agarwal et al. 2015), we created putative miRNA target  
960 sequences by extending 49nt upstream and 1nt downstream of putative miRNA seed match sites.  
961 This would include the 2<sup>nd</sup>-7<sup>th</sup> nucleotides within mature miRNA sequences perfectly matched with  
962 the putative miRNA target sequences, and the lengths of putative miRNA target sequences, which  
963 were all 56nt, equivalent to the maximum length of miRNA target sequences that was identified by a  
964 CLIP-Seq experiment (Scheel et al. 2017).

965 We asked the following two questions:

966 (1) Across tissues, was the allelic imbalance at each heterozygous site within putative miRNA  
967 target sequence associated with the expression level of cognate mature miRNA?

968 (2) Across genes, were genes with heterozygous sites within putative miRNA target sequences  
969 more likely to show allelic imbalance at exons than genes with homozygous sites within  
970 putative miRNA target sequences?



971 To answer question 1, we calculated ASE scores for 459,610 heterozygous sites in 15,091 genes on  
972 30 chromosomes (1-29 and X) in this animal. We selected ASE scores from heterozygous sites within  
973 putative miRNA target sequences. This dataset was combined with the dataset of mature miRNA  
974 read counts from miRNAs that were expressed in the same tissue as where ASE scores were  
975 measured from.

976 Analysis of variance (ANOVA) was used to assess if the expression level of mature miRNAs had an  
977 effect on the allelic imbalance of putative miRNA target sequence. The model was:

$$y = u + x + bz + e$$

978 Where  $y$  was the ASE scores of heterozygous variants at putative miRNA target sequences,  $u$  was  
979 the mean,  $x$  was the effect of the variant names which was a factor variable,  $b$  was the coefficient  
980 for  $z$ ;  $z$  was the read counts across tissues of a cognate miRNA, and  $e$  was the residue.

981 To answer question (2), all 30,831,575 polymorphic sites in the cow's WGS variants from 1000 Bull  
982 Genomes (Daetwyler et al. 2014) (*Bos Taurus* Run 5) were used to identify polymorphic sites within  
983 all putative miRNA target sequences. A label of 0 and 1 was used to distinguish whether a  
984 polymorphic site was homozygous or heterozygous, respectively.

985 A linear regression model was used to assess whether zygosity within putative miRNA target  
986 sequences had an effect on allelic imbalance in exons of miRNA target genes. The model was:

$$y = u + x + e$$

987 Where  $y$  was the ASE scores from heterozygous variants at exons of target genes,  $u$  was the mean,  $x$   
988 was the effect of a factor variable that labelled whether a polymorphic site within a putative miRNA  
989 target sequence was homozygous or heterozygous in this cow, and  $e$  was the residue.

990 A permutation test was performed to examine whether the observed result in question (2) was due  
991 to linkage disequilibrium (LD) between variants within putative miRNA target sequences and that

992 within exons. To construct the null miRNA target sequences, we selected genomic regions that were  
993 within putative miRNA target genes but were neither putative miRNA target sequences nor exons  
994 that were annotated in UMD3.1 (Ensembl release 91). Then we selected WGS variants from 1000  
995 Bull Genomes (Daetwyler et al. 2014) (*Bos Taurus* Run 5) that were within null miRNA target  
996 sequences. We calculated the number of WGS variants that were within the original putative miRNA  
997 target sequences and defined this number as  $N$ . To construct the null dataset, we combined the  
998 dataset from randomly selecting  $N$  variants within null miRNA target sequences with the dataset of  
999 ASE scores from exonic heterozygous sites by the same genes. The null dataset was used to fit the  
1000 same linear regression model as that in question (2). This procedure of constructing the null dataset  
1001 and then fitting linear regression model was repeated 10,000 times. If the coefficient and standard  
1002 error of coefficient from linear regression model that was constructed from the original dataset  
1003 were respectively all bigger and all smaller than those from the 10,000 null datasets, we declared  
1004 the observed result in question (2) was statistically significant.

## 1005 **List of Abbreviations**

1006 ANOVA: An analysis of variance; ASE: Allele-specific expression; CAGE: Cap Analysis Gene Expression;  
1007 CDS: coding sequence; CHIA-PET: chromatin interaction analysis with paired-end tag sequencing;  
1008 CTCF: CCCTC binding factors; ChIP-Seq: chromatin immunoprecipitation followed by high-throughput  
1009 sequencing; DE: Differential Expression; GWAS: Genome Wide Association Study; HITS-CLIP: high-  
1010 throughput sequencing of RNAs isolated by crosslinking and immunoprecipitation, also known as  
1011 CLIP-Seq; H3K27ac: acetylated lysine 27 on histone H3; H3K4me3: tri-methylation of lysine 4 on  
1012 histone H3; INDELS: insertions or deletions; LD: linkage disequilibrium; PRO-Seq: precision run-on  
1013 sequencing; QTLs: quantitative trait loci; ORF: open reading frame; RNA: ribonucleic acid; RNA-Seq:  
1014 RNA Sequencing; SE: Super-enhancers; SINE: short interspersed elements; SNP: single nucleotide  
1015 polymorphism; TAD: Topological Association Domains; WGS: whole genome sequence; mRNA:  
1016 message ribonucleic acid; miRNA: micro ribonucleic acid; pre-miRNA: precursor micro ribonucleic

1017 acid; pri-miRNA: primary micro ribonucleic acid; 5'UTR: five-prime untranslated region; 3P-seq:  
1018 poly(A)-position profiling by sequencing; 3'UTR: three-prime untranslated region.

## 1019 **Declarations**

### 1020 **Ethics approval and consent to participate**

1021 No animal experiments were performed specifically for this work. The data was obtained from  
1022 existing samples, references for these experiments are provided.

### 1023 **Consent for publication**

1024 The authors agree for the publication of this manuscript to the journal RNA.

### 1025 **Data availability**

1026 Micro RNA sequence data has been deposited under European Nucleotide Archive (ENA) accession  
1027 number ERX2749848 - ERX2749881.

### 1028 **Competing interests**

1029 The authors declare no competing interests.

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### 1033 **Author's contributions**

1034 MW performed all the data analysis and wrote the manuscript. MEG, TPH and BJH supervised the  
1035 statistical analysis. CPP and AJC collected samples and sequenced RNA reads. CVJ provided data for  
1036 the allele-specific expression analysis and differentially expression analysis for mRNAs from 18  
1037 tissues from this lactating dairy cow. MW, AJC, JEP, BGC, MEG and BJH conceived experimental  
1038 design and coordination. All authors read, commented and approved the final manuscript.

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1043 the National Centre for Dairy Research and Development at Ellinbank, Victoria, Australia.

## 1044 **Tables**

1045 **Table 1 Comparison of Trimmers and Aligners for Short RNA Sequence Reads.** For the two trimmers  
1046 and each of five aligners used, this table presents the average proportions of trimmed reads that  
1047 were paired and mapped to bovine reference genome and the standard deviation (SD). Also  
1048 presented is the inferred insert sizes averaged across all libraries.

Trimmed	Aligner	Average proportion of reads	
		paired and mapped (SD)	Average insert size (SD)
Cutadapt and Sickle	BWA	97.39% (0.01)	4094 (243)
	Bowtie	1.29% (0.00)	78 (5)
	Bowtie2	96.11% (0.01)	65 (7)
	STAR	75.36% (0.06)	883 (697)
	HISAT2	82.15% (0.03)	2078 (260)
Trimmomatic	BWA	97.45% (0.01)	2079 (677)
	Bowtie	5.72% (0.10)	48 (5)
	Bowtie2	96.11% (0.02)	30 (3)
	STAR	83.02% (0.07)	63 (17)
	HISAT2	84.81% (0.05)	869 (212)

1049

1050 **Table 2 Differentially Expressed miRNAs Observed in Each Tissue.** Differentially expressed miRNAs  
 1051 are defined by P-value <0.01 and an absolute fold change >2 after correction for multiple testing.  
 1052 Novel miRNAs are represented by genomic coordinates. In tissues where no significant up- or down-  
 1053 regulated miRNAs was identified, they were not presented in the table.

Tissue	Regulation	miRNAs of Significant Differential Expression
Adrenal Gland	Up	<i>bta-miR-146b-5p, bta-miR-375-3p, bta-miR-409a-3p, bta-miR-543-3p, bta-miR-7-5p</i>
	down	<i>bta-let-7c-5p, bta-miR-100-5p, bta-miR-181a-3p, bta-miR-183-5p, bta-miR-206-3p, bta-miR-215-5p, bta-miR-30c-5p, bta-miR-32-5p, bta-miR-340-3p, bta-miR-340-5p, bta-miR-532-5p, bta-miR-652-3p, bta-miR-98-3p, bta-miR-98-5p, chr8:83009655-83009675(-), chrX:96382704-96382725(+)</i>
Brain Caudal Lobe	Up	<i>bta-miR-212-5p, bta-miR-219-3p, bta-miR-323-3p</i>
Brain Cerebellum	up	<i>bta-miR-135b-5p, bta-miR-219-3p, bta-miR-219b-5p, bta-miR-323-3p</i>
	down	<i>bta-miR-10a-5p, bta-miR-10b-5p, bta-miR-199b-5p, bta-miR-505-3p, bta-miR-505-5p, chr9:10768323-10768346(+)</i>
Heart	Up	<i>bta-miR-499-5p</i>
Intestinal Lymph Node	down	<i>bta-miR-361-5p</i>
Kidney	down	<i>bta-miR-101-3p, bta-miR-139-5p, bta-miR-150-5p, bta-miR-339b-5p, bta-miR-423-3p, bta-miR-92a-3p</i>
Leg Muscle	Up	<i>bta-miR-206-3p, bta-miR-378-3p, bta-miR-486-5p</i>
Liver	Up	<i>bta-miR-122-3p, bta-miR-122-5p, bta-miR-192-5p, bta-miR-194-5p</i>
	down	<i>bta-miR-30c-5p</i>

Lung	Up	<i>bta-miR-34b-3p, bta-miR-34c-5p</i>
	down	<i>chr1:79250542-79250563(+)</i>
Mammary Gland	down	<i>bta-miR-1271-5p</i>
Ovary	down	<i>chrX:34664642-34664663(+)</i>
Spleen	down	<i>chr8:83009655-83009675(-), chr9:10768323-10768346(+)</i>
Thymus	Up	<i>bta-miR-106a-5p</i>
	down	<i>bta-miR-193b-3p, bta-miR-671-5p, bta-miR-193b-5p, bta-miR-493-5p</i>
Thyroid	Up	<i>bta-miR-135b-5p</i>
	down	<i>chr18:58014874-58014895(-), chr7:39194962-39194983(-)</i>
Tongue	Up	<i>bta-miR-206-3p</i>
White Skin	down	<i>bta-let-7b-3p</i>

1054

1055 **Table 3 Enrichment of miRNA Seed Match Sites within Bovine-specific Histone Modification**

1056 **Signals.** The number of nucleotides inside/outside all putative miRNA seed match sites and  
 1057 inside/outside a histone modification signal. Also, the proportion of nucleotides inside/outside  
 1058 miRNA seed match site that were inside a histone modification signal showing the direction of the  
 1059 test, and the Chi-Square test results showing the significance of the test.

		H3K27ac (Liver) (Villar et al. 2015)			
		Inside	Outside	Total	Prop. Inside
miRNA Seed Match Sites	Inside	532400	3811824	4344224	12.2554%
	Outside	40646950	674559111	715206061	5.6833%
	Total	41179350	678370935	719550285	
Chi-Square Test	$\chi^2$ -value			345673.9	
	P-value			0	

		H3K4me3 (Liver) (Villar et al. 2015)			
		Inside	Outside	Total	Prop. Inside
miRNA Seed Match Sites	Inside	343365	4000859	4344224	7.9039%
	Outside	14275698	700930363	715206061	1.9960%
	Total	14619063	704931222	719550285	
Chi-Square Test	$\chi^2$ -value			757193.9	
	P-value			0	

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		H3K4me3 (Tender Muscle) (Zhao et al. 2015)			
		Inside	Outside	Total	Prop. Inside
miRNA Seed Match Sites	Inside	75891	4268333	4344224	1.7469%
	Outside	9127502	706078559	715206061	1.2762%
	Total	9203393	710346892	719550285	
Chi-Square Test	$\chi^2$ -value			7577.71	
	P-value			0	

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		H3K4me3 (Tough Muscle) (Zhao et al. 2015)			
		Inside	Outside	Total	Prop. Inside
miRNA Seed Match Sites	Inside	89620	4254604	4344224	2.0630%
	Outside	10549060	704657001	715206061	1.4750%
	Total	10638680	708911605	719550285	
Chi-Square Test	$\chi^2$ -value			10248.99	
	P-value			0	

1060

1061 **Table 4 Number of Confirmed Putative Interactions Between miRNA Family and Cognate Targets.**

1062 Presented are the number of interactions between miRNAs and cognate target sequences, target  
 1063 gene names or target transcript IDs in confirmation databases. Also presented are the number of  
 1064 interactions from miRNA families and cognate seed match sites, target gene names or target  
 1065 transcript IDs in our prediction. Last presented are the number of interactions that overlapped  
 1066 between the confirmation set and our prediction set. Genomic coordinates of miRNA target  
 1067 sequences (Scheel et al. 2017) were converted from bostau7 to bostau6 prior to counting and only  
 1068 interactions in kidney were counted. Mammalian target gene names in miRTarBase (Chou et al.  
 1069 2018) and TargetScan (Agarwal et al. 2015) were converted to bovine orthologs and all NA records  
 1070 were removed prior to counting. Apart from CLIP-Seq (Scheel et al. 2017), no tissue information is  
 1071 provided in any other public datasets, and therefore all interactions were counted.

Type	# miRNA	# Target	# Interactions
CLIP-Seq (Scheel et al. 2017)	224 miRNAs	200459 sequences	208688
Our Prediction	238 miRNA families	15999 sites	297802
Overlaps	163 miRNA families	9282 sites	11885
miRTarBase (Chou et al. 2018)	950 miRNAs	8242 gene names	20538
Our Prediction	600 miRNA families	14947 gene names	651674
Overlaps	347 miRNA families	7706 gene names	3173
miRWalk (Dweep and Gretz 2015)	794 miRNAs	17022 transcript IDs	6838356
Our Prediction	600 miRNA families	18196 transcript IDs	775378
Overlaps	358 miRNA families	14909 transcript IDs	229473
TargetScan (Agarwal et al. 2015)	2407 miRNAs	15532 gene names	7440300
Our Prediction	600 miRNA families	14947 gene names	651674
Overlaps	319 miRNA families	14908 gene names	182685

1072



1073 **Table 5 Correlation Between miRNAs and mRNAs of Significant Differential Expression in the same**  
1074 **tissue.** The correlation between the Log2 fold change of mRNA expression (Chamberlain et al. 2015)  
1075 and the Log2 fold change of miRNA expression in this study within each tissue from a single dairy  
1076 lactating cow was tested. The coefficient and p-value from correlation test within each tissue are  
1077 presented. In some tissues, the coefficient and p-value were NA because only one differentially  
1078 expressed (after corrected for multiple testing P-value <0.01 and absolute fold change >2) miRNA  
1079 was found within a tissue.

Tissue	Effect	P-value
Adrenal Gland	0.003091	0.392132
Brain Caudal Lobe	0.745447	9.69x10 <sup>-7</sup>
Brain Cerebellum	-0.00281	0.072024
Heart	NA	NA
Kidney	0.053872	0.106306
Leg Muscle	-4.59879	6.76x10 <sup>-9</sup>
Liver	-0.07229	7.78x10 <sup>-5</sup>
Lung	0.05387	1.15x10 <sup>-9</sup>
Mammary Gland	NA	NA
Ovary	NA	NA
Spleen	-5.2x10 <sup>-17</sup>	1
Thymus	-0.07477	0.001095
Thyroid	-0.00552	0.492063
Tongue	NA	NA
White Skin	NA	NA

1080

1081 **Table 6 Enrichment of Confirmed Putative Interactions Between miRNA Families and Cognate**  
1082 **Targets.** Significance is denoted as “<0.0001” if the putative interactions are more often confirmed

1083 by interactions from a confirmation dataset than all 10,000 randomly-shuffled permutations;  
 1084 otherwise significance is denoted as the ranking of the actual degree of overlap among the list of  
 1085 10,000 random overlaps. Fold change of enrichment is the ratio of the actual number of confirmed  
 1086 putative interactions to the average number of confirmed random interactions from 10,000  
 1087 permutations.

Confirmation Dataset	Significance	Fold Change
miRTarBase (Chou et al. 2018)	<0.0001	1.607254
CLIP-Seq (Scheel et al. 2017)	<0.0001	1.193042
miRWalk (Dweep and Gretz 2015)	<0.0001	1.112938
TargetScan 7.2 (Agarwal et al. 2015)	<0.0001	1.398332

1088

1089 **Table 7 Density of WGS Variants within Genomic Features.** The density of filtered whole genome  
 1090 sequence variants as described by Daetwyler *et al.* (Daetwyler et al. 2017) was the proportion of a  
 1091 genomic feature being polymorphic (including both SNPs and INDELs). The variants were identified  
 1092 from the 1000 Bull Genomes Project (Daetwyler et al. 2014) *Bos Taurus* Run 6.

Genomic Feature	Density
Genome Wide	1.679%
3'UTRs Genome-Wide	1.416%
Known Expressed miRNA Genes	
Precursor	0.710%
Mature	0.619%
Star	0.708%
Novel Expressed miRNA Genes	
Precursor	1.223%
Mature	1.117%
Star	1.225%
Experimentally Identified	miRNA Target Sequences (Scheel et al. 2017)
	1.376%

Computationally Predicted	mRNA Genes Targeted by Expressed miRNAs	1.556%
	miRNA Seed Match Sites	1.375%

1093

1094 **Table 8 Density of Raw WGS Variants with <1% Allele Frequency within Genomic Features.** The  
 1095 density was the proportion of raw single-base-nucleotide substitution variants within a genomic  
 1096 feature with an allele frequency less than 1%. The raw whole genome sequence variants were  
 1097 identified from the 1000 Bull Genomes Project (Daetwyler et al. 2014) (*Bos Taurus* Run 6).

Genomic Feature		Density
Genome Wide		31.095%
Expressed miRNA Genes	Precursor	38.004%
	Mature	38.854%
	Star	38.674%
Experimentally Identified	miRNA Target Sequences (Scheel et al. 2017)	33.492%
Computationally Predicted	miRNA Target Genes	32.407%
	miRNA Seed Match Sites	32.776%

1098

1099 **Table 9 1000 Bull Raw WGS Variants within Seed Match Sites within *TFCP2* and *CD40* mRNA Transcripts.** Weighted context score and weight context score  
 1100 percentiles are statistics from TargetScan (Agarwal et al. 2015) prediction. The lower the weighted context score, the higher the predicted repression effect.  
 1101 The higher the weight context score percentile, the higher the confidence of the prediction. miRNAs are grouped into families and represented by multiple  
 1102 miRNA names connected by the forward slash symbol. miRNA names with a prefix of “miR-” are annotated miRNAs from miRBase. miRNA names with a  
 1103 prefix of “bta-miR-” are expressed known miRNAs in bovine. The rest are expressed novel miRNA sequence detected.

Target Gene	Seed Match Site		Weighted Context		miRNA Family	WGS Variant	
	Genomic Coordinate	Sequence	Score (Percentile)			Position	Allele (Frequency)
<i>TFCP2</i>	chr5:28780088-28780093(+)	AUAUUAU	-0.271 (32)		miR-5011-5p/bta-miR-1277-5p	28780092	A (0.924263); G (0.075737)
	chr5:28798209-28798214(+)	GAAUGA	-0.27 (22)		miR-1298-5p/miR-1298/bta-miR-1298-5p	28798214	A (0.999785); G (0.000215)
	chr5:28806521-28806526(+)	AGGACA	-0.976 (92)		miR-676-3p/miR-676/miR-11995/bta-miR-11995-5p	28806521	A (0.999141); C (0.000860)
	chr5:28806521-28806526(+)	AGGACA	-0.976 (92)		miR-676-3p/miR-676/miR-11995/bta-miR-11995-	28806522	G (0.999141);

				5p		A (0.000860)
	chr5:73427696-73427701(+)	AUGGGU	-0.325 (51)	miR-660-5p/miR-660/miR-6987-3p/bta-miR-660-5p	NA	NA
	chr13:75564023-75564028(+)	UCCCAA	-0.25 (9)	miR-450a-1-3p/miR-450b-3p/miR-6352/GUUGGGAAAACACACUAGAGAA	75564028	A (0.999784); G (0.000217)
	chr13:75568377-75568382(+)	GUGUCA	-0.317 (51)	miR-425-5p/bta-miR-425-5p	75568377	G (0.998482); A (0.001518)
	chr13:75569617-75569622(+)	AUAUUAU	-0.251 (17)	miR-5011-5p/bta-miR-1277-5p	75569617	A (0.461306); G (0.538694)
CD40	chr13:75569618-75569623(+)	UAUAUA	-0.605 (53)	miR-5011-5p/bta-miR-1277-5p	75569623	A (0.999353); T (0.000647)
	chr13:75571520-75571525(+)	GGGCAG	-0.226 (2)	miR-18a-3p/miR-1646/miR-106-3p/miR-7069-3p/bta-miR-18a-3p/bta-miR-18b-3p	75571525	G (0.999784); C (0.000216)
	chr13:75572814-75572819(+)	CAUCUG	-0.291 (45)	miR-1942/miR-7453-5p/bta-miR-219b-5p	75572817	C (0.997623); G (0.002377)
	chr13:75565592-75565597(+)	ACUGGA	-1.667 (89)	miR-145a-5p/miR-145-5p/miR-145/miR-5195-3p/miR-145b/bta-miR-145-5p	NA	NA

1104

## 1105 **Figures**

1106 **Figure 1 Summary of Expressed Known and Novel miRNAs.** Red: known miRNAs. Blue: novel  
1107 miRNAs. (A) Total read counts of mature miRNAs. (B) Read counts of ‘known-antisense’ novel  
1108 mature miRNAs and other novel mature miRNAs. ‘Known-antisense’ novel miRNAs are novel miRNAs  
1109 that overlapped with the antisense strand of known mature miRNA. (C) Number of mature miRNAs  
1110 expressed in each tissue. (D) Number of tissues a mature miRNA was expressed in. (E) Number of  
1111 mature miRNAs identified on chromosome 1-X. (F) Number of precursor miRNAs identified within  
1112 genomic features annotated in UMD3.1.1. Order of annotation is shown in y-axis, i.e. annotated  
1113 miRNAs, CDS, 5’-/3’-UTRs, introns, intergenic and overlapping features. Once a miRNA was assigned  
1114 to a feature, the same miRNA was not counted again in the following features.

1115

1116 **Figure 2 miRNAs Were Expressed in Clusters.** Expressed miRNAs that are within a distance (x-axis)  
1117 on the same DNA strand are merged into a “miRNA cluster”. Line plots shows the number of miRNA  
1118 clusters (y-axis) in each tissue (title) within up to kilo-nucleotide (knt) distance, where distance  
1119 ranges from 0nt to 500knt (A) and 0nt to 3knt (B).

1120

1121 **Figure 3 Mature miRNA Expression Patterns.** (A) A heatmap showing mature miRNAs (y-axis) that  
1122 were significantly differentially expressed (after correction for multiple testing P-value < 0.01 and an  
1123 absolute fold change > 2) in a bovine tissue (x-axis) compared with all other bovine tissues. Colour  
1124 key indicates normalised read counts of the mature miRNA detected in a tissue by miRDeep2. Red:  
1125 Up-regulated; Blue: Down-regulated. (B) A principle component analysis (PCA) plot showing tissue-  
1126 to-tissue distance measured by expression of all mature miRNAs.

1127

1128 **Figure 4 Allele Frequencies for Raw WGS Variants within miRNA Genes, miRNA Targets and the**  
1129 **Entire Genome.** Histogram shows the proportion of alleles (y-axis) within a genomic feature (title)  
1130 that fell within a frequency range (x-axis). The height of each bar was the number of alleles (legend)  
1131 within a frequency range divided by the total number of alleles within the genomic feature (subtitle).  
1132 For each allele present among the 2,333 animals from 1000 Bull Genomes Project (Daetwyler et al.  
1133 2014) (*Bos Taurus* Run 6), including different alleles at the same polymorphic site, its frequency was  
1134 calculated, but only allele frequencies less than 20% from single-base-nucleotide substitution  
1135 variants were plotted.

1136

1137 **Figure 5 Frequency of an  $n$ -th Positional Shift within miRNA Genes, miRNA Targets and the Entire**  
1138 **Genome.** Raw sequence variants (INDELs only) were identified from whole genome sequencing of  
1139 2,333 key ancestor *Bos Taurus* bulls from 1000 Bull Genomes Project (Daetwyler et al. 2014) (Run 6).  
1140 Each bar (y-axis) represented the number of INDELs with an  $n$ -th positional shift (x-axis) divided by  
1141 the size (subtitle) of the genomic feature (title). An  $n$ -th positional shift is the difference between  
1142 the number of nucleotides in the alternative allele to the number of nucleotides in the reference  
1143 allele at the same locus. If  $>1$  alternative allele at a locus was detected, each alternative allele was  
1144 calculated. Only  $n \in (-50, 0) \& (0, 50)$  were shown. The genomic coordinates of experimentally  
1145 identified miRNA target sequences (Scheel et al. 2017) were converted from bostau7 to bostau6  
1146 before calculation.

## 1147 **Supplementary Information**

### 1148 **Supplemental Table S1**

#### 1149 **Short RNA Sequence Reads, Filtering and Alignment**

1150 Trimming summary is given for each sequence library (tissue, technical replicate), a tag of whether  
1151 Illumina adapter combination was detected in raw sequence, in trimmed sequences from cutadapt

1152 (Martin 2011) and sickle (Joshi and Fass 2011), and in trimmed sequences from trimmomatic (Bolger  
1153 et al. 2014) (Illumina adapter contamination detected), total number of reads in raw and trimmed  
1154 libraries (total number of reads), read length of raw and trimmed reads (read length), and  
1155 proportion of guanine and cytosine nucleotides presented in raw and trimmed reads (GC ratio).

1156 Alignment summary is given for each sequence library (tissue, technical replicate), total number of  
1157 reads input to aligner (Total number of reads), number of reads from each end (Read 1, Read 2),  
1158 number and ratio of mapped reads (Mapped reads), number of unmapped reads (Unmapped  
1159 reads), number of paired reads mapped (Reads mapped and paired), reads with a mapping quality  
1160 of 0 (reads mapped and MQ=0), inferred averaged insert size (insert size averaged), paired reads in  
1161 inwards orientation (inward orientated reads) and all other alignment statistics from samtools (Li et  
1162 al. 2009) stats option (version 1.6).

## 1163 **Supplemental Table S2**

### 1164 **Known and Novel miRNAs Expressed in 17 Bovine Tissues**

1165 A table for all expressed known and novel miRNAs that were detected by miRDeep2 (Friedländer et  
1166 al. 2012). Significance threshold was the lowest miRDeep2 score that yielded a signal-to-noise  $\geq 10:1$   
1167 in each library, and miRNAs that passed significance threshold were retained for analysis. miRNA  
1168 type specified whether the record was from a precursor, mature or star miRNA. Records with the  
1169 same rowIndex indicated the mature and star miRNAs were derived from the precursor miRNA. Also  
1170 presented are sequence library (tissue, technical replicate), genomic coordinates of the expressed  
1171 miRNA (chromosome, start, end, strand, Refseq), miRNA duplex origin (miRNA name), miRNA  
1172 sequence, information from miRBase (Kozomara and Griffiths-Jones 2014) (version 22) (miRDeep2  
1173 type, miRBase ID), confidence of the detection (miRDeep2 score, estimated probability of true  
1174 positives, total read count, mature read count, star read count), whether arm switching was  
1175 detected (armSwitch), and whether the known miRNA sequence has been updated to align with the  
1176 annotation in UMD3.1.1.



### 1177 **Supplemental Table S3**

#### 1178 **Differentially Expressed Mature miRNAs**

1179 Presented is results of differentially expression analysis using DESeq2 (Love et al. 2014). Each mature  
1180 miRNA with a sum read count across all tissues  $\geq 10$  was assessed whether the mature miRNA (chr,  
1181 start, end, strand, miRNA name, miRNA sequence) was more often expressed in a lactating cow's  
1182 tissue than the mean expression of all other tissues. The result was presented with P-value after  
1183 correction for multiple testing and the logarithm with a base of 2 of the fold change of  
1184 expression.

### 1185 **Supplemental Table S4**

#### 1186 **Predict miRNA Seed Match Sites**

1187 Presented is putative miRNA seed match sites that were predicted using TargetScan (Agarwal et al.  
1188 2015). Provided were the miRNA seed match sites (chr\_target, seedMatch\_start, seedMatch\_end,  
1189 strand\_target) on bovine protein coding transcripts (Ensembl transcriptId\_target, geneId\_target,  
1190 geneName\_target), and the cognate miRNA\_family.

### 1191 **Supplemental Table S5**

#### 1192 **Examples of Different miRNA Arm Usages Leading to Different mRNA Targets**

1193 Presented is confirmed putative interactions between expressed miRNA and cognate gene targets by  
1194 miRTarBase (Chou et al. 2018) and CLIP-Seq (Scheel et al. 2017).

### 1195 **Supplemental Figure S1**

#### 1196 **Flow Diagram Depicting Steps Taken in This Study**

1197 Presented is a flow diagram of the process to identify expressed miRNAs from deep sequencing  
1198 samples from 17 tissues of a lactating dairy cow using miRDeep2, and to identify putative miRNA  
1199 seed match sites of all expressed miRNAs using TargetScan. Differential expression analysis and

1200 enrichment analysis were also performed to confirm putative interactions between expressed  
1201 miRNAs and cognate targets aligning with known interactions from public repositories before the  
1202 examination of polymorphic sites within miRNA genes and targets. Colours were used to separate  
1203 each analysis, as well as datasets, software and processing steps involved.

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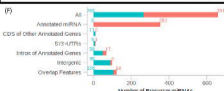
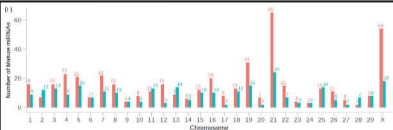
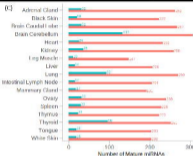
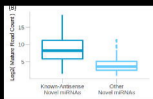
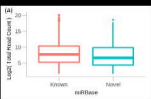
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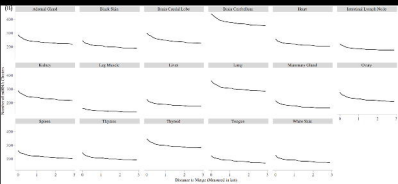
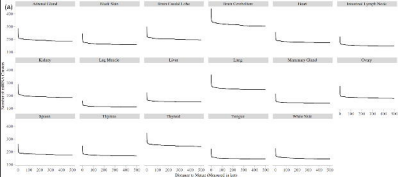
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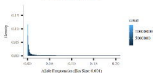






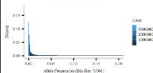
Genome Wide

(| 22,976,488 Alkalis in Total |)



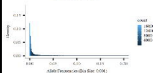
miRNA Target Genes of Computational Prediction

(| 35,826,111 Alkalis in Total |)



miRNA Seed Match Sites of Computational Prediction

(| 11,920,979 Alkalis in Total |)



miRNA Target Sequences of Experimental Identification

(| 281,378 Alkalis in Total |)



Precursor miRNA

(| 1,072 Alkalis in Total |)



Mature miRNA

(| 114 Alkalis in Total |)



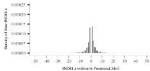
Star miRNA

(| 30 Alkalis in Total |)

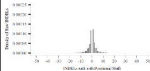


**Genome Wide**

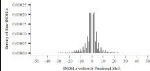
(Total Bases 2,490,922,740 bp)

**miRNA Target Genes of Computational Prediction**

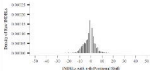
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**miRNA Seed Matrix Sites of Computational Prediction**

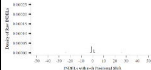
(Total Bases 4,440,612 bp)

**miRNA Target Sequences of Experimental Identification**

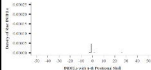
(Total Bases 5,120,609 bp)

**Precursor miRNA**

(Total Bases 3,642 bp)

**Mature miRNA**

(Total Bases 1,149 bp)

**Star miRNA**

(Total Bases 1,540 bp)

