Genome-wide profiling of microRNAs and prediction of mRNA targets in 17 bovine tissues

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Abstract

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

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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

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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 2nd-7th nucleotides at the 5'-end of the mature miRNA. When a

76   target-seed match is extended to 1st or 8th 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.

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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

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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

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

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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

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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   (*uagcaccauuugaaaucgguua*) expressed were all from *chr16:77478646-77478667(+)*. Because *bta-*

197   *miR-29d-3p* and *uagcaccauuugaaaucgguua* only differed in the 19th nucleotide, miRDeep2

198   annotated *uagcaccauuugaaaucgguua* 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,

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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).

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.

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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 2nd to 7th 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. 2nd-8th 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) (version7.2) 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).

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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.

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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 3**Error! Reference source not found.** Here

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

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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

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337   change of miRNA transcription from this study and the log2 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^{-8}) 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

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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

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

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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

Examination of associations among miRNAs, miRNA target regions and

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).

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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 zygosities 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

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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

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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-Liivrand 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   (Dowen 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 ≤30Kb), 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-Liivrand

509   et al. 2017). Those insulator elements separated the TSSs of miRNAs into two groups: miRNAs that

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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 (2nd

520   to 8th 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

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535   interact with 4 to 6,767 putative target genes, which caused an incompletely shuffled dataset that

536   had more permutated 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

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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

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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

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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

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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*'.

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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

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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 Bioloabs 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

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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 ≥5 consecutive base pairs matched with the adapter sequences. Trimmed

723   sequence reads were discarded if final read length was <18nt 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 ≤2 nucleotides matched with the adapter sequence during the standard

726   'seed and extend' approach (Li and Homer 2010) and ≥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 ≥18nt.

732   Trimmed sequence quality for all trimmers was also assessed using FastQC (Andrews 2010) (version

733   0.10.1).

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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 ≥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

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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.

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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.

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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 (2nd-8th 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.

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829　TargetScan 7 did not directly output the genomic coordinates of putative miRNA seed match sites on

830　to UMD3.1 (NBCI) 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".

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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

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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),

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903   TargetScan (Agarwal et al. 2015) and miRWalk (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.

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.

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927   (2) The density of rarer variants with an allele frequency less than α 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 α 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, $n \epsilon (-\infty, +\infty)$, 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.

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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 2nd-7th 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?

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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

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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

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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.

1030   **Funding**

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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1041   genome sequence variants in the *Bos Taurus* genomes. We are immensely grateful to Daisy, the

1042   young dairy cow who was raised at the foothills of the rolling Strzelecki Ranges of West Gippsland,

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)

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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	*bta-miR-146b-5p, bta-miR-375-3p, bta-miR-409a-3p, bta-miR-543-3p, bta-miR-7-5p*
	down	*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(+)*
Brain Caudal Lobe	Up	*bta-miR-212-5p, bta-miR-219-3p, bta-miR-323-3p*
Brain Cerebellum	up	*bta-miR-135b-5p, bta-miR-219-3p, bta-miR-219b-5p, bta-miR-323-3p*
	down	*bta-miR-10a-5p, bta-miR-10b-5p, bta-miR-199b-5p, bta-miR-505-3p, bta-miR-505-5p, chr9:10768323-10768346(+)*
Heart	Up	*bta-miR-499-5p*
Intestinal Lymph Node	down	*bta-miR-361-5p*
Kidney	down	*bta-miR-101-3p, bta-miR-139-5p, bta-miR-150-5p, bta-miR-339b-5p, bta-miR-423-3p, bta-miR-92a-3p*
Leg Muscle	Up	*bta-miR-206-3p, bta-miR-378-3p, bta-miR-486-5p*
Liver	Up	*bta-miR-122-3p, bta-miR-122-5p, bta-miR-192-5p, bta-miR-194-5p*
	down	*bta-miR-30c-5p*

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

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	χ^2-value			345673.9	
	P-value			0	

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		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	χ^2-value			757193.9	
	P-value			0	

		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	χ^2-value			7577.71	
	P-value			0	

		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	χ^2-value			10248.99	
	P-value			0	

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

1062 Presented are the number of interactions between miRNAs and cogante 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

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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	9.69×10^{-7}
Brain Cerebellum	-0.00281	0.072024
Heart	NA	NA
Kidney	0.053872	0.106306
Leg Muscle	-4.59879	6.76×10^{-9}
Liver	-0.07229	7.78×10^{-5}
Lung	0.05387	1.15×10^{-9}
Mammary Gland	NA	NA
Ovary	NA	NA
Spleen	-5.2×10^{-17}	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

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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%

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

1093

Table 8 Density of Raw WGS Variants with <1% Allele Frequency within Genomic Features. The density was the proportion of raw single-base-nucleotide substitution variants within a genomic feature with an allele frequency less than 1%. The raw whole genome sequence variants were 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%

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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 Score (Percentile) | miRNA Family | WGS Variant | |
	Genomic Coordinate	Sequence			Position	Allele (Frequency)
TFCP2	chr5:28780088-28780093(+)	AUAUAU	-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);

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		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
CD40	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(+)	AUAUAU	-0.251 (17)	miR-5011-5p/bta-miR-1277-5p	75569617	A (0.461306); G (0.538694)
	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

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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.

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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

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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 ≥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.

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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 ≥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

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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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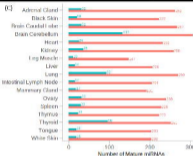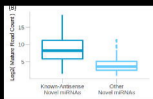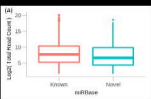1449　　　　(Macropus eugenii). *BMC Genomics* **15**: 1012.

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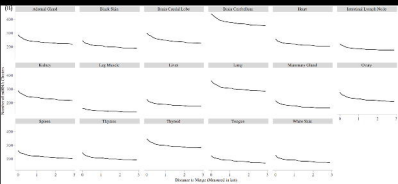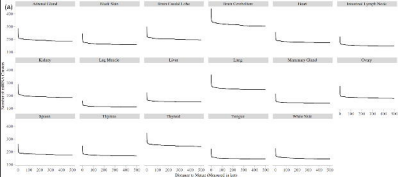1473   in protein coding sequences. *Bioinformatics* **28**: 771-776.

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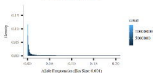1549   healthy and mastitis Holstein cattle by deep sequencing. *Animal Genetics* **45**: 20-27.

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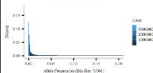1552   *Structural &Amp; Molecular Biology* **17**: 173.

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(a) (b)

(C) (D)

(E)

(F)

(a)

Adrenal Gland	Brush Skin	Distal Caudal Lobe	Distal Cerebellum	Heart	Internal Lymph Node
Kidney	Leg Muscle	Liver	Lung	Mammary Gland	Ovary
Spleen	Thymus	Thyroid	Tongue	White Skin	

(b)

Adrenal Gland	Brush Skin	Brain Caudal Lobe	Brain Cerebellum	Heart	Internal Lymph Node
Kidney	Leg Muscle	Liver	Lung	Mammary Gland	Ovary
Spleen	Thymus	Thyroid	Tongue	White Skin	

Distance to Maps (Minimal is km)

(A)

(B)

Genome Wide (12,039,439 Alleles in Total)

miRNA Target Genes of Computational Prediction (8,488,511 Alleles in Total)

miRNA Seed Match Sites of Computational Prediction (139,031 Alleles in Total)
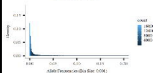
miRNA Target Sequences of Experimental Identification (285,199 Alleles in Total)

Precursor miRNA (1,592 Alleles in Total)
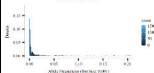
Mature miRNA (194 Alleles in Total)

Star miRNA (362 Alleles in Total)

Genome-Wide
(Total Bases: 2,669,922,345 bp)
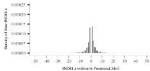
miRNA Target Genes of Computational Prediction
(Total Bases: 736,005,697 bp)
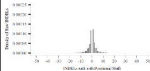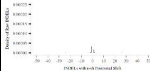
miRNA Seed Match Sites of Computational Prediction
(Total Bases: 4,344,012 bp)
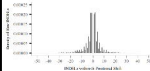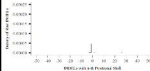
miRNA Target Sequences of Experimental Identification
(Total Bases: 5,128,619 bp)
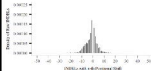
Precursor miRNA
(Total Bases: 78,662 bp)

Mature miRNA
(Total Bases: 12,699 bp)

Star miRNA
(Total Bases: 13,647 bp)