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Effect of breed and dietary composition on the miRNA profile of beef steers divergent for feed efficiency

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Identifying and breeding cattle that are more feed efficient is of great benefit to beef production. Additionally, it is crucial that genes contributing to feed efficiency are robust across varying management settings including dietary source as well as being relevant across contrasting breeds of cattle. The aim of this study was to determine miRNAs that are contributing to the expression of residual feed intake (RFI) across two breeds and dietary sources. miRNA profiling was undertaken in *Longissimus dorsi* tissue of Charolais and Holstein–Friesian steers divergent for RFI phenotype following two contrasting consecutive diets (high-forage and high-concentrate). Ten miRNA were identified as differentially expressed (adj. P < 0.1) across the breed and diet contrasts examined. Of particular interest was the differential expression of miR-2419-5p and miR-2415-3p, both of which were up-regulated in the Low-RFI Charolais steers across each dietary phase. Pathway analysis of target mRNA genes of differentially expressed miRNA revealed enrichment (P < 0.05) for pathways including metabolic related pathways, insulin receptor signalling, adipogenesis as well as pathways related to skeletal muscle growth. These results provide insight into the skeletal muscle miRNAome of beef cattle and their potential molecular regulatory mechanisms relating to feed efficiency in beef cattle.

Genetic selection and breeding of feed efficient cattle is important for the economic sustainability of beef production[1]. Moreover, feed efficient cattle have been shown to produce less methane (g/day[2,3]), thus also benefiting the environmental sustainability of beef production systems. Through a greater understanding of the genomic and molecular control of complex traits such as feed efficiency, particular genes or genomic regions may be identified, which following appropriate validation could be harnessed in genomic selection breeding programs for the genetic selection of feed efficient cattle[4].

However, despite the clear benefits of breeding feed efficient cattle, the main impediment to sustained genetic progress is the process and expense associated with accurately measuring individual animal intake and growth performance. Thus, studies[5–15] have sought to uncover the underlying biology governing feed efficiency primarily through mRNA transcriptional profiling of cattle divergent for the feed efficiency trait; residual feed intake (RFI). However, whilst biological processes related to lipid metabolism and immune function are commonly implicated towards variation in RFI, results across the literature are inconsistent in terms of specific genes contributing to RFI[1,16]. Moreover, although previous transcriptomic studies have revealed an involvement of skeletal muscle tissue, which was targeted in the current study, towards the molecular control of feed efficiency through the identification of differentially expressed mRNA genes, results again are inconsistent depending on individual experimental parameters employed[6,12,14,17]. This is undoubtedly due to the multifaceted nature of the RFI trait coupled with variation in experimental factors across different studies, such as differences in animal breed, developmental stage, and diet, which ultimately confound the outcomes[1].

Thus, the objective of this study was to examine the regulation of transcription, through an evaluation of the miRNA profile in skeletal muscle tissue of beef cattle divergent for RFI. miRNAs are short, single-stranded, endogenous, non-coding RNA molecules which are involved in the regulation of gene expression. Specifically miRNAs orchestrate the degradation or repression of the translation of their target messenger (m)RNAs by directly binding to their 3′ untranslated regions (UTR)[18]. In an effort to overcome the aforementioned impact

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of individual experimental design on the resultant outcome, two contrasting breeds were employed in this study, namely Charolais and Holstein–Friesian. Additionally, in light of knowledge of re-ranking of cattle for RFI when offered either the same diet[19] or diets differing in chemical composition or energy density[20] over successive feed intake recording periods, two contrasting dietary sources were utilised in this study, specifically cattle were first offered a high-forage diet, followed by a high-concentrate diet. We hypothesised that RFI phenotype would affect the miRNAome profile of *longissimus dorsi* muscle of beef cattle under contrasting dietary regimes.

Methods

Animal management

The animal model used in this study has been described previously in Higgins et al.[11] and was originally undertaken to evaluate the within-animal repeatability of feed intake, growth and feed efficiency between varying stages of development in Charolais and Holstein–Friesian beef steers offered contrasting diets over consecutive test periods[21,22]. Briefly, dietary intakes were recorded for 90 Charolais and 77 Holstein–Friesian steers over two consecutive contrasting dietary phases, each of which lasted 70 days, with a 14 day interim acclimatisation period in between. Charolais steers were 451 (± 18.3) whilst Holstein–Friesian were 474 (± 7.7) days of age at the start of the trial. The diets consisted as follows: Diet 1: high-forage (HF; steers were individually offered fresh herbage, harvested twice daily from *Lolium perenne* dominant swards, *ad libitum*); Diet 2: high-concentrate (HC; steers were offered the same concentrate *ad libitum* (86% rolled barley, 6% soya bean meal, 6% molasses, and 2% minerals and vitamins) with a restricted daily allowance of grass silage (10% of dry matter intake (DMI)). All steers were individually fed using an electronic Calan gate system. Additionally, all steers were weighed at the beginning and end of each dietary phase as well as on a fortnightly basis throughout. Mean body weight for Charolais and Holstein–Friesian at the start of HF phase and HC dietary phases were 516 kg (SD = 37.3) and 440 kg (SD = 41.8), and 676 kg (SD = 50.0) and 611 kg (SD = 49.1), respectively[14].

Upon completion of each dietary phase, individual RFI values for each steer were determined (described previously in Higgins et al.[11]). Briefly, the residuals of the regression of DMI data on average daily gain and mid-test metabolic body weight within each breed, were used to compute individual animal RFI coefficients for each feeding phase using the GLM procedure of SAS 9.4 (SAS Inst. INC., Cary, NC). Residual feed intake was calculated for each animal as the difference between actual DMI and expected DMI. At the end of each dietary phase, within breed, steers were ranked for having High-RFI (feed-inefficient; n = 8) and Low-RFI (feed-efficient; n = 8) and were subsequently used for tissue sample collection. Steers selected as both High- and Low-RFI for each breed and over each dietary phase were also tested for biases in live weight, metabolic weight and average daily gain[11,23]. An overview of the animal model is presented in Fig. 1.

Tissue sampling

Longissimus dorsi muscle tissue sample collection is previously outlined in Keogh et al.[14]. Briefly, all steers received local anaesthetic to the site of biopsy collection (5 ml Adrenacaine, Norbrook Laboratories (Ireland) Ltd.). For tissue collection a 6 mm diameter Bergstrom biopsy needle (Jørgen KRUUSE, Veterinary Supplies, Lyon, France) was used. All instruments used for biopsy collection were sterilised, washed with 70% ethanol

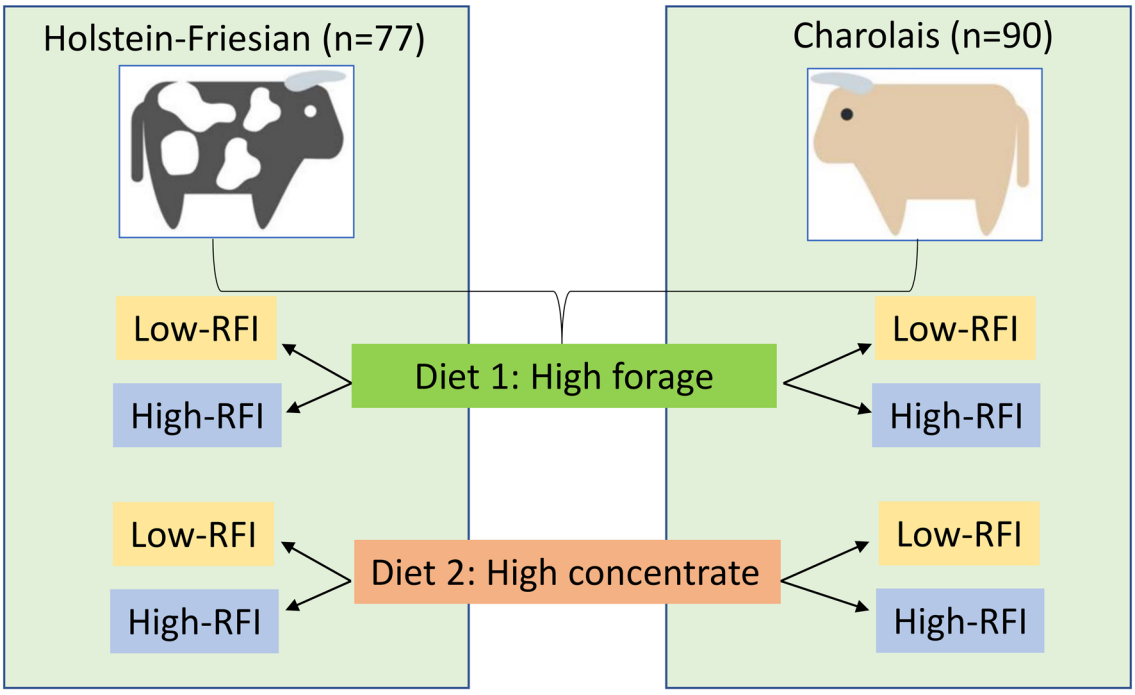

Fig. 1. Overview of animal model.

and treated with RNaseZap (Ambion, Applera Ireland, Dublin, Ireland), prior to use. Care was taken to ensure biopsy samples were consistently harvested at a depth of ~ 2.5 cm into the muscle tissue. Biopsies were washed in sterile DPBS, snap frozen in liquid nitrogen and subsequently stored at − 80 °C for long term storage pending further processing.

RNA isolation and small RNA sequencing

The Qiagen RNeasy Plus Universal Mini kit (Qiagen Ltd, Manchester, UK) was used to isolate total RNA, according to the manufacturer's instructions including steps for the purification of total RNA containing miRNA (Appendix C of the manufacturers protocol). Yield of the resultant RNA was determined by measuring the absorbance at 260 nm with a Nanodrop spectrophotometer (NanoDrop Technologies; Wilmington, DE, USA). The Agilent RNA 6000 Nano LabChip kit (Agilent Technologies Ireland Ltd.) was used on an Agilent 2100 Bioanalyser to determine the quality of the RNA isolated from each sample. All samples were deemed to be of sufficient quality (RNA integrity number (RIN) > 8).

Small RNA sequencing for miRNA profiling was undertaken by a commercial sequencing facility (Macrogen Europe Inc., Amsterdam, The Netherlands). For all samples individual cDNA libraries were prepared using the Illumina Truseq small RNA Library Prep Kit (Illumina, San Diego, CA, United States), according to the manufacturer's instructions. High throughput sequencing was undertaken on an Illumina HiSeq 2500 sequencing platform, incorporating 50 bp single end sequencing for small RNA sequencing.

Bioinformatic analysis

Sequence reads were firstly assessed for quality using FastQC (version 0.11.8[24]). The Illumina 3′ sequencing adaptor was then clipped from all raw sequence reads using Cutadapt (version 1.18[25]). Also using Cutadapt, reads of lengths shorter than 15 bp and longer than 28 bp were removed as short and long reads, respectively. The retained reads were subsequently filtered for other bovine short RNA species including ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs), which were downloaded from the Rfam database via the non-coding RNA database in RNAcentral (https://rnacentral.org/). The final processed sequencing files, specific to miRNA were then re-evaluated for quality again using FastQC (version 0.11.8).

To profile miRNA expression in each sample, the miRDeep2 package (version 2.00.8[26]) modules were used, together with the bovine reference genome (ARS-UCD1.2) and the known bovine mature miRNA sequences and their precursor sequences from the miRBase database (release 22.1[27]). The miRDeep2 mapper module (mapper.pl) was used with default parameters to collapse reads of the sequences into clusters. Bowtie (version 1.1.1) was then employed to align the collapsed reads to the indexed reference genome. Using default parameters and input files including the reference genome, collapsed reads versus reference genome alignment, known bovine mature miRNAs and their precursor sequences (including the hairpin structures) and *Bos taurus* (bta) as the species of interest, the miRDeep2 module (miRDeep2.pl) was used to quantify bovine miRNAs. Through this, the miRDeep2 quantifier module was used to quantify all known expressed miRNAs in the sequence data, producing read counts for each individual sample. The miRNA read counts were then assessed for differential expression using the R (v2.14.1) Bioconductor package, edgeR (version 3.26.7). miRNA read counts were firstly filtered for the removal of lowly expressed genes, whereby any gene with less than one count per million in at least half the number of samples (n = 8) was removed from the analysis. Retained read counts were then normalised using the trimmed mean of M-values normalisation method. Normalised read counts were then analysed with a generalised linear model. The following contrasts were tested for differential expression between the High- and Low-RFI steers: Charolais following a HF diet; Charolais following a HC diet; Holstein–Friesian following a HF diet and; Holstein–Friesian following a HC diet. Genes with a Benjamini–Hochberg false discovery rate of 10% and a fold-change greater than 1.5 were considered differentially expressed.

mRNA targets of the differentially expressed miRNA were predicted using the TargetScan 7.2 Perl scripts (release 7.2; http://www.targetscan.org/vert_72/)[28], downloaded from http://www.targetscan.org/cgi-bin/targetscan/data_download.vert72.cgi. TargetScan predicts miRNA target genes based on a quantitative model that scores candidate target genes based on 14 features including 3′-UTR target-site abundance, predicted seed-pairing stability, identity of the nucleotide at position 1 of the small RNA (sRNA), identity of the nucleotide at the 8th position of the sRNA, identity of the nucleotide at the 8th position of the target site, local AU content near the target site, supplementary pairing at the miRNA 3′ end, predicted structural accessibility, minimum distance of the site from the stop codon or polyadenylation site, probability of target site conservation, open reading frame (ORF) length, 3′-UTR length, number of offset-6mer sites in the 3′ UTR, and the number of 8mer sites in the ORF[28]. Both conserved and non-conserved miRNA target sites were predicted using a file containing all known gene transcripts 3′ UTR sequence alignments and the miRNA family information file, as input files. The targetscan_70_BL_bins.pl and targetscan_70_BL_PCT.pl scripts were used to calculate branch lengths and the probability of the conserved target site. In combination with the RNAplfold (version 2.4.11), the targetscan_70_context_scores.pl script was used to calculate context++ scores for the miRNA target genes based mainly on the 14 attributes listed above. Subsequently, potential target genes for the miRNA reported as differentially expressed across the various contrasts examined were considered for functional enrichment analysis using Ingenuity Pathway Analysis (IPA[29]).

Ethics declaration

This study was conducted at the Teagasc Animal and Grassland Research and Innovation Centre. All procedures involving animals were approved by the Teagasc Animal Ethics Committee and all procedures involving animals in the current study were conducted under an experimental license (AE19132/P029) from the Health Products

Regulatory Authority in accordance with the cruelty to Animals Act 1876 and the European Communities (Amendment of Cruelty to Animals Act 1876) Regulations 2002 and 2005. All experiments were performed in accordance with relevant regulations and the ARRIVE (Animal Research: Reporting on In Vivo Experiments) guideline.

Results

Animal model

Descriptive statistics related to the performance of the animals used in this study are presented in Higgins et al.[10]. During the HF dietary phase, High-RFI Charolais and Holstein–Friesian steers consumed 8 and 11% more than the Low-RFI steers ($P < 0.001$). During the HC dietary phase, High-RFI steers consumed 15 and 17% more, respectively, than their Low-RFI counterparts ($P < 0.001$). Over both dietary phases for each breed there was no significant difference in liveweight[23] or metabolic weight and average daily gain[11].

Sequence alignment and differential expression analysis

An average of 13 M sequence reads were generated across all samples sequenced. After trimming of sequencing reads, approximately 9 M reads on average were retained. Following MirDeep2 pre-processing, an average of 8.8 M sequence reads remained for mapping. Of the 8.8 M reads retained, an average of 92% were mapped to the bovine reference genome (ARS-UCD1.2). After filtering of lowly expressed miRNA in EdgeR, the number of miRNA retained for differential expression in each comparison was as follows: Charolais-HF: 285; Charolais-HC: 285; Holstein–Friesian-HF: 298; Holstein–Friesian-HC: 283. In total, ten miRNA were identified as differentially expressed (adj. P-value 0.1; fold change > 1.5) between High-RFI and Low-RFI steers across the two breeds and contrasting dietary conditions examined (Table 1). Of the differentially expressed miRNA, none were commonly differentially expressed across the four contrasts examined (Fig. 2). However, commonality was evident for some of the contrasts examined; for example, miR-7 was down-regulated in the Low-RFI Charolais steers following the HF diet, but was up-regulated in the Low-RFI Holstein–Friesian steers following the HC diet. Additionally, miR-2419-5p and miR-2419-3p were both up-regulated in the Low-RFI Charolais steers under both HF and HC dietary conditions.

Target gene identification and functional annotation of target genes

Target mRNAs of differentially expressed miRNA were determined using TargetScan. At a weighed context++ score percentile threshold of 99%, 767 target mRNA genes for the ten miRNA reported as differentially expressed were identified (Supplementary Table S1). For functional annotation of target genes a less conservative threshold was used to allow for the generation of enriched pathways and biological functions for each individual differentially expressed miRNA. The predicted target genes with a weighed context++ score percentile of > 75% (Supplementary Table S2) were subsequently analysed for pathway and functional enrichment within IPA. The full list of pathways enriched for each miRNA is presented in Supplementary Table S3. Pathways including PTEN and AMPK signalling were enriched ($P < 0.05$) for the various miRNA identified as differentially expressed between High-RFI and Low-RFI Holstein–Friesian and Charolais steers. Additionally, insulin receptor signalling and adipogenesis pathways as well as processes related to skeletal muscle growth were also implicated towards divergent RFI phenotype in the skeletal muscle of Charolais and Holstein–Friesian cattle. Moreover an evaluation of target mRNA genes and genes identified as differentially expressed in our previous mRNAseq study on the same tissue samples[14] revealed a potential direct relationship between miR-129-3p and its target gene *CREB5* (Fig. 3) as well as miR-7 and *FABP4* (Fig. 4).

Contrast	DE-miRNA	logFC	P-value	FDR
Charolais-HF	miR-338	− 1.04461	4.27E−06	0.001218
	miR-2419-5p	0.961592	0.00018	0.025653
	miR-146b	− 0.73214	0.000772	0.0516
	miR-2415-3p	0.996106	0.000826	0.0516
	miR-146a	− 0.7228	0.000905	0.0516
	miR-7	− 0.6964	0.001813	0.086128
Holstein–Friesian-HF	miR-129-3p	1.16862	0.000149	0.044272
	miR-21-5p	0.852266	0.000348	0.051862
Charolais-HC	miR-2419-5p	1.386478	8.04E−07	0.000229
	miR-2415-3p	1.33551	2.25E−05	0.003206
Holstein–Friesian-HC	miR-150	5.031938	0.000184	0.052194
	miR-7	3.485752	0.000517	0.073203
	miR-15b	4.182449	0.000817	0.077079

Table 1. miRNA differentially expressed in the *Longissimus dorsi* tissue of cattle divergent for RFI phenotype across varied breed types and dietary sources. *Charolais-HF* Charolais cattle under high-forage diet, *Holstein–Friesian-HF* Holstein–Friesian cattle under high-froage diet, *Charolais-HC* Charolais cattle under high-concentrate diet, *Holstein–Friesian-HC* Holstein–Friesian cattle under high-concentrate diet.

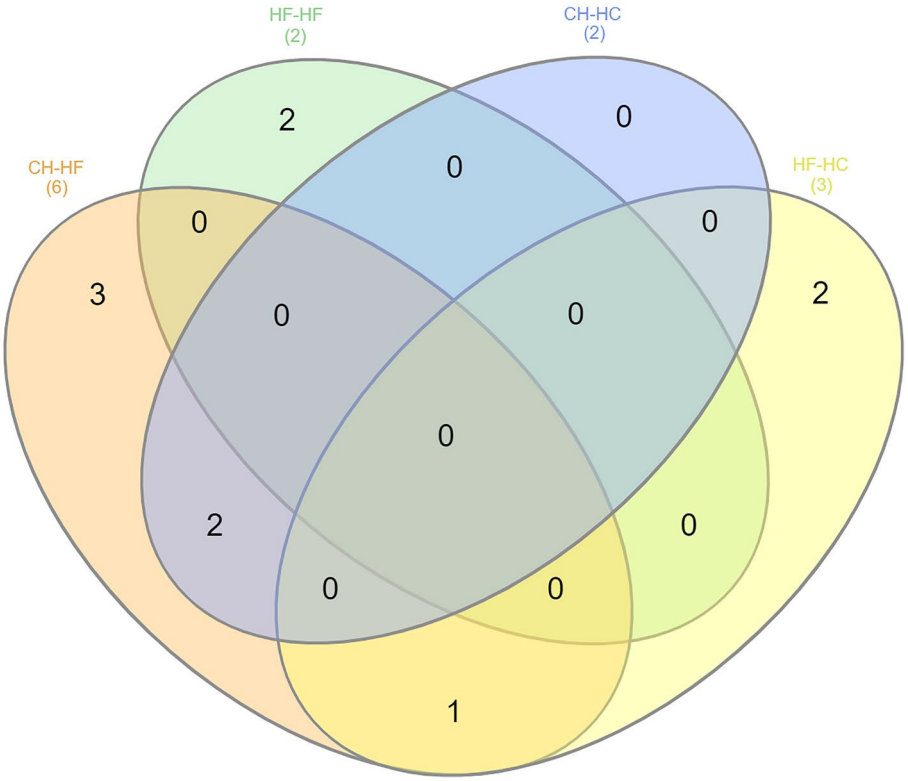

Fig. 2. Venn diagram representing the differentially expressed miRNA between cattle divergent for RFI across varying breed type and offered contrasting dietary composition. *CH-HF* Charolais cattle under high-forage diet, *HF-HF* Holstein–Friesian cattle under high-froage diet, *CH-HC* Charolais cattle under high-concentrate diet, *HF-HC* Holstein–Friesian cattle under high-concentrate diet.

Discussion

Residual feed intake has become the measure of choice for identifying feed efficient cattle, primarily due to the independence of the trait from production traits[1,30]. Indeed, the interest in the trait and potential for genomic selection is evident within the published literature where researchers have examined the underlying biology governing variation in RFI, primarily through transcriptional profiling evaluations[1,16]. However, despite large research endeavours, results within the literature do not consistently point to singular genes or genomic regions as contributory towards variation in RFI. The lack of commonality in results across the literature is undoubtedly due to the variation in experimental design employed amongst the various studies. For example, utilisation of different breeds, dietary sources and undertaking experimental intake periods at different stages of animal development or age can all lead to experiment-specific results confounding the overall outcome. Despite this, biological processes including lipid metabolism and immune response are commonly attributed to variation in RFI across various experimental settings[16]. However, the lack of implication of common genes related to these processes across studies suggests other regulatory genomic regions may be contributing to feed efficiency dependant on both animal specific and environmental factors impacting the feed efficiency outcome. Thus, the current study sought to investigate the contribution of miRNA to variation in RFI in two varied breeds offered consecutive contrasting diets. Results from this study, however, did not identify any singular miRNA as consistently differentially expressed between High- and Low-RFI cattle across both breeds and dietary sources examined. This result highlights the clear impact of both individual animal genotype and imposed management regimes on resultant RFI phenotype and may be attributable to the contrasting body composition of the breeds used as well as the effect of age or stage of development during each dietary phase. Indeed, this finding is similar to the results of our earlier mRNA study using the same tissue samples used in this study[14] as well as in liver tissue[11] where over the same dietary phases no gene was commonly differentially expressed for RFI in either breed over both dietary phases. However although we identified an effect of RFI phenotype in Charolais steers irrespective of diet, apparent through the up-regulation both miR-2419-5p and miR-2415-3p in the Low-RFI Charolais steers across both dietary phase, no miRNA was commonly affected in both breeds at either dietary phase. This result is in contrast to the findings of Mukiibi et al.[31], who in their evaluation of differentially expressed miRNA in the liver tissue of cattle divergent for RFI across three contrasting breeds, reported common differential expression of two miRNA across the three breeds examined. Specifically, miR-449a was up-regulated in the Low-RFI cattle in all three breeds, whilst miR-AB-2 was differentially expressed but followed a different direction of effect based on the breed. The contrasting results between our own study and that of Mukiibi et al.[31] may be directly attributable to the use of different tissues across the studies. The identification of common miRNA in the liver

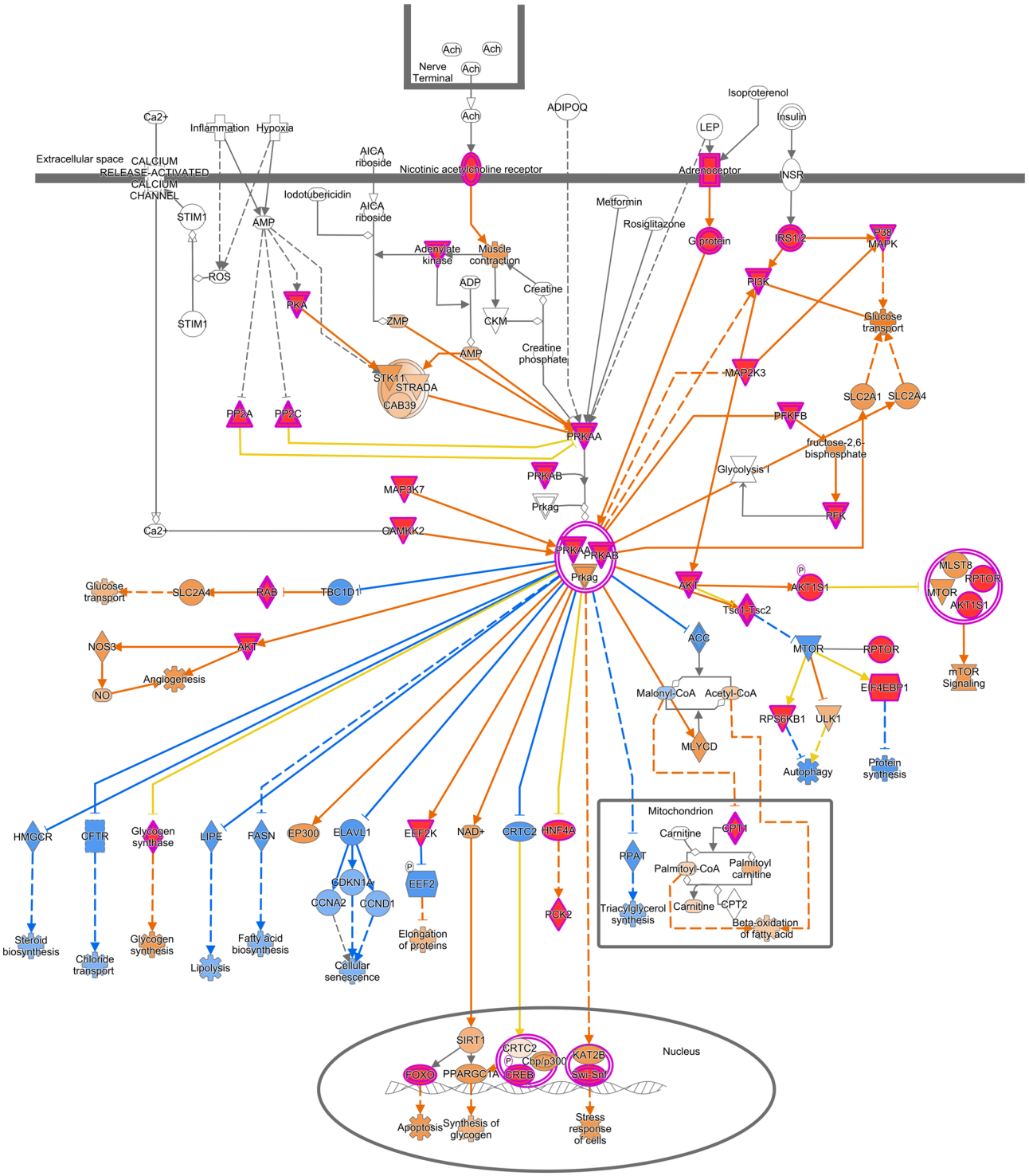

Fig. 3. AMPK signalling pathway enriched based on predicted target mRNA genes of miR-129-3p. Predicted target mRNA genes are highlighted in red and include *CREB5* which displayed lower expression in the Holstein–Friesian steers following the HF diet (Keogh et al., 2023), whilst miR-129-3p followed the opposite direction of effect in the Holstein–Friesian steers at the same dietary phase, indicating a direct relationship between the miRNA and *CREB5* gene.

tissue across three contrasting breeds in Mukiibi et al.[31] potentially suggests a greater role for the liver towards RFI phenotype compared to the skeletal muscle tissue used in the current study, which may be more impacted by individual animal genotype and consequent differences in body growth potential or composition.

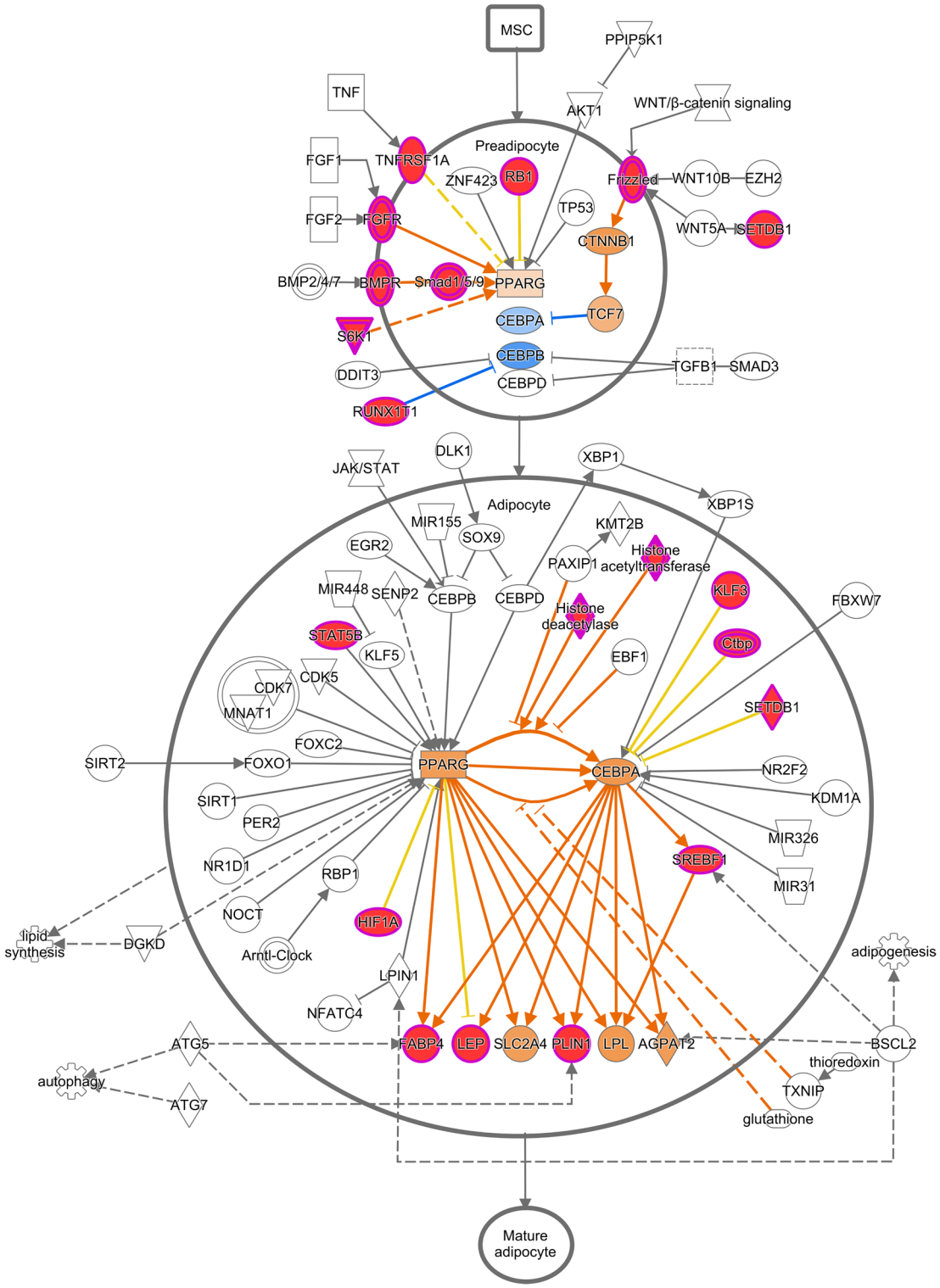

Fig. 4. Adipogenesis pathway enriched based on predicted target mRNA genes of miR-7. Predicted target mRNA genes are highlighted in red and include *FABP4* which displayed lower expressed in the CH steers following the HF diet (Keogh et al., 2023), whilst miR-7 followed the opposite direction of effect in the CH steers at the same dietary phase, indicating a direct relationship between the miRNA and *FABP4* gene.

Despite not identifying any miRNA as common across all the contrasts examined, target genes of the miRNA identified as differentially expressed in this study were involved in common biological processes. These included for example pathways involved in metabolism, as well as adipogenesis, cellular growth and insulin receptor

signalling which were all enriched based on the target genes of multiple miRNA reported as differentially expressed in this current study. The remainder of this discussion will focus on the effect of RFI phenotype of these enriched biological processes.

Metabolic regulation

A contributory factor towards variation in RFI is based on differences in the amount of feed consumed between efficient and inefficient animals, thus the identification of target genes involved in metabolic processes across the various diets and breeds employed in this study is perhaps unsurprising. Of particular interest in the current study was the differential expression of miR-2415-3p, which has target mRNA genes with functions related to metabolic processes. This miRNA was of interest due to its consistent up-regulation across both diets examined in the Charolais steers, suggesting a role in mediating the RFI phenotype across varied dietary regime. However, this result was specific to the Charolais steers, with no difference in miR-2415-3p expression apparent in the Holstein–Friesian steers at either dietary phase. Similarly in their study assessing hepatic miRNA differences between cattle divergent for RFI across various breeds, Mukiibi et al.[31] also reported up-regulation of the same miRNA but only in the liver tissue of Low-RFI Charolais steers. Additionally, two miRNA, miR-338 and miR-146a which were both down-regulated in the Low-RFI Charolais steers following the HF diet in the current study were previously implicated towards a varied response based on feeding different diets. For example, Ojo et al.[32] reported up-regulation of miR-338 in the plasma of Holstein cows when offered a forage-based diet compared to a contemporary group offered a high-grain diet. Similarly, Pacifico et al.[33] observed lower expression of miR-146a in the rumen epithelium of Holstein cows following a high-grain diet in comparison to a primarily forage-based diet. However despite results within the literature suggesting a role for these miRNA dependent on prevailing dietary management, the same result was not observed in the current study where both miRNA were only differentially affected by feed efficiency phenotype in the Charolais steers following the HF diet. The lack of an effect within the Holstein–Friesian steers, may be due to the difference in body composition between the two breeds.

In spite of not identifying a common miRNA as differentially expressed across the contrasts examined in this study, enrichment of the same biological pathways was apparent for the differentially expressed miRNA. For example, the enrichment of the PTEN signalling pathway was of particular relevance as this pathway was enriched based on mRNA targets of all miRNA identified as differentially expressed, with the exception of miR-146b. PTEN is a major negative regulator of the PI3-kinase pathway, which is involved in metabolic, growth and proliferative processes. More recently PTEN has been focused on in relation to its role in metabolic regulation, including roles in glycolysis, gluconeogenesis, glycogen synthesis, lipid metabolism and mitochondrial metabolism[34]. Indeed these processes, in particular lipid metabolism and mitochondrial function have been implicated towards variation in RFI in cattle[1,16,35], highlighting a potential role for this signalling pathway towards variation in RFI. However, the enrichment of PTEN in the current study is based on miRNA that were both up- and down-regulated in the Low-RFI steers, suggesting both positive or negative impacts on the genes of the PTEN signalling pathway. Moreover, despite enrichment of this pathway across the majority of miRNA that were differentially expressed, only one target gene, *FOXO6* was differentially expressed in our complementary mRNAseq analysis of the same tissue samples[14]. Moreover, miR-338 which was predicted to target *FOXO6* was down-regulated in the Charolais steers during the HF diet whilst *FOXO6* was up-regulated in the Holstein–Friesian steers during the same diet[14], highlighting a lack of relationship between the differentially expressed miRNA and the differentially expressed mRNA in the same tissue samples. Target genes of the PTEN pathway were also previously reported as differentially expressed between cattle divergent for RFI including *ITGA9*[6]; *GHR* and *RPS6KB2*[34]; *CCND1*, *BCAR1*, *FGFR3*[11]; *MAPK1*[9]; *CCND1*, *GHR*[10]; *TGFBR3*[8] and; *ITGA3*, *FGFR1* and *FGFR3* in duodenum[36], further implicating the PTEN pathway towards variation in RFI phenotype, which may be mediated by the miRNA identified as differentially expressed in this study.

Similar to PTEN, the AMPK signalling pathway was also enriched from a number of the miRNA identified as differentially expressed between the cattle divergent for RFI across contrasting breed and dietary source. The AMPK protein functions in metabolic regulation and again similar to PTEN also functions in lipid metabolism, glycolysis and mitochondrial homeostasis. Although the AMPK signalling pathway has not previously been attributed to variation in RFI in beef cattle, similar to the current study, de Oliveira et al.[37] reported enrichment of the AMPK signalling pathway based on the target mRNA of a miRNA implicated towards RFI in skeletal muscle of Nelore cattle. The relevance of AMPK towards RFI is however apparent in other species, including chickens[38] and pigs[39]. In the current study a number of mRNA targets of the AMPK signalling pathway were also differentially expressed in our previous mRNA-seq evaluation of the same tissue samples. These included *CREB5* and *PCK2* which were down-regulated in the Low-RFI cattle[14] and *FOXO6* which was up-regulated in the Holstein–Friesian steers. *PCK2* was also down-regulated in the adipose tissue of Angus bulls in the data of Weber et al.[17]. The down-regulation of *CREB5* in the Holstein–Friesian steers during the HF diet was of particular interest, due to the up-regulation of miRNA (miR-129-3p and miR-21-5p) which were predicted to target the expression of this gene, also in the Holstein–Friesian steers during the HF diet, suggesting a potential direct relationship between these miRNA and the *CREB5* gene. *CREB5* belongs to the CREB protein family which is known to regulate cell growth, proliferation and differentiation, downstream of AMPK signalling and based on the results of this study may be affected by variation in RFI phenotype which in turn may be mediated by the miR-129-3p and miR-21-5p miRNA which were affected by RFI phenotype. However results suggest that this relationship between the aforementioned miRNA and *CREB5* is specific to the Holstein–Friesian cattle and only during a high forage diet. Results indicate a role for AMPK signalling towards variation in RFI, mediated at the miRNA and mRNA level. Overall, RFI phenotype clearly affected the metabolism of skeletal muscle, which may be mediated by miRNA, however this is dependent on diet and breed.

Insulin receptor signalling

Despite the documented difference in dietary intake between cattle divergent for RFI a clear contributory role for insulin and glucoregulatory processes towards variation in RFI is not fully apparent. For example, some studies have reported differences in circulating concentrations of insulin and glucose in cattle divergent for RFI whilst others including the study related to the animals used in the current study have not[1,16,23]. Despite conflicting results pertaining to the systemic circulation and regulatory roles of insulin and glucose clearance for cattle divergent for RFI[1,16], transcriptomic evaluations have indicated towards a role for the insulin signalling pathway towards variation in RFI[36,40]. Indeed, a role for the insulin signalling pathway towards variation in RFI was apparent within the results of the current study due to the enrichment of this pathway from the target mRNA of the differentially expressed miRNA. Enrichment of the insulin receptor signalling pathway was apparent for miRNA that were both up-regulated (miR-7, miR-15b, miR-129-3p, miR-150) and down-regulated (miR-7, miR-146a/b, miR-338) in the Low-RFI cattle. However, these miRNA and consequently the insulin signalling pathway were not related to both breeds and diets examined. For example all of the aforementioned up-regulated miRNA were up-regulated in the Holstein–Friesian steers under the HC diet with the exception of miR-129-3p which was up-regulated in the Low-RFI Holstein–Friesian steers during the HF diet. Conversely, all down-regulated miRNA with targets of the insulin signalling pathway were down-regulated in the Charolais steers and only during the HF diet, with no apparent effect on the Charolais steers during the HC diet. These results again highlight the inconsistency in response between cattle divergent for RFI across various breeds and diets, overall suggesting differential molecular regulation of the insulin signalling pathway in skeletal muscle dependent on prevailing diet type. Indeed although we did not identify any of the target genes of the insulin signalling pathway as differentially expressed in our previous mRNAseq study on the same samples[14], specific target genes of the differentially expressed miRNA in this study were previously reported as differentially expressed within the literature. These included *PPP1R3C*, up-regulated in the muscle of Low-RFI cattle[6]; *PRKACA* and *PRKACB* up-regulated in the liver of low-RFI[13] as well as *IRS2* and *PPP1R3C* which were up- and down-regulated, respectively, in the liver of efficient cattle in Higgins et al.[11]. *IRS2* encodes the insulin receptor substrate 2, a cytoplasmic signalling molecule that mediates the control of various cellular processes by insulin, *PPP1R3C* encodes a carbohydrate binding protein that affects glycogen biosynthesis by activating glycogen synthase and limiting glycogen breakdown, dramatically increasing basal and insulin-stimulated glycogen synthesis upon overexpression. *PRKACA* and *PRKACB* both encode subunits of a member of the serine/threonine protein kinase family (PKA), which is involved in the regulation of lipid and glucose metabolism. Overall, results indicate a role for insulin receptor signalling towards variation in RFI, which may be mediated by miRNA regulation, however the results again are not consistent across diets or breeds, similar to the inconsistency within the literature in relation to differences in systemic glucose and insulin concentrations between cattle divergent for RFI.

Adipogenesis and lipid metabolism

A role for lipid metabolism towards variation in RFI is well established from the published literature[1,16], whereby typically feed efficient (Low-RFI) cattle have been shown to display lower expression of genes related to fatty acid metabolism. Similarly, within the current study adipogenesis was identified as an enriched pathway from the mRNA targets of miR-7, miR-129-3p and mir-146a/b, which also had targets enriched for the insulin signalling pathway. Indeed, similar to the miRNA enriched for the insulin signalling pathway, the aforementioned differentially expressed miRNA were inconsistent in their direction of effect across breeds and dietary phases. For example, miR-7 and miR-129-3p were both up-regulated in the Low-RFI Holstein–Friesian steers across the HC and HF diets, respectively, whilst both miR-7 and miR-146a/b were down-regulated in the Low-RFI Charolais steers during the HF diet only. Insulin is a major mediator of both lipogenesis and lipolysis, thus again variation in lipid metabolism towards RFI may be attributable to differential insulin response to systemic glucose clearance. Of the target mRNA genes of the differentially expressed miRNA only one gene, *FABP4*, which encodes a fatty acid binding protein, was also differentially expressed within our previous mRNA study on the same tissue samples[14]. Specifically, *FABP4* which was down-regulated in the Low-RFI Charolais steers following the HF diet was predicted as a target for the miR-7 miRNA which was up-regulated in the same group of steers indicating a direct relationship between miR-7 and *FABP4* due to their differential direction of effect. Indeed, *FABP4* was also down-regulated in the liver of Low-RFI steers in the data of Taiwo et al.[13]. Moreover, additional genes that were predicted targets of the adipogenesis pathway were previously reported as differentially expressed between cattle divergent for RFI. For example *DLK1*, which is involved in the differentiation of adipocytes, was a target mRNA for miR-129-3p and was differentially expressed in the datasets of Mukiibi et al.[10] and Alexandre et al.[6]. Additionally, the *LPIN1* gene, which is involved in triglyceride biosynthesis and adipocyte differentiation was a target mRNA of mir-146a/b. This gene functions in the regulation and control of metabolism of fatty acids at different levels and also acts as a nuclear transcriptional activator to modulate lipid metabolism gene expression. The target mRNA gene of *LPIN1* was of particular interest as this gene was identified as one of only 5 genes that were commonly differentially expressed in the liver across three contrasting breeds of cattle in the data of Mukiibi et al.[10]. Overall, results indicate a clear role for adipogenesis towards mediating variation in RFI phenotype within the *longissimus dorsi* muscle.

In addition to a role towards RFI phenotype, a number of the miRNA reported as differentially expressed in the current study have previously been affected based on biological processes related to fatty acid metabolism or adipogenesis, which is of relevance given that typically within the literature, feed efficient cattle have been shown to display lower expression of genes related to fatty acid metabolism. For example, mir-146b was up-regulated in the muscle tissue of Nelore steers with low genomic estimated breeding values for intramuscular fat deposition[41]. Indeed, miR-146b and miR-146a, which both belong to the same family and were both down-regulated in the Charolais cattle following the HF diet were also both down-regulated in the liver of Low-RFI cattle[42]. Moreover,

although not apparent across diets in the current study, miR-146a was previously reported as down-regulated in the rumen epithelium following a high grain feeding regime[33], which although not reported in the current study suggests a potential role for this miRNA across various diets in addition to the RFI phenotype. Similarly, miR-21-5p which was up-regulated in the Low-RFI Holstein–Friesian cattle following the HF diet was observed as the most expressed miRNA in response to high-grain feeding regime in the rumen epithelium[33]. However, whilst miR-21-5p was up-regulated in Holstein–Friesian steers in the current study, Al-Husseini et al.[42] reported lower expression of this miRNA in the liver tissue of Low-RFI Angus bulls, suggesting differential direction of effect based on either breed or tissue type. Similarly, differential direction of effect was also apparent for miR-7 which was down-regulated in the Low-RFI Charolais steers following the HF diet, but was up-regulated in the Holstein–Friesian cattle following the HC diet, again suggesting differential regulation of this miRNA based on breed and diet offered. This miRNA was also down-regulated in the Low-RFI Angus bulls of Al-Husseini et al.[42]. Moreover, the predicted target mRNA genes of miR-7 pertaining to the adipogenesis pathway highlight an effect of this miRNA on both preadipocyte and adipocyte phases (Fig. 4). Whilst the aforementioned miRNA did not follow the same direction of effect, miR-129-3P, miR-150 and miR-15b were all up-regulated in the Low-RFI Holstein–Friesian cattle. Specifically, miR-129-3p was up-regulated following the HF diet, whilst miR-150 and miR-15b were both up-regulated following the HC diet. All three miRNA were specifically involved in adipogenesis and lipid metabolism. For example, Yu et al.[43] reported differential expression of miR-129-3p between preadipocytes and adipocytes. Moreover, similar to the current study, miR-150 and miR-15b were both up-regulated in the liver of Low-RFI Angus bulls in Al-Husseini et al.[42]. Finally, we observed up-regulation of miR-2419-5p in the Low-RFI Charolais steers following both dietary phases, similarly Mukiibi et al.[31] observed greater expression of miR-2419-5p in the liver of Low-RFI Charolais steers. miR-2419-5p was previously reported to have greater expression in skeletal muscle of Nelore cattle with extreme breeding values for conjugated linoleic acid[44]. Overall, results suggest a clear role for differential miRNA regulation towards adipogenesis and lipid metabolism processes within the *longissimus* tissue of beef cattle divergent for RFI. Indeed, the role for adipogenesis and lipid metabolism may impact the subsequent quality and indeed the fatty acid profile of the resultant meat produced. However, similar to the previous biological processes discussed this effect is not consistent across breeds and dietary phases examined.

Skeletal muscle growth

Skeletal muscle represents a large energy sink within the body[45], thus variation in inherent energy utilisation may be apparent within this tissue type. Indeed, the effect of RFI phenotype within the skeletal muscle tissue, in particular the *longissimus dorsi* is apparent within the literature whereby studies have reported differences in the expression of genes within the *longissimus dorsi*[8,12,14]. Indeed, within the current miRNA based evaluation, differentially expressed miRNA targeting genes involved in growth processes was apparent for each of the comparisons examined with the exception of the HC dietary phase for the Charolais cattle. Pathways including TGF-beta signalling as well as those related to the somatotropic signalling axis (growth hormone and IGF-1 signalling pathways) were enriched based on target genes of differentially expressed miRNA. The TGF-beta signalling pathway in involved in many cellular processes including cell growth and differentiation as well as migration and apoptosis. Indeed the relevance of the TGF-beta system towards feed efficiency in cattle was previously highlighted by Alexandre et al.[6] who identified *TGFB1* as a key regulator mediating feed efficiency in beef cattle. Moreover, *SERPINE1* which was predicted as a target within the TGF-beta signalling pathway was differentially expressed in the corresponding mRNAseq study of the tissues used in this study[14] as well as in the muscle of Nelore steers in Alexandre et al.[6], down-regulated in the Low-RFI cattle in each study.

The somatotropic axis consisting of growth hormone, IGF-1 and their carrier binding proteins is one of the major hormonal systems in the body regulating postnatal growth in mammals. Additionally, the somatotropic axis also plays a key role in the control of the regulation of metabolism and physiological processes, thus an effect of RFI phenotype on the components of the somatotropic axis may be expected; however, results in the literature regarding the role of the somatotropic axis towards RFI are inconclusive. For example, studies have reported higher[46] or lower[47] IGF-1 concentrations, whilst others[48,49] did not observe any difference. Despite this, results from the current study clearly highlight a role for the somatotropic axis towards variation in skeletal muscle between cattle divergent for RFI. This was apparent through the enrichment of IGF-1 and growth hormone signalling pathways in target mRNAs of miRNA differentially expressed across all contrasts examined, with the exception of the HC phase in the Charolais steers. Indeed *CCN2* which is a predicted target gene of miR-129-3p and miR-146a/b and is involved in the IGF-1 signalling pathway was differentially expressed in the same tissue samples at the mRNA level[14]. An evaluation of the target mRNAs within the published literature revealed up-regulation of both *IGF1* and *GHR* in liver tissue of the Low-RFI cattle in Mukiibi et al.[10], whilst in that same study down-regulation of *IGFBP2* was evident in the Low-RFI cattle. *IGFBP2* is involved in regulating the bioactivity and bioavailability of IGF-1, thus down-regulation of this gene in the liver tissue along with up-regulation of both *GHR* and *IGF1* suggests a role for the somatotropic axis towards mediating feed efficiency in beef cattle. Of the mRNA targets identified for the differentially expressed miRNA in this study pertaining to the somatotropic axis, the lower expression of miR-338 in the Low-RFI Charolais following the HF dietary phase was of interest due to the up-regulation of its target gene *FOS*, which is involved in growth proliferation, in the same animals[14], indicating a direct relationship between the miRNA and the target mRNA. Additionally, *FOS* was also reported as up-regulated in Low-RFI cattle in liver tissue[7], skeletal muscle[14] and hypothalamus[6], highlighting a potential role for this gene towards variation in RFI. Overall results from this study and that of the published literature highlight the relevance of biochemical growth processes including the TGF-beta signalling pathway and the somatotropic axis towards RFI in beef cattle which may be mediated through differentially expressed miRNA

identified in this study. However, this effect may be dependent on the breed examined as well as the dietary or management systems employed.

Conclusion

Results in the literature regarding the genomic control governing RFI in beef cattle are inconsistent, suggesting a role for other genomic regions or transcriptional regulators towards variation in RFI. Thus, the aim of this study was to evaluate the regulation of gene expression by miRNA between cattle divergent for RFI across different breed types offered contrasting diets. However, similar to mRNA results, we did not observe any singular miRNA as differentially expressed across all diets and breeds examined in this study. Notwithstanding this outcome, target mRNA of differentially expressed genes revealed the functional control of biological processes related to metabolism, adipogenesis and insulin signalling as well as skeletal muscle growth. Results from this study provide further insight into the skeletal muscle miRNAome expression profiles of beef cattle divergent for RFI and their potential molecular regulatory mechanisms relating to feed efficiency across contrasting breeds and dietary regimes. However, results highlight a clear effect of both breed and dietary composition on RFI phenotype in the skeletal muscle tissue of beef cattle.

Data availability

The sequencing data underlying this article are available in NCBI's Gene Expression Omnibus at [https://www.ncbi.nlm.nih.gov/geo/] and can be accessed with unique GEO ID GSE269311.

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Author contributions

DK and MMcG conceived the animal model, harvested the biological samples and contributed to the statistical analysis. KK undertook laboratory and bioinformatic analysis and wrote the manuscript. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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