ORIGINAL ARTICLE

Resveratrol stimulates microRNA expression during differentiation of bovine primary myoblasts

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Popular scientific summary
- The effect of resveratrol on myoblast differentiation was analyzed.
- MicroRNA expression levels were measured after treatment of primary bovine myoblast with RSV.
- RSV could promote primary bovine myoblast differentiation by stimulating miRNA expression.
- Insulin signaling pathway is the most important pathway where the target genes of the stimulated differentially expressed miRNAs are enriched.

Abstract

Background: Resveratrol (RSV), a phenolic compound, is present in many human dietary sources, such as peanuts, peanut butter, grapes skin, and grape wine. RSV has been widely known for its benefits on human health. Beef from cattle skeletal muscle is one of the main sources of protein for human consumption. Previous studies have also found that pork and chicken qualities are influenced by the feed supplementation with RSV. In addition, our previous study demonstrated the RSV effects on bovine myoblast differentiation using messenger RNA (mRNA) data. In this study, we mainly focused on the influences of RSV on microRNA (miRNA) expression.
Method: We used 20 μM RSV to treat primary bovine myoblasts and extracted RNA for miRNA sequencing. After quality control and alignment for clean reads, we conducted quantification and analysis of differentially expressed (DE) miRNAs in the case (RSV-treated) group versus control (non-RSV treated) group. Next, we predicted the target genes for the DE miRNAs and analyzed them for the enrichments of Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.
Results: Finally, we identified 93 DE miRNAs (adjusted P-value < 0.05), of them 44 were upregulated and 49 were downregulated. Bta-miR-34c was the most significantly upregulated miRNA. *In silico*, prediction results indicated 1,869 target genes for the 93 DE miRNAs. GO enrichment analysis for the genes targeted by DE miRNAs revealed two significant GO terms (adjusted P-value < 0.05), in which the most significant one was stereocilium (GO:0032420). KEGG enrichment analysis showed five significant pathways, and the top significant KEGG pathway was the insulin signaling pathway (bta04910) (adjusted P-value < 0.05).
Conclusions: This study provided an improved understanding of effects of RSV on primary bovine myoblast differentiation through the miRNA modulations. The results suggested that RSV could promote differentiation of primary bovine myoblast by stimulating the miRNA expressions. The target genes of DE miRNAs were significantly enriched in the insulin signaling pathway, thus potentially contributing to improving muscle leanness by increasing the energy metabolism.

Keywords: *biomarker*; *MicroRNA*; *primary bovine myoblast*; *resveratrol*; *bioinformatics*

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MicroRNAs (miRNAs) are small RNA (sRNA) molecules (i.e. 22 nucleotides) that can silence gene expressions by promoting messenger RNA (mRNA) decay or repressing protein synthesis (1). In a review study, Izzotti et al., (2) revealed that 148 miRNAs could be regarded as biomarkers for controlling mutagenesis and carcinogenesis; these miRNAs were modulated by 32 dietary and pharmacological agents, including resveratrol (RSV). Kumar et al., also summarized that RSV could be one of the options for the miRNA-mediated therapeutic strategies (3). In beef cattle, miRNAs have been considered as one of the key regulators for myoblast proliferation, differentiation, and apoptosis (4).

RSV is a polyphenol extracted from grape skin with antioxidant properties, which favors an extended lifespan. Divergent effects of RSV in various cells have been reported, such as the antiproliferation effects on different cancer cells (2, 5), and the pro-differentiation impacts on human lung fibroblasts (6) and embryonic cardiomyoblasts (7). In addition, dietary supplementation of RSV to maternal pigs can improve the meat quality of offsprings due to its effect on muscle fiber characterization and antioxidative activities (8). The same mechanism and same effect were also observed in finishing pig induced by RSV (9). In murine myoblast, RSV could induce myogenesis and hypertrophy (10). Moreover, the fast glycolytic muscle type tends to be more responsive than the slow oxidative muscle to RSV treatment in rat late life (11). RSV can not only potentially modulate the tumor-suppressive miRNA levels to inhibit cancer progressions but also influence the expressions of skeletal muscle development-related miRNAs to promote myoblast differentiations (5, 10). For example, 26 upregulated miRNAs (e.g. miR-21 and miR-27b) and 20 downregulated miRNAs (e.g. miR-20b and miR-149) were observed in the mouse skeletal myoblast (C2C12) after RSV treatment. RSV initiates C2C12 myoblast differentiation by downregulating miR-133b, which could release Srf transcription factor (10).

This study aims to identify the specific miRNAs influenced by RSV treatment on primary bovine myoblast differentiation. Here, three independent replicates for both RSV-treated (case) and no-RSV (control) groups were analyzed for differentially expressed (DE) miRNAs using a next-generation sequencing (NGS) technology. Predictions of miRNA target genes combined with enrichment analysis of Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways assisted to reveal a number of biological processes driven by RSV treatment.

Materials and methods

Culture primary bovine myoblast
According to Miyake's method (12), primary bovine myoblasts were isolated from the fetal beef *longissimus dorsi* muscle and were subsequently cultivated in the high-glucose growth medium (GM). The differentiation of primary bovine myoblasts was induced with differentiation medium (DM) after cells reached nearly 90% confluence. GM was formulated with Dulbecco's modified Eagle's medium (DMEM) containing 1% penicillin–streptomycin and 20% fetal beef serum, while DM was formulated with DMEM containing 2% horse serum. Primary bovine myoblasts were induced for differentiation under constant treatment with 20 µM RSV for 4 days (i.e. 1 µL dimethyl sulfoxide (DMSO) as a solution for RSV attenuated into 5 mL DM). Likewise, the myoblasts in the control group were only treated with 1 µL DMSO without any RSV. The treated cells in two groups were harvested using TRIzol reagent after 4-day differentiation. All the steps of primary bovine myoblast culture were the same as in our previous study, including the RSV treatment (13). Each group had three independent experiments. GM, DM, DMEM, RSV, DMSO and TRIzol were purchased from TransGen (Beijing, China), HyClone (USA), Gibco (USA), Sangon Biotech (Shanghai, China), and Takara (Japan), respectively.

MicroRNA sequencing, quality control and alignment
About 3 µg RNA per cell sample was isolated for the miRNA libraries that were generated using NEBNext (NEB, USA) following the manufacturer's recommendations. We ligated adapters to the 3′-end and 5′-end of miRNA, and then the PCR amplification was performed. Afterwards, the library preparations were sequenced on an Illumina Hiseq platform (Illumina, USA) to generate the 50-bp single-end reads. After removing the raw reads containing ploy N (percentage of uncertain N ≥ 10%) A/T/G/C, with 5′-adapter contaminants, without 3' adapters or insert tags and low-quality reads (percentage of Qphred > 20 ≥ 50%), clean reads were retained. The miRNA tags were mapped to the reference genome of *Bos taurus* (UMD_3.1.1/bosTau8) using Bowtie software (version 0.12.9) (14). Then, the mapped miRNA tags were used to seek the known miRNAs using miRBase20.0 as the reference, and the potential miRNA and the secondary structures were obtained using mirdeep2 software (version 2.0.0.5) (15). Finally, miRNA counts were obtained.

Quantification and differentially expressed analysis for microRNAs
MiRNA expression levels were estimated by transcript per million (TPM) through the following normalization formula (16): Normalized expression = Mapped read count/Total read × 1,000,000. DE analysis of case-control groups was performed using the R package DESeq (version 1.18.0) (17). The *P*-values were adjusted using the Benjamini and Hochberg method. DE miRNAs were defined when the adjusted *P*-values were <0.05 as the threshold. In addition, we calculated fold changes (FCs)

based on the TPM between the case and control group to define the upregulated (i.e. $\log_2(FCs) > 0$) and downregulated (i.e. $\log_2(FCs) < 0$) miRNAs.

DE microRNA expressions validation by qRT-PCR

The primary bovine myoblast samples were divided into two copies, with one used for miRNA sequencing and the other for the quantitative reverse transcription-polymerase chain reaction (qRT-PCR) identification. Three upregulated DE miRNAs and three downregulated DE miRNAs were randomly selected to identify the sequencing results in myoblast samples. *U6* was used as the normal reference gene for qRT-PCR. The method of $2^{-\Delta\Delta Ct}$ was used to calculate the relative miRNAs expression level. All the primer details are listed in Table S1.

Target gene prediction and enrichment analysis of GO and KEGG pathway

The target gene predictions for DE miRNAs were performed using miRanda software (version 3.3) (18). R package *clusterProfiler* (version 3.6) was applied to test the statistical enrichment of the target genes in GO terms and KEGG pathways (19). The thresholds of the adjusted *P*-value < 0.1 for the significant GO terms and the adjusted *P*- value < 0.05 for the KEGG pathways enrichment were used in this study.

Results

Statistical alignment with bovine reference genome for sRNAs

On average, 13,162,918 clean reads retained after quality control and 11,539,765 (84.34%) of them were mapped to the sRNA of bovine reference genome. Generally, the length region of sRNA in animals is 18-35 nucleotides, while the miRNA length region focuses on 21-22 nucleotides. After the annotation of miRNAs to the miRBase20.0 dataset, 192,090 (1.40%) and 117,651 (0.86%) of the aligned reads were mapped to the mRNA exon and intron regions, respectively (Table 1). In addition, among the mapped known miRNAs, 520 mature miRNAs were mapped and 109 novel miRNAs were found, respectively.

The length distributions (18nt–35nt) of sRNAs in RSV1, RSV2, RSV3, DMSO1, DMSO2 and DMSO3 samples were similar (Fig. 1). The major length of sRNAs in this study was 20nt–24nt, which was typical of sRNA of dicer-processed products (Fig. 1). Obviously, 22nt was the most frequent length, followed by 23nt, 21nt, and 24nt in the six samples (Fig. 1 and Table S2).

Differentially expressed microRNAs

It was reported that RSV influenced muscle development, cancer, inflammation, and metabolic disease because of its regulation capability to miRNA expressions (10, 20–23). After statistical testing, 93 DE miRNAs were identified, where 44 were upregulated and 49 were downregulated (adjusted *P*-value < 0.05) (Fig. 2A and Supplementary file 1). The clusters of 93 DE miRNAs showed a good division between two groups where the upper cluster of the case group showed higher expression levels than the control group, while this pattern was reversed in the lower cluster (Fig. 2B). In this study, bta-miR-34c (adjusted *P*-value = 1.23E-20) was the most significantly upregulated miRNA (Table S3), which indicated its

Table 1. Statistics of alignment with reference genome of *Bos taurus* (UMD_3.1.1/bosTau8) for small RNAs (sRNA) and miRBase20.0 dataset for microRNAs

Sample	Total read	Clean read after QC	Clean read of sRNA	Mapped read sRNA	Mapped of degraded mRNA exon	Mapped of degraded mRNA intron	Mapped miRNA mature number	Mapped novel miRNA mature number
RSV 1	19,504,521 (100.00%)	18,755,102 (96.16%)	18,213,020 (93.38%)	17,209,702 (88.23%)	276,326 (1.42%)	159,772 (0.82%)	526	130
RSV 2	15,608,847 (100.00%)	15,107,306 (96.79%)	14,618,490 (93.66%)	13,834,886 (88.63%)	383,402 (2.46%)	172,697 (1.11%)	521	112
RSV 3	11,625,598 (100.00%)	11,171,267 (96.09%)	10,591,418 (91.10%)	9,360,007 (80.51%)	141,676 (1.22%)	100,723 (0.87%)	520	104
DMSO 1	11,519,021 (100.00%)	11,085,847 (96.24%)	10,710,129 (92.98%)	9,486,889 (82.36%)	115,203 (1.00%)	87,593 (0.76%)	512	100
DMSO 2	12,865,220 (100.00%)	12,348,288 (95.98%)	11,771,402 (91.50%)	10,423,246 (81.02%)	126,473 (0.98%)	103,870 (0.81%)	524	113
DMSO 3	10,968,870 (100.00%)	10,509,695 (95.81%)	10,131,100 (92.36%)	8,923,861 (81.36%)	109,461 (1.00%)	81,253 (0.74%)	516	96
Mean	13,682,013 (100.00%)	13,162,918 (96.20%)	12,672,593 (92.62%)	11,539,765 (84.34%)	192,090 (1.40%)	117,651 (0.86%)	520	109
SD	3,303,028	3,194,911	3,160,276	3,300,636	112,501	38,753	5	12

Note: SD indicates standard error. RSV1, RSV2, and RSV3 indicate the case groups that were treated with resveratrol (RSV). DMSO1, DMSO2, and DMSO3 indicate the control groups that were only treated with DMSO with RSV.
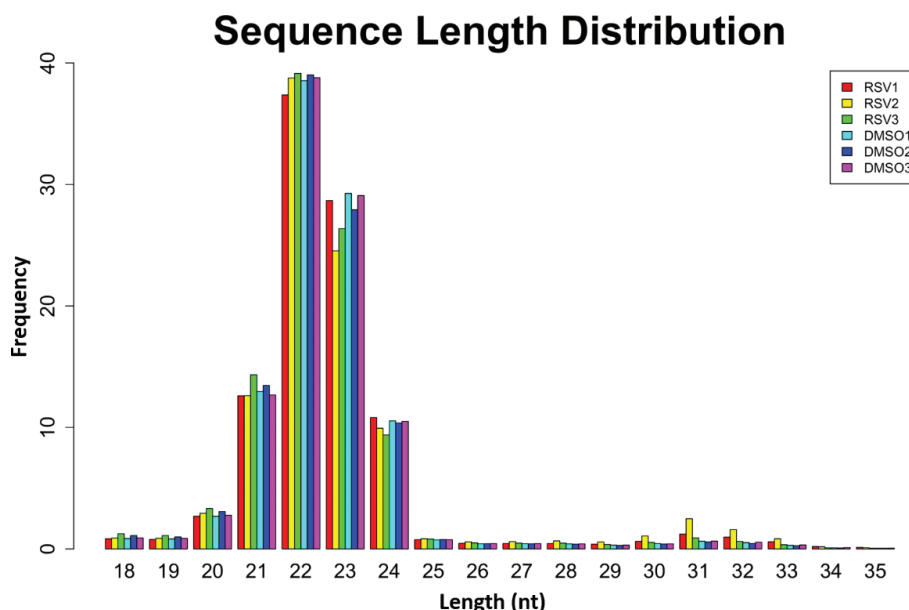
Fig. 1. Length distribution and frequency of the sequences in the six samples.

special response to RSV. Meanwhile, we validated six selected DE miRNAs (i.e. bta-miR-432, bta-miR-382, bta-miR-127, bta-miR-744, bta-miR-125b, and bta-miR-27b) by qRT-PCR. As shown in Fig. 2C and D, bta-miR-432, bta-miR-382, and bta-miR-127 were significantly upregulated, while the bta-miR-744, bta-miR-125b, and bta-miR-27b were significantly downregulated in the RSV group, which were completely consistent with the results of miRNA sequencing.

Target genes of differentially expressed microRNAs
A single miRNA can potentially target many functional genes, and vice versa that a single gene involved in muscle development can be targeted by many different miRNAs, so the mechanism of RSV stimulated of muscle development by miRNAs is complex (18). In this study, 11,494 target genes of RSV-regulated miRNAs were predicted using miRanda software. Figure S1A shows that 4,795 target genes were targeted by one specific miRNA, whereas 2,739 target genes were targeted by two miRNAs and 315 target genes were targeted by more than 10 miRNAs, which indicates their strong cooperative translational interaction (Fig. S1A). We found 173 miRNAs corresponding to target more than 10 genes (Fig. S1B) that also demonstrated the target genes multiplicity regulated by one miRNAs (18). For example, the upregulated DE miR-432 could target 23 genes (Supplementary file 2). Additionally, the downregulated DE bta-miR-744 targeted 1491 genes, including some key genes for myoblast differentiation, such as the myogenic differentiation 1 (*MYOD1*) and myogenic factor 6 (*MYF6*) (Supplementary file 2).

Significant enrichments of GO and KEGG pathway of target genes for differentially expressed microRNAs
We used 1,869 target genes of RSV-regulated DE miRNAs for GO enrichment analysis. It showed that the target genes were significantly enriched in stereocilium (GO:0032420) and stereocilium bundle (GO:0032421) cellular component (adjusted *P*-value < 0.05) (Supplementary file 3). The results of KEGG pathway analysis revealed five significant pathways (adjusted *P*-value < 0.05) (Supplementary file 3). Here, 25, 35, and 39 target genes were enriched in the top three significant pathways, which include insulin signaling pathway (bta04910), ras signaling pathway (bta04014), and mitogen-activated protein kinase (MAPK) signaling pathway (bta04010), respectively, indicating that RSV-regulated myoblast differentiation could be mainly mediated by these three pathways (Fig. 4).

Discussion

RSV addition benefits meat production and quality
Generally, three main factors are the genetic background of cattle breed, nutritional supply, and human management, which contribute to beef yield and beef quality by affecting the contents of muscle tissues and intramuscular fat (IMF) (24–26). Researchers place more emphasis on maintaining excellent genetic inherent characteristics for the traits and developing an optimal feed formula in economic animals (27).

RSV is an important phenolic compound present in the wine grape pomace (28). The wine grape pomace has been regarded as a bioactive dietary component in the diet of rumen animal because of its antioxidative ability (29).
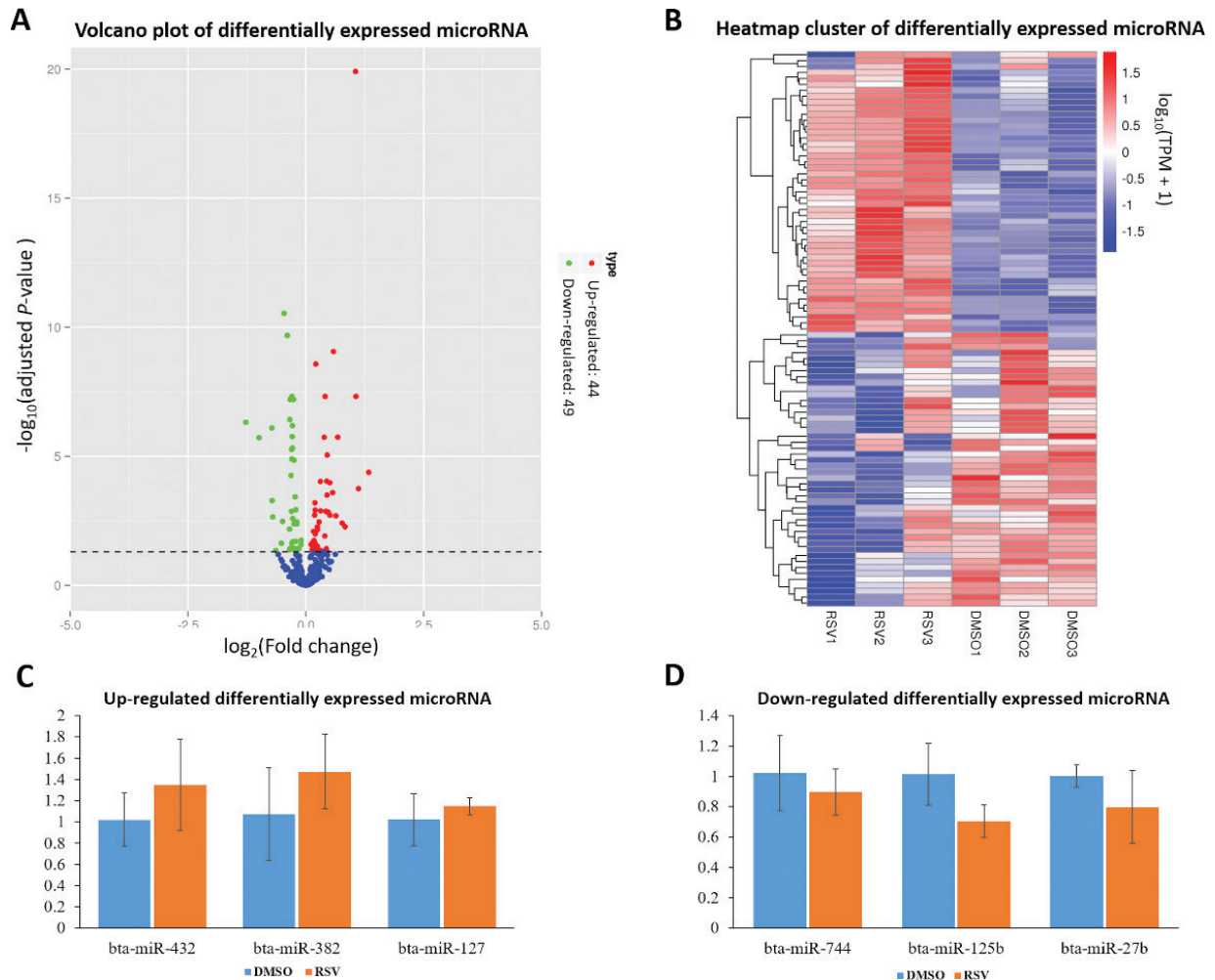
Fig. 2. Differentially expressed (DE) analysis of microRNAs (miRNAs). (A) Volcano plot of upregulated and downregulated DE miRNAs. (B) Cluster of DE miRNAs in three replicates of case and control groups. Note: the colors from blue to red indicate the miRNA expression levels from low to high after the transform of $\log_{10}(TPM + 1)$. RSV1, RSV2, and RSV3 indicate the case groups that were treated with resveratrol (RSV). DMSO1, DMSO2, and DMSO3 indicate the control groups that were only treated with DMSO with RSV. (C) qRT-PCR validation for upregulated DE miRNAs, including bta-miR-432, bta-miR-382, and bta-miR-127. (D) qRT-PCR validation for downregulated DE miRNAs, including bta-miR-744, bta-miR-125b, and bta-miR-27b.
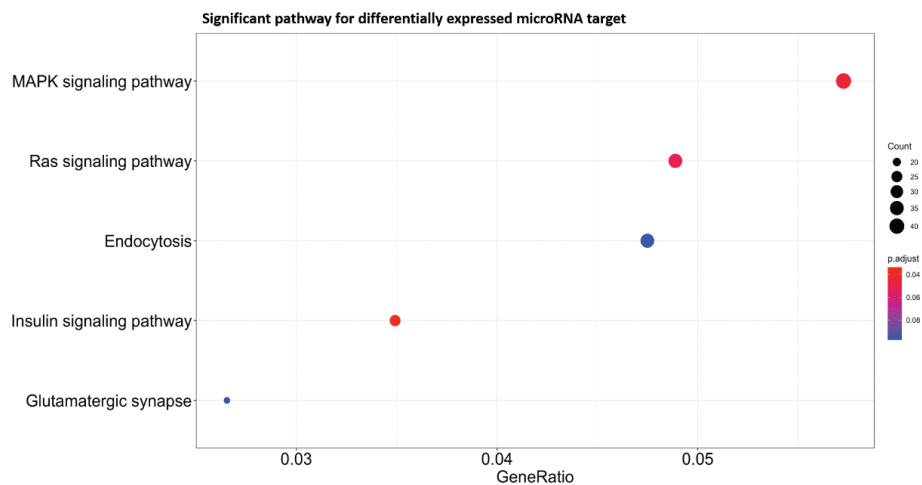

Fig. 3. Significant pathways for the targets of differentially expressed (DE) miRNAs.

Addition of wine grape pomace to animals benefits the feed efficiency and meat tenderness (29). In the previous studies, RSV has been reported to protect against impaired meat quality of heat-stressed and transport-stressed broilers (30, 31), and to improve the meat quality of growth-retarded pigs (32). The antioxidant ability of RSV also improved the flavor, texture, and color of fresh meat (33). RSV combined with short-term caloric restriction protected aging-induced muscle loss (11) and enhanced the effect of exercise in reversing sarcopenia (34).

RSV promotes myoblast differentiation through regulating microRNAs

According to Kaminski et al., 20 μM RSV had no significant toxic effect on C2C12 myoblast with less than 2% of apoptotic cells (10). Our previous study revealed that RSV (20 μM) regulated gene expressions at mRNA levels during the later differentiation period of primary bovine myoblast (13). In this study, we generated miRNA sequence data to demonstrate RSV effects on primary bovine myoblast differentiation using the same RSV treatment method. The results of this study showed that the most upregulated DE miRNA induced by RSV was bta-miR-34c (Table S3), which was also an DE miRNA in two developmental stages of cattle, that is, fetal and adult stage (35) and in both fast-type and slow-type cattle muscle tissues (36). Based on the miRbase database, the mature sequence of miR-34c is conserved among human, cattle, pig, and mouse species. Hsa-miR-34c was regarded as a tumor suppressor (37) and an inhibitor for osteoblast proliferation and differentiation (38). Mmu-miR-34c inhibited C2C12 myoblast proliferation but promoted C2C12 myoblast differentiation (39). Ssc-miR-34c has been confirmed to promote pig satellite cell differentiation by targeting Notch1 (40). It has been reported that RSV stimulated myogenin expression, enhanced the level of light-chain myosin in modulating cardiomyoblast differentiation (7), and promoted C2C12 myoblast differentiation (10). Thus, the upregulation of bta-miR-34c could act as an RSV-induced biomarker to promote primary bovine myoblast differentiation. Meanwhile, the addition of RSV could contribute to the skeletal muscle development in fetus and to subsequent beef production and quality by regulating the miRNA expressions.

Differentially expressed microRNAs serve as potential biomarkers for beef production and quality

MicroRNAs are increasingly used as potential biomarkers for human and animal diseases (41–43), as miRNAs are relatively stable than mRNAs under the defined conditions, especially for exposure to ribonucleases in vitro (44). The stability of miRNAs and its low cost of detections support their roles in a wide range of applications as biomarkers (44, 45).

Myoblasts differentiated into myofibers regulated by myogenic regulatory factors (i.e. Myf5, MyoD, and myogenin) can be divided into three main stages, namely, proliferation, differentiation, and cell fusion (46). A cultured primary bovine myoblast in vitro can simulate bovine embryonic muscle development. As the regulators for myogenesis, miRNAs play the functional roles in myoblast proliferation and differentiation by regulating the expressions of specific muscle development-related genes (47), which may ultimately influence beef production. The target genes of miRNAs participated in signaling pathways that finely regulate muscle and fat content, thus contributing to high beef quality, like the well-marbled beef (26, 48, 49). This study used 1,869 target genes for the 93 DE miRNAs that were mainly enriched in three significant KEGG pathways and two significant GO terms (Fig. 3). The top significant KEGG pathway, insulin signaling pathway (bta04910), was reported to regulate glucose uptake in skeletal muscle and improve muscle leanness (26). Phosphoenolpyruvate carboxykinase 1 gene (PCK1) is one of the target genes enriched in the insulin signalling pathway, and its expression level has been confirmed to positively associate with the IMF content, a key factor in evaluating beef quality (50). Another significant pathway is the MAPK signaling pathway, which played an important role in cell proliferation, differentiation, survival, and death (51). DE genes between well-marbled and lean-marbled beef were also enriched in MAPK signaling pathway (26). Moreover, five targets (metallothionein 3 gene (MT3), ghrelin obestatin prepropeptide gene (GHRL), insulin gene (INS), protein kinase C gamma (PRKCG), and neudesin neurotrophic factor (NENF)) were enriched in the biological process term regulation of response to food (GO:0031095) that were associated with body weight or appetite (52–54). Therefore, we suggested that the RSV-induced DE miRNAs could be used as biomarkers to improve beef production and quality through targeting the key genes that are essential for the important pathways related to muscle developments.

Bioinformatics serves as a potential tool in determining beef production and quality

The ability of RSV to delay cardiovascular alterations, as well as its positive influence on different cancer types, neurodegenerative events, and anti-inflammatory effects, by modulating various signal transduction cascades has been widely studied (20, 55, 56). This study mainly focused on transcriptomic analysis of effects of RSV on the growth and development of bovine skeletal muscle. In this study, we found that DE miRNAs could be considered as the potential biomarkers to monitor dietary or nutraceutical effects of RSV on bovine skeletal muscle growth and development. Obviously, bioinformatics application can be

extended to genomics, epigenomics, proteomics, metabolomics, and other omics data that can be also useful for the identifications of candidate biomarkers in beef production and quality (57). For example, genomic selection is used for cultivating high-yielding beef varieties (58), together with epigenomics (i.e. DNA methylation) analysis of the modification of gene expressions, to regulate cattle growth and beef quality (59, 60). Metabolomics data are strongly related to the animal cellular processes as the end products, so it could be used to monitor the cattle health and product quality (61, 62). The applications of bioinformatics are also beneficial for the research and development of new feed additives derived from natural products (63, 64), and to help detect and suppress outbreaks of foodborne viruses to ensure food quality (63, 64).

Conclusion

Overall, this study provided an improved understanding of the molecular mechanisms underlying the impact of RSV on miRNA expression to regulate primary bovine myoblast. The RSV-stimulated miRNAs could serve as potential biomarkers to monitor dietary or nutraceutical effects of RSV on skeletal muscle growth and development.

Acknowledgments

Competing interests and funding

Data availability

Not applicable.

Ethics and consent

All the animal procedures were carried out according to the protocols approved by the College of Animal Science and Technology, Northwest A&F University, China. All the experimental animals were approved by the Institutional Animal Care and Use Committee in the College of Animal Science and Technology, Northwest A&F University, China.

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