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RESEARCH ARTICLE

Identification of circulating miRNA involved in meat yield of Korean cattle

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Abstract

Cattle plays an important role in providing essential nutrients through meat production. Thus, we focused on epigenetic factors associated with meat yield. To investigate circulating miRNAs that are involved with meat yield and connect biofluids and *longissimus dorsi* (LD) muscle in Korean cattle, we performed analyses of the carcass characteristics, miRNA array, qPCR, and bioinformatics. Carcass characteristics relative to the yield grade (YG) showed that the yield index and rib eye area were the highest, whereas the backfat thickness was the lowest for YG A (equal to high YG) cattle among the three YGs. miRNA array sorted the circulating miRNAs that connect biofluids and LD muscle. miRNA qPCR showed that miR-15a ($r = 0.84$), miR-26b ($r = 0.91$), and miR-29c ($r = 0.92$) had positive relationships with biofluids and LD muscle. In YG A cattle, miR-26b was considered to be a circulating miRNA connecting biofluids and LD muscle because the target genes of miR-26b were more involved with myogenesis. Then, miR-26b-targeted genes, DIAPH3 and YOD1, were downregulated in YG A cattle. Our results suggest that miR-15a, miR-26b, and miR-29c are upregulated in biofluids and LD muscle, whereas DIAPH3 and YOD1 are downregulated in the LD muscle of finishing cattle steers.

Keywords: circulating miRNA; Korean cattle; *longissimus dorsi* muscle; meat yield; microarray

Introduction

Feeding trials, castration, and breeding are used to improve marbling in cattle. However, high marbling has a negative effect on the meat yield because of the increased backfat thickness (BF). Thus, recent studies have investigated genetic factors related to simultaneous improvement of the meat quality and yield. For example, rs41719435 in DNA-protein kinase (located on BTA14) was positively associated with fat thickness and yield grade (YG) (Rempel et al., 2012). It was also reported that polymorphisms in hormone-sensitive lipase and leptin are important factors for the carcass yield and meat quality in beef cattle (Fang et al., 2013; Tian et al., 2013). The carcass weight, a determining factor for the yield index (YI), may be related to the proliferation of myoblasts in cattle. Thus, the proliferation rates of myoblasts were shown to have a positive relationship with the live and carcass weight in cattle (Coles et al., 2015). These studies demonstrate that genetic factors and myogenesis could be important keys to obtaining a better meat yield in cattle.

Blood contains cells, plasma, protein, glucose, mineral ions, hormones, carbon dioxide, and exosomes. A recent study showed that peptides, lipids, microRNA (miRNA), mRNA, and DNA from exosomes may be useful biomarkers for determining cancer, inflammation, and certain cardiac disorders (Barteneva et al., 2013; Rak, 2013). Exosomal miRNAs can shuttle between cells to communicate and exchange genetic material (Zhao et al., 2015). In addition, a recent study showed that exosomal miRNA can move into

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Abbreviations: BF, backfat thickness; DIAPH3, diaphanous-related formin 3; GEO, Gene Expression Omnibus; KEGG pathway, Kyoto Encyclopedia of Genes and Genomes pathway; LD muscle, *longissimus dorsi* muscle; miRNA, microRNA; REA, rib eye area; RN18S1, 18S ribosomal RNA; SW, slaughter weight; YG, yield grade; YI, yield index; YOD1, YOD1 deubiquitinase

muscle cells to regulate gene expression. In particular, it was demonstrated that during myogenesis, exosomal miRNAs moved into cells, where they affected the Wnt signaling pathway (Forterre et al., 2014). Furthermore, miRNAs may regulate gene expression in muscle cells; thus, miR-23a was found to repress atrogin-1 and muscle RING-finger protein-1 in the muscle tissue (Hudson et al., 2014). It was also shown that miR-27a/b, miR-208a/b, and miR-499 can repress myostatin (Hitachi and Tsuchida, 2014), and that miR-151-3p can repress ATP2a2 in the slow muscle of C2C12 myotubes (Wei et al., 2014).

A recent study analyzed miRNAs in cattle, finding miR-1, miR-133a, miR-206, and miR-378 to be abundant in the *longissimus dorsi* (LD) muscle of Chinese Qinchuan beef cattle (Sun et al., 2014). In addition, the association between the genomic context characteristics of miRNAs and gene expression was analyzed using the subcutaneous adipose tissue of British-continental crossbred steers (Romao et al., 2014). Furthermore, the profiles of differentially expressed miRNAs and a target gene in the intramuscular and subcutaneous adipose tissues of adult beef cattle were determined using microarrays and bioinformatics (Wang et al., 2013). miRNAs can affect economic traits in farm animals. Thus, single nucleotide polymorphisms in miRNA target sites or miRNA gene promoters may contribute to variations in the production or health traits of farm animals (Wang et al., 2013). This evidence suggests that miRNA could not only regulate gene expression but also could be involved in the reproduction and health of cattle.

Nutrients from consumed feed enter target tissues and affect their gene expression, after which molecules or biofluids (miRNAs and peptides) are signaled to move into nearby cells. There have been no previous reports of circulating miRNAs moving into neighboring cells and affecting gene expression in the tissues of cattle, so this study focused on screening for circulating miRNAs involved with the meat yield in Korean cattle.

Materials and methods

Animal care and use

All of the experiments involving Korean cattle were approved by the Animal Care and Use Committee in Seoul National University (SNU-151102-1), Korea.

Sample collection

Blood samples (20 mL) were collected from the jugular veins of 31 Korean cattle steer (aged 29–30 months) at the National Agricultural Cooperative Federation, Gyeonggi-do. Samples of blood (5 mL) were then transferred to a Serum BD Vacutainer (TUBE SST II PLH 16 × 100 8.5 PLBL CE GLD) and a plasma BD Vacutainer (TUBE PST II PLH 16 × 100 8.0 PLBL L/GN). Each vacutainer tube was incubated at room temperature for 20 min and then centrifuged at 1,500*g* for 10 min. The supernatant (serum) was transferred into biofluids precipitation process immediately. LD muscle samples were collected immediately after slaughter at an Agricultural Cooperative's joint market in Eumseong, Chungcheongbuk-do. The LD muscle samples were preserved in Allprotect solution (Qiagen, USA).

Carcass characteristic analysis

We collected animal data from the Korea Institute for Animal Products Quality Evaluation (KAPE) website (http://www.ekape.or.kr). In Korea, the YI (YI, %) is based on the rib eye area (REA, cm^2), BF (mm), and the slaughter weight (SW, kg). YI determines the YG, and KAPE classifies the YG as A, B, and C grades. In this study, we denoted the A, B, and C grades as YG A (n = 10), YG B (n = 11), and YG C (n = 10), respectively, where YG A was above 67.5% of YI, YG B ranged from 62.7% to 67.5%, and YG C was below 62.7% of YI in Korean cattle. Thus, we analyzed the miRNAs based on the different YGs.

$$\begin{aligned}\text{Yield index}(\%) = 68.184 &- [0.625 \times \text{backfat thickness (mm)}] \\ &+ [0.13 \times \text{rib eye area } (cm^2)] \\ &- [0.024 \times \text{slaughter weight (kg)}]\end{aligned}$$

Total RNA extraction

Biofluids were precipitated using a miRCURY^{TM} exosome precipitation kit (Exiqon, Denmark). After trimming the fat tissue, the LD muscle was ground in liquid nitrogen for removing exonuclease such as DNase and RNase. Total RNA was extracted from the biofluid pellets and the ground tissue using Trizol LS reagent (MCB, USA). We crosschecked the amount of RNA by NanoDrop spectrophotometer (Thermo, USA) and Bioanalyzer 2100 (Agilent, USA). We detected an RNA peak at 25–200 nucleotides and an exosomal RNA amount range from 170 pg to 510 pg. We also performed a qPCR analysis of miR-93 (Bae et al., 2015) for validating the existence of miRNAs in the biofluids. LD muscle RNA contained 28S, 18S, and 5.8S ribosomal RNA peaks (Figure 1). Thus, RNA purity was standardized by as Eikman's protocols for extraction and preservation of mRNAs and miRNAs (Eikmans et al., 2013). We pooled the total RNA grouped by YG for the biofluids and LD muscle. Total RNA was stored at −70°C before miRNA analysis.

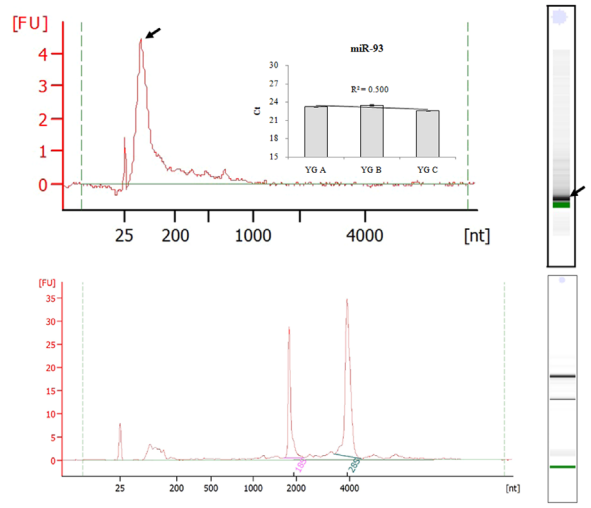

Figure 1 RNA preparation and validation of miRNA presence in the biofluids and LD muscle from Korean cattle. The exosomal RNA peak was detected from 50 nt to 200 nt (black arrow), while the LD muscle RNA had 28S, 18S, and 5.8S ribosomal RNA peaks. miR-93 expression was validated among YG A and YG B and YG C for identifying the existence of miRNA in the biofluid. Thus, miR-93 showed a similar expression pattern ($R^2 = 0.5$) in the biofluid of the three grade. LD muscle: longissimus dorsi muscle; YG A: yield grade A, YG B: yield grade B, YG C: yield grade C.

Bovine miRNA array

The miRNAs of six pooling samples grouped by YG for the biofluids and LD muscle were characterized by microarray. The Agilent bovine miRNA array comprised 806 miRNA groups from miRBase v21 (genome-build-id = UMD3.1, genome-build-accession = NCBI_Assembly: GCA_000003055.3). In total, 100 ng of RNA was incubated with 10 × calf intestinal phosphatase buffer, labeling spike-in, and calf intestinal phosphatase for 30 min at 37°C. The reaction mixture was then denatured and chilled with 100% dimethyl sulfoxide for 5 min at 100°C. A ligation reaction was performed using 10 × T4 RNA ligase buffer, cyanine3-pCp, and T4 RNA ligase for 2 h at 16°C. The unlabeled cyanine3-pCp was removed from the ligation mixture using a Micro Bio-Spin6 column (Bio-Rad, USA). Cyanine3-labeled complementary RNA was incubated with 10 × GE blocking agent and 2 × Hi-RPM hybridization buffer for 5 min at 100°C. The reaction mixtures were then hybridized with the bovine miRNA array (8× 60 K, Agilent) for 20 h at 55°C, after which the hybridized array was washed with gene expression wash buffers 1 and 2. The array image was scanned using an Agilent Scanner B. The signal density on the array was extracted using Feature extraction software, where the signal density was normalized using Genespring ver 13.0 (Agilent). Significant miRNAs were determined when the normalized values exceeded 1.

Bioinformatics analysis

Genes targeted by bovine miRNA were predicted using TargetScanHuman Release 6.2 (Lewis et al., 2005; Grimson et al., 2007; Friedman et al., 2009; Shin et al., 2010; Garcia et al., 2011; Nam et al., 2014; Agarwal et al., 2015), and the Gene Ontology and KEGG pathway were analyzed using Database for Annotation, Visualization, and Integrated Discovery (DAVID) Bioinformatics Resources v6.7 (Huang et al., 2009).

Mature miRNA qPCR

The bovine mature miRNA sequence was determined using the miRBase version 21 database. The bovine miRNA primers were designed using the miRprimer2 program (Busk, 2014). cDNA was synthesized from exosomal RNA using a miScript II RT Kit (Qiagen). miRNA expression was determined with a miScript SYBR Green PCR Kit (Qiagen) and RotorgeneQ. miR-93 (Bae et al., 2015) and RN18S1 were used as the reference miRNA of biofluids and LD muscle, respectively, because continuous miR-93 expression was validated in the blood of Korean cattle (Figure 1). The relative miRNA expression level was calculated using the $2^{-\Delta\Delta}$Ct method. The primers are listed in Table S1.

Quantitative reverse transcription PCR

RNA (1 μg) was reverse transcribed by high capacity cDNA reverse transcription (Applied Biosystems, USA) and PCR

Table 1 Comparison of the carcass characteristics grouped by the three yield grades in Korean cattle

Meat yield grade (number of animals)	YG A (n = 10)	YG B (n = 11)	YG C (n = 10)
Yield index* (%)	68.4 ± 0.4^{a}	64.9 ± 0.37^{b}	60.9 ± 0.4^{c}
Rib eye area (cm^2)	97.2 ± 2.3^{a}	95.2 ± 2.1^{ab}	89.0 ± 2.3^{b}
Backfat thickness (mm)	7.7 ± 0.63^{a}	12.5 ± 0.58^{b}	17.6 ± 0.63^{c}
Slaughter weight (kg)	449.5 ± 9.58	461.5 ± 8.75	460.0 ± 9.6

Different superscript letters indicate significant differences ($P < 0.05$) among the yield grades. Mean ± standard error. YG A: yield grade A; YG B: yield grade B; YG C: yield grade C.

*Yield index (%) = 68.184 − [0.625 × backfat thickness (mm)] + [0.13 × rib eye area (cm^2)] − [0.024 × slaughter weight (kg)].

was performed in duplicate using a QuantiNova SYBR Green PCR Kit (Qiagen, Hilden) with a Rotorgene Q PCR system (Qiagen). The relative abundances of specific messenger RNAs were calculated by normalization against that of *gapdh* and the relative mRNA expression level was calculated using the $2^{-\Delta\Delta}$Ct method. The primers are listed in Table S2.

Gene Expression Omnibus (GEO) database submission

The bovine miRNA expression datasets were submitted to GEO at the NCBI. The assigned GEO submission number is GSE73389 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE73389).

Statistical analysis

All of the statistical analyses were performed using SAS release 8.02 (SAS Institute, Inc., Cary, NC, USA). Differences in gene expression among three or more groups were tested by ANOVA. Pearson's correlation coefficient analysis was performed to test for linear relationships in the miRNA expression levels of biofluids and LD muscle. The data were expressed as the mean ± SEM.

Results

Carcass characteristics

We compared the YI, REA, BF, and SW for 31 Korean cattle steers relative to the YG. Cattle with YG A had a YI of 68.4% ± 0.94%, REA of 97.2 ± 8.02 cm^2, BT of 7.7 ± 1.16 mm, and SW of 449.5 ± 23.20 kg. Cattle with YG B had a YI of 64.9% ± 1.16%, REA of 95.2 ± 6.26 cm^2, BT of 12.5 ± 1.81 mm, and SW of 454.7 ± 38.20 kg. Cattle with YG C had a YI of 60.9% ± 1.58%, REA of 89.0 ± 6.46 cm^2, BT of 17.6 ± 2.72 mm, and SW of 460.0 ± 25.25 kg (Table 1). Thus, the carcass characteristics showed that YI and REA were the highest, whereas BF was the lowest for YG A. However, SW did not differ significantly among the three YGs.

Identification of circulating miRNAs connecting biofluids and LD muscle

We analyzed miRNAs from biofluids and LD muscle using a bovine miRNA array (Agilent), which included 809 probes. We then performed principal components analysis (PCA) to compare the miRNA expression patterns of YG A, YG B, and YG C. In both the biofluids and LD muscle, the expression pattern of YG A differed from that of both YG B and YG C (Figure 2a). Thus, the PCA analysis showed that the miRNA expression level depended on the YG. We considered a value greater than 1 to be a significant normalized miRNA value (Table 2).

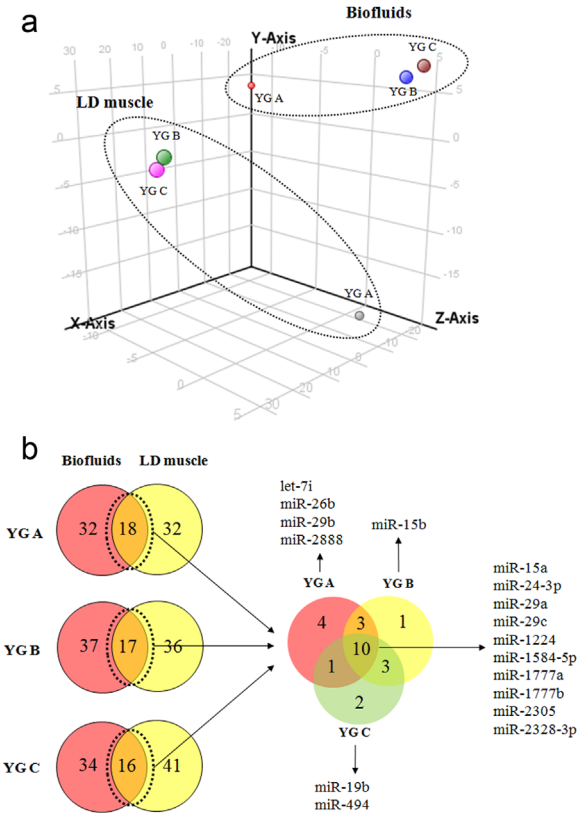

Figure 2 miRNA expression pattern analysis and Venn diagram analysis in the biofluids and LD muscle from Korean cattle. (a) Principal component analysis was performed to compare the miRNA expression patterns in YG A, YG B, and YG C. In both the biofluids and LD muscle, the expression pattern for YG A differed from that for both YG B and YG C. (b) We performed a Venn diagram analysis to determine the circulating miRNAs connecting the biofluids and LD muscle relative to the YG. Thus, we sorted common miRNAs that occurred in both the biofluids and LD muscle. We then performed a Venn diagram analysis of common miRNAs. The miRNAs associated with YG A were let-7i, miR-26b, miR-29b, and miR-2888; that associated with YG B was miR-15b; and those associated with YG C were miR-19b and miR-494. The common miRNAs were miR-15a, miR-24-3p, miR-29a, miR-29c, miR-1224, miR-1584-5p, miR-1777a, miR-1777b, miR-2305, and miR-2328-3p. LD muscle: longissimus dorsi muscle; YG A: yield grade A, YG B: yield grade B, YG C: yield grade C.

We performed a Venn diagram analysis to determine the circulating miRNAs connecting the biofluids and LD muscle relative to the YG, where we sorted the common miRNAs that occurred in both the biofluids and LD muscle. We then performed a Venn diagram analysis of the common miRNAs. The miRNAs related to YG A were let-7i, miR-26b, miR-29b, and miR-2888; miR-15b was related to YG B; and miR-19b and miR-494 were related to YG C. The common miRNAs were miR-15a, miR-24-3p, miR-29a, miR-29c, miR-1224, miR-1584-5p, miR-1777a, miR-1777b, miR-2305, and miR-2328-3p (Figure 2b).

Table 2 Profiles of biofluid and LD muscle miRNA in Korean cattle

	miRNA name*	
Meat yield grade	Biofluids	LD muscle
YG A	miR-15a, -15b, -16b, -19a, -19b, -20a, -21-5p, -23a, -24-3p, -25, -26b, -27a-3p, -27b, -29a, -29b, -29c, -92a, -101, -106b, -122, -142-5p, -223, -451, -486, -671, -1224, 1246, -1343-5p, -1584-5p, -1777a, -1777b, -1835, -2305, -2316, -2328-3p, -2348, -2374, -2389, -2412, -2455, -2881, -2887, -2888, -2893, -3141, -3154, -6528, -7865, -8550, let-7i	miR-1, -10b, -15a, -22-3p, -23a, -23b-3p, -24-3p, -26a, -26b, -27b, -29a, -29b, -29c, -30b-5p, -30c, -98, -101, -125b, -126-3p, -133a, -133b, 151-5p, -193a-3p, -199a-3p, -206, -331-3p, -362-5p, -365-3p, -378b, -378c, -499, -1224, -1260b, -1584-5p, -1777a, -1777b, -2305, -2328-3p, -2478, -2888, -2893, -3613b, let-7a-5p, let-7b, -let-7c, let-7d, let-7e, let-7f, let-7g, let-7i,
YG B	miR-15a, -15b, -16b, -19a, -19b, -20a, -23a, -24-3p, -25, -27a-3p, -27b, -29a, -29c, -92a, -122, -140, -106b, -142-5p, -223, -451, -486, -494, -685_v14.0, -1224, -1246, -1260b, -1343-5p, -1584-5p, -1777a, -1777b, -1835, -2305, -2309, -2316, -2328-3p, -2348, -2374, -2389, -2412, -2413, -2428, -2455, -2478, -2881, -2887, -2888, -2892, -2893, -3141, -3154, -4444, -6528, -7865, -8550	miR-1, -10b, -15a, -15b, -22-5p, -23a, -23b-3p, -24-3p, -26a, -26b, -27a-3p, -27b, -29a, -29b, -29c, -30b-5p, -30c, -99a-5p, -100, -101, -125b, -126-3p, -128, -133a, -133b, -145, -151-5p, -199a-3p, -206, 331-3p, -365-3p, -378b, -378c, -486, -499, -1224, -1260b, -1584-5p, -1777a, -1777b, -2305, -2328-3p, -2478, -2893, -3613b, -4286, let-7a-5p, let-7b, let-7c, -let-7d, let-7f, let-7g, let-7e, let-7i,
YG C	miR-15a, -15b, -16b, -19a, -19b, -20a, -24-3p, -25, -29a, -29c, -92a, -101, -106b, -122, -140, 142-5p, -185, -223, -451, -486, -494, -685_v14.0, -1224, -1246, -1260b, -1343-5p, -1584-5p, -1777a, -1777b, -1835, -2305, -2309, -2316, -2328-3p, -2348, -2374, -2389, -2412, -2413, -2428, -2455, -2478, -2881, -2887, -2888, -2893, -3141, -6528, -7865, -8550	miR-1, -10b, -15a, -19b, -22-5p, -23a, -23b-3p, -24-3p, -26a, -26b, -27a-3p, -27b, -29a, -29b, -29c, -30b-5p, -30c, -30d, -99a-5p, -100, -101, -125b, -126-3p, -128, -133a, -133b, -145, -148a, -151-5p, -193a-3p, -195, -199a-3p, -206, -331-3p, -365-3p, -378b, -378c, -486, -494, -499, -1224, -1260b, -1584-5p, -1777a, -1777b, -2305, -2328-3p, -2478, -3613b, -4286, let-7a-5p, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let-7i

YG A: yield grade A; YG B: yield grade B; YG C: yield grade C; LD muscle: *longissimus dorsi* muscle.
*The unique miRNAs were considered that normalized value was above 1.

Table 3 Mature miRNA qPCR analysis of biofluids and LD muscle from Korean cattle

	Biofluids			LD muscle			
miRNA name	YG A	YG B	YG C	YG A	YG B	YG C	Correlation*
let-7i	0.7 ± 0.05	1.0 ± 0.11	0.7 ± 0.07	139.1 ± 28.11^{a}	1.0 ± 0.48^{b}	2.3 ± 0.83^{b}	−0.61
miR-26b	4.2 ± 0.25^{a}	1.6 ± 0.19^{b}	1.0 ± 0.10^{c}	61.6 ± 12.56^{a}	1.0 ± 0.12^{b}	1.2 ± 0.20^{b}	0.91
miR-29b	1.9 ± 0.04	2.5 ± 0.09	1.0 ± 0.08	15.4 ± 3.22^{a}	1.2 ± 0.08^{b}	1.0 ± 0.16^{b}	−0.83
miR-2888	2.4 ± 0.76	1.9 ± 1.28	1.0 ± 0.05	320.7 ± 75.85^{a}	1.0 ± 0.38^{b}	2.1 ± 0.70^{b}	0.51
miR-15b	1.9 ± 0.87	1.0 ± 0.28	2.1 ± 1.49	163.1 ± 7.2^{a}	1.0 ± 1.07^{b}	2.0 ± 1.22^{b}	0.37
miR-19b	1.1 ± 0.47	2.1 ± 0.65	1.0 ± 0.70	14.8 ± 0.29^{a}	1.0 ± 0.33^{b}	1.3 ± 0.11^{b}	−0.67
miR-494	4.0 ± 5.16	1.0 ± 1.22	7.3 ± 5.33	70.0 ± 12.38^{a}	1.2 ± 2.62^{b}	1.0 ± 0.49^{b}	0.28
miR-15a	2.8 ± 0.77^{a}	1.1 ± 0.09^{b}	1.0 ± 0.35^{b}	18.9 ± 4.50^{a}	1.5 ± 0.16^{b}	1.0 ± 0.16^{b}	0.84
miR-24-3p	1.3 ± 0.07	1.5 ± 0.17	1.0 ± 0.08	48.7 ± 15.92^{a}	1.0 ± 0.27^{b}	2.0 ± 0.29^{b}	−0.48
miR-29a	1.2 ± 0.03	1.0 ± 0.14	1.1 ± 0.04	63.6 ± 20.25^{a}	1.0 ± 0.27^{b}	3.3 ± 1.86^{b}	0.58
miR-29c	3.6 ± 1.30^{a}	1.0 ± 0.12^{b}	1.0 ± 0.36^{b}	25.7 ± 5.32^{a}	1.0 ± 0.05^{b}	1.3 ± 0.25^{b}	0.92
miR-1224	1.0 ± 0.72	1.0 ± 2.23	5.7 ± 5.53	ND	ND	ND	–
miR-1584-5p	2.3 ± 1.10	4.2 ± 1.34	1.0 ± 0.28	ND	ND	ND	–
miR-1777a	1.8 ± 0.09	1.3 ± 0.29	1.0 ± 0.07	ND	ND	ND	–
miR-1777b	1.1 ± 0.48	1.0 ± 0.12	1.1 ± 0.79	179.8 ± 19.46^{a}	1.0 ± 0.24^{b}	1.9 ± 0.36^{b}	0.06
miR-2305	2.1 ± 0.26	1.0 ± 0.13	1.4 ± 0.07	ND	ND	ND	–
miR-2328-3p	2.1 ± 0.66	1.7 ± 0.20	1.0 ± 0.26	ND	ND	ND	–

ND: not detected; YG A: yield grade A; YG B: yield grade B; YG C: yield grade C; LD muscle: *longissimus dorsi* muscle.
*The degree of association was measured by the correlation coefficient. Different superscript letters indicate significant differences ($P < 0.05$) among the yield grades.

Validation of circulating miRNAs by mature miRNA qPCR

We validated the miRNAs by mature miRNA qPCR analysis of the biofluids and LD muscle (Table 3). We also performed a correlation analysis based on the miRNA expression patterns in the biofluids and LD muscle. In both the biofluids and LD muscle, the expression levels of miR-15a, miR-26b, and miR-29c were highest in YG A. Correlation analysis detected a linear relationship between miR-15a ($r = 0.84$), miR-26b ($r = 0.91$), and miR-29c ($r = 0.92$) with the biofluids and LD muscle.

Bioinformatics analysis of circulating miRNA target genes

We performed TargetScanHuman and DAVID analysis to predict the genes and biological processes targeted by the circulating miRNA. We also analyzed the Gene Ontology and KEGG pathways of the targeted genes (Table 4). Targeted genes for miR-15a were BCL2L2, DLEU7, TRIAP1, HSPA4L, FGF7, SULT1B1, TTC14, CNIH3, and ATP1B4. Targeted genes for miR-29c were TIMM8B, SYPL2, TRAF4, and BCL2L10. But, biological function of targeted genes for miR-15a and miR-29c was not analyzed because of the low abundance for genes. Thus, Gene Ontology and KEGG pathway for the targeted genes of miR-26b was performed.

Gene Ontology analysis identified the following biological processes ($P < 0.01$): protein folding, proteolysis involved in cellular protein catabolic process, protein catabolic process, cellular protein catabolic process, modification-dependent protein catabolic process, modification-dependent macromolecule catabolic process, glycoprotein biosynthetic process, glycoprotein metabolic process, small GTPase-mediated signal transduction, cellular macromolecule catabolic process, and macromolecule catabolic process. The KEGG pathway analysis showed regulation of the actin cytoskeleton and biosynthesis of unsaturated fatty acids. Thus, we performed qPCR analysis of the miRNA target genes, including regulation of actin cytoskeleton and biosynthesis of unsaturated fatty acids.

Table 4 Gene Ontology and KEGG pathway analysis of the genes targeted by miR-26b

Term	*P*-value*
Biological process	
GO:0006457, protein folding	0.001
GO:0051603, proteolysis involved in cellular protein catabolic process	0.001
GO:0030163, protein catabolic process	0.001
GO:0044257, cellular protein catabolic process	0.001
GO:0019941, modification-dependent protein catabolic process	0.002
GO:0043632, modification-dependent macromolecule catabolic process	0.002
GO:0009101, glycoprotein biosynthetic process	0.005
GO:0009100, glycoprotein metabolic process	0.006
GO:0007264, small GTPase mediated signal transduction	0.007
GO:0044265, cellular macromolecule catabolic process	0.008
GO:0009057, macromolecule catabolic process	0.010
KEGG pathway	
hsa04810: Regulation of actin cytoskeleton	0.039
hsa01040: Biosynthesis of unsaturated fatty acids	0.040

*The *P*-value denotes enrichment for targeted genes involved in the indicated biological processes and KEGG pathways. A smaller *P*-value represents greater enrichment.

Validation of circulating miRNA target genes

We performed qPCR analysis of the target genes for miR-26b in LD muscle from finishing Korean cattle steers. The qPCR analysis showed that the expression levels of the diaphanous-related formin 3 (DIAPH3) and YOD1 deubiquitinase (YOD1) genes were lower ($P < 0.05$) in YG A than YG C, whereas the expression levels of the slingshot protein phosphatase 3 (SSH3) and p21 protein-activated kinase 2 (PAK2) genes were higher ($P < 0.05$) in YG A than YG C (Table 5).

Discussion

The relationship between meat quality and the YG has been reported previously in Korean cattle. For example, carcass assessments for Hanwoo bulls, cows, and steers showed that the meat quality increased as the YG decreased (Park et al., 2002). However, a decrease in the YG had a negative effect on the efficiency of beef production and the beef price. Thus, we investigated circulating miRNAs connecting biofluids and LD muscle to identify an epigenetic factor related to improved meat yield in cattle.

Microarray and miRNA qPCR analyses verified a circulating miR-26b between biofluids and LD muscle in finishing Korean cattle steer. There have been no reports of the functions of miR-26b in cattle. However, in other species, including humans and the mouse, it was reported that miR-26b may be involved in cell maintenance functions, such as apoptosis, adipocyte differentiation, cell cycle, inflammatory response, and glucose uptake. In particular, the suppression of miR-26b protects cardiomyocytes from hypoxia-induced apoptosis (Wang et al., 2015). Moreover, miR-26b promotes adipocyte differentiation and attenuates cell proliferation by arresting the G1/S transition (Xu et al., 2015). It has been shown that miR-26b also participates in the inflammatory response of lipopolysaccharide-stimulated bovine alveolar macrophages by modulating the NF-κB pathway via the targeting of phosphatase and tensin homolog (Zhang et al., 2015). Additionally, miR-26b promotes the insulin-stimulated uptake of glucose and increases insulin-stimulated

Table 5 qPCR analysis of genes targeted by miR-26 in LD muscle of Korean cattle

Gene symbol	Gene name	YG A	YG C	*P*-value
Regulation of actin cytoskeleton				
FGF7	Fibroblast growth factor 7	1.6 ± 0.29	1.0 ± 0.58	0.271
SSH3	Slingshot protein phosphatase 3	2.4 ± 0.01	1.0 ± 0.14	0.016
vMRAS	Muscle RAS oncogene homolog	1.1 ± 0.29	1.0 ± 0.33	0.887
DIAPH3	Diaphanous-related formin 3	0.4 ± 0.10	1.0 ± 0.40	0.011
IQGAP2	IQ motif containing GTPase activating protein 2	2.1 ± 0.52	1.0 ± 0.52	0.201
ITGA10	Integrin, alpha 10	0.9 ± 0.24	1.0 ± 0.31	0.860
ABI2	Abl-interactor 2	0.5 ± 0.05	1.0 ± 0.09	0.612
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog	1.1 ± 0.15	1.0 ± 0.36	0.802
PAK2	p21 protein (Cdc42/Rac)-activated kinase 2	1.2 ± 0.01	1.0 ± 0.05	0.041
CHRM3	Cholinergic receptor, muscarinic 3	1.3 ± 0.26	1.0 ± 0.38	0.418
CFL2	Cofilin 2 (muscle)	1.1 ± 0.04	1.0 ± 0.03	0.081
PIK3R3	Phosphoinositide-3-kinase, regulatory subunit 3	1.1 ± 0.11	1.0 ± 0.23	0.611
Biosynthesis of unsaturated fatty acids				
ACOX1	Acyl-CoA oxidase 1, palmitoyl	1.1 ± 0.22	1.0 ± 0.05	0.632
PTPLA	Protein tyrosine phosphatase-like (proline instead of catalytic arginine), member A	1.2 ± 0.08	1.0 ± 0.15	0.226
YOD1	YOD1 deubiquitinase	0.6 ± 0.22	1.0 ± 0.11	0.020

Mean ± standard error. YG A: yield grade A; YG C: yield grade C.

glucose transporter type 4 translocation to the plasma membrane in human mature adipocytes (Xu et al., 2015). Therefore, these results suggest that miR-26b may be involved with cell maintenance in the skeletal muscle of cattle.

qPCR analysis for miRNA-target genes showed that DIAPH3 and YOD1 were downregulated in LD muscle of cattle with YG A. A recent study showed that the microtubule polymerization state controls myofibroblast differentiation by regulating the localization of DIAPH3 (Sandbo et al., 2013). In addition, it has been shown that YOD1 is involved in protein homeostasis via deubiquitinating enzymes by conjugating p97 in the endoplasmic reticulum (Ernst et al., 2009; Liu and Ye, 2012). Overall, our results suggest that miR-26b may participate in myofibroblast and protein homeostasis by regulating DIAPH3 and YOD1 in LD muscle of finishing cattle.

Conclusions

This study is the first to detect the presence of a circulating miRNA in bovine LD muscle and to identify the corresponding target genes. We also suggest that this circulating miRNA may act by regulating the transcriptome of LD muscle in finishing Korean cattle steer. Therefore, this circulating miRNA could be used as an biomarker to improve the meat yield in cattle.

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Authors' contributions

Surim Lee, Seungju Park, Jaekyoung Cheong, Jongyoul Ko, Jinjong Bong, and Myunggi Baik contributed to data acquisition. Jinjong Bong contributed to data mining, bioinformatic analyses, and statistical analysis. Jinjong Bong contributed to interpretation of results and drafted the manuscript. All authors approved the final version of the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Table S1. List of mature miRNA primers employed in this study.

Table S2. List of qPCR primers used for miR-26b-targeted genes.