Mar 17, 2025

Screening and validation of key microRNAs regulating muscle development in Hanper Sheep

PLOS One

DOI
https://dx.doi.org/10.17504/protocols.io.5qpvoox5xv4o/v1

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DOI: https://dx.doi.org/10.17504/protocols.io.5qpvoox5xv4o/v1

External link: https://doi.org/10.1371/journal.pone.0325054

Protocol Citation: yunxia zhi, Boxin Hu, Shujun Tian, Ying Bai, Xiaoyong Chen 2025. Screening and validation of key microRNAs regulating muscle development in Hanper Sheep. **protocols.io** https://dx.doi.org/10.17504/protocols.io.5qpvoox5xv4o/v1

Manuscript citation:
Zhi Y, Hu B, Tian S, Bai Y, Chen X (2025) Screening and validation of key microRNAs regulating muscle development in Hanper sheep. PLOS One 20(6). doi: 10.1371/journal.pone.0325054

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Protocol status: Working
We use this protocol and it's working

Created: March 17, 2025

Last Modified: March 17, 2025

Protocol Integer ID: 124464

Keywords: muscle development, functional enrichment, microRNA, Hanper sheep, muscle development in hanper sheep background, longissimus dorsi muscle tissue of hanper sheep, meat quality in sheep, muscle fiber, expressed mirna, identifying mirna, essential for muscle growth, discovery of new mirna, longissimus dorsi muscle tissue, diameter of muscle fiber, meat characteristic, influence muscle development, muscle fiber diameter, regulating muscle development, muscle fiber area, muscle growth, cellular mechanisms behind muscle growth, validation of key microrna, genetic quality of livestock, longissimus dorsi of hanper sheep, hanper sheep, mirna expression pattern, mirna, hanper sheep background, improving meat quality, key microrna, new mirna, economic significance of meat characteristic, livestock, muscle, meat quality, sheep, biological functions of these target gene, underpinning muscle, key pathways such as tgf, bioinformatics tool, target gene, target mrna, first time in sheep

Funders Acknowledgements:
Talents Introduction Fund of Hebei Agricultural University
Grant ID: YJ201938

Abstract

Background/Objectives: In sheep farming, the economic significance of meat characteristics is substantial, and advancing the genetic quality of livestock relies heavily on understanding the cellular mechanisms behind muscle growth and its regulation. This study examined miRNA expression patterns in the longissimus dorsi muscle tissue of *Hanper* sheep of various ages, with the goal of determining their biological functions and identifying miRNAs and their target mRNAs that influence muscle development and meat quality. Methods: Using the Image-Pro Plus 6.0 program and HE and fluorescent staining procedures, we measured the diameter of muscle fibers in the longissimus dorsi of *Hanper* sheep at three distinct ages (1, 7, and 13 months) in order to calculate the average fiber size. For the analysis of muscle fiber area, one-way ANOVA was conducted using SPSS 25.0, with LSD tests applied afterward to compare the different groups. Transcriptome sequencing was conducted to identify miRNAs, and bioinformatics tools were applied to predict their target genes. GO and KEGG functional annotations were used to analyze the biological functions of these target genes. RT-qPCR was performed to validate the expression levels of differentially expressed miRNAs. Results: Muscle fiber diameter and area increased progressively with age, as indicated by HE and fluorescence staining. Four novel miRNAs identified for the first time in sheep were among the 116 differentially expressed miRNAs that were found. These miRNAs were found to be involved in key pathways such as TGF-β, mTOR, Wnt, and MAPK, which regulate muscle growth and development. It was determined that three new miRNA-mRNA pairs—oar-miR-133/*MSC*, oar-miR-148a/*FST*, and oar-miR-410-3p/*NIN*—may be essential for muscle growth. RT-qPCR results confirmed the expression trends observed in the transcriptome sequencing data. Conclusions: Our knowledge of the fundamental molecular mechanisms underpinning muscle growth and development is improved by the discovery of new miRNAs and the target genes that correspond to them. These findings may serve as new breeding targets for improving meat quality in sheep.

Materials

	A	B	C	D	E
	Category	Item	Brand / Supplier	Catalog Number	Notes
	Sample collection	Hanper rams (1, 7, 13 months old)	Hebei Liansheng Agricultural Development Co., Ltd.	-	Healthy, similar body weight, no genetic kinship
		Liquid nitrogen	General Laboratory Supply	-	For rapid freezing of muscle samples
		Ultra-low temperature freezer (-80°C)	-	-	For sample storage
		Slaughter operation standard	NY/T 3469-2019	-	Humane slaughter of livestock
	Measurement of muscle fiber area	4% Neutral paraformaldehyde solution	Servicebio or equivalent	G1101	Tissue fixation
		Paraffin wax	Leica Biosystems or equivalent	-	Embedding fixed tissue
		Microtome (5 µm slices)	Leica or equivalent	-	Tissue sectioning
		Hematoxylin and Eosin (HE) staining kit	Servicebio or equivalent	G1120	Includes all HE staining reagents
		Neutral resin mounting medium	Servicebio or equivalent	G8590	For slide sealing
		Microscope (15×40 magnification)	Olympus / Nikon / Leica	-	Observation and image capture
		Eyepiece micrometer	-	-	Fiber diameter measurement
		Image-Pro Plus software (Version 6.0)	Media Cybernetics, USA	-	Image analysis

	A	B	C	D	E
		SPSS statistical software (Version 25.0)	IBM	-	Statistical analysis
	Total RNA extraction and miRNA sequencing	TRIzol Reagent Kit	Invitrogen, Carlsbad, CA, USA	15596026	Total RNA extraction
		1% Agarose gel electrophoresis reagents	Sangon Biotech / Thermo Fisher	-	RNA integrity check
		NanoPhotometer‰ spectrophotometer	IMPLEN, CA, USA	-	RNA purity detection
		Qubit‰ RNA Assay Kit	Life Technologies, CA, USA	Q32852	RNA concentration measurement
		Qubit‰ 2.0 Fluorometer	Life Technologies, CA, USA	Q32866	For RNA quantification
		RNA Nano 6000 Assay Kit	Agilent Technologies, CA, USA	5067-1511	RNA integrity evaluation
		Agilent Bioanalyzer 2100 system	Agilent Technologies, CA, USA	G2939BA	RNA quality assessment
	Small RNA Library Preparation & Sequencing	NEBNext‰ Multiplex Small RNA Library Prep Set for Illumina‰	NEB, USA	E7560S	Small RNA library construction
		High Sensitivity DNA Chips	Agilent Technologies, CA, USA	5067-4626	Library quality check
		cBot Cluster Generation System	Illumina	-	Library clustering
		TruSeq SR Cluster Kit v3-cBot-HS	Illumina	GD-401-3001	Cluster generation

	A	B	C	D	E
		Illumina HiSeq 2500 Sequencing Platform	Illumina	-	50 bp single-end sequencing
	Data processing	Bowtie (v1.2.3)	SourceForge	-	Small RNA sequence alignment
		mirdeep2	GitHub	-	Known/novel miRNA prediction
		miREvo	GitHub	-	Novel miRNA prediction
		srna-tools-cli	GitHub	-	Small RNA annotation
		MiRBase (v20.0)	-	-	Known miRNA database
		RepeatMasker & Rfam databases	-	-	Non-miRNA filtering
		miRanda	-	-	Target gene prediction
		TargetScan	-	-	Target gene prediction (cross-validation)
	Differential expression miRNA analyses and functional pathway enrichment	DESeq (R package v1.8.3)	Bioconductor	-	Differential expression analysis
		GOseq	Bioconductor	-	GO enrichment analysis
		KOBAS	http://kobas.cbi.pku.edu.cn/	-	KEGG pathway enrichment analysis
		Cytoscape	https://cytoscape.org/	-	miRNA-mRNA interaction network
	RT-qPCR Validation	M5 miRNA cDNA Synthesis Kit	Mei5 Biotechnology Co., Ltd.	M51103-500	miRNA reverse transcription

	A	B	C	D	E
		M5 miRNA qPCR Assay Kit	Mei5 Biotechnology Co., Ltd.	M52002-500	qPCR detection for miRNA
		ABI Prism 7500 Real-Time PCR System	Thermo Fisher Scientific, USA	4351104	qPCR amplification
		RNase-free water	Thermo Fisher Scientific, USA	AM9937	PCR-grade water
		Primers (Forward & Reverse)	-	See Table 1	Specific to each miRNA and U6 (reference)

Table 1. Primer sequences utilized in quantitative fluorescence PCR.

	A	B	C
	Primer	**Primer sequence（5'to3'）**	**Length, nt**
	oar-miR-133	TTGGTCCCCTTCAACCAGCTGT	22
	oar-miR-148a	TCAGTGCACTACAGAACTTTGT	22
	oar-miR-191	CAACGGAATCCCAAAAGCAGCT	22
	oar-miR-27a	TTCACAGTGGCTAAGTTCCGC	21
	oar-miR-30a-5p	TGTAAACATCCTCGACTGGAAGC	23
	oar-miR-3957-3p	ACGCACAGCACCTCACTGAGCT	22
	oar-miR-410-3p	AATATAACACAGATGGCCTGT	21
	oar-miR-541-5p	AAAGGATTCTGCTGTCGGTCCC	25

	A	B	C
		ACT	
	U6	AGTGCAGGGTCCGAGGTATT	20

Sample collection

1 In this study, we selected three uncastrated Hanper rams at three different developmental stages (1 month, 7 months, and 13 months old “M1, M7, and M13”) from the Hebei Liansheng Agricultural Development Co., Ltd. sheep farm. At each stage, three rams with similar body weights, good health status, and no genetic kinship were chosen. To ensure animal welfare during transport, the rams underwent a pre-shipment
quarantine in a separate area. Careful handling practices were implemented throughout the journey, avoiding excessive confinement and unnecessary force to mitigate both stress responses and injuries. After arriving at the slaughterhouse, the sheep were humanely slaughtered according to the “Livestock and Poultry Slaughtering Operation Regulations for Sheep” (NY/T 3469-2019). Following the butchering process, longissimus dorsi muscle specimens were promptly excised and subjected to rapid freezing using liquid nitrogen. To maintain sample integrity, they were later transferred to an ultra-low temperature environment of -80°C for preservation.

Measurement of muscle fiber area

2 The methods of fluorescence staining and HE staining were applied. The longissimus dorsi muscle tissue was maintained in a 4% neutral paraformaldehyde solution prior to being stained with HE staining, following Guo's protocol [1]. Briefly, 5μm-thick paraffin slices were created by removing the fixed muscle tissue. Fix a small piece of tissue with wax, cut it into thin slices with a slicer, then per-form HE staining, staining steps are dewaxing, covering water, hematoxylin staining, 5% acetic acid differentiation, returning blue, eosin staining, dehydration, dropping neutral resin. The stained samples were viewed under a microscope with a 15×40 zoom, using an eyepiece micrometer for accurate measurements. Fifteen images were captured from diverse, brightly lit areas across different focal points. Measuring their diameter and calculating the average using Image-Pro Plus 6.0 software. Data related to the muscle fiber areas were processed with SPSS 25.0 software, employing a one-way analysis to assess variance. Multiple comparisons were conducted using the LSD method to pinpoint any notable differences between the groups.

Total RNA extraction and miRNA sequencing

3 Following the comprehensive directions for the process, the Trizol reagent Kit (Invitrogen, Carlsbad, CA, USA) was used to extract RNA from longissimus dorsi muscle tissue. RNA degradation and contamination were assessed using 1% agarose gel electrophoresis. RNA purity was determined with a NanoPhotometer®

spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using the Qubit® RNA Assay Kit on a Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was evaluated with the RNA Nano 6000 Assay Kit on the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). For each sample, 3 μg of total RNA was used as the starting material for small RNA library construction. Libraries were prepared using the NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA.), following the manufacturer's recommended protocol, with index sequences added to distinguish between different samples. Finally, library quality was assessed on the Agilent Bioanalyzer 2100 system using High Sensitivity DNA Chips. Clustering of the index-coded samples was performed on a cBot Cluster Generation System using the TruSeq SR Cluster Kit v3-cBot-HS (Illumina), according to the manufacturer's instructions. After cluster generation, sequencing of the prepared libraries was carried out on the Illumina HiSeq 2500 platform, generating 50 bp single-end reads.

Data processing

4 After quality control, sequences within a specific length range were selected from the clean reads for subsequent analyses. Bowtie (version 1.2.3) [2]was used to align small RNA tags to the reference genome (Ovis aries Oar_v4.0) with no mismatches allowed. The small RNA sequences mapped to the genome were used for the identification of known miRNAs. MiRBase 20.0 was used as the reference database, and miRNA prediction along with secondary structure visualization was performed using mirdeep2 [3] and srna-tools-cli. The first nucleotide bias of miRNAs with specific lengths and the base preference at each position of all miRNAs were analyzed. To eliminate small RNA tags originating from protein-coding genes, repetitive sequences, rRNA, tRNA, snRNA, and snoRNA, the small RNA tags were aligned to the RepeatMasker database, Rfam database, or species-specific datasets. The hairpin structure of miRNA precursors was used to predict novel miRNAs. In this study, a combination of miREvo [4] and mirdeep2 [3] was used to predict unannotated small RNA tags by analyzing secondary structures, Dicer cleavage sites, and minimum free energy (MFE). Custom scripts were used to calculate the counts of the identified miRNAs, and both the first nucleotide bias for miRNAs of specific lengths and the nucleotide preference at each position for all miRNAs were analyzed separately. Target gene prediction was conducted using miRanda [5]. The expression levels of miRNAs were normalized and estimated using TPM (Transcripts Per Million), according to the following formula [6]: Normalized expression = mapped readcount/Total reads*1, 000, 000

Differential expression miRNA analyses and functional pathway enrichment

5 The expression levels of miRNAs were normalized using TPM (Transcripts Per Million). Differentially expressed miRNAs (DEMs) between the longissimus dorsi muscle tissues of Hanper sheep at 1 month, 7 months, and 13 months were identified using the DESeq R package (version 1.8.3). P-values were adjusted for multiple hypothesis testing using the Benjamini & Hochberg method to control the false discovery rate (FDR). By default, an adjusted P-value (FDR) < 0.05 was considered the threshold for significant differential expression. The criteria for selecting DEMs were as follows: TPM > 1, $|\log2(FC)| \geq 1$, P-value or FDR < 0.05, and at least three biological replicates [7].
Gene Ontology (GO) enrichment analysis was performed on the predicted target genes of the DEMs. GO enrichment was analyzed using the GOseq software, based on the Wallenius non-central hypergeometric distribution [8]. The significance thresholds for GO enrichment were set at P < 0.05 or FDR < 0.05. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was conducted using the KOBAS software [9] [10] to evaluate the statistical enrichment of candidate target genes in KEGG pathways and identify important biological pathways potentially regulated by miRNAs. The significance thresholds for KEGG enrichment were P < 0.05 or FDR < 0.05. Target gene prediction was performed through cross-validation using multiple databases, including miRanda and TargetScan, and further filtered by RNA-seq data to remove genes with low expression. Additionally, to explore the interactions between miRNAs and muscle growth-related genes, an interaction network diagram was constructed using Cytoscape software, revealing regulatory relationships at the molecular level.

RT-qPCR verification

6 RNA isolated from the longissimus dorsi muscle was processed for reverse transcription with the M5 miRNA cDNA Synthesis Kit, a tool from Mei5 Biotechnology Co., Ltd, specializing in small RNA first strand synthesis. Using U6 (Small Nuclear RNA) as an internal reference. Table 1 presents the primer specifications. The quantitative PCR process was conducted following the protocols of the M5 miRNA qPCR Assay Kit, a system designed for miRNA detection using fluorescent methods (Mei5 Biotechnology Co., Ltd). The reaction system was as follows: 2 × M5 miRNA qPCR Mix10μL, 7.2uL of RNase-free water, 2μL of cDNA template, 0.4μL of forward primer, and 0.4μL of reverse primer. An ABI Prism 7500 real-time PCR machine (Thermo Fisher Scientific, Waltham, MA, USA)

was used for all qPCR reactions. To determine the target gene's expression, the relative quantification approach of $2^{-\Delta\Delta Ct}$ was used.

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Acknowledgements

This research was supported by the Talents Introduction Fund of Hebei Agricultural University, grant number "YJ201938".