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Article

Effects of Low and High Maternal Protein Intake on Fetal Skeletal Muscle miRNAome in Sheep

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Simple Summary: Prenatal maternal feeding is critical for fetal development. miRNAs may impart unique metabolic and immunologic effects on developing fetuses. The fetal muscle miRNAome was found to be altered via maternal protein intake levels. oar-miR-3957-5p and miR-329a-3p were up-regulated in low and standard protein diets vs a high protein alternative diet. Many of the putative miRNA target genes and genetic pathways identified were involved in known muscle disease and immune function.

Abstract: Prenatal maternal feeding plays an important role in fetal development and has potential to cause long-lasting epigenetic modifications. MicroRNAs (miRNAs) are non-coding, single-stranded RNAs that serve as one epigenetic mechanism. Though miRNAs have crucial roles in fetal programming, growth, and development, there are limited data regarding maternal diet and sheep miRNA expression. Therefore, weanalyzed high and low maternal dietary protein for miRNA expression in sheep fetal *longissimus dorsi*. Pregnant ewes were fed an isoenergetic high-protein (HP, 160–270 g/day), low-protein (LP, 73–112 g/day), or standard protein diet (SP, 119–198 g/day) during pregnancy. miRNA expression profiles were evaluated using the Affymetrix GeneChip miRNA 4.0 Array. Twelve up-regulated, differentially expressed miRNAs (DE miRNAs) were identified targeting 65 genes. The oar-3957-5p miRNA was highly up-regulated in LP and SP compared to HP. Previous transcriptome analysis identified integrin and non-receptor protein tyrosine phosphatase genes targeted by miRNAs detected in the current experiment. A total of 28 GO terms and 10 pathway-based gene sets were significantly ($p_{adj} < 0.05$) enriched in target genes. Most genes targeted by identified miRNAs are involved in immune and muscle disease pathways. Our study shows dietary protein intake during pregnancy affects fetal skeletal muscle epigenetics via miRNA expression.

Keywords: *Ovis aries*; nutrimiromics; dietary protein; prenatal life; gestation

1. Introduction

In all mammals, maternal nutrition in pregnancy plays a critical role in intrauterine development, fetal survival, growth, and organogenesis [1,2]. Therefore, maternal under- or over-nutrition during gestation may cause low offspring growth and performance [3] with significant influence on the economic viability of livestock industry [4,5].

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Dietary nutrients, especially macronutrients, can influence the rate of gene transcription directly through interactions with the genomic regulatory elements or indirectly affecting and/or modulating important signaling pathways [6,7]. The understanding of how nutrition affects an individual's phenotype has greatly benefited from developments in molecular biology and genomics, and this area is called nutrigenomics. The term "epigenetics" describes heritable phenotypic alterations that occur without a change in the DNA sequence [8]. A few examples of epigenetic mechanisms are those that modify the chromatin structure, such as DNA methylation and histone modifications, and as well as those that control the activity of proteins, like microRNAs (miRNAs). The term "Nutrimiromics" has been used to refer to the study of interactions between nutrients and food components with the organism's genome via modification of gene expression due to epigenetic processes related to miRNAs [9].

Protein intake has a variety of nutritional and biological effects. Protein plays a vital role in the regulation of food intake, glucose and lipid metabolism, blood pressure, bone metabolism, and immunological function in addition to nutritional importance as a supply of amino acids for protein synthesis [10]. Proteins' physiological functions are influenced by their physico-chemical characteristics, amino acid make-up, and bioactive peptides encoded in their amino acid sequences [10]. Furthermore, research has been done on how proteins affect fetal and post-natal development [11]. To date, more experiments have focused on protein restriction compared high protein about maternal nutritional effects on the fetus and infant [12,13]. Blumfield and Collins [13], stated that altered maternal protein intake during pregnancy may have detrimental effects to the offspring, and optimal macronutrient ratio is crucial for the pre- and post-natal environment. Additionally, protein content is one of the most expensive and limiting feed characteristics for livestock nutrition. As a feed ingredient, protein content is usually provided from human-edible ingredients like soybean, other oilseeds, and grains [14]. Thus, careful management of these limiting feedstuffs is critical for sustainable agriculture in the context of growing world population and increasing livestock product demand [15]. Furthermore, balanced protein consumption may help to control nitrogen pollution to aid both environmental and economical sustainability [16].

Previous research has examined extreme undernutrition or overnutrition conditions to test the effects of maternal nutrition on fetal development in livestock and model animals, including fetal programming of muscle and fat tissues [12,17,18]. However, few studies have examined potential effects of maternal protein intake on the overall miRNA expression of fetal skeletal muscle. We have previously reported that maternal diets differing in protein content alter fetal skeletal transcriptome, and maternal high protein compared standard protein triggered pathways related to the immune system and disease in sheep skeletal muscle [18]. On the other hand, the mechanisms by which these diets affect fetal miRNAome in sheep skeletal muscle are not known. The objective of this study was to investigate the impact of different maternal dietary protein levels during mid-to-late gestation on the total miRNA expression of fetal skeletal muscle in sheep.

2. Materials and Methods

2.1. Experimental Animals and Diets

Details of ewes and diets were explained detailed previously [18]. Briefly, three distinct protein diets (standard, high, and low protein) were used in the study to assess the effect of maternal dietary protein on the miRNAome of fetal skeletal muscle. Twelve Akkaraman ewes aged two were divided into three treatment groups at random, with four ewes in each group. Ewe specifications are presented in Sohel et al. [18]. After a 15-day adaptation period, the ewe's daily dry matter (DM) feed consumption was assessed and compared with the US National Research Council (NRC) [19] recommendations for ewes. Three alternative feed regimens with variable protein content were created based on content of wheat straw, alfalfa, soybean meal, barley, and maize grain, alfalfa and soybean meal were the main protein sources in the diet, whereas barley and corn were the main sources of energy. Wheat straw and alfalfa were additional sources of cellulose and energy. The protein content of the diets was changed by substituting different amounts of soybean meal and alfalfa. As recommended by NRC [19], the ewes were fed from 0 to 30 days of gestation. Ewes were

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fed the following diets from day 31 to day 105 of gestation: diet 1 was a conventional diet with an NRC level of the nutritional content of feeds (daily DM feed intake ranged from 1252.3 to 1862.4 g, crude protein ranged from 119.4 to 198.6 g, and kcal ME/kg ranged from 2506.0 to 3909.3 g), diet 2: low-protein diet (1247.9–1726.4 g DM feed intake daily; 73.1–112.2 g crude protein; 2520.1–3649.0 kcal ME/kg); 3: high-protein diet (1252.4-629.8 g DM feed intake daily; 160.0–271.6 g crude protein; 2513.0–3880.9 kcal ME/kg), and diet 3: high-protein diet (daily, DM feed intake 1252.4 to 1829.8 g, crude protein 160.0 to 271.6 g and 2513.0 to 3880.9 kcal ME/kg). The diets included alfalfa, wheat straw, barley, corn, and soybean meal. The chemical composition of the materials for feed was ascertained in the feed analysis laboratory in accordance with A.O.A.C. (1990). Diets were made in an isoenergetic fashion. Specific information about diets was reported previously [18].

2.2. Estrus Synchronization, Semen Preparation, and Artificial Insemination

During the annual mating season, estrus synchronization was achieved using progesterone-infused pessaries (EaziBreed CIDR; 1.38 mg progesterone; Zoetis, Turkey) for 12 days, 2 mL of PGF2 (5 mg/mL Dinoprost tromethamine; Dinolytic; Zoetis, Turkey) treatment on day 12, and 400 IU of eCG (Folligon, 1000 IU/vial PMSG, MSD Animal Health, Turkiye) at the time of pessary removal. Four 1- to 3-year-old rams were utilized in the investigation, and the semen was extracted using the artificial vaginal procedure while an estrus ewe was present. A phase-contrast microscope with a heated plate set at 37°C was used to measure the motility of the artificial vaginal method-obtained sperm. For the insemination of ewes, the ejaculates of four rams with a motility of 70% or higher were combined. To achieve homogeneity, the appropriate ejaculates were blended based on their spermatological motility. The density of mixed ejaculates was determined using the hemocytometry method. To create 0.5 ml of semen containing 150×10^6 motile spermatozoa, the semen was diluted with Trisma base, D-Fructose, Citric acid, and skimmed milk [20]. Then, a water bath set at 35 °C was used to keep the semen. Using diluted fresh semen, artificial insemination was carried out intravaginally 48 hours following sponge removal.

2.3. Necropsy, Sample Collection and Isolation of miRNA

On the 105th day of pregnancy, the dams had necropsies. A total of 14 fetuses were gathered from 12 dams. To avoid growth bias resulting from multiple births, twin birth fetuses were not included in the experiment. In the conventional protein group and the low protein group, respectively, there were two twin pregnancies. To make sure that each experimental group contained 3 fetuses, a fetus from the HP group was also removed. A sample of muscle tissue was taken from the fetal left *longissimus dorsi* muscles. Prior to miRNA extraction, all tissues were snap-frozen in liquid nitrogen and stored at a temperature of −80 °C. Thirty milligrams of each skeletal muscle tissue were homogenized in QIAzol Lysis reagent (Qiagen, Hilden, Germany) with liquid nitrogen using a homogenizer (Precellys Bertin, France). Using a miRNeasy mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions, total RNA containing miRNA was isolated from tissue homogenates. The manufacturer's recommendations were followed for the on-column DNA digestion stage using the RNase-Free DNase Set (Qiagen, Hilden, Germany). With the help of a BioSpec-nano micro-volume UV-Vis spectrophotometer (Shimadzu Co., Kyoto, Japan), the purity and concentration of the total RNA were assessed.

2.4. Microarray Hybridization and Data Analysis

The miRNA profile was determined from the Affymetrix Microarray system using the GeneChip miRNA 4.0 Array (Affymetrix, USA). The miRNA microarray contains 30,434 mature miRNAs from all organisms, miRBase set Release 20.0. Following the manufacturer's instructions, 500 ng of total RNA was labeled using an Affymetrix FlashTag Biotin HSR RNA Labeling Kit. Following hybridization, the chip arrays were washed and stained using AGCC Fluidics Control Software on a Fluidics Station 450. After that, a high-resolution Affymetrix GeneChip Scanner 3000 was used to scan the array's fluorescence. The Affymetrix GeneChip Command Console program produced

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probe cell intensity files (*CEL files), which were imported into probe-level summary files (*CHP files) for data extraction. Software called Expression Console and Transcriptome Analysis Console were used to analyze these *CHP files. After image analysis, the data were normalized. Data was compared using tests including the t test, analysis of variance (ANOVA), and significance analysis of microarray after $\log_2$ translation of the normalized signal intensities of arrays. The identification of miRNA-targeted genes was performed using TargetScan [21] (http://www.targetscan.org; accessed on 08 January 2024) with selection criteria of cumulative weighted value ≤ −0.75 for Targetscan. Genes targeted by multiple miRNAs were assembled from individual miRNA target lists. ConsensusPathDB [22] (http://cpdb.molgen.mpg.de/; accessed on 10 January 2024) was used to perform gene ontology (GO) annotation and enrichment analysis from three ontologies (molecular function, biological process, and cellular component) on the frequently identified target genes. The threshold for significance was set at $p < 0.01$ for both of these analyses. The predicted target gene lists were examined using the default collections of the KEGG, Reactome, and BioCarta (http://cgap.nci.nih.gov/Pathways/BioCarta_Pathways) databases.

3. Results

Microarray analysis was used to compare the expression profiles of miRNAs in the fetal longissimus dorsi muscle samples collected from ewes fed with different protein (standard-SP, high-HP, and low protein-LP) diets. As presented in Table 1, 12 different miRNAs were differentially expressed (>2-fold, p < 0.01, FDR <0.05) in the different diet groups as per the Affymetrix GeneChip miRNA 4.0 Array. Chromosomal locations and mature miRNA sequences of these 12 prominent miRNAs identified using Transcriptome Analysis Console commercial software are shown in Table 1.

Table 1. The list of differentially expressed miRNAs with a fold change ≥ 2 and FDR = 0 in fetal skeletal muscles derived from high (HP), low (LP), and standard protein (SP) group.

Transcript ID (Array Design)	miRBase ID	Fold change	*p*-value	Style	Mature sequence	OAR
LP vs. HP						
oar-miR-3957-5p	MIMAT0019325	4.58	0.04	up	cucggagaguggagcugugggugu	18
oar-miR-329a-3p	MIMAT0019266	2.96	0.04	up	aacacaccugguuaaccuuuuu	18
SP vs. HP						
oar-miR-3957-5p	MIMAT0019325	4.25	0.005	up	cucggagaguggagcugugggugu	18
oar-miR-432	MIMAT0001416	2.47	0.007	up	ucuuggaguaggucauugggugg	18
oar-miR-200c	MIMAT0030044	2.24	0.009	up	uaauacugccggguaaugaugg	3
oar-miR-362	MIMAT0030060	2.24	0.01	up	aauccuuggaaccuaggugugagu	X
oar-miR-409-3p	MIMAT0019328	2.2	0.01	up	cgaauguugcucggugaaccccu	18
oar-let-7c	MIMAT0014964	2.18	0.01	up	ugagguaguagguuguauggguu	1
oar-miR-493-3p	MIMAT0019238	2.17	0.01	up	ugaaggucuacugugugccagg	18
oar-miR-181a	MIMAT0014973	2.12	0.02	up	aacauucaacgcugucggugagu	12
oar-miR-379-5p	MIMAT0019247	2.11	0.02	up	ugguagacuauggaacguaggc	18
oar-miR-134-5p	MIMAT0019308	2.1	0.03	up	ugugacugguugaccagaggg	18
oar-miR-127	MIMAT0001415	2.04	0.04	up	aucggauccgucugagcuuggcu	18
SP vs. LP						
oar-miR-200c	MIMAT0030044	2.45	0.01	up	uaauacugccggguaaugaugg	3

We identified 2 significantly up-regulated miRNAs in the HP group compared to the SP group. (Table 1). The 2 miRNAs were oar-miR-3957-5p and oar-miR-329a-3p. The fold change of those miRNAs was 4.58, and 2.96, respectively. miRNA oar-miR-3957-5p was also upregulated in SP group compared to HP group with the fold change 4.25. One of the remarkable results of our miRNAome analysis was the identification of nine miRNAs on OAR18 in which they clustered together in a tight locus (Figure S1). The miRNA oar-miR-200c showed up-regulation in SP group compared to LP group. The differentially expressed miRNAs were grouped using a hierarchical clustering technique,

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and the outcomes demonstrated that the various diet groups could be readily distinguished by the hierarchical grouping of all covered ovine miRNAs (Figure S2).

Next, we analyzed the target genes that may be regulated by these 12 miRNAs in two layers. First, we identified genes targeted by multiple miRNAs (Table S1). In silico analyses identified that 32 genes found to be targeted by two miRNAs and no mRNA found to be targeted by three or more miRNAs (Table S1). In the second layer, a total of 65 genes were targeted by at least one of 12 miRNAs (Table S2). Hypergeometric distribution testing was used to examine the gene ontology (GO) of their anticipated target genes to investigate the roles of differentially regulated miRNAs among 3 distinct diet groups. GO terms for genes that targeted by 2 miRNAs were identified as being related to 19 biological processes and 6 molecular functions (Table 2). GO terms for genes that are targeted by single miRNA were identified as being related to 20 biological processes, involved in the creation of 5 molecular functions, and expected to take part in 3 cellular components (Table 3).

Table 2. GO terms of 32 genes targeted by multiple miRNAs with ConsensusPathDB (category level 4) (adjusted p<0.01).

Category	Gene ontology term	Set size	Candidates contained	p-value	q-value
Biological process	GO:0048247 lymphocyte chemotaxis	65	3 (4.6%)	9.74e-05	0.00792
	GO:0002548 monocyte chemotaxis	71	3 (4.2%)	0.000127	0.00792
	GO:0051171 regulation of nitrogen compound metabolic process	6033	17 (0.3%)	0.000574	0.0199
	GO:0071621 granulocyte chemotaxis	124	3 (2.4%)	0.000655	0.0199
	GO:0080090 regulation of primary metabolic process	6227	17 (0.3%)	0.000863	0.0199
	GO:0031323 regulation of cellular metabolic process	6297	17 (0.3%)	0.000996	0.0199
	GO:0097530 granulocyte migration	149	3 (2.0%)	0.00111	0.0199
	GO:0006796 phosphate-containing compound metabolic process	3342	11 (0.3%)	0.0032	0.0419
	GO:0044271 cellular nitrogen compound biosynthetic process	5019	14 (0.3%)	0.0032	0.0419
	GO:0009059 macromolecule biosynthetic process	5040	14 (0.3%)	0.00335	0.0419
	GO:0060255 regulation of macromolecule metabolic process	6339	16 (0.3%)	0.0037	0.042
	GO:0044267 cellular protein metabolic process	5282	14 (0.3%)	0.00531	0.0553
	GO:0034976 response to endoplasmic reticulum stress	298	3 (1.0%)	0.00779	0.0602
	GO:0034654 nucleobase-containing compound biosynthetic process	4307	12 (0.3%)	0.00786	0.0602
	GO:0060326 cell chemotaxis	305	3 (1.0%)	0.00837	0.0602
	GO:0048638 regulation of developmental growth	306	3 (1.0%)	0.00845	0.0602
	GO:0018130 heterocycle biosynthetic process	4372	12 (0.3%)	0.00889	0.0602
	GO:0019438 aromatic compound biosynthetic process	4383	12 (0.3%)	0.00908	0.0602
	GO:0034645 cellular macromolecule biosynthetic process	4976	13 (0.3%)	0.00916	0.0602
Molecular function	GO:0005125 cytokine activity	237	4 (1.7%)	0.000299	0.00459
	GO:0005126 cytokine receptor binding	273	4 (1.5%)	0.00051	0.00459
	GO:0019887 protein kinase regulator activity	190	3 (1.6%)	0.00224	0.0134
	GO:0048018 receptor ligand activity	493	4 (0.8%)	0.00445	0.0164
	GO:0019210 kinase inhibitor activity	73	2 (2.7%)	0.00456	0.0134

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GO:0001664	G protein-coupled receptor binding	294	3 (1.0%)	0.00757	0.0227

Table 3. Pathway analysis of 32 genes targeted by multiple miRNAs with ConsensusPathDB (adjusted $p<0.01$).

Pathway name	Set size	Candidates contained	*p*-value	*q*-value	Pathway source
Chemokine receptors bind chemokines	62	3 (4.8%)	0.000117	0.00397	Reactome
COVID-19 adverse outcome pathway	15	2 (13.3%)	0.000241	0.00397	Wikipathways
Rheumatoid arthritis - Homo sapiens (human)	93	3 (3.3%)	0.000378	0.00397	KEGG
Viral protein interaction with cytokine and cytokine receptor - Homo sapiens (human)	100	3 (3.0%)	0.000483	0.00397	KEGG
Chagas disease - Homo sapiens (human)	102	3 (2.9%)	0.000512	0.00397	KEGG
Toll-like receptor signaling pathway - Homo sapiens (human)	104	3 (2.9%)	0.000542	0.00397	KEGG
IL-7 signaling pathway	25	2 (8.0%)	0.000683	0.00429	Wikipathways
Cytokine-cytokine receptor interaction - Homo sapiens (human)	295	4 (1.4%)	0.00102	0.00558	KEGG
Cyclin D associated events in G1	44	2 (4.5%)	0.00212	0.00931	Reactome
G1 Phase	44	2 (4.5%)	0.00212	0.00931	Reactome
Chemokine signaling pathway - Homo sapiens (human)	192	3 (1.6%)	0.00316	0.0126	KEGG
Peptide ligand-binding receptors	207	3 (1.4%)	0.00391	0.0143	Reactome
Lipid and atherosclerosis - Homo sapiens (human)	215	3 (1.4%)	0.00435	0.0146	KEGG
Human cytomegalovirus infection - Homo sapiens (human)	225	3 (1.3%)	0.00493	0.0146	KEGG
DNA damage response	68	2 (2.9%)	0.00498	0.0146	Wikipathways
E2F transcription factor network	75	2 (2.7%)	0.00603	0.0166	PID

GO functional enrichment analysis and pathway enrichment analysis were performed on 32 genes targeted by multiple miRNAs and separately on 65 genes targeted by at least one miRNA (Tables 2 and 3). GO terms showed that genes targeted by multiple miRNAs were mainly involved in biological processes (BP) lymphocyte chemotaxis, monocyte chemotaxis, regulation of nitrogen compound metabolic process, granulocyte chemotaxis and other categories that control immune system (Table 2). In terms of molecular function (MF), targeted mRNAs were involved in cytokine activity, cytokine receptor binding, protein kinase regulator activity (Table 2). We conducted a pathway enrichment study using public pathway databases to better understand the roles of these projected target genes, and 31 pathways were found (Table 3). Pathway analysis showed that targeted genes by multiple miRNAs were significantly enriched in the Chemokine receptors bind chemokines, Rheumatoid arthritis - Homo sapiens (human), Viral protein interaction with cytokine and cytokine receptor - Homo sapiens (human) and Toll-like receptor signaling pathway - Homo sapiens (human) (Table 3).

GO functional enrichment analysis for genes targeted by single mRNAs in terms of BP, the primary categories for the functions were cellular macromolecule biosynthetic process, macromolecule biosynthetic process, regulation of biosynthetic process and cellular nitrogen compound biosynthetic process cell (Table 4). In terms of MF, targeted mRNAs were involved in DNA binding, regulatory region nucleic acid binding and metal ion binding (Table 4). In terms of CC, targeted mRNAs were involved in nuclear lumen, nucleus, nucleoplasm (Table 4). Pathway

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analysis showed that targeted genes by single miRNAs were significantly enriched in the Rheumatoid arthritis - Homo sapiens (human), Transferrin endocytosis and recycling, Oncogene Induced Senescence and G1 to S cell cycle control (Table 5).

Table 4. Gene ontology terms of 65 genes with ConsensusPathDB (category level 4) (adjusted p<0.01).

Category level	GO term		Set size	Candidates contained	*p*-value	*q*-value
Biological process	GO:0034645	cellular macromolecule biosynthetic process	4976	30 (0.6%)	6.54e-06	0.000634
	GO:0009059	macromolecule biosynthetic process	5040	30 (0.6%)	8.62e-06	0.000634
	GO:0009889	regulation of biosynthetic process	4310	27 (0.6%)	1.38e-05	0.000676
	GO:0044271	cellular nitrogen compound biosynthetic process	5019	29 (0.6%)	2.59e-05	0.000881
	GO:0010467	gene expression	5644	31 (0.5%)	2.99e-05	0.000881
	GO:0051171	regulation of nitrogen compound metabolic process	6033	32 (0.5%)	4.06e-05	0.000996
	GO:0080090	regulation of primary metabolic process	6227	32 (0.5%)	8.1e-05	0.0017
	GO:0031323	regulation of cellular metabolic process	6297	32 (0.5%)	0.000103	0.00189
	GO:0060255	regulation of macromolecule metabolic process	6339	32 (0.5%)	0.000119	0.00195
	GO:0018130	heterocycle biosynthetic process	4372	25 (0.6%)	0.000176	0.00259
	GO:0090304	nucleic acid metabolic process	5270	28 (0.5%)	0.0002	0.00267
	GO:1901362	organic cyclic compound biosynthetic process	4529	25 (0.6%)	0.000316	0.00387
	GO:0034654	nucleobase-containing compound biosynthetic process	4307	24 (0.6%)	0.000393	0.00445
	GO:0019438	aromatic compound biosynthetic process	4383	24 (0.5%)	0.000516	0.00542
	GO:0009892	negative regulation of metabolic process	3076	18 (0.6%)	0.00172	0.0169
	GO:0050779	RNA destabilization	36	2 (5.6%)	0.00478	0.0421
	GO:0009893	positive regulation of metabolic process	3654	19 (0.5%)	0.00487	0.0421
	GO:0051101	regulation of DNA binding	124	3 (2.4%)	0.00543	0.0439
	GO:0072175	epithelial tube formation	126	3 (2.4%)	0.00568	0.0439
	GO:0016331	morphogenesis of embryonic epithelium	141	3 (2.1%)	0.00774	0.0569
Molecular function	GO:0003677	DNA binding	2515	22 (0.9%)	6.1e-07	7.63e-06
	GO:0001067	regulatory region nucleic acid binding	1535	17 (1.1%)	7.63e-07	7.63e-06
	GO:0046872	metal ion binding	4259	23 (0.5%)	0.000869	0.00579
	GO:0001228	DNA-binding transcription activator activity, RNA polymerase II-specific	444	6 (1.4%)	0.00164	0.00822
	GO:0004879	nuclear receptor activity	52	2 (3.8%)	0.00978	0.0391
Cellular component	GO:0031981	nuclear lumen	4461	24 (0.5%)	0.000678	0.0183
	GO:0005634	nucleus	7626	33 (0.4%)	0.00198	0.0183
	GO:0005654	nucleoplasm	4103	21 (0.5%)	0.00341	0.0307

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Table 5. Pathway analysis of 65 genes with ConsensusPathDB (adjusted p<0.01).

Pathway name	Set size	Candidates contained	p-value	q-value	Pathway source
Rheumatoid arthritis - Homo sapiens (human)	93	8 (8.7%)	1.69e-05	0.00776	KEGG
Transferrin endocytosis and recycling	31	4 (12.9%)	0.000533	0.0765	Reactome
Oncogene Induced Senescence	32	4 (12.5%)	0.000603	0.0765	Reactome
Small cell lung cancer - Homo sapiens (human)	92	6 (6.5%)	0.000935	0.0765	KEGG
G1 to S cell cycle control	64	5 (7.8%)	0.00112	0.0765	Wikipathways
Small cell lung cancer	96	6 (6.2%)	0.00117	0.0765	Wikipathways
Adenosine ribonucleotides de novo biosynthesis	38	4 (10.5%)	0.00117	0.0765	HumanCyc
RND1 GTPase cycle	42	4 (9.5%)	0.00171	0.0776	Reactome
Human cytomegalovirus infection - Homo sapiens (human)	225	9 (4.0%)	0.00185	0.0776	KEGG
RND2 GTPase cycle	43	4 (9.3%)	0.00186	0.0776	Reactome
Cyclin D associated events in G1	44	4 (9.1%)	0.00203	0.0776	Reactome
G1 Phase	44	4 (9.1%)	0.00203	0.0776	Reactome
Cyclins and cell cycle regulation	23	3 (13.0%)	0.00269	0.0948	BioCarta
Spinal Cord Injury	117	6 (5.1%)	0.00319	0.104	Wikipathways
Cell cycle: g1/s check point	25	3 (12.0%)	0.00343	0.105	BioCarta
Insulin receptor recycling	26	3 (11.5%)	0.00384	0.109	Reactome
Collecting duct acid secretion - Homo sapiens (human)	27	3 (11.1%)	0.00429	0.109	KEGG
Constitutive Signaling by AKT1 E17K in Cancer	27	3 (11.1%)	0.00429	0.109	Reactome
Lipid and atherosclerosis - Homo sapiens (human)	215	8 (3.7%)	0.00508	0.119	KEGG
Iron uptake and transport	57	4 (7.0%)	0.00523	0.119	Reactome
Superpathway of purine nucleotide salvage	59	4 (6.8%)	0.00591	0.119	HumanCyc
Purine nucleotides de novo biosynthesis	59	4 (6.8%)	0.00591	0.119	HumanCyc
Oxidative phosphorylation - Homo sapiens (human)	133	6 (4.5%)	0.00597	0.119	KEGG
Prion disease - Homo sapiens (human)	273	9 (3.3%)	0.00661	0.126	KEGG
Chemokine receptors bind chemokines	62	4 (6.5%)	0.00704	0.129	Reactome
Vitamin D Receptor Pathway	184	7 (3.8%)	0.00765	0.13	Wikipathways
Viral protein interaction with cytokine and cytokine receptor - Homo sapiens (human)	100	5 (5.0%)	0.00776	0.13	KEGG
Ectoderm Differentiation	142	6 (4.2%)	0.00814	0.13	Wikipathways
Amino acids regulate mTORC1	34	3 (8.8%)	0.00824	0.13	Reactome
ROS and RNS production in phagocytes	35	3 (8.6%)	0.00893	0.135	Reactome
Toll-like receptor signaling pathway - Homo sapiens (human)	104	5 (4.8%)	0.00912	0.135	KEGG

4. Discussion

Proteins are vital for the structure, function, and regulation of the body's tissues and organs in all living organisms; in addition, they are the most expensive and often most limiting ingredients in livestock feed formulation [23]. Excess fed protein can contribute to excreted nitrogen with undesirable nitrate leaching to groundwater and atmospheric ammonia emissions [24]. Despite the importance of protein for all living organisms, ruminants use dietary protein for production rather inefficiently (as defined by g N in product/g N intake) [25]. This creates opportunity to utilize technologies such as dynamically alter nutrient intakes via mid-infrared spectroscopy of milk

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composition [24,26]. Additionally, feedomics may assist in modifying nutrition for monetary and long-term gains. The key components of the feedomics study are nutrigenomics, nutrigenetics, and nutritional epigenetics, which aid in establishing a link between genetic variation and nutrient-driven epigenetic changes that are proposed as the main barrier to meeting nutritional demands [27]. Maternal nutrition is a major intrauterine environmental factor that alters expression of the fetal genome in ways that may have lifelong consequences. Such fetal programming has been shown to be regulated by epigenetic modifications, including DNA methylation, histone modifications, and as well as miRNAs [12].

In addition, we have identified that miR-3957-5p was upregulated in both LP and SP groups vs HP group (Table 1). Pokharel et al. [28] detected miR-3957-5p in the tissue biopsies of *corpus luteum* and endometrium of pregnant ewes. Prior work demonstrated an abundance of miRNAs in packed form as extracellular vesicles to maintain feto-maternal communication in multiple mammals [29–31]. In the current study, together with miR-3957-5p, other miRNAs revealed a large cluster of miRNAs on chromosome 18 (Table 1) which may co-regulate different biological processes [32]. This is consistent with Pokharel et al. [28] who also detected similar miRNAs in the same clustered locus. This miRNA cluster is highly conserved among placental mammals and known to be regulated by a maternally imprinted *DLK1-DIO3* region, which has been associated with severe muscle disease caused by impaired expression of dystrophin called Duchenne muscular dystrophy [33–35]. Additionally, two growth QTL associated with sheep body weight [36] and average daily gain [37] were found coinciding with the miRNA cluster on OAR18. In the same experimental design subjected to current study, Sohel et al. [18] showed that a high-protein diet consumed by the mother predominantly changed the expression of mRNAs involved in myogenesis and immunity in the developing fetus's skeletal muscle. Both Shandilya et al. [38] and Sharma et al. [39,40] showed that sheep exposed to bacterial lipopolysaccharide challenge exhibited differentially regulated miRNAs such as miR-3957-5p, miR-329a-3p, miR-379-5p (Table 1) which regulate biological processes such as stress and immune responses.

A critical layer of analysis involves miRNA targeting the same genes (Table S2). This is an established part of miRNA biology and a useful way to highlight important genes and pathways [41,42]. Multiple miRNAs regulating the same target gene are naturally cooperative since miRNAs act to inhibit targets; this heterotypic regulation is believed to offer extra regulatory specificity [43]. And specially, there is ongoing debate over whether clustered miRNAs with different seed sequences co-evolved due to shared functions [43]. In the current study, two members of miRNA cluster that are oar-miR-3957-5p and oar-miR-432 (Table S2; Figure S1) are cooperatively targeting Calcium/calmodulin dependent protein kinase IG (*CAMK1G*) and Ras protein specific guanine nucleotide releasing factor 1 (*RASGRF1*) genes. Calcium/calmodulin-dependent protein kinases are known to be key regulators of calcium signaling in health and disease [44] and it has been observed that T cells and B cells produce less IFN-γ when *CaMKIV* is inhibited or silent [45]. Futhermore experiments showed that in myoblasts and myotubes treated with IFN-γ, calcium/calmodulin-dependent protein kinase IV specifically suppresses the production of co-stimulatory molecules and pro-inflammatory cytokines/chemokines [46]. In terms of *RASGRF1*, it is an intrinsic key mediator for brain-derived neurotrophic factor (BDNF)-induced R-Ras activation and R-Ras-mediated axonal morphological regulation [47]. One of the nerve growth factor family's members, BDNF, is primarily produced by the brain and its main role involves synaptic modulation, neurogenesis, neuron survival, immune regulation, myocardial contraction, and angiogenesis in the brain [48]. On the other hand, previous experiments had shown that BDNF has a significant regulatory role in myogenic differentiation in skeletal muscle which acts as a retrograde survival factor for motor neurons in the neuromuscular system [49,50]. Additionally, on the same miRNA cluster, oar-miR-379-5p and oar-miR-134-5p were found to be cooperatively targeting Glutamyl-TRNA Amidotransferase Subunit C (*GATC*) (Table S2). *GATC* was associated with mitochondrial respiratory dysfunction which cause skeletal muscle atrophy, is a state of uncontrolled inflammation and oxidative stress [51]. Multiple target analysis showed a complex network of mutual interactions between let-7c, miRNAs on various chromosomes and mRNAs (Table S2). In literature role of Let-7c and other Let-7 family members's

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in myogenesis [52] and ovine fetal skeletal muscle development related to maternal obesity had been shown [53].

A second outcome involves immune genes. Chemokine ligands (CCLs), *CCL3*, *CCL3L1*, and *CCL3L3* are all targeted by both oar-miR-432 and let-7c (Table S2). *CCL3* is critical for trophoblast invasion and maintenance of pregnancy [54,55]. While others have studied dietary impacts on sheep, to our knowledge, this is the first demonstration that maternal diet protein intakes in the context of isoenergetic dietary formulations could impact *CCL3* and maintenance of pregnancy. In addition, *IL7* is targeted by both oar-miR-432 and oar-miR-200c (Table S2). This is particularly striking because *IL7* influences development of the offspring's T cell immunity [56,57]. These examples highlight the importance of maternal protein intake on multiple critical outcomes for fetal health and lifetime wellbeing.

Rheumatoid arthritis - *Homo sapiens* (human) pathways were identified in different protein intake groups among both layers of target prediction (Tables 3 and 5). Rheumatoid arthritis (RA) is a chronic, inflammatory, systemic autoimmune disease that causes skeletal muscle loss and weakness in RA individuals [58,59]. Similarly, transferrin endocytosis and recycling pathway is associated with iron homeostasis, is a key regulator of innate and adaptive immunity and plays a decisive role in inflammatory processes [60]. These findings were consistent with the findings of transcriptome analysis [18], which revealed that many detected genes are involved in pathways related to the immune system and diseases.

5. Conclusions

In summary, muscle biology is crucial for economic and nutritional value of sheep, and long-term optimization of sheep production in requires enhanced understanding of the molecular mechanisms that control muscle growth and metabolism. To the best of our knowledge, this is the first study that investigates the nutrimiromics of sheep fetal skeletal muscle based on differing maternal protein intake. In addition, experiments that investigate effect of nutrients and bioactive compounds that regulate miRNAs in the fetal period are very limited. The findings of our research show that maternal diet in terms of different protein levels may affect fetal miRNAome that epigenetically affect the skeletal muscle transcriptome in vivo [18]. The discovery of multiple miRNAs that target mRNAs is a significant development that may enable us to further comprehend the network that connects miRNAs and their targets. Since the maternal high-protein diet specifically changed the expression of miRNAs involved in number of pathways and biological processes that are unique to disease and immunity, these results may have provided insight into the reversible nature of epigenetic modifications, which hold promise for the use of miRNAs in the diagnosis, prevention, and treatment of health conditions in the offspring.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: miRNA cluster on OAR18; Figure S2: Heat map of the microarray miRNA expression profile in fetal skelatal muscle derived from high, low, and standard protein groups; Table S1: List of targeted mRNAs by single miRNA; Table S2: List of targeted mRNAs by multiple miRNA.

Author Contributions: MUC, MMHS, and BA conceived and designed the study. KG and MA contributed insemination and necropsy. KG, MA, KA, and MK performed the sample collection. YK and MK carried out animal nutrition experiments. MUC, MMHS, and BA contributed genetic experiments. MUC and MMHS performed the research and analyzed the data. SNW contributed to analysis. MUC and BA drafted the paper. All authors have read and approved the manuscript.

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