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ANIMAL GENETICS

Investigation of Expression Levels of Transcription Factor Genes in Native Sheep Breeds of Türkiye

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Abstract—This study was aimed to investigate the expression levels of transcription factor (TF) genes (*MYF6, MYOD1, MYF5, MYOG*) and proteins associated with muscle growth in the *longissimus dorsi* (LD) and *gluteal* (GL) muscles in sheep. For this aim, two fat-tailed sheep breeds (Akkaraman ($n = 10$), Awassi ($n = 10$)) and two thin-tailed sheep breeds (Kivircik ($n = 10$) and Karayaka ($n = 10$)) from Türkiye's native sheep breeds were examined. The expression level was analyzed for MYF6, MYOD1, MYF5, and MYOG genes and proteins that RNAs and proteins were isolated from fresh tissues. As a result of the statistical analysis, in the LD tissue, respectively, *MYOG* and *MYF5* genes in the Karayaka sheep breed; *MYOD1* gene in Akkaraman sheep breed; *MYF5* gene in Awassi sheep breed were found to be significant ($P < 0.05$). In GL tissue, respectively, *MYOG* and *MYF6* genes in Akkaraman sheep breed; *MYOD1* gene in Karayaka sheep breed; *MYF6* gene in Akkaraman and Awassi sheep breed were significant ($P < 0.05$). The *MYOG* (fold change 6.87) and *MYOD1* (fold change 15.41) genes were upregulated in the GL muscle of the fat-tailed Akkaraman sheep breed. In addition, in the thin-tailed Karayaka sheep breed, down-regulation of *MYOD1* (fold change –0.22) gene in LD muscle and up-regulation of *MYOD1* (fold change 6.67) gene in GL muscle was found. As a result, it can be considered that *MYOG* and *MYOD1* genes as potential candidate genes in molecular selection studies for Akkaraman sheep breed in terms of muscle development.

Keywords: fat-tailed sheep, gene expression, MRF gene family, thin-tailed sheep breed

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INTRODUCTION

Improving meat yield characteristics such as rapid growth, high carcass yield, increasing carcass and meat quality are among the most important topics in breeding studies regarding the livestock animals raised for meat yield. In recent years, there has been an increasing interest in the use of genetic markers in the studies conducted for this purpose. Studies in sheep, which includes 25% of the known mammalian livestock animal breeds, are relatively limited [1]. Sheep offer great opportunities in meeting the future demand for food of animal origin and responding to changing market needs because they are more flexible livestock animals compared to cattle due to their adaptability to very different geographies [2].

In addition to being resistant to bad environmental conditions and having the capacity to make high use of low-yielding pastures, fat-tailed sheep breeds store excess calories as tail fat, which also has commercial value, in the season when grass is abundant. Due to these characteristics, fat-tailed sheep breeds have adapted well to Anatolian geography, where steppe climate conditions prevail in general, compared to thin-tailed breeds Ertugrul et al. (Ertugrul et al., 2009). For this reason, 87% of domestic sheep population in Türkiye consists of fat-tailed sheep breeds [4]. With narrower breeding areas, there are also thin-tailed domestic sheep breeds such as Kivircik and Karayaka, which store fat in body cavities and between muscle fibers instead of the tail, and therefore have higher quality meat compared to fat-tailed sheep breeds [3, 5].

The Akkaraman breed, one of the fat-tailed sheep breeds in Türkiye, is mainly bred in the Central Anatolia Region, where sheep breeding is most intense, but this breed is also common in the transition regions close to this area. Therefore, it is the sheep breed with the largest population size in Türkiye [3]. Another fat-tailed breed bred in Türkiye, the Awassi breed, is bred in the Southeastern Anatolia Region in Türkiye, Iraq, Syria, Jordan, Israel, and Palestine [6]. The Kivircik breed, one of the thin-tailed domestic sheep breeds in

Türkiye, is bred in the Marmara Region and North Aegean region of Türkiye and is superior to other domestic sheep breeds in terms of meat quality [7]. Another thin-tailed sheep breed is the Karayaka breed, which, like the Kivircik breed, is better compared to the fat-tailed breeds in terms of meat quality and taste and is bred in the inner parts of the Black Sea Region [3].

One of the important challenges in molecular biology in understanding gene regulation is to reveal genetic information that control gene regulation, which is the basis of all biological processes and phenotype [8]. Transcription factors, an important family of gene regulatory proteins, play an important role in the growth, development, and evolution of higher organisms [9]. Therefore, investigating the transcriptome profile of transcription regulatory proteins, especially in different muscle tissues, provide useful information to improve meat production and quality in, especially sheep.

The myogenic regulatory factor (MRF) gene family, which includes myogenin (MYOG), myoblast detection protein-1 (MYOD1), myogenic factor 5 (MYF5) and myogenic factor 6 (MYF6), has important roles in the muscle growth and development [10, 11]. During this process, *MYOD1, MYF5* and *MRF6* control the formation of myogenic cells [12], while *MYOG* is required for terminal differentiation of skeletal muscle [10].

Skeletal muscle growth and development is a complex process supported by transcriptome regulation involving regulatory networks and signaling pathways [13]. To date, *MRF*s [14] in livestock animals were associated with growth hormone [15], insulin-like growth factors [16] and *myostatin* genes [17] were reported to be associated with muscle growth and development. The transcriptome analyzes to be performed in different species and different muscle groups will contribute to the understanding of the effects of the candidate genes responsible for muscle development or the previously determined candidate genes in different muscle groups.

The aim of the study was to investigate *MYOG, MYOD1, MYF5* and *MYF6* genes and protein expression levels in *longissimus dorsi* (LD) and *gluteal* (GL) muscles in two fat-tailed sheep breeds (Akkaraman and Awassi) and two thin-tailed sheep breeds (Kivircik and Karayaka) from among the sheep breeds in Türkiye.

MATERIALS AND METHODS

Animal Material

The animal material of the study consisted of 5-month-old four different sheep breed ($n = 10$ Kivircik lambs, $n = 10$ Karayaka lambs, $n = 10$ Akkaraman lambs and $n = 10$ Awassi lambs). Fresh LD and GL samples were taken on the day of slaughter and frozen in liquid nitrogen. It was stored at −80°C until protein and RNA isolation.

RNA Isolation and RT-qPCR

LD and GL tissue samples were homogenized with the help of liquid nitrogen and take in sterile 1.5 mL plastic tubes. RNA isolation was performed with trizol (Roche, Mannheim, Germany) from LD and GL tissue samples [18]. The concentration of the RNAs were determined with a nanodrop device (Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek, USA).

DNase (Invitrogen DNA-free DNA removal kit, Invitrogen, Thermo Fisher Scientific, Carlsbad, US) was applied to RNA samples to prevent DNA contamination. DNase-treated RNA samples were checked on a 1% agarose gel. c-DNA synthesis was performed with these RNA samples by First Strand c-DNA Synthesis Kit (Roche Ltd., Mannheim, Germany) and gene expression analysis was determined by RT-qPCR [19]. The Ct values of target genes designed with Primer 3 software [20] were normalized with housekeeping gene [21] and normalized data was applied to the $2^{-\Delta\Delta Ct}$ methods for statistical analyses [22].

Western Blot Analysis

Protein isolation was performed on ice with Cell lysis buffer (Cell Signaling Technology, USA) on the muscle tissue samples taken fresh from the slaughterhouse on the study day and frozen in liquid nitrogen. The concentration of isolated proteins was determined by the Lowry method [23]. Marker and protein samples, whose concentration was adjusted according to protein size, were loaded into 8–12% SDS-polyacrylamide (SDS-PAGE) gel as 60 μg protein and electrophoresis was carried out in vertical electrophoresis (MiniProtean Tetra System, BioRad, Hercules, CA, USA) system in running buffer at 70 V for two hours. After the electrophoresis, the gel was transferred to methanol-activated Polyvinylidene difluoride (PVDF) membranes (Immobilon-PSQ Transfer membrane, Millipore) and the transfer process was applied at 4 C for one hour at 100 V to the membranes placed in the transfer tank. The membrane obtained as a result of the transfer process was incubated for one hour in an orbital shaker for one hour in blotting solution with 5% skim milk powder (Tris buffer saline-Tween 20 (TBS-T): 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20). The membranes were kept in the primary antibody solution (1 : 500 dilution ratio) overnight at 4°C for each protein (MYOD1 (LSC130926 (LSBio, Lynnwood, Washington, USA)), MYOG (LS-C113013 (LSBio, Lynnwood, Washington, USA)), MYF5 (LSBIO LS-C29704 (LSBio, Lynnwood, Washington, USA)), MYF6 (LS-C191948 (LSBio, Lynnwood, Washington, USA)) and B-Actin (4967S (Cell Signaling Technology, Danvers, Massachusetts, USA))) planned to be studied after

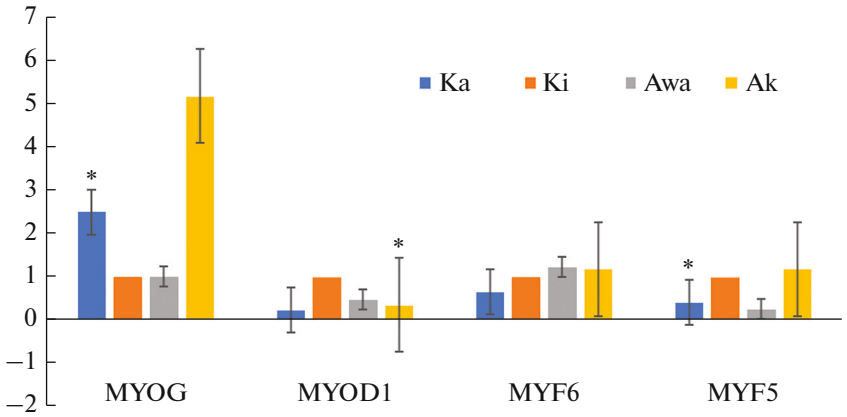

Fig. 1. Expression levels of the investigated genes in Longissimus dorsi muscle by breed.

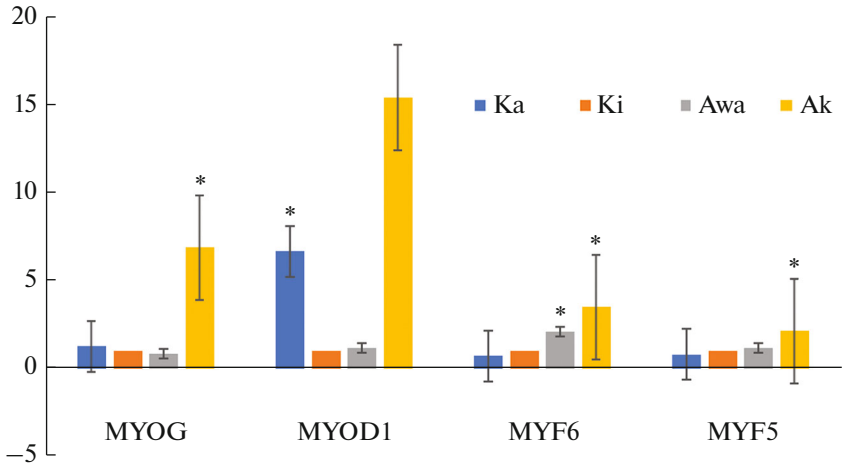

Fig. 2. Gene expression levels investigated by breed in Gluteal Muscle tissue.

blotting. The following day, the membrane was incubated with (HRP)-Conjugated secondary antibodies for one hour at room temperature. To control the primary antibody incubation, chemiluminescence dye was applied to the membrane and incubated for five minutes at room temperature in the dark, imaging was done in the BioRad (ChemiDoc™ XRS + System with Image Lab™) system, the band sizes were evaluated, and the data were normalized with the β-Actin.

Statistical Analyzes

Histogram, q-q plots, and Kolmogorov–Smirnov test were used to test the conformity of the study data to normal distribution. The expression differences of the genes by tissues between the breeds were tested with One-Way ANOVA. Statistical significance control of the difference in expression levels of genes between the tissues within the breed was tested with the Paired Sample *t*-test. Windows 14.01 SPSS software was used in the statistical analysis of the data and the significance level was accepted as $P < 0.05$.

RESULTS

MYOG gene expression level was upregulated ($P < 0.05$) in Karayaka breed in LD muscle, while the expression levels of *MYOD1* was downregulated in Akkaraman breed and *MYF5* genes were downregulated in Awassi and Karayaka breeds ($P < 0.05$) (Fig. 1).

The expression of *MYOG, MYF5* and *MYF6* genes were upregulated in Akkaraman breed, *MYOD1* genes in Karayaka breed and *MYF6* genes in Awassi breed ($P < 0.05$) (Fig. 2).

Longissimus dorsi tissues showed that the expression levels of *MYOG, MYOD1, MYF5* and *MYF6* genes and the expression levels of MYOG, MYOD1, MYF5 and MYF6 proteins in these muscles changed (increased and/or decreased) harmoniously in all breeds (Fig. 3).

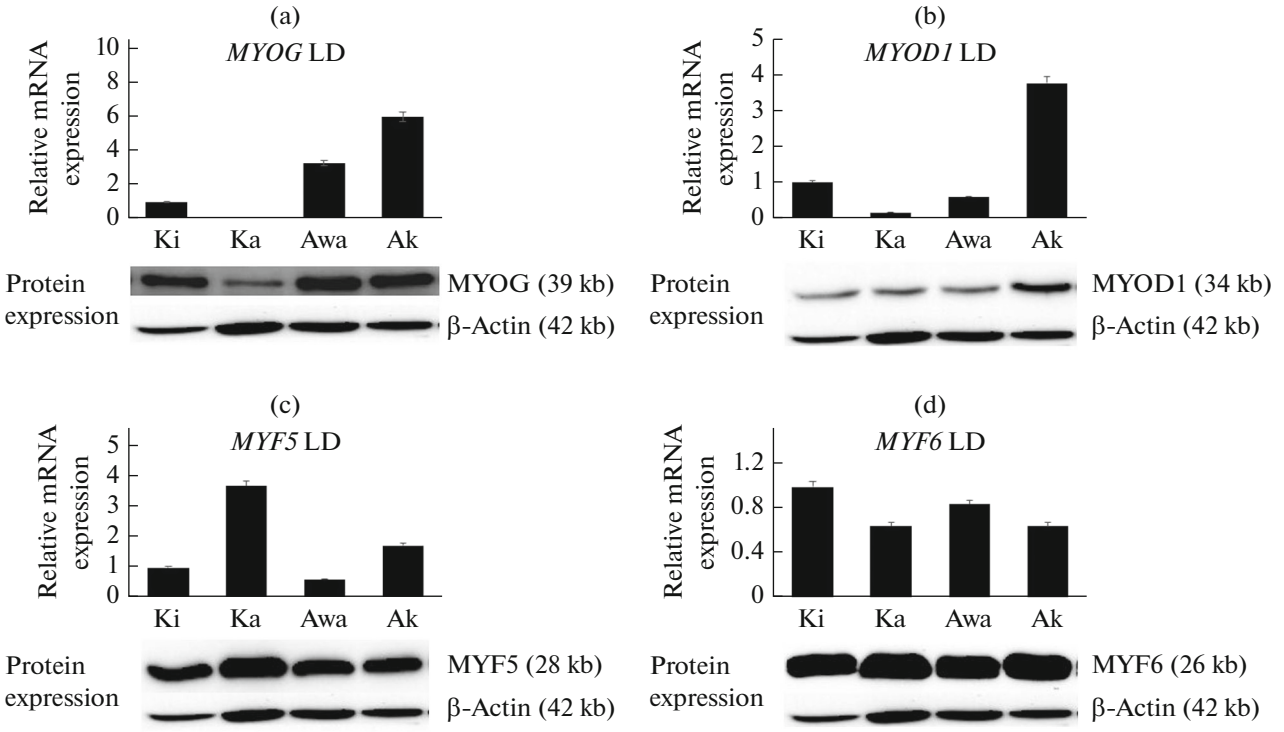

Fig. 3. Comparison of *gluteal muscle* tissue gene expression and western blot (a) MYOG, (b) MYOD1 ; (c) MYF5; (d) MYF6; Ki: Kıvırcık, Ka: Karayaka; Awa: Awassi; Ak: Akkaraman.

Gluteal tissues showed that the expression levels of *MYOG, MYOD1, MYF5* and *MYF6* genes and the expression levels of MYOG, MYOD1, MYF5 and MYF6 proteins in these muscles changed (increased and/or decreased) harmoniously in all breeds (Fig. 4).

DISCUSSION

This study aimed to present the gene and protein expression profiles of the four members of the *myogenic regulatory* gene family *MYOG, MYOD1, MYF5* and *MYF6* in the LD and GL muscles of the fat-tailed and thin-tailed native Turkish sheep breeds and to explore the potential of using the obtained data in breeding studies to increase the meat yield in sheep.

Siqin et al. [24] reported that the *MYOG* and *MYF6* gene expression levels in the LD muscle were stable during postnatal development in the fat-tailed Wuzhumuqin sheep breed. A study investigating these effects on muscle development in short-tailed Hu sheep found that the expression level of the *MYOG* gene was downregulated in the LD muscle, whereas the expression level was upregulated in the *gastrocnemius* and *soleus muscles*, reporting the dependence of the expression level of the *MYOG* gene on the muscle groups as one of the reasons for this difference [25]. In support of this view, this study found that the *MYOG* gene and protein expression level was statistically significantly ($P < 0.05$) upregulated in the LD muscle in the thin-tailed Karayaka sheep breed, while the expression level of the same gene and protein was downregulated in the GL muscle ($P < 0.05$). On the other hand, it was determined that the *MYOG* gene expression level was upregulated in GL muscle tissue in Akkaraman breed, a fat-tailed breed. This supports the knowledge that the activity of the *MYOG* gene, which plays a key role in regulating the development of muscle cells during the formation of skeletal muscle, changes specifically for the muscle group [26].

The increase in the number of satellite cells responsible for muscle cell development differs from breed to breed, species to species, and muscle group to muscle group [27–29]. *MYOD1* gene is involved in satellite cell increase in different ways [30, 31]. It was determined that the expression level of *MYOD1* gene in pigs varies according to age, breed and muscle type, and it was reported that this may be related to the number of satellite cells in different breeds and different muscle groups [32]. The cited study suggested that new candidate genes related to meat yield could be revealed by investigating *MYOD1* gene and closely related *MYF5* and *MYF6* gene expression levels in different pig breeds or at different developmental stages of pigs [32]. This situation can be considered to be the case for all livestock animals.

Sun et al. [13] investigated the transcriptome profile of *MYOD1* and *MYOG* genes with the aim of improving sheep meat yield and quality in thin-tail

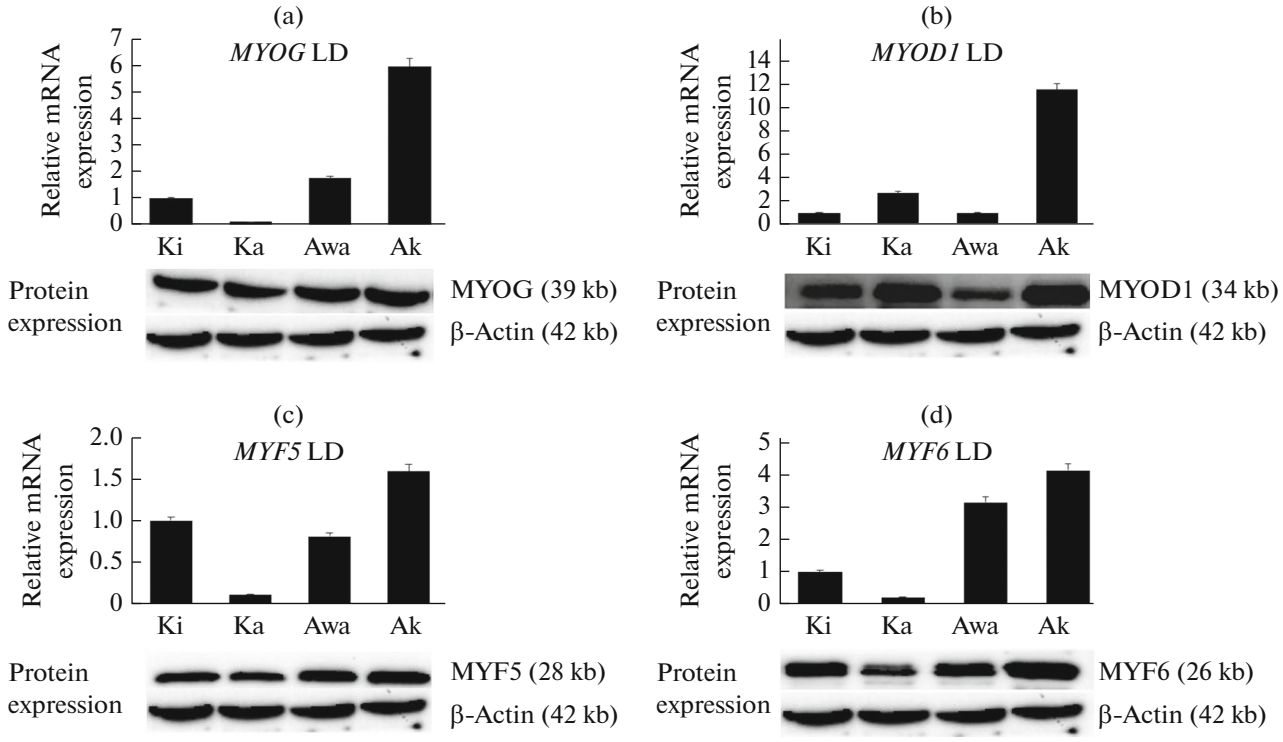

Fig. 4. Comparison of *longissimus dorsi* muscle tissue gene expression and western blot (a) MYOG, (b) MYOD1; (c) MYF5; (d) MYF6; Ki: Kıvırcık, Ka: Karayaka; Awa: Awassi; Ak: Akkaraman.

Merino and Han sheep breeds and reported that the gene expression levels of these genes were downregulated in muscle tissue. The present study presented that the *MYOG* gene expression was statistically significantly higher ($P < 0.05$) in the LD muscle in the Karayaka breed, one of the sheep breeds that was examined in this study. On the other hand, *MYOG* and *MYOD1* gene expression levels were found to be upregulated in the GL muscle in the fat-tailed Akkaraman breed. It was observed in the present study that the expression level of *MYOD1* gene decreased statistically significantly in the LD tissue of the fat-tailed Akkaraman breed samples ($P < 0.05$). This difference may be due to the nutritional difference and the geographical region where the animals are breed. These expression differences obtained in this study may be due to the different tail phenotype. Again, Sun et al. [13] reported that *MYF6* gene expression was upregulated in Merino and Han sheep which they investigated. Similarly, in this study, it was also observed that *MYF6* gene and protein expression levels were statistically significantly higher in GL muscle tissue of Akkaraman and Awassi breeds ($P < 0.05$).

Ropka-Molik et al. [29] reported that the expression level of *MYF6* in body muscles in different pig breeds did not change significantly. In this study, the expression level of *MYF6* gene was found to increase statistically significantly in fat-tailed Akkaraman and Awassi breeds ($p < 0.05$). This situation might be due to difference in species. Fan et al. [33] investigated the expression level of *MYF6* gene and protein in the skeletal muscle tissue of Duroc breed pigs with high meat fat ratio and Pietrain breed pigs with low meat fat ratio; and reported that *MYF6* gene and protein expression levels were statistically high in the Pietrain breed. In the present study, the fact that the expression level of *MYF6* gene and protein was upregulated in Akkaraman and Awassi breeds, the two fat-tailed breeds with high body fat accumulation in the tail, suggested that there may be a relationship between the expression level of this gene and fat metabolism. Studies on the expression patterns of major myogenic regulatory factors in pigs, an important source of red meat, may clarify the genetic basis of the postnatal myogenesis process and its effects on high or low muscle levels in different breeds of pigs [32]. This case may be valid for all livestock animals.

According to the results obtained in the study, it was observed that the gene and protein expression profiles of *MYOG, MYOD1, MYF5* and *MYF6* genes in both fat-tailed and thin-tailed sheep breeds were showed parallel increases and/or decreases in LD and GL muscles. With this the potential of using *MYOG, MYOD1* gene and protein expressions as markers in

Table 1. Information on the genes whose expressions were examined

Gene	Gene ID	PS	A	Reference
MYOD1	443405	F: 5'-GCTTGAGCAAAGTCAACGAG-3' R: 5'-GCCTTCGATATAGCGGATTG-3'	116 bp	[20]
MYOG	443158	F: 5'-ACTACCTGCCTGTCCACCT-3' R: 5'-CCCAGCCCCTTGTCTTCAA-3'	106 bp	[20]
MYF5	443159	F: 5'-CAGCAGTTTTGACAGCGTCT-3' R: 5'-TGATCCGATCCACTATGCTG-3'	107 bp	[20]
MYF6	100188930	F: 5'-CAGACCCAAGCAGGAAAATC-3' R: 5'-AATGCTTGTCCCTCCTTCCT-3'	124 bp	[20]
β-Actin	443052	F: 5-GGACCTGACGGACTACCTCATG-3 R: 5-GGCCATCTCCTGCTCGAAGT-3'	136 bp	[21]

MYOD1: Myoblast detection protein-1; *MYOG:* Myogenin; *MYF5:* Myogenic factor 5; *MYF6:* Myogenic factor 6; *β-Actin*: Actin beta; PS: Primer sequence; A: Amplicon size; bp: Base pair.

selection studies to improve meat yield in different muscle groups in Akkaraman sheep breed.

AUTHOR CONTRIBUTION

KA designed the study and supervised the experiments. DB contributed to animal material. FD contributed to gene expression and western blot analyses. MHS and MUÇ analyzed statistical data. The manuscript was prepared by FD and edited by KA and BA. All authors contributed to the manuscript and approved the submitted version.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Erciyes University (09.04.2014/14-78).

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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