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Research Article

Seasonal Gene Expression Profile Responsible for Hair Follicle Development in Angora Goats

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ABSTRACT

Mammals have physiological reprogramming adaptation ability to changing seasonal light and temperature, through their biological clocks maintained by circadian rhythm, photoperiodism and thermoperiodism. Seasonal differences do not only affect vital activities of animals like migration, reproduction, and sleeping, but also cause dramatic changes in their economically important characteristics (e.g. fur quality and fattening level). Mohair is constituted of non-medullary hairs produced by secondary hair follicles in Angora goats and the effects of seasonal differences on mohair structure and related genes are still unknown. We examined the gene expression levels of *BMP-2, FGF-5, HOXC13, KAP9.2 and TGFBR2* normalized with *GAPDH* in skin biopsies taken from Angora goats (n=20) in two different follicle development stages; telogen (in February) and anagen (in June). *HOXC13* showed overexpression in anagen phase ($P<0.005$) whereas expression was undetectable in telogen phase. *BMP-2* ($P<0.005$), *FGF-5* ($P<0.005$) and *TGFBR2* ($P<0.01$) were significantly upregulated in anagen, while *KAP9.2* expression showed no difference between two phases. This is the first study on hair follicle-related genes in the Angora goat. Additionally, depending the role of HOXC13 in pathways, it suggests that its overexpression may be one of the main factors associated with the non-medullary hair structure in Angora goats.

Keywords: Anagen, Hair follicle development, Mohair goat, Secondary hair follicles, Telogen

Introduction

Goat fiber is the most luxurious fiber in the world and is divided into two major products; Cashmere and Mohair, which are obtained from unique breeds [1]. However, many goat breeds yield less-valuable goat hair that is used mainly to produce felts, carpets, and tents. Due to its silky-like structure, heat resistance, easy dyeability and unique luster with a fine texture, mohair is one of the most preferred raw materials for the textile industry [2,3]. Mohair is produced by the Angora goat that originated from the district called Angora in Anatolia (present-day Ankara, the capital city of Türkiye).

The phylogenetic studies showed that maternal and paternal origins of the Angora goats were common with the other native goat breeds and the results pointed out Türkiye as the area of domestication and breeding center [3-7]. The beauty and eye-catching features of Angora goats have been well described in written records found in Sumerian cuneiforms [2]. During the Ottoman Empire period, the Angora goat was presented as a precious gift to other kingdoms and empires. Nowadays, Angora goats have been raised foremost in Turkey, United States, New Zealand and Argentina, for mohair production [8]. Although weaving with mohair is known as one of the oldest handicrafts of Anatolia and women have played an important role in this tradition for thousands of years, breeding Angora goats and producing of mohair are becoming values being more neglected and forgotten in Türkiye [8,9].

Animals show specific seasonal adaptation features according to light and temperature changes, and these physiological mechanisms are mainly coordinated by circadian rhythm, thermoperiodism and photoperiodism [10,11]. These factors do not only affect many vital activities such as hibernation and reproduction, but also affect the traits of economic importance e.g. fiber quality, fattening and milk yield [11]. Fiber production and

hair follicle (HF) development are mainly controlled by increased levels of melatonin hormone secretion during shortened daylight in autumn and winter [10,11]. There are two types of HF; primary hair follicles (PHF) and secondary hair follicles (SHF), the latter provides the non-medullary characteristics of Mohair [12].

There are three phases in the hair follicle development cycle: anagen (growth), catagen (regression), and telogen (quiescence) [13,14]. In goats, SHF forming the cashmere and mohair fibers remain in the active anagen phase between June and November (approximately 185 days), followed by the catagen phase between December and January (approximately 60 days), then from February to the end of May, the telogen phase proceeds (approximately 120 days) [15,16]. Differentiated gene expressions are also closely linked with hair follicle proliferation, growing and falling phases in the epidermal and mesenchymal cells. Despite the differentially expressed genes in follicle development phases that have been previously described and several genes were pointed out as key genes in the regulation of HF morphogenesis in Cashmere goats [17-19]. It has been shown that Homeobox C13 *(HOXC13)* was essential for hair shaft differentiation [20]. Bone Morphogenetic Protein-2 *(BMP-2),* Fibroblast Growth Factor-5 *(FGF-5)* and Transforming Growth Factor Beta Receptor-2 *(TGFBR2)* were associated with hair growth cycle regulation [18,19], while Keratin-Associated Protein-9.2 *(KAP9.2)* was responsible for keratinization [21]. However, there is no information on these genes for the mohair.

In the present study, it was aimed to examine the gene expression levels of *BMP-2, FGF-5, HOXC13, KAP9.2* and *TGFBR2* genes in the skin biopsies from Angora goats between two different follicle development periods to shed light on mohair development.

Material and Methods

Ethical Statement

All the procedures were carried out in accordance with the approval of the Animal Welfare Act. Ethical Committee of Ankara University, with the approval number 2014-18-137.

Sample Collection

In this study, skin biopsies were collected from 20 female Angora goats (2-3 years old), without at least 3 generations of common relatives according to their official records and best representing nationally registered breed characteristics in terms of hair, body size, and morphological characteristics. The goats were sampled from Güdül district of Ankara, which is mainly breeding area of Angora goats under the breeders condition and governmental inspection to protect breed characteristics. Before sampling hairs were trimmed and the skin surface was disinfected biopsies were taken from the right thoracic region using a 5 mm diameter sterile punch biopsy and the skin was sutured with a disposable alloy stamp after the biopsy procedure. The time schedule of the sampling has been determined considering the months with the highest and lowest annual temperatures and day lengths according of the studied geographic location [22] *(Fig. 1)*. According these parameters, telogen and anagen follicle development phases were sampled in February and June, respectively.

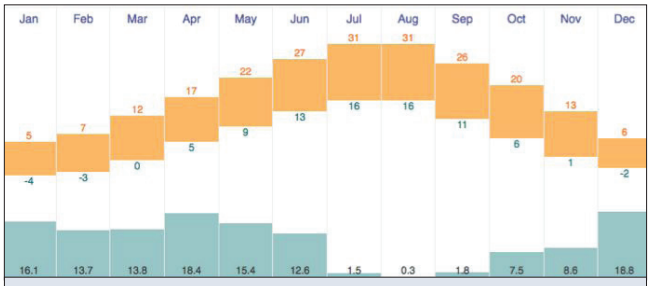

Fig 1. Annual weather averages near Ankara. The data was based on weather reports collected during 1985-2015. Orange lines represent lowest and highest temperatures (°C), green lines represent precipitation levels (mm)

Nucleic Acid Extraction and cDNA Synthesis

Skin samples (approximately 50 mg) were stored in cryovials immersed into liquid nitrogen (-196°C) to inhibit/stop RNAse activity. Tissue samples were homogenized using a pestle and mortar in liquid nitrogen and RNAs were extracted using PureZol, a monophasic combination of phenol and guanidine isothiocyanate (Biorad, USA, Cat no 7326890), according to the manufacturer's instructions. Obtained RNAs were treated with DNase (ThermoFisher, Germany, Cat no EN0525) and 1µg of total RNA was converted to cDNA in a reverse transcription (RT) reaction using the iScript cDNA Synthesis Kit (Biorad, USA, Cat no 1708891). Nucleic acids were measured by NanoDrop C2000 (ThermoFisher, Germany), visualized in Safe-View (NBS Biologicals, England, Cat no NBS-SV1) stained 1% agarose gel electrophoresis. The A260/A280 ratio of 1.8-2.1 was used to indicative assess RNA purity, while the integrity of the RNA was evaluated by 28S/18S ratio close to 2 to be indicative of intact RNA.

Histopathological Analysis

For histopathologic analyses, the frozen samples (half parts of skin biopsies) were directly transferred into cold 10% buffered formalin and fixated for 24 h. Following tissue processing procedure, tissues were dehydrated in degraded alcohol series, and cleared in xylene series, and they were embedded in paraffin (Leica TP1020). Paraffin

Table 1. *Designed oligonucleotides, amplicon length and Genebank accession numbers*

Gene	Sense Primer (5'-3') Antisense Primer (5'-3') TaqMan Probe (bold letters)	Amplicon Size, bp	Accession Number
BMP-2	ACACAGTGCGCAGCTTTCAC AAGAAGAATCGCCGGGTTGT **TCCCACTCATTTCCGGCAGTTCT**	82	NM_001287564.1
FGF-5	CCTCAGCACGTCTCTACCCA GACTTCTCCGAGGTGCGGAA **TCAAGCAATCGGAGCAGCCGGAACT**	145	XM_013964679.2
HOXC13	GCCCACCTCTGGAAGTCTCC TTGCTGGCTGCGTACTCCTT **TGCGCCCGCGCCTGTAGCTGT**	140	XM_018047656.1
KAP9.2	TGACCACCTGCTGTCAACCC CAGCTGGACCCACTGAAGGT **CCACAGCTGCTGGACCCACAGCAGGT**	70	XM_018065084.1
TGFBR2	ATCACGGCCATCTGCGAGAA GCAGACCGTCTCCAGTGTGA **CAGCCACGCAGACCTCCTCCGGC**	87	XM_018067217.1
*GAPDH**	GCATCGTGGAGGGACTTATG CAGTAGAAGCAGGGATGATGTT **ATCACTGCCACCCAGAAGACTGTG**	129	AJ431207.1

** Internal control gene*

blocks were sectioned at a thickness 6-8 µm and stained with hematoxylin-eosin [23]. Histopathologic evaluation was performed using a light microscope (Trinocular Olympus BX51 microscope attached with DP25 digital camera) for the hair follicle development phases and hair morphologic structures.

q-PCR and Measurement of Expression Levels

To analyze seasonal effects on the molecular regulation of hair follicle cycling, *BMP-2, FGF-5, HOXC13, KAP9.2* and *TGFBR2* genes were selected based on Wnt, activin/BMP, and TGFB signaling pathways according to the previous comparative transcriptomic study in skin of Cashmere goats [19]. The specific oligonucleotides and TaqMan probes were designed according to mRNA sequences of the genes in concordance with exon-exon junction regions by using Genscript [24] *(Table 1)*. *GAPDH* was used as a reference gene to normalize the gene expressions [25].

Quantitative real-time PCR (qPCR) was performed in duplicate using the CFX96 Connect real time system (Biorad, USA). A 20 µL reaction mix containing 20 ng of cDNA template, 1x SsoAdvanced Universal Probes Super mix (Biorad, USA, Cat no 1725280), 0.25 µM each primer and 0.2 µM specific Taqman probe were amplified at the following conditions, initial denaturation at 95°C for 3 min followed by 40 cycles of 95°C for 10 sec, annealing at 68°C for 10 sec, and extension at 72°C for 10 sec. Amplification was completed by an additional cycle at 72°C for 30 sec. To evaluate the amplification specificity of TaqMan primers, melting curves were generated in the range of 65°C to 95°C, with the temperature increasing at a rate of 0.5°C/sec. Differentially expressed gene levels were analyzed by using relative gene expression analysis with $2^{\Delta\Delta Ct}$ [26]. A Wilcoxon two-group test was performed to determine the significance of the differences between the two groups, due to the small sample size. In order to further explore the molecular mechanism of the proteins in hair follicle development, a biological pathway was evaluated from wikipathway [27].

Results

Histological Findings

The follicle development cycle was classified as anagen (growth), catagen (regression), and telogen (quiescence). Anagen follicles in the deep dermis have fully developed sebaceous glands and well-identified inner and outer root sheaths *(Fig. 2-A)*. The catagen phase frequently showed a transitional period from growth to rest periods and characterized dermal papilla condensation and thickness in basement membranes of follicle epithelia. The shrinking outer root sheath is highly degenerative and had a characteristic hyaline vitreous membrane *(Fig. 2-B)*. Well-defined distinct inner and outer root sheaths indicated the anagen *(Fig. 2-C)*. Follicles characterized by wrinkled inner root sheath appearing as an amorphous keratin mass (trichilemmal keratinization) were considered as telogen phase *(Fig. 2-D)*.

qRT-PCR Analysis and Statistics

Statistical analyses were performed on log fold-changes (ddCTs), and fold changes ($2^{\Delta\Delta Ct}$) were illustrated as bar graphic *(Fig. 3)*. To compare anagen and telogen,

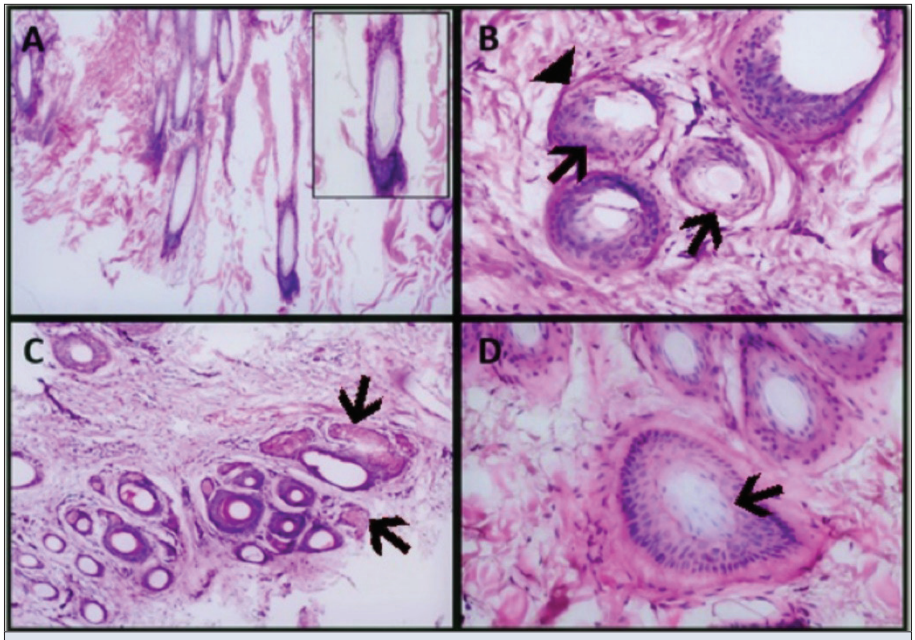

Fig 2. Classification of hair cycle stages in Angora goats using transverse hair follicle sections. Results were examined by HE staining. **A.** Deep dermis anagen (growth phase) follicles, well-defined inner and outer root sheats, **B.** Degenerative outer root sheaths *(black arrows)* and hyaline membrane *(arrowhead)* in the catagen phase, **C.** Well defined distinct inner and outer root sheaths indicate anagen. Advanced sebaceous glands *(black arrows),* **D.** Telogen phase characterized by an inner root sheath that appears as an amorphous keratin mass *(black arrow)*

descriptive statistics and ddCTs of the genes normalized to *GAPDH* were presented in *Table 2*. It was determined that *BMP-2* (P<0.005), *FGF-5* (P<0.005) and *TGFBR2* (P<0.01) were significantly upregulated in anagen, while no significant change was observed between two phases for *KAP9.2* gene expression (P>0.05). A high level of expression was determined for *HOXC13* in the anagen phase (P<0.005), whereas expression was not detected in the telogen phase. To estimate the relative quantification, Ct values were accepted as 40 for this gene in the telogen phase.

Discussion

Mohair production and intense Angora goat breeding are currently made in many countries in the world and the processed mohair products have been considered exclusively unique and their economic value is at the utmost level. Albeit Turkey has excellent preciousness as being the first goat domestication and mohair goat production site [3-7], due to improper agricultural policies and socio-economic conditions, losing its advantages in terms of qualified mohair production.

The ratio of primary/secondary follicles per mm² in Angora goat skin is between 5.3 to 9.1. The hair of the Angora goat differs from other wool fibers by their homogenous mohair production, which is relatively close to each other, due to the low amount of medullary fibers and the high number of secondary hair follicles that solely produce mohair. The cuticle, cortex, and medulla layers of mohair fiber are the same as in other fibers. The cells in

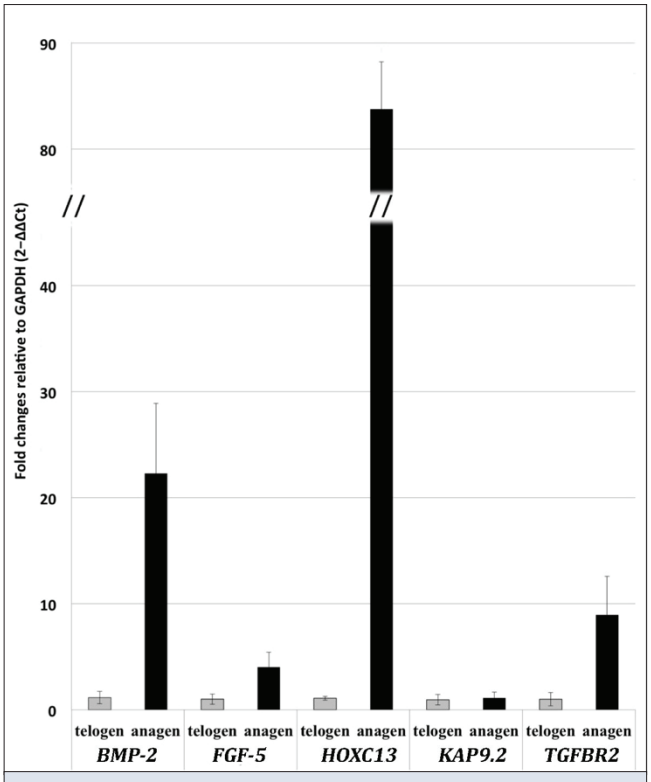

Fig 3. Bar graph presentation of fold change of *BMP-2, FGF-5, HOXC13, KAP9.2* and *TGFBR2* genes in June samples (anagen) compared to February samples (telogen). All Ct values are normalized to *GAPDH* and expressed as change from their respective controls

Table 2. *Log fold-changes (ddCTs) of the genes normalized to GAPDH in anagen compared to telogen phase. The mean ddCT values and standard deviations were estimated on two technical replicates in each condition*

ddCT Descriptive Statistics	***BMP-2***	***FGF-5***	***HOXC13***	***KAP9.2***	***TGFBR2***
Mean	4.4268	2.0423	6.3797	-0.1104	3.0485
Standart deviation	0.4020	0.5233	0.5479	1.1275	0.6171
P-value	0.0026**	0.0034**	0.0049**	0.4473	0.0051*

*Significant difference is indicated by asterix, *P<0.01; **P<0.005*

the cuticle layer are thin, wide in morphology and do not fold over each other, causing the mohair to be brighter and softer. The fact that the cortex layer has a small number of orthocortex cells in the mohair fiber enables the fiber to take dye easily. Mohair fiber has a discontinuous type of medulla. The quality of the mohair decreases when the medulla fiber ratio exceeds 4% [28]. In the present study, it was demonstrated that the hair follicles were not containing a prominent medulla typical of Angora goat mohair. Skin biopsies collected during winter months showed that telogen phase hair follicles histology, while their morphology changed to anagen phase in the summer months. In this study, skin biopsies were stored in liquid nitrogen and then they were transferred to frozen sectioning for histologic analysis. Thus, albeit the section quality was enough for the histologic evaluation of follicle structures, especially collagen fibers and epithelia were not in the desired consistency and showed some understaining features with hematoxylin eosin. It is also suggested that skin biopsies frozen in liquid nitrogen might be suitable for prolonged periods, at least 6 months, for histologic evaluation, if they are forwarded to the frozen sectioning.

Homoebox *(Hox)* genes are evolutionarily conserved transcription factors that regulate cell fate during embryonic development. In mammals *Hox* genes are clustered into four groups through a-d and genes are divided into 13 paralogous groups [29]. In the later embryonic stages in mice, *HOXC13* expression was found in all body hair follicles, in the filiform papillae of tongue epithelium and the footpad epidermis, while in the postnatal stage, expression was determined in anagen hair follicles, mainly in the matrix of the hair bulb and the precortical region of the hair shaft [30], proving its importance in hair growth cycle regulation. In Cashmere goats, though the measured expression of *HOXC13* was higher in telogen, expression was determined in both phases and even higher expression was determined in low fleece yielding Cashmere goats [21]. However other studies [20,31] found that *HOXC13* expression was higher in anagen, compared to catagen and telogen. In the presented study, in contradiction to previous findings, *HOXC13* gene expression was measured only in the anagen phase. Moreover, Tkatchenko et al.[32] speculated that overexpression of *HOXC13* inhibits hair follicle specific gene/genes. The downregulation caused by inhibition acts as negative feedback circuits. In concordance with the non-medullary SHF results of microscopy, we speculate that overexpression of *HOXC13* might lead to the same effect, for non-medullary HFs in mohair goats. The biological pathway [27] showed that *HOXC13* was related indirectly via *FOXQ1* and interestingly *FOXQ1*-null mice showed non-medullary HFs and satin hairs [33]. Non-medullary HFs resulting in silky and satin texture of mohair are important characteristics of Angora goat [22,34]. To understand the genetic mechanism behind this formation, HOXC13 and FOXQ1 proteins should be structurally investigated and those amounts should be measured in different phases of HF development in Angora goats.

Transforming Growth Factor Beta proteins (TGFBs) have vital importance in the regulation of the transcription of genes related to cell proliferation, cell cycle arrest, wound healing, immunosuppression, and tumorigenesis [35]. The lack of TGFB2 protein in mice results delay in HF morphogenesis and also decreases the number of HF [36]. Oshimori and Fuchs [37] also showed that conditional loss of TGFBR2 led to a prolonged telogen phase and delayed anagen initiation in TGFB2 signaling-deficient mice. Consistent with the literature, we found that *TGFBR2* was present in telogen, however, significant expression was measured in the anagen (P<0.01).

BMPs belong to the TGFB superfamily and TGFB/BMP interactions have been shown to play a central role in hair shaft growth and differentiation [18]. *BMP-2* expression is important for early embryonic development, maintaining homeostasis and cell fate in adults [38]. Also, in HF stem cells TGFB and BMP activation cause proliferating keratinocytes to transiently withdraw from the cell cycle thus changing the HF phase [18,39]. Li et al.[31] reported that *BMP-2* was highly expressed during the anagen by analyzing with time-course RNA-seq analysis on skin biopsies of Inner Mongolia cashmere goats. In the comparison of anagen and telogen phases for the expression of *BMP-2* gene, we have determined a strong upregulation in the anagen (P<0.01). Similarly, Su et al.[40] also determined an upregulation in secondary hair follicles in early anagen of Cashmere goats. Obtaining data from Angora goats is compatible with this knowledge.

FGF-5 is a signaling protein during the hair growth cycle, which inhibits hair growth by blocking papilla cell activation [41]. The *FGF-5* gene is associated with the Angora phenotype (long hair coat) in mice [42], long furred breeds of cats [43] and dogs [44], and as well as trichomegaly in humans [45]. Silencing of the *FGF-5* gene showed an increase in not only hair length but also the number of SHF in Cashmere goats [46]. Guo et al.[47] reported that one novel SNP (c.-253G>A) in the 5′-UTR of *FGF-5* resulted in a premature protein and was likely a causal variant for the long hair phenotype of cashmere goats. In the present study, *FGF-5* showed a moderate upregulation in the anagen ($P<0.01$). This result was consistent with the study of Zhang et al.[48]. Deep sequencing of *FGF-5* and revealing possible variants in Angora goats might contribute to the understanding of the angora phenotype.

According to relative gene expressions, all genes upregulated during the anagen stage except for *KAP9.2* gene. As a member of a KAP family consisting of 27 families with 100 genes, *KAP9.2* gene is responsible for keratinization [49]. Liu et al.[50] analyzed expression levels of *KAP9.2* in anagen, catagen, and late telogen in sheep and showed statistically significant differences between HF development phases. Even though studies determined the importance of *KAP9.2* expression in Cashmere goats as well [21], unlikely, our data didn't show any differences in the expression level of this gene in Angora goats between anagen and telogen phases. Wang et al.[21] indicated that lower expression of *KAP9.2* gene during anagen stages resulting higher yields in cashmere goats than that of their catagen stages. Thus, this is the clear indication of *KAP9.2* gene activity shows seasonal differences in cashmere goats. Moreover, it is also evidenced that the goat hairs in anagen stages having low *KAP9.2* gene expression yield high-quality cashmere, than that of individuals having higher *KAP9.2* gene activity in anagen stages. The fine and non-medullary structure of Angora goat's hair, has enhanced its quality and well-curling nature. Low levels of *KAP9.2* during both anagen and telogen stages in the present study can be explained by the quite different hair morphology of Angora goat breed, which has no hair medulla and fiber pigmentation. As far as the authors' knowledge, *KAP9.2* gene analysis and its correlation between the hair developmental stages in Angora goats have not been studied before, and this finding is indeed interesting as it can be considered for the breed genetic difference and may give great information on the development of Angora hair's constant quality. However, the authors believe that this novelty needs confirmation in the future.

In conclusion, the comparison of some gene expression levels in anagen and telogen phases; *FGF-5, TGFBR2* and *BMP-2* expressions are significantly up-regulated in anagen of the skin biopsies from Angora goats. Between the two phases for *KAP9.2* expression showed no difference. The *HOXC13* was strongly overexpressed in anagen and was undetectable in telogen. This is the first study on hair follicle-related genes in the Angora goats. The comparative studies including different geographical zones and further analyses based on omics technologies can improve our understanding of mohair regulation and secondary hair follicle formation.

Availability of Data and Materials

The authors declare that the data and materials are available on request from the corresponding author (B. Çınar Kul).

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Competing Interest

The authors declared that there is no competing interest.

Author Contributions

Experimental design was performed by BCK, NB and OK, material preparation and analysis were performed by BCK, NB, MYA, OSC, OO, MB, OK, results were interpreted by BCK and OK, the first draft of the manuscript was written by BCK and OK, all authors contributed to the final version of the manuscript.

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