Seasonal Gene Expression Profile Responsible For Hair Follicle Development In Angora Goats

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Abstract

Background: Mammals have physiological reprogramming adaptation ability to changing seasonal light and temperature, through their biological clocks maintained by circadian rhythm, photoperiodism and thermoperiodism. These seasonal differences do not only affect vital activities of animals like migration, reproduction, sleeping, but also cause dramatic changes in their economically important characters (e.g. fur quality, fattening levels and milk yield). 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.

Methods and Results: 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 and anagen, sampled in February and June, respectively. HOXC13 showed high level of expression in anagen phase whereas expression was undetectable in telogen phase. TGFBR2, FGF-5, and BMP-2 were significantly upregulated in anagen, while KAP9.2 expression showed no difference between two phases.

Conclusions: This is the first study on hair follicle-related genes in the angora goat and revealed that these genes differ between geographic zones and/or breeds. Additionally, we speculate that overexpression of HOXC13 might be one of the underlying factors associated with non-medullary hair nature, making the mohair more shiny and silky in Angora goats.

Introduction

Goat fiber is the most luxurious fiber in the world and 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 the 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 material for textile industry [2]. Mohair is produced by the Angora goat originated from the district called Angora in Anatolia (present day Ankara, capital city of Turkey).

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 Turkey as the area of domestication and breeding center [3–5]. The beauty and eye-catching features of Angora goats have been well described in written records found in Sumerian cuneiforms dated 12th century, and 16th century voyagers journals [2]. During the Ottoman Empire period, Angora goat had been presented as a precious gift to other kingdoms and empires. In nowadays, Angora goats have been raised foremost in Turkey, United States, New Zealand and Argentina, for mohair production [6]. 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 the mohair are becoming values being more neglected and forgotten in Turkey [6–7].

Animals show specific seasonal adaptation features according to light and temperature changes, and these physiological mechanisms mainly coordinated by circadian rhythm, thermoperiodism and photoperiodism (Lin et al., 2009; Dibner et al, 2010). These factors do not only affect many vital activities such as hibernation and reproduction, also affect the traits of economic importantance e.g. fiber quality, fattening and milk yield. Fiber production and hair follicle (HF) development are mainly controlled by increased levels of melatonin hormone secretion during shortened daylights in autumn and winter [8–9]. There are two types of HF; primary hair follicles (PHF) and secondary hair follicles (SHF), the latter provides the non-medullary characteristics of Mohair.

There are basically three phases in the hair follicle development cycle: anagen (growth), catagen (regression), and telogen (quiescence) [10–12]. 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) [13–15]. Differentiated gene expressions are also closely linked with the hair follicle proliferation, growing and falling phases in the epidermal and mesenchymal cells. Despite the differentially expressed genes in follicle development phases have been previously described and several genes were pointed out as key genes in regulation of HF morphogenesis in Cashmere goats [15–17]). It has been shown that, Homeobox C13 (HOXC13) was essential for hair shaft differentiation [18]. 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 [16, 17], while Keratin-Associated Protein-9.2 (KAP9.2) was responsible for keratinization [19]. 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 BMP2, FGF5, 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.

Materials And Methods

Sample collection

In this study, skin biopsies were collected from 20 unrelated female goats (2–3 years old) representing specific phenotypes of Angora goats in February and June corresponding telogen and anagen follicle development phases, respectively. Prior to sampling hairs were trimmed and skin surface was disinfected biopsies were taken from the right thoracal region using 5 mm diameter sterile punch biopsy and skin sutured with disposable alloy stamp after biopsy procedure. Since it is known that day length regulates hair growth by mechanisms proceeding through melatonin due to its relationship with photoperiodism [20] and thermoregulation [21], 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). All the procedures were carried out in accordance with the approval of Animal Welfare Act. Ethical Committee of the Ankara University, with the 2014-18-137 numbered report.

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 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 manufacturer's instructions. Obtained RNAs were treated with DNase (ThermoFisher, Germany, Cat no EN0525) and 1 µg of total RNA 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.

Histopathological analysis

For histopathologic analysis, remaining part of the skin samples stored in liquid nitrogen were firstly transferred to − 80°C for 2 hours, then frozen sectioning was performed and tissue sections were obtained in 6-8µm in thickness. They were routinely stained with hematoxylin-eosin and evaluated for hair follicle development phases and hair morphologic structures using light microscope (Trinocular Olympus BX51 microscope attached with DP25 digital camera).

q-PCR and Measurement of Expression Levels

To analyze seasonal effects on the molecular regulation of the hair development, BMP-2, FGF-5, HOXC13, KAP9.2 and TGFBR2 genes were selected based on Wnt, activin/BMP, and TGFB signaling pathways. Genes were selected from different functional pathways to minimize the possibility of co-regulation. The specific oligonucleotides and TaqMan probes were designed according mRNA sequences of the genes in concordance with to exon-exon junction regions by using Genscript [23] and BiSearch [24] (Table 1). GAPDH was used as a reference gene to normalize the gene expressions [25].

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.

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. Significant difference is indicated by asterisk, *P < 0,01; **P < 0,005.

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*

Quantitative real-time PCR (qPCR) were performed in duplicate using the CFX96 Connect real time system (Biorad, USA). A 20 µl reaction mix containing 20ng of cDNA template, 1× SsoAdvanced Universal Probes Super mix (Biorad, USA, Cat no 1725280), 0.25µM each primers and 0.2µM specific Taqman probe were amplified at following conditions, initial denaturation at 95°C for 3 min followed by 40 cycles of 95°C for 10s, annealing at 68°C for 10s, and extension at 72°C for 10s. Amplification was completed by an additional cycle at 72°C for 30s. 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/s. Differentially expressed gene levels were analyzed by using relative gene expression analysis with $2^{\Delta\Delta Ct}$ [26]. Wilcoxon two group test was performed to determine the significance of the differences between two groups, due to small sample size [27].

Results

Histological Findings

Follicles development cycle was classified as anagen (growth), catagen (regression), and telogen (quiescence). Anagen follicles in the deep dermis have had 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). 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

Statistical analyses were performed on log fold-changes (ddCTs), while fold changes ($2^{\Delta\Delta Ct}$) were used to show the relative expressions (Fig. 3). To compare anagen and telogen, descriptive statistics and ddCTs of the genes normalized to GAPDH were presented in Table 1. The mean ddCT values and standard deviations were estimated on two technical replicates in each phase. It was determined that BMP-2 (P-value = 0,0026), FGF-5 (P-value = 0,0034) and TGFBR2 (P-value = 0,0051) were significantly upregulated in

anagen, while no significant change was observed between two phases for KAP9.2 gene expression (P-value = 0,4473). High level of expression (P-value = 0,0049) was determined for HOXC13 in anagen phase, whereas expression was not detected in telogen phase. To estimate of the relative quantification, Ct values were accepted as 40 for this gene in telogen phase.

In order to further explore the molecular mechanism of the genes in hair follicule development, biological pathway was performed and revealed that HOXC13 was related with directly hair follicule differentiation and indirectly via FOXQ1 (Fig. 4).

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 as exclusively unique and their economic value is in utmost level. Albeit Turkey has an excellent preciousness as being the first goat domestication and mohair goat production site [3–5], due to improper agricultural policies and socio-economic conditions, loosing its advantages in terms of qualified mohair production.

The ratio of primary/secondary follicles per mm^2 in Angora goat skin is between 5,3 to 9,1. Angora goat finesse are characterized 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 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 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 quailty of the mohair decreases when medulla fiber ratio exceeds 4% [28]. In the present study, it was demonstrated that the hair follicles were not containing prominent medulla typical to 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 for follicle structures, especially collagen fibers and epithelia were not in the desired consistency and showed some understaining features with hematoxylin eosin. It is also suggesting that skin biopsies freezed in liquid nitrogen might be suitable for prolonged periods, at least 6 months, for histologic evaluation, if they are forwarded to the frozen sectioning.

Hox or Homoebox genes are evolutionarily conserved transcription factors that regulates cell fate during embryonic development. In mammals Hox genes are clustered into four groups through a-d and genes divided into 13 paralogous groups [29]. In the later embryonic stages in mouse, HOXC13 expression found in all body hair follicles, in the filiform papillae of tongue epithelium and in footpad epidermis, and in the postnatal stage expression 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 measured expression of HOXC13 was higher in telogen, expression was determined in both phases and even higher expression determined in low fleece yielding Cashmere goats [19]. However another studies [18, 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 anagen phase. Moreover, Tkatchenko et al. (2001) [32] speculated that overexpression of HOXC13 inhibits hair follicule 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 same effect, for non-medullary HFs in mohair goats. Biological pathway revealed that HOXC13 was related with indirectly via FOXQ1 and interestingly FOXQ1-null mice showed non-medullary HFs and satin hairs [33]. Non-medullary HFs resulting silky and satin texture of mohair are important characteristics of Angora goat [20, 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 regulation of the transcription of genes related to cell proliferation, cell cycle arrest, wound healing, immunosuppression, and tumorigenesis [35]. The lack of TGFB2 in mice results a delay in HF morphogenesis and also decrease the number of HF [36]. Oshimori and Fuchs (2012) [37] also showed that conditional loss TGFBR2 led to prolonged telogen phase and delayed anagen initiation in TGFB2 signaling–deficient mice. Consistent with literature, we found that TGFBR2 was present in telogen, however significant expression measured in anagen (P-value = 0,0051).

BMPs belong to the TGFB superfamily and TGFB/BMP interactions have been shown to play a central role in hair shaft growth and differentiation [16]. 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 HF phase [16, 39]. Li et al. (2022) [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 comparison of anagen and telogen phases for expression of BMP-2 gene, we have determined a strong upregulation in anagen (P-value = 0,0026). Similarly, Su et al., (2009) [40] also determined an upregulation in secondary hair follicles in early anagen of Cashmere goats. Obtained 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]. 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 increase in not only hair length but also the number of SHF in Cashmere goats [46]. Guo et al. (2019) [47] reported that one novel SNP (c.-253G > A) in the 5'-UTR of FGF-5 resulted a premature protein and was likely a causal variant for long hair phenotype of cashmere goats. In the present study, FGF-5 showed a moderate upregulation in anagen (P-value = 0,0034). 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 up regulated during anagen stage except for KAP9.2 gene. As a member of a KAP family consisting 27 families with 100 genes, KAP9.2 gene is responsible for keratinization [48, 49]. Liu et al. (2015) [50] analyzed expression levels of KAP9.2 in anagen, catagen, and late telogen in sheep and showed statistically significant difference between HF development phases. Even tough studies determined the importance of KAP9.2 expression in Cashmere goats as well [19], unlikely, our data didn't show any differences of this gene expression level in Angora goats between anagen and telogen phases.

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 two phases for KAP9.2 expression showed no difference. The HOXC13 was strongly overexpressed in anagen and was undetectable in telogen. Based on the result obtained from this study, we speculate that overexpression of HOXC13 by triggering negative feedback circuits might be one of the underlying factors associated with non-medullary hair nature, resulting more shiny and silky mohair in Angora goats, by triggering negative feedback circuits. This is the first study on hair follicle-related genes in the Angora goats and revealed that these genes differ between geographic zones and/or breeds. The comparative studies including different geographical zones and further analyses based on omics technologies can improve our understanding of mohair regulation and secondary hair follicule formation.

Declarations

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Author Contributions

Experimental design was performed by Bengi CINAR KUL, Nuket BILGEN and Oguz KUL, material preparation and analysis were performed by Bengi CINAR KUL, Nuket BILGEN, Mustafa Yenal AKKURT, Ozge Sebnem CILDIR, Ozge OZMEN, Merve BISKIN, Oguz KUL, results were interpreted by Bengi CINAR KUL and Oguz KUL, first draft of the manuscript was written by Bengi CINAR KUL and Oguz KUL, all authors commented on previous versions of the manuscript.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Ethics approval

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

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Figures

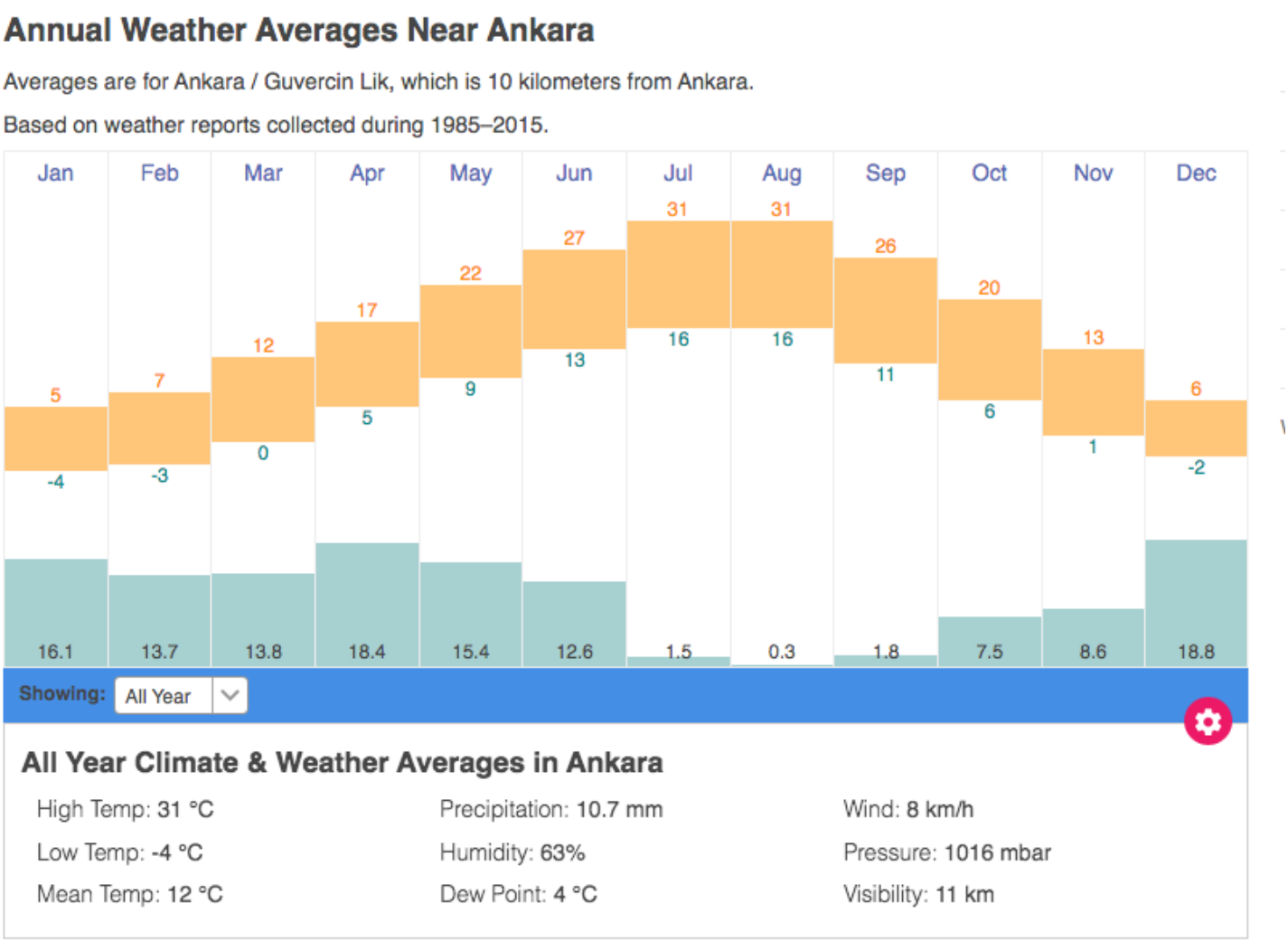

Figure 1

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

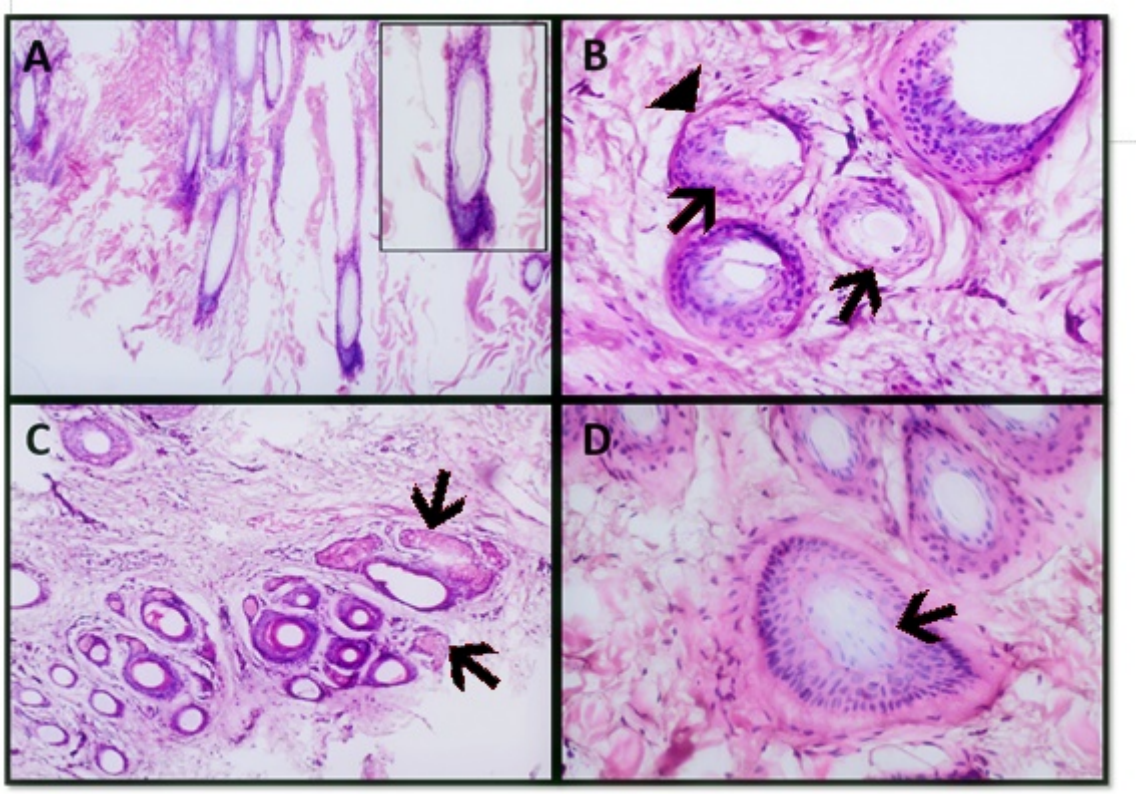

Figure 2

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).

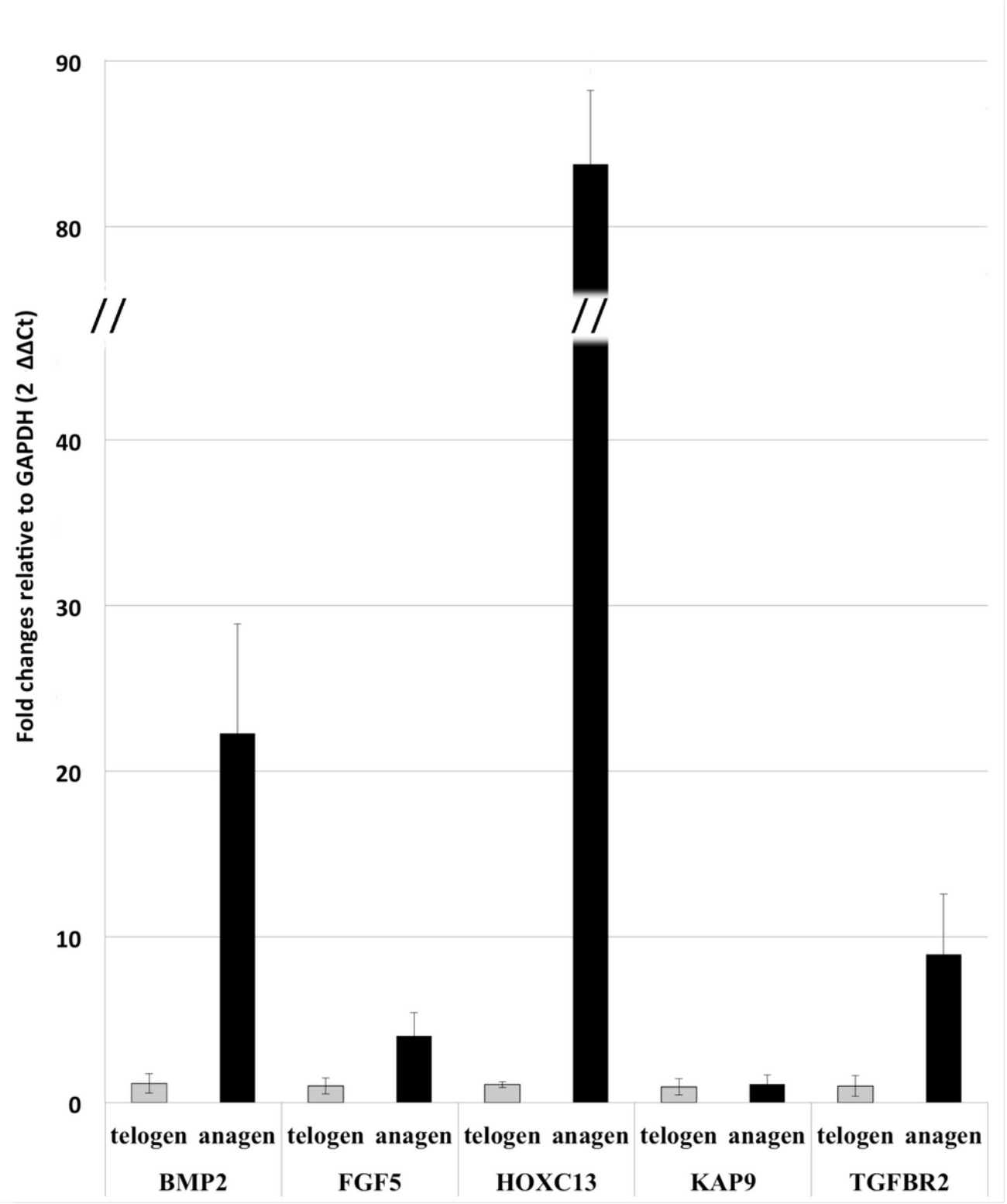

Figure 3

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.

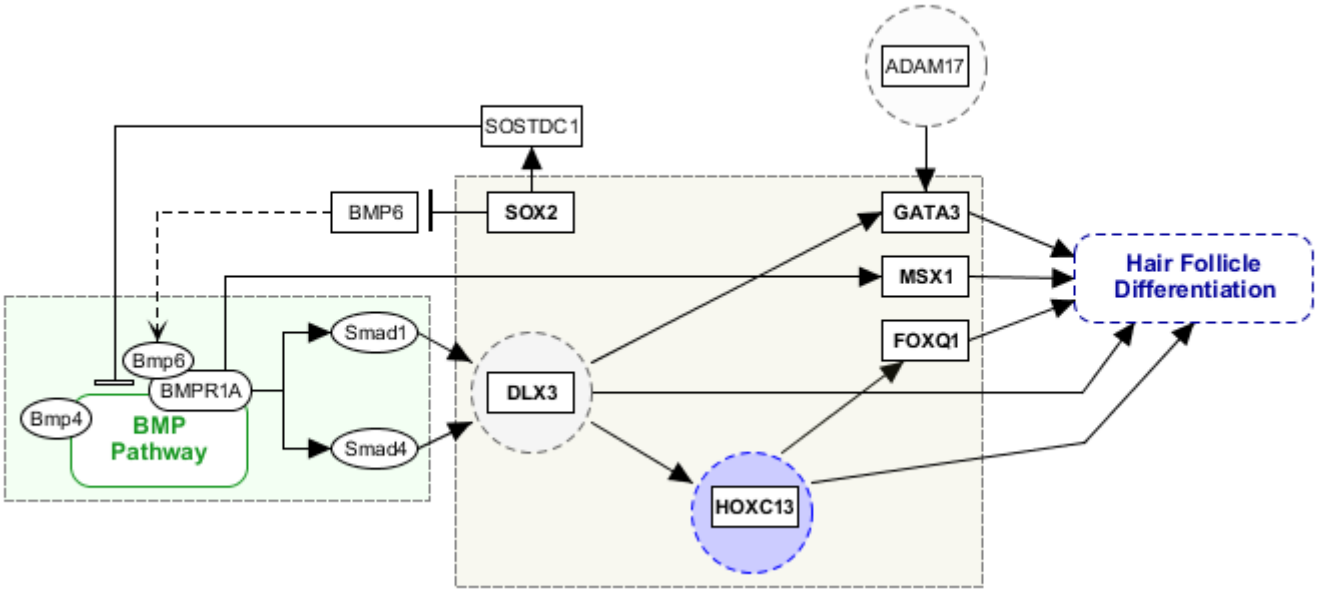

Figure 4

HOXC13 network for hair follicle differantation. Modified from: Trindade D., Slenter D., Ehrhart F., Mélius J., Hanspers K., Willighagen E., Weitz E., Winckers L. Hair follicle development: cytodifferentiation -part3. Last accession date:21.10.20021 https://www.wikipathways.org/index.php/Pathway:WP2840#nogo2