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Expression of the *RORα* gene in Inner Mongolian cashmere goat hair follicles

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ABSTRACT. The expression of retinoid-acid-related orphan receptor α (*RORα*) was evaluated at the mRNA level using real-time polymerase chain reaction (qRT-PCR), and its expression localization was determined by *in situ* hybridization of adult Inner Mongolian cashmere goats at different times of the year. *In situ* hybridization demonstrated that *RORα* was expressed in secondary hair follicles of the hair shaft, inner root sheath, outer root sheath, medulla, and other parts that are target organs of the *RORα* receptor gene. qRT-PCR results showed that there was no significant difference in the *RORα* mRNA abundance in February, April, August, and October ($P > 0.05$), and the only difference occurred in December relative to February, August, and October ($P < 0.05$). This difference revealed that melatonin possibly promotes cashmere growth through the nuclear receptor *RORα*. This

study provides a good foundation for future studies on the relationship between the melatonin receptor and cashmere growth; in addition, it provides new insights for increased cashmere production and quality.

Key words: *RORα*; *RORα* expression; Cashmere goats; *In situ* hybridization; Real-time polymerase chain reaction

INTRODUCTION

Retinoid-acid-related orphan receptor α (*RORα*) belongs to the orphan nuclear receptor (ONR) family, which includes unique members of the nuclear receptor superfamily (Aranda and Pascual, 2001) that is widely distributed in all body tissues. By entering the nucleus to directly activate target gene transcription, ONRs participate in a variety of important physiological processes (Giguère et al., 1995; Medvedev et al., 1997; Moraitis and Giguère, 1999). Besides, ONRs play important roles in development and cell differentiation by maintaining body homeostasis, and they are involved in advanced neural control (Dzhagalov et al., 2004; Boukhtouche et al., 2006; Jaradat et al., 2006; Gold et al., 2007).

Previous studies have shown that melatonin can promote hair growth in goats (Wuliji et al., 2003b; Abecia et al., 2005), but the signal pathway and regulation mechanism involved are still poorly understood. Because *RORα* is one of the melatonin receptors (Pozo et al., 2004), we studied *RORα* expression in skin hair follicles to provide new insights into the mechanism of melatonin's function in promoting hair growth.

MATERIAL AND METHODS

Experimental animals and skin samples

Male Inner Mongolian cashmere goats from a goat stud farm (stud farm, Inner Mongolia, Erdos) were used in this study. Prior to sample collection, procedures for skin sampling from adult animals were approved by the National Animal Care Standard (GB14925-2001), and sample collection was performed under their supervision. Skin biopsy samples measuring 1 cm^2 were harvested from the lateral side of the body of 6 adult goats using a scalpel during the months of February, April, August, October, and December (a total of 30 samples were obtained during 5 months); all the samples were frozen in liquid nitrogen for real-time polymerase chain reaction (qRT-PCR) or fixed with 4% paraformaldehyde solution for paraffin section preparation.

RNA isolation and cDNA synthesis

Total RNA was extracted from skin using RNA extraction kits (Takara Inc., Madison, WI, USA). The RNA concentration and purity were determined using the $A_{260/280}$ absorbance and ratios. Total RNA was used for cDNA synthesis. Reverse transcription was employed to obtain the first-strand cDNA, which was used as the template for PCR amplification. The PCR products were separated by 1.2% agarose gel electrophoresis. The fragment corresponding to the *RORα* cDNA was purified, cloned, and then sequenced.

RORα expression and localization

The plasmid DNA containing a 417-bp fragment was obtained by cloning and was used as the template for amplifying the *RORα* gene; universal primers (SP6 and T7) were used. The PCR products were purified and used as the template for the preparation of sense and antisense probes. RNA *in situ* hybridization was performed using a digoxigenin-labeled riboprobe.

RORα expression levels

qRT-PCR was employed to quantify the relative gene copy number of *RORα* expression in goat skin. The primers used for upstream qRT-PCR amplification are shown in Table 1. Beta actin (β-actin) was used for normalization as the housekeeping gene. Relative expression levels of *RORα* were calculated with respect to the housekeeping gene.

Statistical analysis

Sequence data were analyzed using Sequencher Version 4.1 (Gene Codes Corp., Ann Arbor, MI, USA) and aligned using CLUSTAL W. Homology searches were performed on the network server of the National Center for Biotechnology Information. Experimental data were analyzed by one-way analysis of variance using the SPSS13.0 statistical software for Windows (Chicago, IL, USA). The results are reported as means ± SE, and the least significant difference test was used for *post hoc* multiple comparisons. Every group is described as the expression of cDNA in cashmere goat skin tissue during different months. Samples were harvested from 6 individual cashmere goats in February, April, August, October, and December. The experiment was performed in triplicate. Different letters indicate a significant difference with $P < 0.05$.

RESULTS

Amplification of the *RORα* cDNA fragments

The designed primers (Table 1) were used to amplify the *RORα* cDNA fragments from the skin samples. The product length was 417 bp. The results of the qRT-PCR amplifications are shown in Figure 1. After cloning and sequencing, the *RORα* products were aligned with the *RORα* sequences for other species obtained from GenBank. The homology between goat *RORα* and other species was 98.3%, as shown in Figure 2. This result confirms that the qRT-PCR fragment is the *RORα* cDNA fragment from goat.

In situ hybridization analysis

To characterize the location of *RORα* mRNA expression in hair follicles, skin samples collected in February, April, August, October, and December from adult Inner Mongolian cashmere goats were used for *in situ* hybridization following the standard protocol. The localization of *RORα* expression in hair follicles over 5 months was mainly distributed in the inner root sheath, medulla of primary hair follicles, and inner root sheath of secondary follicles in April. Stronger expression signals were present in the cortex and the outer root sheath of primary follicles in August. There were obvious expression signals in primary follicles and the

secondary follicles in October, and in the outer root sheath of primary and secondary follicles in December, as shown in Figure 3.

Table 1. Polymerase chain reaction (PCR) primers used for real-time PCR amplification.

Gene		Primers	Product size (bp)
RORα	Forward	5'-GCCGCTGACTCCCACCTAT-3'	417
	Reverse	5'-ATCACTCCCGCTGCTTG-3'	
β-actin	Forward	5'-GTCACCAACTGGGACGACA-3'	208
	Reverse	5'-AGGCGTACAGGGACAGCA-3'	

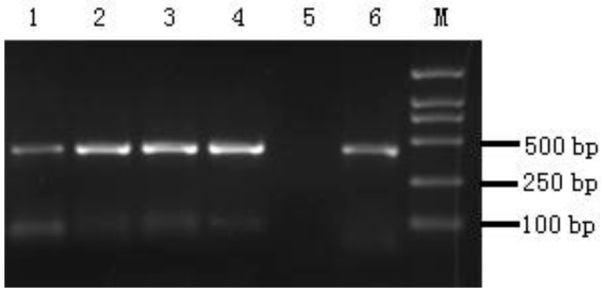

Figure 1. Real-time polymerase chain reaction of RORα in Inner Mongolian cashmere goats. *Lanes 1-4* = samples from 4 different individual cashmere goats; *lane 5* = negative control; *lane 6* = positive control; *lane M* = DL2000 marker.

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Query  54    GCCGCTGACTCCCACCTATAGCATCTCGGCCAACGGGCTCACGGAGCTTCACGATGACCT  113
             ||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  641   GCCGCTGACTCCCACCTATAACATCTCGGCCAACGGGCTCACGGAGCTTCACGATGACCT  700

Query  114   CAGCAACTACATCGACGGGCACACCCCCGAGGGCAGCAAGGCAGACTCCGCCGTCAGCAG  173
             ||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  701   CAGCAACTACATCGACGGGCACACCCCCGAGGGCAGCAAGGCAGACTCTGCCGTCAGCAG  760

Query  174   CTTCTACCTGGACATCCAGCCTTCCCCGGACCAGTCAGGTCTTGATATCAATGGAATCAA  233
             |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  761   CTTCTACCTGGACATACAGCCTTCCCCGGACCAGTCAGGTCTTGATATCAATGGAATCAA  820

Query  234   ACCAGAACCCATATGTGACTACACACCAGCATCAGGCTTCTTCCCCTACTGCTCTTTCAC  293
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  821   ACCAGAACCCATATGTGACTACACACCAGCATCAGGCTTCTTCCCCTACTGCTCTTTCAC  880

Query  294   CAACGGAGAGACTTCCCCAACTGTGTCCATGGCCGAACTAGAACACCTTGCACAGAATAT  353
             ||| ||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  881   CAATGGAGAGACTTCCCCAACTGTGTCCATGGCAGAACTAGAACACCTTGCACAGAATAT  940

Query  354   ATCTAAATCACATCTGGAAACTTGCCAATACTTGAGAGAAGAGCTCCAGCAGATAACGTG  413
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  941   ATCTAAATCACATCTGGAAACTTGCCAATACTTGAGAGAAGAGCTCCAGCAGATAACGTG  1000

Query  414   GCAGACTTTTCTACAGGAGGAGATTGAGAATTATCAAAACAAGCAGCGGGAGGTGAT  470
             |||||| ||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1001  GCAGACCTTTCTGCAGGAGGAGATTGAGAATTATCAAAACAAGCAGCGGGAGGTGAT  1057
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Figure 2. Partial sequence alignment of the goat *RORα* cDNA and the bovine *RORα* sequence.

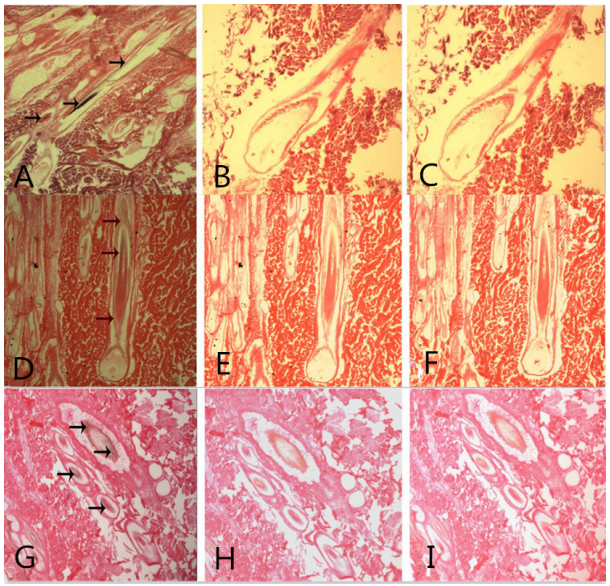

Figure 3. *RORα* mRNA *in situ* hybridization of the skin. **A. D.** and **G.** *In situ* hybridization of the skin with an anti-sense probe in months 2, 8, 12, respectively. **B. E.** and **H.** *In situ* hybridization of the skin with sense probe in months 2, 8, 12, respectively. **C. F.** and **I.** Control (40X).

RORα mRNA expression in different months

To determine the variation of *RORα* expression with the seasons, qRT-PCR was employed. The relative expression levels of *RORα* were normalized with respect to β-actin. The expression of *RORα* mRNA was the highest in February and the lowest in December ($P < 0.05$). There was no significant difference in *RORα* mRNA abundance in February, April, August, and October ($P > 0.05$), but a large change was noted in December compared to February, August, and October ($P < 0.05$), as shown in Figure 4.

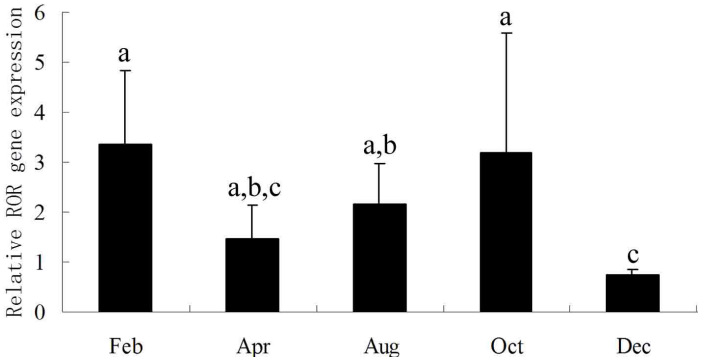

Figure 4. mRNA expression levels of Inner Mongolian cashmere goat *RORα* measured by qRT- PCR. The expression levels were quantified in months 2, 4, 8, 10, and 12 of the year in adult Inner Mongolian cashmere goats. The quantification of the target gene expression was performed 3 times. Results are reported as means ± SE.

DISCUSSION

Expression of *RORα* and interaction with melatonin

The role of *RORα* in the network that regulates the biological circadian rhythm has recently been of interest. For some time, it was thought that *RORα* had no natural ligand; however, after numerous experiments, it was determined that cholesterol and its derivatives are the natural ligands of *RORα* (Bitsch et al., 2003; Kallen et al., 2004). So far, a large number of tumors, including breast cancer, prostate cancer, and colon cancer, have been reported to be associated with *RORα* functions. The regulation of *RORα* at the transcriptional level is affected by melatonin (Wiesenberg et al., 1998; Winczyk et al., 2002; Karasek et al., 2003), and it has been shown that there is a direct interaction between them. *RORα* and melatonin control the proliferation of the breast cancer cell line MCF27 through the Ca^{2+}/CaM signaling pathway (Nakahara et al., 2001; Korkmaz et al., 2009). The proliferation role of melatonin in colon cancer cells in mice can be canceled by *RORα*, which acts as an auxiliary inhibitor. Moreover, *RORα* functions as a tumor-related gene with transcriptional activation that is affected by melatonin (Dai et al., 2001).

In this study, we used qRT-PCR to detect the expression of *RORα* mRNA of Inner Mongolian cashmere goat skin tissue during different months. The data indicate that the skin tissue may be the target organ of melatonin. Our previous study showed that the mRNA of melatonin's membrane receptors MTNR1a and MTNR1b was not expressed in Inner Mongolian cashmere goat skin tissue (unpublished results), whereas, the mRNA of melatonin's nuclear receptor *RORα* was expressed. We hypothesize that melatonin interacts with *RORα* through the skin structure and directly participates in the regulation of cashmere growth. The confirmation of this conclusion needs to be further proven at the protein level.

Localized expression of *RORα* in goat skin and hair follicles

Little is known about the role of *RORα* in hair follicle development in cashmere goats. RORβ is only expressed in the central nervous system and pituitary nodule, and it is mainly concentrated in the retina, pineal gland, suprachiasmatic nucleus, and pituitary nodules (Schaeren-Wiemers et al., 1997). To determine the cellular localization of *RORα* mRNA in hair follicles, we performed *in situ* hybridization of adult Inner Mongolian cashmere goat hair follicles. Results revealed that *RORα* was expressed strongly in the hair shaft of secondary follicles in February, while, in April, it was expressed strongly in the inner root sheath and medulla of primary hair follicles and the inner root sheath of secondary follicles. There was a stronger expression signal in the hair shaft cortex and the outer root sheath of secondary follicles in August. The expression signal could be seen in the shaft of primary and secondary follicles in October and the outer root sheath of the primary and secondary follicles in December. From these results, we conclude that melatonin accelerates cashmere growth by primary and secondary follicles. The phenotypes that will appear when melatonin is over- or under-expressed need to be determined.

Differential expression of *RORα* in goat skin tissue

qRT-PCR results showed that *RORα* mRNA expression was activated and that the

expression pattern varied in months 2, 4, 8, 10, and 12 of the year. However, there was no significant difference ($P > 0.05$) in the *RORα* mRNA abundance in February, April, August, and October, and the only difference was observed in December ($P < 0.05$) relative to February, August, and October, indicating that the expression of *RORα* changes during the development cycle of skin tissue.

The secretion of melatonin is closely related to light and shows obvious cyclical changes (Bedrosian et al., 2013). With regard to day and night cycles and alternating day length changes in a year, melatonin secretion demonstrates marked cyclic variations: less secretion in the day and more secretion at night (Hedlund et al., 1997). After the summer solstice, with the changing daylight time from long to short, melatonin secretion gradually increases. After the winter solstice, the days become longer, and melatonin secretion is gradually reduced (Casao et al., 2010).

Cashmere growth is seasonal and begins at the end of the summer solstice when the light changes from short to long, and it reaches a peak in September and October. Meanwhile, cashmere grows slower in November, and it almost stops growing in December. After the winter solstice, cashmere growth gradually declines as the days lengthen. Melatonin secreted by the pineal gland regulates cashmere growth. The growth of cashmere can be promoted by a high concentration of melatonin (Ibraheem et al., 1994).

A study showed that prolactin could affect the growth of hair follicles (Rose et al., 1998). In addition, the concentration of prolactin correlated with cashmere traits (Santiago-Moreno et al., 2004). Rhind et al. (2004) demonstrated that prolactin's seasonal changes might affect the growth of cashmere fiber. Lan et al. (2009) reported that a novel missense single nucleotide polymorphism within the goat prolactin gene was associated with cashmere fiber length.

The secretion of melatonin and prolactin have a complex relationship. Some scholars believe that melatonin regulates cashmere growth by affecting the secretion of prolactin (Nixon et al., 1993). Melatonin can inhibit the secretion of prolactin. The content of prolactin in the blood of Australian cashmere goats with melatonin treatment was decreased significantly (Klören and Norton, 1995). Wuliji et al. (2003a) implanted and used oral melatonin treatments on the Spanish goats. Over the treatment period of 4 to 6 weeks, the prolactin concentration in these treatment groups was significantly lower than that in the control group (Wuliji et al., 2003a). Santiago-Moreno et al. (2004) implanted melatonin into the European argali, and the annual plasma prolactin concentration decreased. Prolactin showed periodic secretion throughout the year, indicating that the growth and removal of hair, at least in part, are related to cyclical plasma prolactin concentration changes. Ibraheem et al. (1994) found that exogenous prolactin can stimulate the growth of the secondary follicle and hair shaft when cultured *in vitro*. Pearson et al. (1996) reported that changes in the prolactin concentration can stimulate the activities and recession of hair follicles and that hair follicles can respond to prolactin secretion when it is above or below a certain baseline.

Lardone's latest study showed that the melatonin of human lymphocytes collaborates with membrane receptors (MTNR1a) and nuclear receptors (*RORα*) to activate IL-2 expression (Lardone et al., 2009). Moreover, the regulation of *RORα* expression by melatonin was dose-dependent. *RORα* mRNA expression decreases while the concentration of melatonin increases. Consistent with these findings, *RORα* expression was increased while the secretion of melatonin decreased in August and October. In contrast, *RORα* expression was reduced while the secretion of melatonin increased in December and April (Viganò et al., 2001). The result of February is not in accordance with this finding, which may result from RNA degradation

during qRT-PCR. Because mRNA expression is not absolutely positively related to protein expression levels in some cases, whether the mRNA expression level is consistent with protein expression needs to be addressed. In addition, the role of the nuclear receptor *RORα* in promoting cashmere growth and the relationship of *RORα* mRNA expression on protein level in different growth periods of cashmere goat hair follicles need to be further addressed.

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