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A Study on Melatonin Receptor Genes Expression in Embryonic Goose Skin Feather Follicles

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ABSTRACT

Background: In recent years, it has been reported that melatonin plays an important role in the development of skin hair follicles in mammals. The down we often use is mainly from the skin feather follicles of geese, but their production is not high. Therefore, the study of skin feather follicles is of great significance.

Methods: The skin feather follicle tissues of goose dorsal skin at three developmental nodes (E14, E18 and E28) were collected; the differential expression and distribution of melatonin receptor genes were detected by fluorescence quantitative PCR, Western Blot and immunohistochemical methods, respectively. The results showed that melatonin receptor gene was expressed in varying degrees at three time points of goose embryonic skin feather follicle development. The expression sites and Protein expression level of *Mel1a* and *RORα* were regularly changed by immunohistochemistry and Western Blot.

Result: Our research showed that melatonin receptor genes can play an important regulatory role in the development of goose skin feather follicles.

Key words: Development, Goose, *Mel1a*, *Mel1b*, *Mel1c*, Melatonin, *RORα*, Skin feather follicles.

INTRODUCTION

With the continuous improvement of people's consumption level of waterfowl, the market demand for its products has been increasing, among which the demand for goose down accounts for a large proportion (Pingel and Germany 2011). The increase in goose down yield is closely related to the development of skin feather follicles, therefore, research on goose has also focused more attention on the improvement of goose skin feather follicles (Sun *et al.,* 2020). Studies have shown that melatonin can promote the development of hair follicles (Chetan *et al.,* 2021; Nixon *et al.* 1993). In recent years, researchers have used real-time fluorescent quantitative PCR, nucleic acid *in situ* hybridization and other technologies to detect the expression of melatonin receptors in various animal tissues and in the skin feather follicles. The different expression levels of the receptors indicate that their combination is also differently (Kobayashi *et al.,* 2005; Slominski *et al.,* 2012).

MATERIALS AND METHODS

Ethical statement

The Goose Industry Research and Development Centre of Jilin Agricultural University, enforcing the Regulations for the Administration of Affairs Concerning Experimental Animals, approved all the experimental procedures to ensure animal welfare (Permission number: GR(J)18-011. Date: 22 May 2018).

Animals and sample collection

Goose eggs were collected from the Jilin white goose provenance base research center of Jilin Agricultural University. The whole experiment starts in March, 2019 and ends in November, 2020. Jilin white goose are raised in semi-dense plots and fed with standard type of goose fodder with free access to water under natural conditions. 200 fertile eggs (120-130 g) of Jilin White geese were randomly selected and incubated at 37.8°C, moisture was controlled at 68-70%. Cut and remove the dorsal skin of day 14, day 18 and day 28 embryos using ophthalmic scissors and forceps under sterile conditions to a size of 1×1 cm^2.

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Total RNA extraction and complementary DNA (cDNA) synthesis

RNA was extracted from the embryonic dorsal skin of Jilin White Goose by triazole method on 14th day, 18th day and 28th day respectively. DNA enzyme treatment and total RNA pollution treatment were carried out in each group, total RNA quality and concentration were evaluated with 2100 Bioanalyzer (Agilent Technologies, USA), the purity and degradation were also evaluated on 1.0% agarose gels. The first-strand cDNA was synthesized by reverse transcription Kit (ABP Biosciences, Maryland, USA). Firstly, RNA template, primer and enzyme free water were mixed and incubated at 42°C for 2 min and then other components were added. And incubated at 25°C for 10 min, 42°C for 15 min, 85°C for 5 min and finally cooled up on ice. The final product was stored in the -20°C refrigerator.

Quantitative real-time PCR

The CDs sequence of goose gene was found by NCBI and the primer pairs were designed (Table 1). Transgen Biotechnology (Beijing, China) and bio rad CFX real-time PCR system and software (bio rad, California, USA) were used to detect the quantitative expression of *AANAT*, *ASMT*, *Mel1a*, *Mel1b*, *Mel1c* and *RORα* in skin tissues, respectively. The total volume of qPCR reaction was 20 μl, including 2 μl cDNA, 0.6 μl forward and reverse primers (10 μmol), 10 μl SYBR Green qPCR supermax and 6.8 μl nuclease free water. Amplifcation conditions were as follows: pre-denaturation at 94°C for 30 s, 45 cycles of amplifcation (94°C for 5 s and 60°C for 30 s). The relative expression was detected 3 times in each sample. B-actin was used as a control and $2^{-\Delta\Delta CT}$ was used to calculate the gene expression level.

Immunohistochemistry

First, the skin was fixed for 48 h with a solution of 4% paraformaldehyde in PBS (pH 7.4) at 4°C. Then, the skin with feather follicles was cut and the samples were immersed in different concentrations of ethanol (70%, 80%, 90% and 100%) and twice in 100% xylene for 10 min each time. The treated samples were embedded in paraffin, cut into 5 μm thickness, stretched and baked for 3 h at 65°C and then soaked in xylene twice. Next, the treated tissue sections were washed with PBS solution for 15 min (in three times). After boiling the sodium citrate solution (pH=6), the heating was turned off and the slices were placed in the solution for twice, for 15 min each time, with an interval of 15 min between the 2 times. The tissue sections were washed 3 times with PBS solution for 5 min each time. The blocking of tissue sections was carried out with goat serum blocking solution at room temperature and incubated with the first antibody of *Mel1a* and *RORα* 20 min later (4°C overnight). Finally, the sections were incubated in the second antibody (room temperature, 50 min). After that, the tissues were washed with PBS solution three times for 5 min each time and then stained with DAB (5-10 min). The dyed part was washed with running water for 10 min and then dyed again with hematoxylin for about 2 min. The slices were decolorized with 1% hydrochloric acid ethanol for 10-15 s, quickly removed and placed in distilled water and then placed in phosphate buffered saline containing Tween-20 (PBST) with pH 7.4 for 5-10 min. The dyed part was washed again with running water for 15 min, then dehydrated and transparent with different concentrations of ethanol and xylene. Finally, the glass slides were examined with bx53 electric fluorescence illumination microscope (Olympus Tokyo).

Western blot analysis

Firstly, 200 μL RIPA lysate and about 30 mg skin follicle tissue sample were added into the EP tube and carry out high-speed homogenization at 4°C. Place the sample on ice for 2 h to lyse, then centrifuge it at 12000 rpm at 4°C for 5min and aspirate the supernatant. Then centrifuge again, aspirate the clear sample in the middle, until the final clear

Table 1: Target genes primers sequence.

Gene		Primers sequence	Product size (bp)
Mel1a	F	5′-TGTACCGCAACAAGAAGCTG-3′	73 bp
	R	5′-GCTACCCATTCTCTCTGGACAC-3′	
Mel1b	F	5′-GTAGCCATCGATCCTTTGGA-3′	224 bp
	R	5′-GGCTTGCTCTTCTGACCATC-3′	
Mel1c	F	5′-CTGGTCCTGGTCTCGTGCGTAT-3′	139 bp
	R	5′-GATGTGTCAAGCATGATCCGTC-3′	
RORα	F	5′-ACCGCTGCCAACACTGTAGATTAC-3′	150 bp
	R	5′-GATGATCTCGCTGCTGCTGCTG-3′	
AANAT	F	5′-GCCATCAGCGTGTTCGAGAT-3′	90 bp
	R	5′-CAGGAAGTGGCGGATCTCAT-3′	
ASMT	F	5′-TTGGAGCAAGTCCCTGTGTC-3′	120 bp
	R	5′-ATTCCATCGCACTGGCATCT-3′	
β-actin	F	5′-ACCAGTTCCTGGTGTTAGGTT-3′	124 bp
	R	5′-CCGCTTACTCAACAAATCAAAGGAT-3′	

F: Denotes forward primers and R: Denotes reverse primers.

and transparent protein solution is obtained and store it in the -80°C refrigerator. The protein samples are then prepared and gels are prepared. The conditions of electrophoresis are: the constant pressure of the concentrated gel is 90 V for 20 min, the constant pressure of the separating gel is 160 V and the electrophoresis can be stopped when the bromophenol blue reaches below the platinum wire in the electrophoresis tank and add crushed ice outside the electrophoresis tank during electrophoresis. Then put it into the transfer tank filled with pre-cooled transfer liquid and put crushed ice in the outer tank, constant current 300 mA, 50 min. The membrane was then dyed with Ponceau red. Wash the membrane with TBST for 5 min, change into the blocking buffer and block with skimmed milk powder for 1 h at room temperature. After that, the primary antibody was incubated on a shaker under the conditions of 4°C, overnight. After the primary antibody was incubated, 5 ml TBST was added to wash the membrane for 5×3 min. And then incubate in the secondary antibody diluent for 40 min with gentle shaking at room temperature. After repeated washing, add the configured ECL luminescent liquid, plus the appropriate size film, press the box to expose for 30 s and then develop for 1-3 min and fix for 2 min after banding appears.

Statistical analysis

SPSS 23.0 was used for statistical processing of the data. Single factor analysis of variance (One-way ANOVA) was used among groups and least square significant difference (LSD) test was used to compare the means. The significant difference of the data was considered as $p<0.05$.

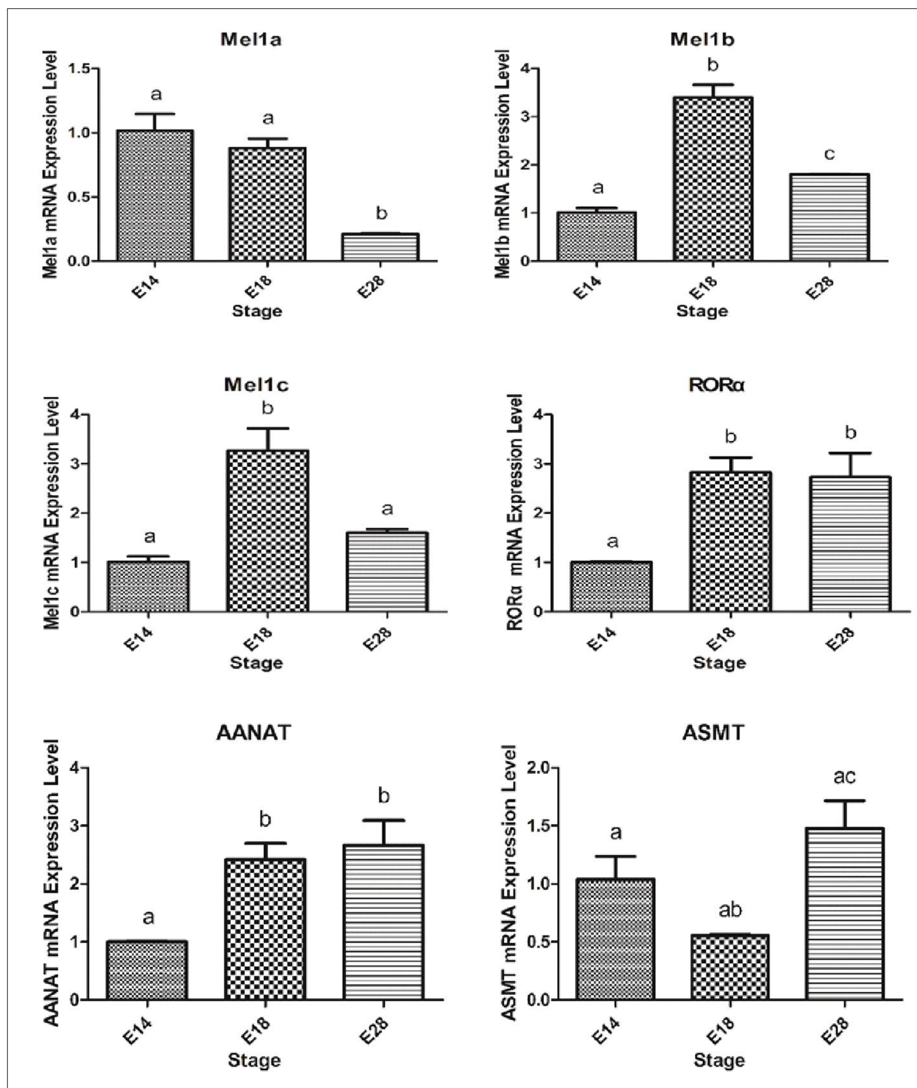

Fig 1: Expression of genes encoding melatonin receptor *Mel1a*, *Mel1b*, *Mel1c*, *RORα* and the key enzyme of melatonin synthesis: *AANAT*, *ASMT* in the skin with feather follicles of the goose. The data is represented as means±SEM; n=9 geese and the different letters indicate the significant difference of mRNA expression between different stages of feather follicles development ($p<0.05$) reported in arbitrary units (AU) normalized with *β-actin.* The vertical ordinate represents relative mRNA expression level of each target gene. The horizontal ordinate indicates the skin feather follicle samples at different times of embryonic stage.

RESULTS AND DISCUSSION

mRNA expression levels of target genes in skin feather follicles

The four receptor genes and two key synthesis enzyme of melatonin all have continuous differential expression of mRNA levels in the back skin feather follicle tissue of goose embryos, as the result showed in Fig 1. The results indicated that the expression of *Mel1a* gene showed a decreasing trend at the three embryonic stages and the expression level at E28 was significantly lower than that at E18 ($P<0.05$) and extremely very significantly lower than that at E14 ($P<0.01$). Moreover, the expression levels of *Mel1b* gene and *Mel1c* gene both increased first and then decreased and the expression levels at E18 were also significantly higher than those at E14 and E28 ($P<0.01$). The expression level of *RORα* gene as-well showed a trend of first increasing and then decreasing expression levels. The expression level at E14 was significantly lower than that at E18 ($P<0.01$) as well as that at E28 ($P<0.05$), while at E18 and at E28 the expression level difference was not significant ($P>0.05$).

The skin feather follicle is an important accessory organ of the skin, which plays a vital role in the growth and development of feathers (Lucy *et al.*, 2021). As for the expression of *Mel1a*, *Mel1b*, *Mel1c* and *RORα*, it may be related to the occurrence and development of primary feather follicles and secondary feather follicles. During the development of goose embryonic skin feather follicles, Melatonin transfers information through receptors to realize the regulation of skin feather follicles development. In addition, the expression of the two genes, AANAT and ASMT, was detected in the tissue, indicating that not only mammals, but also the skin feather follicles of poultry are also important melatonin synthesis sites *in vitro* and this is consistent with the view point of Hardeland *et al.*, (2011).

Immunolocalization of *Mel1a* and *RORα* proteins in skin feather follicles development

As shown in Fig 2, the melatonin receptor *Mel1a* protein and *RORα* protein were located for the first time during the development of goose skin feather follicles. After observation, it was found that the back skin of the 14th day

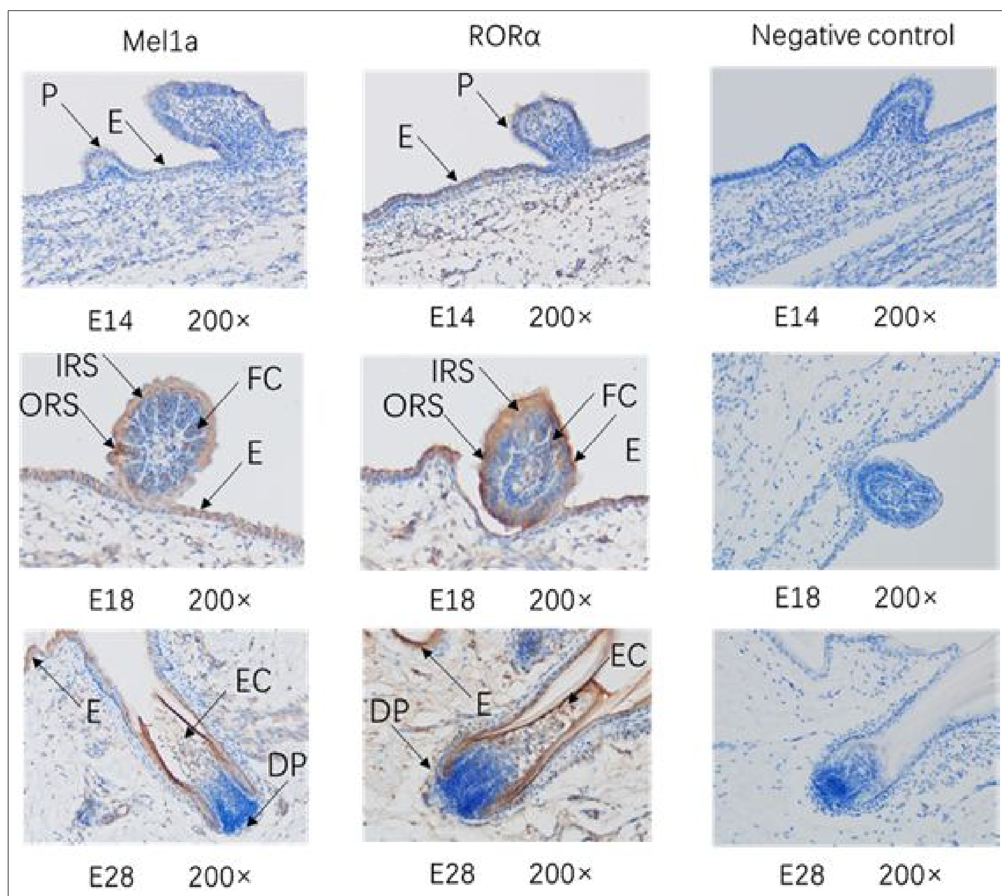

Fig 2: Localization of *Mel1a* protein and *RORα* protein during the development of skin feather follicles of the goose.
All dorsal skin samples were stained with DAB. Longitudinal section (200×) showed that dense dermis and villi began to develop in E14 and E18 and at E28. The positive expression site begins to migrate.
E: Epidermis, P: Placode, ORS: Outer root sheath, IRS: Inner root sheath, FC: Feather crest, EC: Epidermal collar, DP: Dermal papilla.

old goose embryo had grown obvious feather buds and a large number of cells were enriched in the front of the feather buds. At this time, the positive expression sites of *Mel1a* protein and *RORα* protein were mainly concentrated in the epidermis and the placode. At E18, the skin feather follicles are developed more fully. First, the fibroblasts in the dermis at the base of the skin feather follicles gradually proliferated to form the hair papilla and then the internal structures such as the Epidermal Collar and feather crest of the skin feather follicles have gradually formed and the skin feather follicles cavity gradually decreased until it disappears. At this stage, secondary feather follicles have begun to develop around the primary feather follicle. Furthermore, the positive expression of *Mel1a* protein and *RORα* protein in the epidermis has a tendency to increase, while *RORα* protein is more significant. In addition, both proteins are highly expressed in the outer heel sheath and there is also a small amount of expression in the inner root sheath and feather crest. At E28, the primary feather follicle has formed a complete feather follicle tissue. However, at this period the positive expression of *Mel1a* protein and *RORα* protein in the outer root sheath, inner root sheath and epidermis are significantly increased and there is also a higher expression in the Epidermal Collar, but in the dermal papilla, the positive expression of *RORα* protein is slightly higher.

At present, although it has been reported that *Mel1a* protein is expressed in the inner root sheath of skin feather follicles, the research on it has mainly focused on ovaries and follicles (Slominski *et al.*, 2005; He *et al.*, 2014; Sundaresan *et al.*, 2009; Lee *et al.*, 2001). Zhao *et al.*, (2015) found that *RORα* was expressed in the hair stem, inner root sheath, outer root sheath and medulla of cashmere goats. However, during the anagen period, as the skin feather follicles continue to develop and mature, the overall expression site of *RORα* has not change too much. The *RORα* protein expression sites in goose skin feather follicles tissue were consistent with those reported for cashmere goats, but its overall expression showed a trend from outside to inside and the same is true for *Mel1a*.

Protein expression levels of *Mel1a* and *RORα* genes in skin feather follicles

Fig 3A shows the visualization results of the NC film of *Mel1a* and *RORα* proteins, it is fully confirmed that in the embryonic skin feather follicles of goose, the two receptor proteins of *Mel1a* and *RORα* of melatonin are quantitatively expressed. The histogram result is shown in Fig 3B. Analysis of the results found that in the embryonic back skin hair follicles of Jilin white goose, the expression of *Mel1a* and *RORα* receptor proteins of melatonin both showed a gradual upward trend and the expression levels of both receptor proteins were the lowest at E14 and both were significantly lower than the expression levels of E18 and E28 ($P<0.01$) and the expression level of *Mel1a* protein was slightly lower. At E18, the expression levels of both receptor proteins were significantly higher ($P<0.01$). At E28, the expression levels of the two receptor proteins reached the highest and were significantly higher than that at E18 ($P<0.05$). Studies have

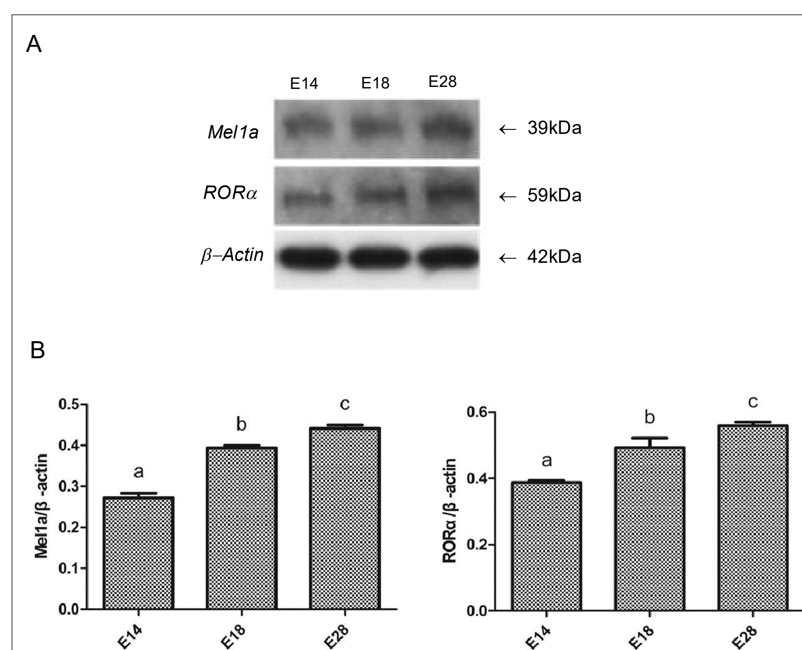

Fig 3: Western blot analysis of *Mel1a* and *RORα* proteins.

A: The upper two panels show the measurable quantities of protein bands in each stage of development. The lower panel indicates the *β-actin* as an internal control in all samples. B: Each sample is shown as mean±SEM of the ratio of the relative density of *Mel1a* and *RORα* to *β-actin*. The different superscripts indicate statistically significant differences ($p<0.05$).

shown that *Mel1a* receptors may be related to the circadian rhythm of organisms and it is speculated that melatonin may play an important role in the biological function of circadian secretion through *Mel1a* receptors (Zawilska ***et al.,*** 2009). And the expression of *RORα* protein has a positive effect on the growth of villi (Zhao ***et al.,*** 2015).

In addition, in this study, the expression of *Mel1a* and *RORα* at the mRNA and protein levels has a certain degree of deviation. It is speculated that this situation may be related to genetic differences. According to the genetic central dogma, DNA has to undergo a series of transcription and translation in the process of protein formation and the transmission of gene genetic information in this seemingly simple but very complex process may be due to its own reasons or the influence of uncertain factors, resulting in the deviation of mRNA level and protein level expression (Lu *et al.,* 2019).

CONCLUSION

In conclusion, by detecting the differential expression and localization of melatonin receptor in goose embryo skin feather follicle tissue, it can be demonstrated that melatonin receptor has an important regulatory role in the development of goose embryo skin feather follicle tissue. It provides a theoretical basis for the further study of the mechanism of melatonin on goose skin feather follicles.

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Conflict of interest: None.

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