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Candidate genes for the development of hair follicles in Hu sheep

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ABSTRACT. The aim of this study was to detect candidate genes for the development of hair follicles in the Hu sheep breed. Seven genes have been detected in large, medium, and small wave follicles of Hu sheep using gene chip technology. The histological features of the follicles of newborn Hu-lambs were combined with fluorescence quantitative PCR technology to detect the correlation between the expression of the seven genes and hair follicle development. Among the genes studied, matrix metalloproteinase 2 (*MMP2*), bone morphogenetic protein-7 (*BMP7*), and sideroflexin 1 (*SFXN1*) showed a significantly different pattern of expression in large, medium, and small wave follicles ($P < 0.05$). The expression of *MMP2* had a significant positive correlation with secondary follicles in large waves ($P < 0.05$), while the expression

of *BMP7* had a significant correlation with primary follicle diameter in small wave follicles, and a highly significant positive correlation with the number of secondary follicles in the small waves ($P < 0.01$). The expression of *SFXN1* was significantly and positively correlated with the diameters of small wave primary follicles; it also showed a highly significant positive correlation with secondary follicle diameters. Although other genes are associated with hair follicles, their expression in large, medium, and small wave follicles was not significant. We propose that *BMP7*, *MMP2*, and *SFXN1* genes could be important candidate genes for use in breeding Hu lambs with early coat development.

Key words: Hu sheep; Hair follicle; Patterns

INTRODUCTION

The Hu sheep breed is particularly noted for the characteristics of the lambskin obtained from lambs slaughtered within 3 days of birth. The skins from these lambs have soft white hair with a wavy pattern; the wavy patterns gradually disappear at 3 days after birth and the quality of lambskin diminishes. There are great differences in the lambskin qualities in Hu sheep: the pattern of the hair follicles varies, with three types of waves, large, medium, and small. The average width of the small wave is 0.5-1.25 cm, the medium wave is 1.25-2.0 cm, and the large wave is more than 2.0 cm. In terms of quality, the small waves are better than medium and large waves. Additionally, different lambs from the same ewe may show different patterns of wool curl and patterns. However, genetic factors are known to play an important role in the patterns of hair follicle formation in lambskin. The hair follicles on lambskin can be divided into myelinated wool primary follicles and non-myelinated wool secondary follicles. Identification of genes associated with hair follicle development will provide greater insights into the generation of the different patterns in Hu lambskin. Here, we studied individuals with three distinctively different patterns as the material for comparison.

A previous study of differentially expressed genes in Hu lambskin based on microarray technology and bioinformatic analysis identified *BMP7*, *MMP2*, *CDKNIC*, *MT3*, *SFXN1*, *SNAI*, and *IL13RA1* as candidate genes involved in the formation of different wool patterns (Sun et al., 2013). Bone morphogenetic protein-7 (*BMP7*) is a member of the transforming growth factor-β (*TGF-β*) superfamily. In vivo and in vitro experiments have confirmed that TGF-β can stimulate hair follicle epithelial cells (Oshimori and Fuchs, 2012). Noramly and Morgan (1998) reported that *BMP1* and *BMP7* expression are related to the size of the feather germ and its spatial distribution. These observations suggest that this protein family may play a potential role in the development of hair follicles. *BMP2*, *BMP4*, and *BMP7* have been found to be expressed in developing hair follicles (Thomadakis et al., 1999). Genetic studies confirmed that BMP signaling plays an important role in the control of cell differentiation and apoptosis (Botchkarev and Sharov, 2004). Recent studies on hair follicles concentrated mainly on the roles of *BMP2* and *BMP4*; the function of *BMP7* in cancer has been examined (Qian et al., 2009) and the whole sequence has been cloned (Zhao et al., 2009). Matrix metalloproteinase 2 (*MMP2*) is involved in physiological processes including cell proliferation, differentiation,

bone formation, and collagen catabolism, and is a negative regulator of cell adhesion. It can shear and process insulin-like growth factor and then trigger activity of insulin-like growth factor (*IGF*). It plays an important role in cell proliferation, differentiation, and inhibition of apoptosis (Sternlicht and Werb, 2001). *CDKNIC*, *MT3*, *SFXN1*, *SNAI*, and *IL13RA1* genes are all involved in cell proliferation, differentiation and apoptosis (Sun et al., 2013). *MT3* is also involved in esophageal cancer (Tian et al., 2004), *CDKNIC* in colorectal cancer (Zeng et al., 2011), and *SNA1* in breast cancer (Yu, 2010); whether these genes are also related to the growth and development of hair follicles is not yet clear.

Although some research has examined the growth cycle of hair follicles of goats (Sun et al., 1984; Li et al., 2005), there is no detailed information on gene expression during hair follicle development in skin tissue of newborn Hu sheep. Here, we investigated expression changes in *BMP7*, *MMP2*, *CDKNIC*, *MT3*, *SFXN11*, *SNAI*, and *IL13RA1* in newborn Hu lambs and examined the relationship between expression of these genes and large, medium, and small waves follicles during hair development These analyses are expected to elucidate the role of these genes on hair follicle development and to provide a basis for improvement in breeding high quality lambs.

MATERIAL AND METHODS

The research was carried out in Suzhou stud farm and Yangzhou University, Jiangsu Province Key Laboratory-Animal Genetic and Molecular Design Laboratory.

Experimental populations

Fifteen 2-day-old Hu lambs were selected from Suzhou stud farm in Jiangsu Province: 6 groups of full-sib individuals, in which 3 groups showed small and large waves, whereas the other groups showed large, medium, and small waves. The waves were measured on the skin of the back. An approximately 1.5 cm^2 skin was cut off and then cut into small pieces and placed into an RNA-free tubes and stored with RNA preservation solution in a 4°C refrigerator overnight, and then stored at -70°C. Another piece of skin was attached to cardboard and fixed in 4% formaldehyde.

Reagents and instruments

The PrimeScript RT reagent kit and the SYBR Premix Ex Taq™ II kit were purchased from Takara Biotechnology Co. Ltd. (Dalian); RNA preservation solution was purchased from Tiangen Biochemical Science and Technology Co. Ltd. (Beijing, China). The ABI 7500 Real-Time PCR instrument was purchased from the Applied Biosystems. Formaldehyde, alcohol, paraffin, and iodine were purchased from Sangon Biological Engineering Co. Ltd (Shanghai, China).

Paraffin sections

Skin biopsies of large, medium, and small waves were used to prepare conventional paraffin sections. After fixation, the tissues were dehydrated, cleared, embedded, sectioned, and hematoxylin and eosin stained by standard means. Hair follicle structure was observed

under the optical microscope, and images were captured with a scanning microscopic imaging system. Each slice selected three different horizons. The numbers of primary and secondary hair follicles and their diameters were determined. SPSS17.0 software was used for univariate analysis of variance (ANOVA) of the data. Significance was set at $P < 0.05$.

Real-time PCR

RNA was extracted from pieces of Hu lamb skin using conventional methods (Sun et al., 2013). cDNA was prepared using a PrimeSYBR RT Reagent Kit and Perfect Real Time kit (TaKaRa). The reaction mixture contained 2 µL, 5X PrimeScript Buffer, 0.5 µL PrimeScript RT Enzyme Mix I, 0.5 µL oligo dT primer, 0.5 µL random 6 mers, 4 µL total RNA, and 2.5 µL RNase free dH_20. Amplification conditions were 37°C for 15 min, 85°C for 5 s. The primers were designed using the Oligo7 software and are described in Table 1. To synthesize the first strand of cDNA, a standard PCR amplification was used. *GAPDH* was used as the reference gene and the SYBR Green I method was used for quantitative testing. A standard curve was established using cDNA gradient dilution, and each sample was tested 3 times in the 7500 PCR instrument for RT-PCR. The relative expression of the target gene was calculated according to the $2^{-\Delta\Delta Ct}$ method.

Table 1. Sequence information for genes and primers for RT-PCR.

Gene name	Primer sequences (5'→3')	Fragment size (bp)
GAPDH	F: GTTCCACGGCACAGTCAAGG R: ACTCAGCACCAGCATCACCC	127
BMP7	F: TGAGTTCCGCATTTACAAGG R: GTGGCTGTGATGTCAAAAAC	177
MMP2	F: GTACCCCAAGCCGCTGACC R: TCCAGAATTTGTCTCCAGCGAAG	116
MT3	F: CTCCTGCACCTGCTCCGACTC R: TCCAGAATTTGTCTCCAGCGAAG	99
CDKNIC	F: GGCACCTCACTCGCATCTG R: AAGCGCGAAGAGACTGCAAG	127
SFXN1	F: CAAACAAGCCATCACGCAAG R: GCAAAAGCCAACTATTCCAAC	160
IL13RA1	F: GTGGAAAAGTGCATCTCGC R: TACTTGGACGCTGTGCTGTT	273
SNAI1	F: GCCCGCCGGAGACCCAATTA R: GAGCCAAGAGAGTCCCAGATGAG	161

RESULTS

Total RNA quality test

The concentration of RNA was measured using a Nano Drop ND-1000 Spectrophotometer (Nano Drop Technologies, Wilmington, USA) and samples with a purity (A_{260}/A_{280}) of >1.8 were used. Clear bands at 18S and 28S were detected on a gel (Figure 1).

Augmented product

The dissolution curves showed relatively sharp single peaks for the seven target genes and *GAPDH*, indicating the high quality of the designed primers and the optimization of the PCR (Figure 2).

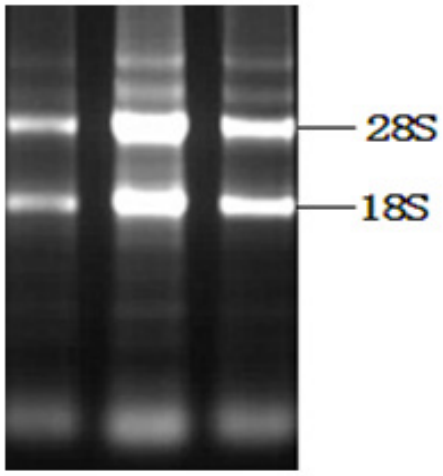

Figure 1. Total RNA from 2-day-old Hu lambskin.

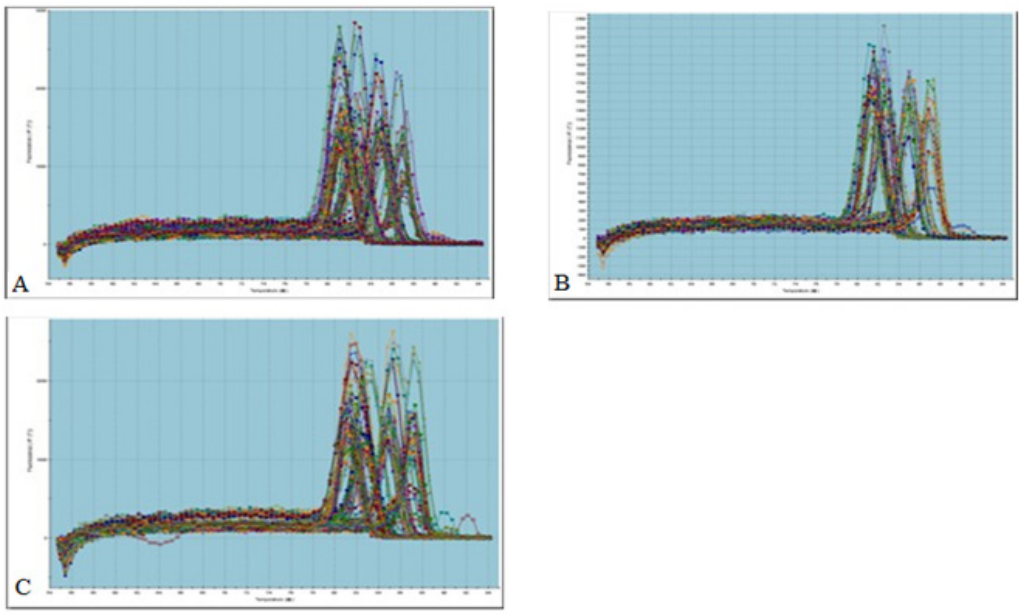

Figure 2. Dissolution curves for *GAPDH* and the seven tested genes.

Structural characteristics of hair follicles in Hu sheep

Transverse section images of large, medium, and small waves are shown in Figure 3.

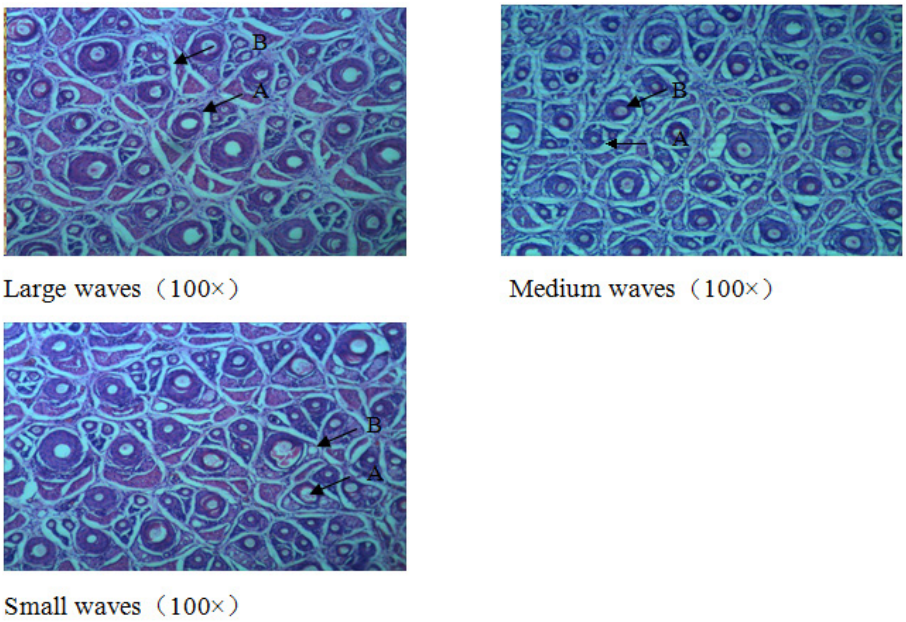

Figure 3. Transverse section images of large, medium, and small wave follicles.

The diameters of primary and secondary follicles and the number of primary and secondary follicles are given in Table 2.

Table 2. Characteristics of large, medium, and small wave follicles.

Group	No. of individuals	No. of primary follicles	No. of secondary follicles	Diameter of primary follicles (μm)	Diameter of secondary follicles (μm)
Large waves	6	38.33 ± 2.13^{a}	18.44 ± 0.84^{A}	80.02 ± 1.42^{A}	44.73 ± 0.69^{A}
Medium waves	3	37.22 ± 1.50^{a}	13.56 ± 0.53^{B}	72.41 ± 2.32^{B}	35.91 ± 1.22^{B}
Small waves	6	37.67 ± 0.75^{a}	17.39 ± 0.57^{A}	73.95 ± 0.80^{B}	43.38 ± 1.26^{A}

Means with different lower-case letters within the same column indicate significant differences between different rows. Means with different capital superscripts within the same column indicate highly significant differences between different rows. Means with the same lower-case letter within the same column indicate no significant differences between different rows.

The diameters of primary follicles were significantly larger in large waves compared to medium and small waves ($P < 0.01$); there were no significant differences in size between primary follicles of medium and small waves ($P > 0.05$). The diameter of secondary follicles in medium waves was significantly smaller than in large and small waves ($P < 0.01$); there was no significant difference in size between large and small waves ($P > 0.05$). The number of primary follicles among large, medium, and small waves did not differ significantly ($P > 0.05$); however, the number of primary follicles in large waves was higher than in the medium and small waves. The number of secondary follicles in medium waves was significantly lower than in large and small waves ($P < 0.01$); large and small waves did not differ significantly ($P > 0.05$) (Table 2). Most hair follicle groups in three different patterns consisted of 3 follicles, the

central primary follicle had the largest diameter, and the side primary follicles were somewhat smaller (Figure 3).

Gene expression in large, medium, and small waves

cDNAs from large, medium, and small waves were prepared as described above. The level of expression of *BMP7* and *SFXN1* was significantly higher in medium and large waves compared to small waves ($P < 0.05$). The level of expression of *MMP2* was significantly higher in medium waves than small and large waves ($P < 0.01$). None of the other tested genes showed any significant differences in expression among large, medium, and small waves (Table 3).

Table 3. Relative expression of 7 genes among 3 groups.

Genes name	Relative expression of large waves	Relative expression of medium waves	Relative expression of small waves
BMP7	1.18 ± 0.07^{a}	1.19 ± 0.14^{a}	1^{b}
MMP2	0.99 ± 0.81^{B}	1.71 ± 0.24^{A}	1^{B}
CDKNIC	0.97 ± 0.11^{a}	0.93 ± 0.089^{a}	1^{a}
MT3	0.99 ± 0.11^{a}	0.93 ± 0.089^{a}	1^{a}
SFXN1	1.21 ± 0.08^{a}	1.35 ± 0.13^{a}	1^{b}
SNAI	1.07 ± 0.08^{a}	1.17 ± 0.14^{a}	1^{a}
IL13RA1	1.17 ± 0.11^{a}	1.34 ± 0.18^{a}	1^{a}

Means with different lower-case letters within the same column indicate significant differences between different rows. Means with different capital superscripts within the same column indicate extremely significant differences between different rows. Means with the same lower-case letter within the same column indicate no significant differences between different rows. The diameters of large wave secondary follicles.

Correlation between expression of tested genes and follicle characteristics

The diameters and numbers of primary and secondary follicles are crucial to the formation of the wave patterns. We examined the correlation between the level of expression of the seven tested genes and hair follicle characteristics in large, medium, and small wave groups. All seven genes showed significant correlations with some of the hair follicle characteristics, suggesting that all of them may be involved in hair follicle development (Table 4).

Table 4. Correlation between the expressed genes and the follicle characteristics.

Index	BMP7	CDKNIC	MT-3	MMP2	IL13R1	SFXN1	SNA1
Diameters of large waves primary follicles	0.037	0.230	0.409	-0.113	-0.104	-0.496*	-0.195
Diameters of large waves secondary follicles	0.389	0.593**	0.516**	0.383	0.311	0.358	0.244
Diameters of small waves primary follicles	0.501*	0.323	0.055	0.340	0.499*	0.507*	0.573*
Diameters of small waves secondary follicles	0.174	-0.424	0.205	0.102	0.102	0.623**	0.147
Diameters of medium waves primary follicles	-0.667*	-0.603	0.119	-0.375	-0.737*	-0.801**	-0.737*
Diameters of medium waves secondary follicles	-0.515	-0.205	0.183	-0.109	-0.424	-0.285	-0.566
Number of large waves primary follicles	-0.157	-0.475**	-0.681**	0.198	-0.085	0.325	0.321
Number of large waves secondary follicles	0.319	0.180	0.033	0.500*	0.431	0.449	0.567*
Number of small waves primary follicles	-0.395	-0.602**	-0.768**	-0.293	-0.202	-0.372	-0.029
Number of small waves secondary follicles	0.650**	0.250	0.274	0.431	0.541*	0.327	0.493*
Number of medium waves primary follicles	0.410	0.364	-0.066	0.390	0.447	0.680*	0.609
Number of medium waves secondary follicles	-0.771*	-0.264	0.487	-0.039	-0.670*	-0.410	-0.643

DISCUSSION

As a constituent of the skin, the hair follicle is the tissue regulator that directly controls hair growth, development, differentiation, and cyclical adjustment. Wool has two types of fiber, marrowless velvet and medulla developed hair; myelinated hair occurs in primary follicles, and growing un-myelinated hair occurs in secondary follicles (Li et al., 2005). Secondary follicles differentiate from primary hair follicles. Primary follicles have a complete set of associated structures, such as a large hair ball deep in the dermis, and are surrounded by sebaceous glands, sweat glands, arrectores pilorum, and other ancillary structures. However, in secondary follicles, the hair ball is located at a shallow position in the dermis; secondary follicles have small diameters, no sweat gland, or erector muscle, and only a small number of sebaceous glands located in a further shallow position (Du, 1982). In the present study, we showed that the primary and secondary follicles in skin of 2-day-old Hu lambs were fully developed, and that most waves consisted of 3 follicles. The centrally located primary follicle had the largest diameter, while the lateral primary follicles had smaller diameters; these were surrounded by 1 or 2 secondary follicles. The density of the primary and secondary follicles determines the fineness and pattern of the wool (Lin et al., 2000; Botchkarev et al., 2002; Purvis and Swan, 2002): the larger the diameter of the hair follicles, then the more rough and poor texture of the wool. Our analyses showed that the diameter of the large-wave hair follicles was greater than those of the medium and small waves. The diameters of the primary and secondary small-wave follicles were intermediate between large and small waves. The wool quality of small waves is considered far superior to that of large and medium waves, which are merely acceptable materials for preparation of wool products.

The transforming growth factor-β (*TGF-β*) superfamily member, *BMP7*, is expressed in hair follicle development. There is evidence that *BMP2*, *BMP4*, and *BMP* receptor *IA* are expressed in epidermal cells that synthesize keratin in the hair bulb (Wozney et al., 1988). Moreover, the expression of *BMP2* in telogen was approximately 25-fold higher than in the proliferation phase of development of hair follicles of Inner Mongolia Cashmere goats (Su et al., 2008). On the other hand, *BMP4* showed a lower level of expression in telogen and a much elevated level in the proliferation phase of secondary hair follicles, suggesting that *BMP2* and *BMP4* had an inhibiting effect on hair follicle development (Wu et al., 2009). In the present study, we showed that the expression of *BMP7* in small wave follicles was significantly higher than large wave follicles during the same period, suggesting that *BMP7* might be involved in the development of hair follicles and the regulation of hair growth. The *MMP2* and *SFXN1* genes varied in expression among large, medium, and small wave follicles. We speculate that these genes might have a role in the development of hair follicles, but their exact function will need to be clarified in future studies.

The pattern of expression of BMP7 was consistent with the follicle number and diameter characteristics of primary and secondary hair follicles. In combination with the results of the differences in expression of *BMP7* in large, medium, and small waves, we suggest that *BMP7* may have a role in the regulation of hair follicle development. The pattern of *MMP2* expression showed that the number of hair follicles had significant difference in small wave large and medium waves, and was consistent with the biopsy results. Similarly, *SFXN1* expression showed that the diameter of hair follicle had significant difference in small wave large and medium waves. Although *SFXN1* expression was inconsistent with the biopsy

results. This may have been due to insufficient sample size since the tissues were obtained from full-sib lambs, we suggest that *MMP2* and *SFXN1* may also have a role in the regulation of hair follicle development Finally, we conclude that the *BMP7*, *MMP2* and *SFXN1* genes, which were expressed in large, medium and small waves, were candidates genes for hair follicle development.

Conflicts of interest

The authors declare no conflict of interest.

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