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GHR in Sheep Skin's Localization and Related Function Research

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Abstract: The abundance of GHR (Growth Hormone Receptor) expression and the expression spot in the sheep skin were researched by using relative quota RT-PCR and the organization in situ hybridization. The mRNA level of GHR in the epilated group skin obviously went down in 0th-3rd days and rose to the level before the epilating again in the 6th day. There were not obvious changes in the later. Compared with the control group there was no obvious changes. Though, GHR mRNA in the epilated group obviously was lower than the control group in the 3rd day. But the hybridization signal demonstrated that the closest spot is hair-follicle's wool nipple and exterior and interior root-sheath on the cut sheet. The dermis and various kinds of different cuticle cells have the very weak hybrid signal or not have. GHR has effect on the hair-follicle cell and it is possibly related to the wool character regulation.

Key words: Growth hormone receptor, RT-PCR, organization *in situ* hybridization, sheep, hair-follicle

INTRODUCTION

The wool's growth is a very complex physiological biochemistry process. First, hair-follicle's formation appeared in the embryo and experienced the growing period, the catagen and resting stage of wool cycle process again and again. It is influenced by the inheritance, the environment, the nutrition and so on. For example, traumatogenic ways such as epilating, shaving and scraping wools in the skin surface or using chemical reagent (depilatory) may induce the priming of the hair-follicle's growing period and promote hair-follicle's growth.

On the control of the sacculation, it is generally held that the first signal of the formation of hair follicles is from the mesenchymal (Paus *et al*., 1999; Botchkarev *et al*., 1999). The cells which enter growth period from hair follicles should have certain characteristics: characteristics similar to stem cells's. To maintain a particular rhythm they can produce the signaling molecule and send it to the surrounding cells in the epidermis or dermis. Stenn and Paus (2001) and others considered that the starting of growing hair follicles can be spontaneous from the resting stage or induced by artificially. The artificial induction commonly uses the traumatic way such as epilating, fiercely shaving and scraping the skin surface or the use of chemical reagents (such as hair removal agent) and other ways to stimulate hair follicles to produce a signal. Chase and Eaton (1959) further prove that the growth of hair follicles is stimulated by the removal of at least 1,000 hair follicles. But the growth of hair follicles can not be activated by the signal from which a hair follicle only removed. In this way, starting the growth of hair follicles must be stimulated to achieve a minimum threshold. It is remarkable that this trauma signal is only confined to the wound but not extended. At the same time, Stenn and other consider the induced way could not accurately reflect the normal conditions for spontaneous process of the growth of hair follicles but it is supposed that the two cases are similar in certain aspects of the way to starting route. Considering these reports, hair follicles of the sheep was studied at the level of molecule regulation by the way of epilating and shaving.

GH (Growth Hormone) is an important endocrine factor which regulates animals growth and the three substance metabolism processes. Ward and Brown (1998) and others improved the construction of gene *GH* and published the sequences (MTSGH10) which could less and stably increase the concentration in the blood GH (20%) but the pulse-release disappearing, it could improve the speed of living body weight gain. Adams *et al*. (2002) and others used MTSGH10 GH transgenic Merino and Dorset sheep to study and research wool growth. Results showed that: the production of Merino wools increased and Dorset wools reduced. Data showed that sebaceous gland contained a large number of GHR while it could excrete GH. When the sebaceous gland secretion of GH excessed, the growth of wools would be inhibited. Piper *et al*. (2001) and others found that the Merino wools

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production increased and at equal pace the coefficient of variation in the diameter of wool also increased. The reason was that GH played different roles in the primary and secondary hair follicles. However, GH is a biomacromolecule, it can not directly permeate the cellular membrane and must combine with the GHR of the target cell surface. GH interacts with a Receptor (GHR) (Frank and Fuchs, 2008) belonging to the cytokine/haemat opoietin receptor superfamily. This receptor superfamily present common structural features in the extracellular hormone-binding domain. The binding of a single hormone molecule to a GHR monomer induces receptor homodimerization and activation of the tyrosine activity of JAK2 associated kinase, triggering a phosphorylation cascade, undergoing the trophic signal. This signal takes place through activation of the STATs family transcription factors which translocate to the nucleus where they modify transcription of specific genes. Growth Hormone (GH) and Insulin-like Growth Factor-1 (IGF-1) are associated with many anabolic and metabolic functions. GH alone is gluconeogenic and lipolytic. However, it is difficult to dissociate most of the effects of GH from IGF-1. Both hormones have been associated with amino acid uptake, protein synthesis, inhibition of proteolysis and mineral metabolism. According to the somatomedin hypothesis, many of the actions of GH are carried out through the action of hepatic IGF-1. Indeed, plasma GH is known to cause the release of hepatic IGF-1 into the blood stream which accounts for 75% of circulating IGF-1. Dicks *et al*. (1996) and others found that the distribution of IGF-1R existed in cutis vera cum hair papilla, the inner and outer root sheath and the cortex gland of Angora goats and Kashmir goats. The wool fiber is mainly composed of the hard keratin protein and keratin related protein. Hard keratin is called microfilament keratin (Intermediate Filament proteins, IFs) which in the cortex of hair fiber and widely existed in animal hair, feathers, claws and nails and which is coded by type I (acid) and type II (alkaline) belong to two multigene families. Keratin is a protein associated wool fiber matrix keratin, composed of about 100 different keratins. According to its amino acid content and molecular mass size it can be divided into three families which include ultra-high molecular weight sulfoprotein, high sulfur rich protein and glycine/protein tyrosine. High-sulfur proteins (including KAP1-5) and high-glycine/protein tyrosine (including KAP6, 7, 8) are multi-gene families with intronless to form gene cluster on chromosome and their expression are all controlled by genetic. For example, the research by Rufaut *et al*. (1999) and others showed that the structures of the hair follicles were affected by KAP3.2 and Dsg1.

This research analyzed relations of GHR and the wool character regulation by relative quota RT-PCR and organization *in situ* hybridization to research sheep hair-follicle's expression spot and by epilating and shaving wools to stimulate its change of expression quantity.

MATERIALS AND METHODS

Sample gathering and RNA extraction: Refer by Li (2006), 36 China Merino sheep were selected in the same varieties and body situation. They were divided into 6 groups stochastically, epilated and shaved wool separately in the shoulder to draw an item randomly each time and adopt integumentum sample which is 1×2 cm in the area of epilating field, shaving hair field and control plot when separately in 0, 3, 6, 9 and 12 days when wool grown to 1 cm then adopted the last sampling (about 50 days). After the sampling, it was immediately put in the liquid nitrogen quick-freeze and took over to -80°C the refrigerator to preserve.

Slice manufacture of organization *in situ* hybridization slide silicification and diaper

Silicification: The cover glasses were spreaded out and boiled in 0.1 mol L^{-1} HCL under the condition of ventilating in 20 min. Until cooling the hydrochloric acide were fallen over. The slides were poaching with the deionized water and natural drying. The cover glasses were soaked in Dimethyl Dichloro Silicon (DMDCS) several times under the condition of ventilating and collected in the one heat-resisting petri plate (or culture dish) after drying and with the deionized water thoroughly rinsed several times. The cover glasses loaded with culture dish were securely wrapped with the aluminum foil in 180°C the oven 4 h over night and took them out. After cooling the ambient temperature they were carried on the following processing.

Invest: Slides (mounts or laps) which had prepared after over 160°C to bake well and cooled to the room temperature were soaked up and down in 0.5% (also 0.1%) the PLL fluid dips several times and dispersed the vertical stroke to place on the rack in air natural drying and 4°C then pre-emergency.

Preparation of paraffin section: Blow the sheep skin surface and rapidly adopt the skin from the sheep shoulder, the abdomen, the thigh with the surgery instruments for example, the surgical scissors, the

Table 1: RT-PCR condition for skin GHR, IGF-1, IGF-1R, KAP3.2 and KAP6-1 mRNA in sheep

Groups	GHR	IGF-1	IGF-1R	KAP3.2	KAP6-1
PCR condition	0.8 mol L^{-1} $MgCl_2$ 94°C 30 sec, 56°C 45 sec, 72°C 30 sec and 30 cycles	0.8 mol L^{-1} $MgCl_2$ 94°C 30 sec, 56°C 45 sec, 72°C 60 sec and 31 cycles	0.8 mol L^{-1} $MgCl_2$ 94°C 30 sec, 56°C 45 sec, 72°C 30 sec and 31 cycles	0.8 mol L^{-1} $MgCl_2$ 94°C 30 sec,56°C 45 sec, 72°C 30 sec and 30 cycles	0.8 mol L^{-1} $MgCl_2$ 94°C 30 sec, 61°C 45 sec, 72°C 30 sec and 30 cycles
β-actin/Motive gene primer	1:7	1:6.5	1:2	1:1.9	1:1.4

tweezers which were treated with DMDCS and cut them into 0.5×0.5×0.2 cm size then carry on the paraffin wax embedding then using HE dyeing.

Organization *in situ* hybridization

The disposal of tissue slice: The tissue slices were baked under 65°C in 2 h and took out the slices rapidly put in the xylene 10 min×3 times. They were progressively put in ethyl alcohol (100, 95, 90, 80 and 70%) and entered the water. They were washed with the methyl alcohol 5 min with repetatur semel. PBS flushed them 2 times fast, affected with the 0.2 mol hydrochloric acid under room temperature in the 10 min separately, affected with the 0.2% Trionx-100 in the 15 min, affected with including 0.2 mol glycine PBS under the room temperature in the 15 min, digested with proteinase K under 37°C in the 15-30 min, affected with including 0.2 mol glycine PBS under the room temperature for 15 min, affected with including 5 mmol $MgCl_2$ PBS for 10 min with repetatur semel. They were fixed with the 4% paraformaldehyde under the room temperature for 15 min, effected with including 5 mmol $Mgcl_2$ PBS for 10 min with repetatur semel. They were progressively put in alcohol (70, 80, 90, 95 and 100%) for dehydration, after air drying to preserve for sparing.

Prehybridization and hybridisation: The slices were preliminarily dipped in 2×SSC, simply washed for 15 min, incubated with the 50% deionized formamide (4×SSC disposition, V/V) in 37°C for 15 min, pre-hybridized in 42°C for 30 min to 2 h then hybridized in the hybridization fluid for 20 h. Afterward the cover glass was gently removed from them which were washed in 2×SSC for 1-2 min and then in 2×SSC/50% deionized formamide in 42°C for 10 min, washed in 1×SSC/50% deionized formamide in 37°C for 30 min with repetatur semel, fostered in 4×SSC 10 $mmol^{-1}$ DTT under the room temperature for 1 h, washed in 0.1×SSC in 37-40°C for 30 min×2 times. The slides were put in PBS until carrying on the next step operation.

Hybridization signal examination: The slides were washed in 2×SSC under 40°C for 5 min for three times repeatly, washed in 0.1×SSC under 40°C for 30 min with repetatur semel, washed in 1×the washing buffer solution under the room temperature for 5 min, fostered in 1×baffling the buffer solution under the room temperature for 30 min, deleted periphery organization's buffer solution, fostered with adding the anti-digoxin alkalinity phosphatase immune body (1×to baffling buffer solution 1:1000 time of dilution) under the room temperature for 2 h, washed in 1×the washing buffer solution under the room temperature for 15 min with repetatur semel, neutralized in 1×under neutralizing buffer solution under room temperature for 5 min, deleted periphery organization's buffer solution, colorationed with adding the NBT/BCIP colored fluid under the room temperature and evading the light for 2 h~over night. Observing the hybrid response result under the microscope, the positive reaction was the dark blue.

Relative quota RT-PCR examines the mRNA abundance of GHR

PCR reaction condition: Then with the 2.0% agarose gel detection, the gray zone was analyzed by LabWork 3.0 which is an image analysis software. The result was the ratio between the net with gray and of the net with gray of β-actin. Data were analysed by ANOVA in STATISTICA Software (For Windows Version 5.1, StatSoft Inc., USA) and significant differences were tested and identified by LSD (Table 1).

RESULTS AND DISCUSSION

Sheep hair-follicle activity analysis: Because the slice observation is limited by the position of cuting, the hair-follicle accurate growth phase which locates in the vegetative cycle cannot be determined, only then can be determined the hair-follicle active condition through the statistical analysis (Fig. 1).

Total of hair-follicle was collected from 5 slices following to the slices, respectively below sebaceous glands. Taking 10 complete hair-follicles as the statistical scope in each slice and the active hair-follicles had the red in root sheath's hair-follicle. The hair-follicle activity equation is as follows. In this research primary follicle activity (%): 90±3.3:

$$\text{Hair-follicle activity (\%)} = \frac{\text{The numbers of red in root sheath hair-follicle}}{\text{The total of hair-follicle}}$$

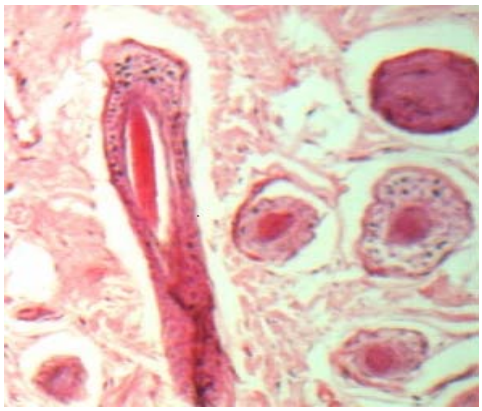

Fig. 1: HE (x100)

Table 2: Growth hormone acceptor mRNA in sheep hair-follicle's localization statistics

Hair papilla	Exterior and interior root-sheath	Scarfskin	Sweatglands
+++	+++	++	+

Few or not have the mark + weak mark +++ strong marks

Growth hormone acceptor mRNA in sheep hair-follicle's localization: The expression situation of the *GHR* gene in the sheep hair-follicle was detected by organization hybridization *in situ*. The test result indicated that the expression of the *GHR* gene existed in the sheep hair-follicle.

The hybridized signal assumed amethyst from (Fig. 2a and b). The hair-follicle's wool nipple and exterior and interior sheath were the closest spot on the slice marks. The dermis and kinds of different cuticle cell have the very weak hybridized signal or not (Table 2).

Plucking and shaving to *GHR*, *IGF-1*, *IGF-1R* gene expression influence in the skin: As shown in Fig. 3, GHR mRNA level of the plucking group skin significantly decreased in the 0th to 3rd days and rose to the level before the plucking to the 6th day. Then there were no significant changes. Compared with the control group, GHR mRNA of shaving group in the skin were not significant changes but plucking group was significantly lower than shaving group and the control group in the 3rd day.

As shown in Fig. 4, IGF-1 mRNA level of the epilated group skin significantly changed that was significantly reduced in the 3rd and 9th days, significantly increased in the 6th and 12th days. This of the skin in the shaving group had gradually rising trend in 0-6 days, reached its peak in the 6th day then gradually reduced, reached its peak in the 12th day and declined again. In the 3rd day, plucking groups were significantly lower than shaving

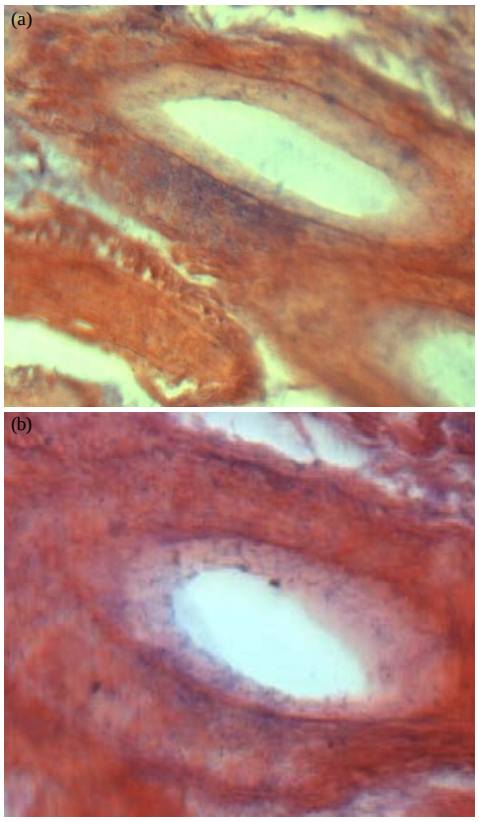

Fig. 2: Localization of GHR mRNA in wool follicles

and control groups. But plucking groups and the group were higher than shaving in the 6th and 12th days and significantly differenced in the 6th days.

As shown in Fig. 5, IGF-1R the mRNA level of the skin in the plucking group didnot change obviously in 0-3 days, obviously reduced in the 6th day, hereafter gradually rose and maintained at the corresponding level again but is lower than the control group. This of the skin in the shaving group presented peak value two times in the 6 and 12th days but didnot change in the other time. In the 6th and 12th days this of the plucking group was obviously lower than shaving group and the control group but the shaving group was obviously higher than the plucking group and the control group.

Plucking and shaving influence *KAP3.2*, *KAP6-1* gene expression in the skin: As shown in Fig. 6, the relative abundance of KAP3.2 mRNA in plucking group skin in the 0-3 days was significantly reduced then gradually increased in the 3rd day and restored to the level before plucking ($p>0.05$). KAP3.2 mRNA of shaving group skin and the control group had the same basic expression and with no significant changes. In the 3rd day, this of

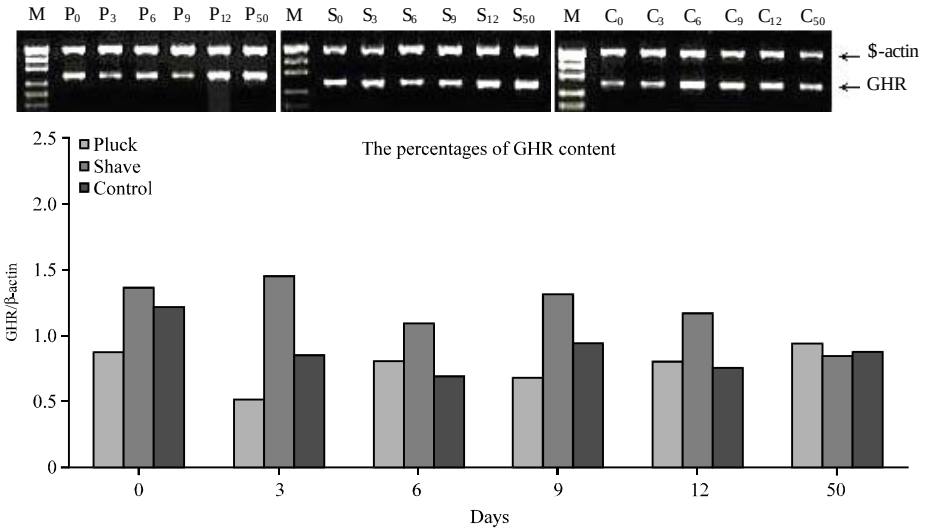

Fig. 3: Effect of plucking or shaving on relative abundance of skin GHR mRNA; Up: representative agarose gel electrophoresis photo of RT-PCR result; P_0~P_{50} represent plucking skin days 0~50, respectively; S_0~S_{50} represent shaving skin days 0~50, respectively; C_0~C_{50} represent control skin days 0~50, respectively; M: Marker (pUC19 DNA/MspI); Down: result analysis of GHR mRNA in different treatment skin, n = 6

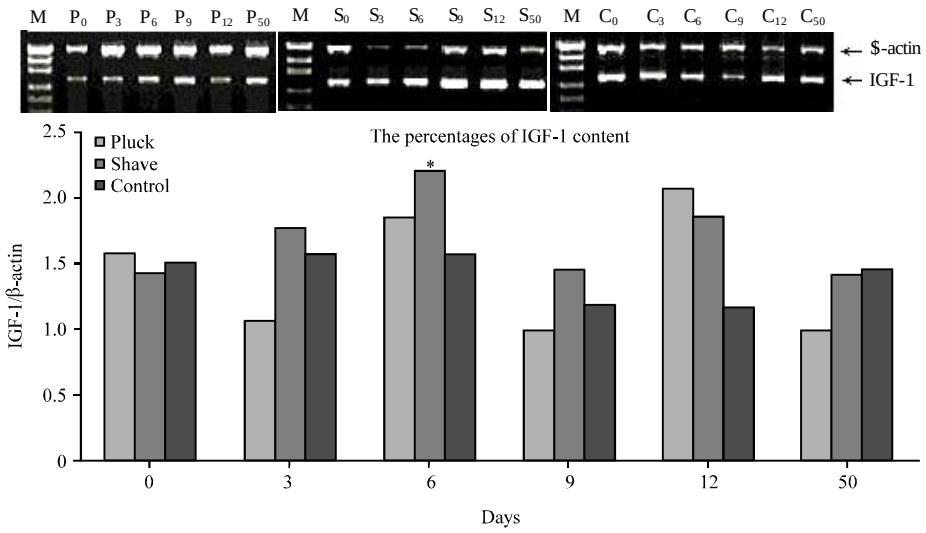

Fig. 4: Effect of plucking or shaving on relative abundance of skin IGF-1 mRNA; Up: representative agarose gel electrophoresis photo of RT-PCR result; P_0~P_{50} represent plucking skin days 0~50, respectively; S_0-S_{50} represent shaving skin days 0~50, respectively; C_0-C_{50} represent control skin days 0-50, respectively; M: Marker (pUC19 DNA/MspI); Down: result analysis of IGF-1 mRNA in different treatment skin, n = 6 *p<0.05

plucking group was significantly lower than shaving group and the control group with no differences in the other time.

As shown in Fig. 7, KAP6-1 the mRNA level in the plucking group skin obviously reduced in the 0th~3rd days, gradually rose in the 3rd~9th days, hereafter relatively maintained steady. This of the shaving group skin did not obviously change in the 0~6 days went up in the 6~9th days, hereafter gradually receded. In the 3rd day, KAP6-1 the mRNA level of plucking group was obviously lower than shaving group and the control group with no differences in the other time.

The growth of wool is an actual process that various growth factors or other factors effectively regulate and controll the keratin synthesis of hair follicles, hair papilla which is the birthplace of the growth of hair fibers is the

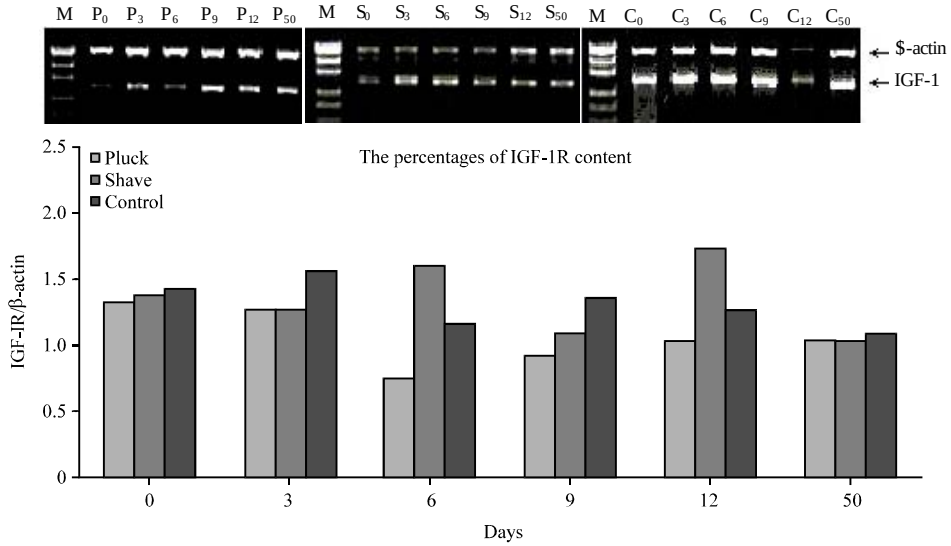

Fig. 5: Effect of plucking or shaving on relative abundance of skin IGF-1R mRNA; Up: representative agarose gel electrophoresis photo of RT-PCR result; P_0~P_{50} and represent plucking skin day s 0~50, respectively; S_0~S_{50} represent shaving skin days 0~50, respectively; C_0~C_{50} represent control skin days 0~50, respectively; M: Marker (pUC19 DNA/MspI); Down: result analysis of IGF-1R mRNA in different treatment skin, n = 6

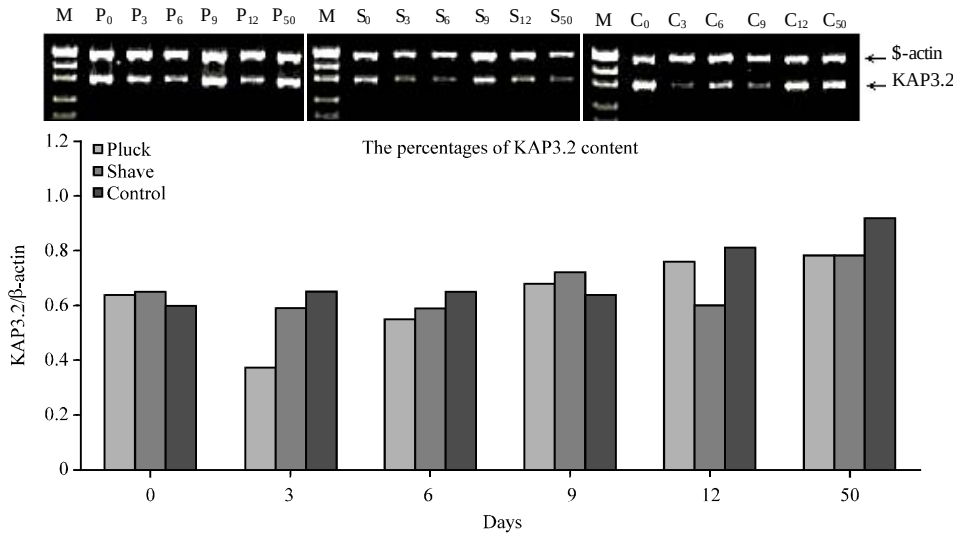

Fig. 6: Effect of plucking or shaving on relative abundance of skin KAP3.2 mRNA; Up: representative agarose gel electrophoresis photo of RT-PCR result; P_0~P_{50} represent plucking skin days 0~50, respectively; S_0~S_{50} represent shaving skin days 0~50, respectively; C_0~C_{50} represent control skin days 0~50, respectively; M: Marker (pUC19 DNA/MspI); Down: result analysis of KAP3.2 mRNA in different treatment skin, n = 6

only part of the organization that is rich in blood supply and plays a crucial role in the proliferation of matrix-induced cell and the growth of hair.

Growth hormone receptor is a receptor on the cell membrane, it indicates that the target cells are acted by the hormone role. Positioning study of the Growth Hormone (GH) mRNA in cattle breast, liver tissue in mice and rabbits had been reported. Glimm *et al*. (1992) and others ascertained that the milk gland in the breast-feeding cattle had rich GHR by Northern blot and hybridization *in situ* and GHR focus on the distribution of breast epithelial cells of cattle. GH plays a direct role in the growth and function of the cattle breast and the epithelial cells in the mammary gland are the target cells which GH acts in. The action about GH to the growth of wool also had been reported in the literature

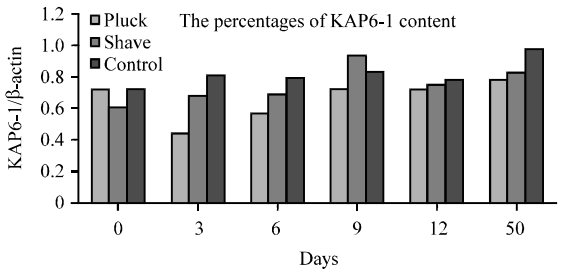

Fig. 7: Effect of plucking or shaving on relative abundance of skin KAP6-1 mRNA; Up: representative agarose gel electrophoresis photo of RT-PCR result; P_0~P_{50} represent plucking skin days 0~50, respectively; S_0~S_{50} represent shaving skin days 0~50, respectively; C_0-C_{50} represent control skin days 0~50, respectively; M: Marker (pUC19 DNA/MspI); Down: result analysis of KAP6-1 mRNA in different treatment skin, n = 6

but the mode of action had not been determined. The results of this study show that GH plays a direct role in the growth of the sheep hair follicles, the target point of GH playing role is the follicle cells. And mainly expressing in the hair papilla and parts exterior and interior root sheath and had agreement with major part of IGF production.

The start-up of the follicle growth cycle is generally spontaneous from the rest period but it may also be induced by a manual start. Shaving and scratching the surface of the skin or the use of chemical reagents (hair removal agent) are likely to produce a stimulating signal to the follicle growth. The research shows that the IGF-1 expression quantity can significantly increased by shaving but the expression quantity of the GHR, IGF-1R, KAP3.2 and KAP6-1 are not significantly impacted. The expression quantity of GHR, IGF-1, KAP3.2 and KAP6-1 in the skin significantly decreased after plucking 3 days and IGF-1R also significantly decreased in the 6th day, but *GHR*, *IGF-1*, *IGF-1R*, *KAP3.2* and *KAP6-1* gene expression did not significantly change in the other time. The gene expression of related the growth of hair follicles could be influenced by plucking and shaving which may relate with the extent of the injury.

CONCLUSION

All in all, the expression sites of GHR demonstrated that it might play an important role in the regulation and control of bristle traits. Shaving significantly affects nothing but the IGF-1 according to the report is matched with speculated regulation of the wool characters. Because shaving the hair follicles will not cause serious injury and plucking will reduce expression level of related gene, the later expression will be improved.

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