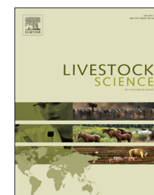
Livestock Science 191 (2016) 29–36

Contents lists available at ScienceDirect

Livestock Science

journal homepage: www.elsevier.com/locate/livsci



Expression of nerve growth factor in skin tissues and its effect on the proliferation of outer root sheath cells in cashmere goats

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ARTICLE INFO

Article history:
Received 26 March 2016
Received in revised form
2 July 2016
Accepted 4 July 2016

Keywords:
NGF
ORS cells
Proliferation
Cashmere goats
Hair follicle cycling

ABSTRACT

Nerve growth factor (NGF) is widely expressed in the nervous system and best understood for its neuronal function. The present study was designed to describe the characteristics of hair follicles and the expression pattern of NGF and its receptor, tyrosine kinase receptor (TrkA), in the skin of Liaoning cashmere goats during hair follicle cycling. Results of hematoxylin and eosin staining revealed that there was a high ratio of secondary to primary follicles, with more active secondary follicles during anagen phase than other phases. The results of quantitative real-time polymerase chain reaction and western blot showed that both NGF and TrkA mRNA and protein levels were persistently detected in the skin tissue during hair follicle cycling. Furthermore, NGF and TrkA mRNA and protein levels during anagen were significantly higher than that during catagen. Using immunohistochemistry, the especially high immunoreactivities of TrkA in the outer root sheath (ORS) and NGF in the internal root sheath (IRS) and ORS were observed during anagen of hair follicles. Meanwhile, NGF and TrkA immunoreactivities were also observed in the epidermis of cashmere goats at similar expression levels as hair follicles. ORS cells were isolated from secondary follicle, and NGF treatment experiments were also studied. Result show that 20 ng/mL or 100 ng/mL NGF could significantly promote the proliferation of ORS cells in vitro, but the promotion could be blocked by the addition of 20 ng/mL K252a, the inhibitor of TrkA. The present study indicates that NGF might be involved in the reconstruction and growth of hair follicles through TrkA tyrosine kinase dependent pathways.

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1. Introduction

The goat cashmere industry is an important part of the animal husbandry industry. Cashmere yield can be highly variable as a result of genetic and environmental factors (Lupton, 1996). There is a great need in the cashmere industry for generating goat breeds that give high yields of cashmere by traditional genetic and breeding methods. Therefore, it is important to explore possible factors that affect the growth and development of hair follicles and to clarify the regulatory mechanism of hair follicle cycling and growth. Hair follicle development displays periodical morphological changes and is governed by complex programs of gene expression (Li et al., 2012; Wang et al., 2012). Recent studies have showed that melatonin (Fu et al., 2014), insulin-like growth factor 1 (Bai et al., 2013), and vascular endothelial growth factor (Gu et al., 2013) play an important role in the development of hair follicles in cashmere goats.

Nerve growth factor (NGF), a member of the neurotrophin family, is a small secreted protein that is widely expressed in the nervous system and best understood for its role in the growth, maintenance, and survival of neurons (Sofroniew et al., 2001; Huang and Reichardt, 2001). NGF exerts its biological activity by binding the high-affinity transmembrane TrkA receptor or the low-affinity neurotrophin p75NTR receptor (Huang and Reichardt, 2003). In addition to its well-known neuronal roles, recent studies have showed that it has important non-neuronal roles (Wang et al., 2013; Li et al., 2010; Abir et al., 2005). In rats, NGF and its receptors are expressed during hair follicle development, while NGF levels can affect also the morphology of rat and mouse hair follicles (Botchkareva et al., 2000; Eva et al., 2006). Although it has been reported that NGF and its receptor TrkA play crucial roles in hair follicle cycling, no study has explored the expression, distribution, and function of NGF and TrkA in the hair follicle of cashmere goats.

Hair follicles of cashmere goats change periodically in the same manner that other mammals undergo cycling of growth, regression, and rest phases, which are known as anagen, catagen, and

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http://dx.doi.org/10.1016/j.livsci.2016.07.002
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Please cite this article as: Ma, W., et al., Expression of nerve growth factor in skin tissues and its effect on the proliferation of outer root sheath cells in cashmere goats. Livestock Science (2016), http://dx.doi.org/10.1016/j.livsci.2016.07.002

telogen phases, respectively (Hardy, 1992; Botchkarev and Kishimoto, 2003). In this study, the histological changes of hair follicles of cashmere goats during all cycling phases were observed using hematoxylin-eosin (H&E) staining. In addition, the NGF and TrkA transcripts and proteins were measured in the hair follicle of skin during hair follicle cycling using quantitative real-time-polymerase chain reaction (qRT-PCR) and western blot, and immunofluorescence (IF) to determine the location of NGF and TrkA proteins. The proliferation and differentiation of ORS cells play an important role in hair follicle elongation (Lee and Tumbar, 2012). To investigate the role of NGF in ORS cells, the proliferation of cultured ORS cells treated with exogenous NGF or the inhibitor of its receptor were also studied. From this, we can determine whether NGF and TrkA are potent genetic markers in goat cashmere traits.

2. Materials and methods

2.1. Sample collection

Skin samples were collected by biopsy punch (1 cm diameter) from the mid-side of four adult female Liaoning Cashmere goat (Institute of Animal Husbandry of Liaoning Province, Liaoyang, China, 41°28′N, 123°17′E) aged 3 years in May, September, and January, representing the three hair follicle developmental stages: anagen, catagen, and telogen, respectively. In order to avoid the differences between individuals, skin samples of three season were from the same individual. Samples for total RNA and protein analysis were stored at −196 °C, and those for H&E staining and IF were mounted in optimal cutting temperature (OCT) compound and stored at −196 °C until use.

2.2. H&E staining

The tissues mounted in the OCT compound were cut using a cryostat (CM3050 S, Leica Biosystems, Germany), and 9-μm-thick sections were obtained. H&E staining of these sections was performed according to the classic H&E staining method (Kiernan, 2008).

2.3. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

All kits and reagents used in the experiments were purchased from Takara Biotechnology (Dalian, China), unless otherwise noted. Total RNA was extracted from skin using Trizol reagent (Invitrogen, Co, Foster, USA) and reverse transcription of RNA was performed using PrimeScript™ RT reagent Kit. cDNA was stored at −80 °C until used. PCR was performed in reaction mixtures, with a final volume of 25 μL containing 150 ng of cDNA, 2.5 μL of 10× Buffer, 10 pmol of each primer, and 0.2 units of rTaq polymerase. Cycling conditions for NGF and TrkA were denaturing at 94 °C for 5 min, followed by 40 cycles of PCR (95 °C for 20 s, 57 °C for 20 s, and 72 °C for 20 s). RNA samples served as negative controls. PCR products were separated in 1.5% agarose gel, and then sequenced by Huada Company (Beijing, China). Specific primers for each gene are shown in Table 1.

2.4. qRT-PCR

To compare the relative abundance of mRNA transcripts of *NGF* and *TrkA* genes in the skin at various developmental stages of hair follicles from each group, qRT-PCR was performed using SYBR premix Ex Taq™ II (Takara). The *GAPDH* gene was used as the internal reference gene to normalize the amount of expressed NGF and TrkA mRNAs, and each sample was tested in triplicate. Cycling conditions for *NGF*, *TrkA*, and *GAPDH* genes were as follows: denaturing at 95 °C for 5 min, followed by 45 cycles of PCR (95 °C for 20 s, 57 °C for 20 s, and 72 °C for 10 s). The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to determine the relative levels of NGF and TrkA mRNAs in each sample.

Table 1
Primers used for RT-PCR for detection of NGF, TrkA, and GAPDH.

Gene	Primer (5′→3′)	Size (bp)	Reference sequence
NGF	F: 5′ AAGCGGCGACTGCGTTCA 3′ R: 5′ TCCCAGCACCATCACCTCC 3′	249	KF914670.1
TrkA	F:5′ TCGGCGATTTCGGTATG 3′ R:5′ GTGGTGAACTTGCGGTAGAG 3′	121	XM_005677331.1
GAPDH	F: 5′GGGTCATCATCTCTGCACCT 3′ R: 5′ ACAGTCTTCTGGGTGGCAGT 3′	211	AJ431207.1

2.5. Western blot analysis

All kits and reagents used in the experiments were purchased from Beyotime Biotechnology (Haimen, Jiangsu, China) unless otherwise noted. Total skin proteins were isolated using Cell Lysis Buffer with 1 mM phenylmethylsulfonyl fluoride. Concentration of total protein for each sample was determined with Bicinchoninic Acid (BCA) protein assay kits. Equal amounts of 20 μg proteins were separated by SDS-PAGE, and the separated proteins were transferred onto polyvinylidene fluoride membranes (Millipore Co., Billerica, MA, USA). After blocking with Tris-buffered saline containing Tween-20 (TBST) and 5% non-fat milk, the membranes were incubated with rabbit anti-mouse polyclonal antibodies specific to NGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (diluted 1:100 in TBST) and TrkA (Santa Cruz Biotechnology) (diluted 1:100 in TBST) at 4 °C overnight, washed with TBST (3×5 min) and incubated for 1 h with an HRP-conjugated goat anti-rabbit IgG secondary antibody (diluted 1:500 in TBST) (Boster, Wuhan, China). The western blot results were visualized using Bioanalytical Imaging System C500 (Azure Biosystems, Inc., Dublin, CA, USA). For the negative control, primary antibodies were replaced by PBS.

2.6. Immunofluorescence of NGF and TrkA in skin tissues

Sections of 9-μm thickness were cut and mounted on lysine-coated slides using a microtome cryostat (CM3050 S, Leica Biosystems, Germany). After washing with PBS (3×5 min), the slides were blocked with 10% normal goat serum for 30 min at 37 °C. They were then incubated with rabbit anti-mouse polyclonal antibodies specific to NGF and TrkA (Santa Cruz Biotechnology) (1:400) at 4 °C overnight. After washing with PBS (3×5 min), the slides were exposed to goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (Boster, China) (1:100) for 30 min at 37 °C. Negative controls were prepared using PBS instead of primary antibodies. Finally, the slides were washed with PBS and examined under a fluorescence microscope (Olympus X71, Japan). Sections of each sample were examined in triplicate for both positive antibody staining and negative controls.

2.7. Isolation and culture of ORS cells

Skin tissues in a bottle filled with 4 °C phosphate buffer saline (PBS) (Boster Inc., Wuhan, China) containing antibiotics were transported to the laboratory immediately. The PBS used in this procedure was supplemented with antibiotics containing 100 IU/mL penicillin and 0.1 mg/mL streptomycin (Boster Inc.,

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