Acta Histochemica 113 (2011) 608–612

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

Immunolocalization of Wnt5a during the hair cycle and its role in hair shaft growth in mice

YiZhan Xing, Wei Xu, Ke Yang, XiaoHua Lian, Tian Yang*

Department of Cell Biology, Third Military Medical University, Chongqing 400038, People's Republic of China

ARTICLE INFO

Article history:
Received 26 January 2010
Received in revised form 15 June 2010
Accepted 20 June 2010

Keywords:
Hair follicle
Wnt5a
Non-canonical Wnt
Hair shaft growth
Immunolocalization
Mouse

ABSTRACT

Previous studies have shown that the Wnt signaling pathway plays an important role in the growth and development of hair follicles. It has been generally accepted that Wnt5a, a non-canonical Wnt gene, inhibits the Wnt/β-catenin signaling pathway. Several reports have addressed its mRNA expression in embryonic and postnatal hair follicles, but its exact role in the growth of hair follicles is currently unknown. In this study, we investigated the immunolocalization of Wnt5a protein in pelages of the dorsal skin and whisker follicles of mice. We found that in the anagen phase, dermal papilla cells showed the highest staining levels of Wnt5a protein, while in the catagen and the telogen phases the staining levels were lower. During the growth stage, Wnt5a protein was prominently located in the matrix and precortex cells in addition to the inner root sheath, outer root sheath and the dermal papilla. As the hair cycle progresses, the immunostaining of Wnt5a was gradually decreased in the catagen phase and was located in the bulge and secondary hair germ in the telogen phase. This Wnt5a immunostaining profile was consistent between dorsal skin pelages and whisker follicles. Furthermore, in an *in vitro* study using whisker follicle organ culture, we demonstrated that the growth of the hair shaft was significantly inhibited by adenovirus Wnt5a. Our findings suggest that Wnt5a is a dynamic factor in the hair cycle and it is important for the regulation of hair shaft growth.

Introduction

The skin of mammals is a complex organ and serves as a major barrier against external injury from the surrounding environment. The epidermis is constantly self-renewing throughout life, including the hair follicles, which also undergo cyclical phases of hair growth (anagen), regression (catagen) and rest (telogen) (Hardy, 1992). The hair cycle is strictly regulated by various growth factors, hormones and signal molecules, of which Wnt protein is one of the most important factors (Stenn and Paus, 2001).

Up to now, 19 Wnt members have been identified in mice and humans (Cadigan and Liu, 2006; Gordon and Nusse, 2006). They are classified into two groups: canonical Wnts and non-canonical Wnts (Cadigan, 2008), which activate different signaling pathways, namely the canonical Wnt/β-catenin signaling pathway and the non-canonical Wnt signaling pathway, respectively. In most cases, non-canonical Wnts could antagonize the canonical Wnt/β-catenin signaling pathway. Some studies have indicated that the canonical Wnt/β-catenin signaling pathway could promote the growth and development of hair follicles (Kuhl et al., 2001; Lo Celso et al., 2004). However, the effects of non-canonical Wnt signaling pathway in these processes are almost unknown. Wnt5a is such a typical non-canonical Wnt, which has an essential role in morphogenesis of several organs, including convergent movement of gastrulation in *Xenopus* (Kuhl et al., 2001), outgrowth of diverse structures (Yamaguchi et al., 1999) and elongation of the small intestine in mouse (Cervantes et al., 2009). Though it has been shown that Wnt5a mRNA was located in the dermis during morphogenesis and in hair follicles of postnatal skin of mice (Reddy et al., 2001), it is still not clear on which cells it has an effect because Wnt family members are secretory proteins. A recent immunohistochemical study has also shown that Wnt5a protein is expressed in human skin (Romanowska et al., 2009). However, there is no immunostaining of Wnt5a protein during the complete hair cycle of mouse and the role of Wnt5a in hair follicle growth is still largely unknown.

To address these questions, we first investigated the immunolocalization of Wnt5a protein and found there was a similar distribution pattern between pelages and whisker follicles. Then in order to explore the role that Wnt5a plays in the growth of hair follicles, we further studied whether the growth of hair follicles could be inhibited by Wnt5a or not, using an *in vitro* organ culture system. Our results provide the first detailed information of Wnt5a protein immunolocalization during a complete hair cycle

Abbreviations: Bu, bulge; DP, dermal papilla; IHC, immunohistochemistry; HG, secondary hair germ; HS, hair shaft; IRS, inner root sheath; ORS, outer root sheath.

* Corresponding author.
E-mail address: tiany@163.net (T. Yang).

0065-1281/$ – see front matter © 2010 Elsevier GmbH. All rights reserved.
doi:10.1016/j.acthis.2010.06.006

and these demonstrate that Wnt5a plays a role in regulation of hair shaft growth.

Materials and methods

Animals

Female C57BL/6J mice used in this study were purchased from the animal facilities of the Third Military Medical University. All the animal-related procedures were strictly in accordance with approved institutional animal care and maintenance protocols. Mice at different ages ($n = 16$), including P0, P7, P16, P21, were sampled according to a previous report (Muller-Rover et al., 2001).

Immunohistochemistry (IHC)

The IHC analysis was performed as described previously (Anderson et al., 1996). Samples of the back skin and whiskers were excised and fixed in 10% formalin at 4 °C for 20 h. After dehydration through a graded series of ethanol concentrations, the tissues were embedded in paraffin and 5 μm sections were cut. Tissue sections were deparaffinized with xylene and rehydrated through an ethanol series, and incubated in 3% H_2O_2 for 15 min to block the endogenous peroxidase activity. The sections were blocked with 2% rabbit serum for 1 h at room temperature and then incubated with a 1:200 dilution of rabbit anti-Wnt5a (Santa Cruz, CA, USA) at 4 °C overnight. After washing, sections were incubated in HRP-labeled conjugated polymer (ZhongShan, Xuanwu zone, Beijing, China) for 30 min at 37 °C and stained with 3,3′-diaminobenzidine tetrahydrochloride hydrate (DAB) (Sigma–Aldrich, St. Louis, MO, USA). The immunostained slides were lightly counterstained with hematoxylin (Invitrogen, Carlsbad, CA, USA) for 5 s, washed in running tap water for 20 min, dehydrated through an ethanol series, cleared with xylene and mounted with Canada balsam (Sigma–Aldrich, St. Louis, MO, USA). All steps were performed at room temperature unless otherwise specified. All dilutions and thorough washes between steps were performed using PBS unless otherwise specified. Negative controls were carried out using the same procedure, but PBS or normal serum was used instead of the primary antibody. Images were recorded using a DFC300FX digital camera (Leica Microsystems, Berlin, Germany) connected to a DMIRB microscope (Nikon, Tokyo, Japan).

Preparation of adenovirus

The recombinant adenovirus GFP (Ad-GFP) and adenovirus Wnt5a (Ad-Wnt5a) were propagated in 293 cells, purified by cesium chloride (Amresco, Solon, OH, USA) density gradient centrifugation and dialysis, and stored at −70 °C (He et al., 1998; Luo et al., 2007). Adenovirus particle concentration was determined by spectrophotometric analysis using a validated assay based on Adenovirus Reference Material obtained from the ATCC (American Type Culture Collection, Manassas, VA, USA).

Hair follicle organ culture and hair growth measurement

Organ culture of whisker follicles of 7-day-old female C57BL/6J mouse were carried out using a procedure modified from that described previously (Buhl et al., 1989). Whisker pads were cut and whole whisker follicles were dissected out of the specimens under a MZ125 dissection microscope (Leica Microsystems, Berlin, Germany). Isolated early- and mid-anagen whisker follicles were maintained in 24-well plates with 0.5 ml incubation medium at 37 °C and under 5% CO_2 in a humidified incubator. The basal medium was serum-free Williams' E medium (Gibco, Carlsbad, CA, USA) supplemented with 100 U/ml penicillin–100 μg/ml streptomycin (Gibco), 10 μg/ml insulin (Sigma–Aldrich), 0.4 μg/ml hydrocortisone (Sigma–Aldrich) and 2 mmol/L L-glutamine (Invitrogen). All follicles were arranged into three groups, which were cultured in basal medium (control), basal medium supplemented with Ad-GFP or Ad-Wnt5a (10^8 PFU), respectively. Each group had 10 hair follicles. After 4 days, the length of the hair shaft protruding from the hair follicle was measured under a MZ125 dissection microscope (Leica Microsystems, Berlin, Germany).

Statistical analysis

All experiments were performed three times with similar results. The data are reported as the mean ± SEM. Statistical significance of hair shaft length data was determined using Student's t-test. A value of $p < 0.05$ was considered statistically significant.

Results

Immunolocalization of Wnt5a protein in back skin pelages at various stages of the hair cycle

To investigate the effects of Wnt5a in postnatal hair cycle, we first performed an immunolocalization analysis in the back skin of mice of different times. At P0, moderate levels of Wnt5a protein were found in multiple layers of developing hair follicle (Fig. 1A and E). At P7, the intensity of Wnt5a protein staining in the epithelial part of the hair follicles increased remarkably, including hair shaft (HS), inner root sheath (IRS), outer root sheath (ORS) and matrix, while little difference was found in the dermal papilla (DP) compared with that at P0 (Fig. 1B and F). At P16, hair follicles entered the catagen phase. Reduced Wnt5a protein distribution was detected in the shortened hair follicle, such as DP, epithelial strand of each hair follicle (Fig. 1C and G). At P21, the lowest level of Wnt5a protein was found in the bulge region and secondary hair germ (HG), with weak positive cells in DP (Fig. 1D and H).

Immunolocalization of Wnt5a protein in whisker follicles at various stages of the hair cycle

It is well-known that whiskers are specialized hair follicles and differ from pelage in terms of hair cycle and structure (Hardy, 1992). To evaluate whether the immunostaining of Wnt5a protein differs between these two types of hair follicles or not, we examined its immunolocalization in whisker follicles of three hair cycle stages. Positive cells were detected in multiple layers of whisker follicles in anagen, including matrix, IRS, ORS, precortex cells, DP, with the highest expression levels in anagen (Fig. 2A and D), lower in catagen (Fig. 2B and E) and the lowest in telogen (Fig. 2C and F), which was in accorded with the profile of pelage.

Hair follicle growth inhibition by Wnt5a

To determine the role of Wnt5a in anagen, we further used whisker follicles in *in vitro* organ culture. The isolated whisker follicles were cultured in William's E medium (control) or basal medium containing Ad-GFP or Ad-Wnt5a (Fig. 3A and D). After 4 days, we examined elongation length of hair shaft of the cultured hair follicles and adenovirus infection efficiency shown by green fluorescence (Fig. 3C and F). Prolonged hair shaft was observed in the control (Fig. 3B) and Ad-GFP groups (data not shown). In contrast, most whisker follicles treated with Ad-Wnt5a had a shorter hair shaft (Fig. 3E). The mean length of the hair shafts after 4 days, determined by analysis of digital images, was 0.75 ± 0.583, 0.68 ± 0.362 and 0.22 ± 0.457 mm for follicles cultured in the control, with Ad-GFP and with Ad-Wnt5a, respectively (Fig. 3H).