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Expression and tissue distribution of hepatocyte growth factor (HGF) and its receptor (c-Met) in alpacas (*Vicugna pacos*) skins associated with white and brown coat colors

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ABSTRACT

Hepatocyte growth factor (HGF)/c-Met signaling has been considered as a key pathway in both melanocyte development and melanogenesis. To understand better the expression patterns and tissue distribution characterization of HGF and its receptor c-Met in skin of white versus brown alpaca (*Vicugna pacos*), we detected the tissue distribution of HGF and c-Met using immunohistochemistry and analyzed the expression patterns by using Western blot and quantitative real time PCR (qPCR). Immunohistochemistry analysis demonstrated that HGF staining robustly increased in the dermal papilla and mesenchymal cells of white alpaca skin compared with that of brown. However, c-Met staining showed strongly positive result, particularly inhair matrix and root sheath in brown alpaca skin. Western blot and qPCR results suggested that HGF and c-Met were expressed at significantly high levels in white and brown alpaca skins, respectively, and protein and transcripts possessed the same expression pattern in white and brown alpaca skins. The results suggested that HGF/c-Met signaling functions in alpaca coat color formation offer essential theoretical basis for further exploration of the role of HGF/c-Met signaling in pigment formation.

Introduction

In adult animals, coat color is dependent on pigment-producing melanocytes that localize at the dermal border and transfer melanin-containing organelles to adjacent keratinocytes that are pushed upward as they proliferate (Barsh, 2007). Melanocyte generates two distinct types of melanin, namely, the yellow to reddish pheomelanins and the black to brown eumelanins (Ito and Wakamatsu, 2008, Pape et al., 2008). Melanin synthesis is regulated by more than 350 genes in mammals, including melanogenic enzymes, transcription factors, hormones, neurotransmitters, cytokines, and growth factors (Slominski et al., 2004, Yamaguchi and Hearing, 2009, Montoliu et al., 2015). However, the molecular and cellular mechanisms regulating coat color in wool-producing animal have not been completely elucidated.

Alpacas have more than 22 natural coat colors; thus, the species is therefore suited for studies on the mechanism of natural coat color formation (Lupton et al., 2006, Mcgregor, 2006). Previous studies, including those we conducted, demonstrated that the three pigmentation critical enzymes, namely, tyrosinase (Tyr), tyrosinase-related protein 1 (Tyrp1), tyrosinase-related protein 2 (DCT), and the critical factors, which include melanocortin-1 receptor protein (Mc1r), inducible nitric oxide synthase, micropthalmia-associated transcription factor (MITF), solute carrier family 7 member 11(Slc7a11), and β-catenin, were stimulators of melanogenesis in alpaca and agouti signaling proteins (Asip), miR-25 and miR-137 were inhibitors of melanogenesis in alpaca (Feeley and Munyard, 2009, Zhu, 2010, Chandramohan, 2013, Dong, 2012, Xue et al., 2014).

Although the role of numerous genes in regulating the coat color of mice, dog, cat, and sheep has been verified (Kaelin and Barsh, 2013, Fan et al., 2013, Sturm, 2009, Montoliu et al., 2015), minimal knowledge on the potential role of hepatocyte growth factor (HGF)/c-Met signaling in the forming of alpaca coat color is available. HGF is a polypeptide growth factor consisting of heavy α and light β chains linked by a disulfide bond; the receptor for HGF is a 145 kDa protein with tyrosine kinase activity that is the product of the c-Met proto-oncogene (Hirobe et al., 2004). In normal skin, c-Met present on epithelial cells and melanocytes

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control multiple aspects of melanocyte function in response to HGF, which is upregulated by ultraviolet radiation (UVR) exposure in keratinocytes and fibroblasts (Mildner, 2007, Soong, 2012). In normal melanocytes, HGF/c-Met signaling has been observed to play a role not only for survival, proliferation, and differentiation of melanocyte precursors, but also for melanogenesis by regulating Tyr and MITF (Kos, 1999, Mcgill, 2006, Cheli et al., 2010). Although previous evidence has indicated that HGF/c-Met has a function in melanocyte development and melanogenesis regulation, the expression and localization of HGF/c-Met in alpacas have not been fully elucidated. In this study, we observed the localization of HGF/c-Met protein within the skin tissue using immunohistochemistry and analyzed its transcript and protein expression level in the skin of white and brown alpacas. Results indicated that HGF/c-Met signaling may have a function in coat-color regulation and melanogenesis in alpacas.

Materials and methods

Animals and tissue collection

Housing and care of alpacas and collection of skin samples that were used in the experiments were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals (http://www.cioms. ch/frame 1985 texts of guidelines.htm) and approved by the Animal Experimentation Ethics Committee of Shanxi Agricultural University, Taigu, China. Three pieces of skin were harvested from the hindquarters of each alpaca (3 white and 3 brown) by 8 mm (biopsy punches Miltex, Japan). Two biopsies were immediately stored in liquid nitrogen for RNA and protein extractions, and the third biopsy was fixed in Bouin's solution for 24 h at 4 °C and then extensively washed in 70% ethanol. Subsequently, the fixed samples were dehydrated in a graded series of ethanol (85%, 95%, and 100%), cleared in xylene, and embedded in paraffin wax. Sections of skin tissue (5 μm) were prepared and mounted onto 2% 3-aminopropyltriethoxysilane-coated slides for immunohistochemical localization of HGF and c-Met.

Immunohistochemistry of HGF and c-Met in the skin

A polyclonal rabbit anti-HGF (or c-Met) antibody was utilized for the localization studies. All incubations were performed in a humidified chamber. Sections of paraffin-embedded skin were deparaffinized and rehydrated in a graded series of ethanol (100%, 95%, 90%, 85%, 80%, and 70%). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 10 min. After three rinses (5 min each) in phosphate-buffered saline (PBS, pH 7.4), sections were incubated with 5% bovine serum albumin (BSA) in PBS at room temperature for 20 min to block the non-specific binding, then incubated overnight at 4 °C in the presence of polyclonal rabbit anti-HGF (Cat. No. bs-1025R; 1:50 in PBS, Beijing Biosynthesis Biotechnology Co., Beijing, China) or anti-c-Met (Cat. No. bs-0668R; 1:50 in PBS, Beijing Biosynthesis Biotechnology Co., Beijing, China). For determination of non-specific staining, the primary antibody was replaced by non-immune bovine serum. After rinsing, HGF and c-Met staining was carried out according to the HGF and c-Met programs. In the HGF program, sections were incubated in biotinylated goat anti-rabbit second antibody (Boster, Wuhan, China) at 37 °C for 30 min, and then rinsed three times in PBS (5 min each). Immunoreactivity was visualized by incubating sections in the presence of 5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitroblue Tetrazolium (BCIP/NBT, 1:20, Boster, Wuhan, China) substrate at RT for 20 min. The sections were then rinsed and counterstained with nuclear fast red (Boster, Wuhan, China), and coverslips were sealed with neutral balsam. In the c-Met program, sections were incubated with polymerized HRP-conjugated goat anti-rabbit secondary antibody (Cat. No. bs-0295G; 1:100 in PBS, Beijing Biosynthesis Biotechnology Co., Beijing, China) at 37 °C for 30 min, and then rinsed three times in PBS (5 min each). Immunoreactivity was visualized by incubating sections in the presence of 3,3′′-diaminobenzidine (DAB, Beijing Biosynthesis Biotechnology Co., Beijing, China) substrate at RT for 5 min. The sections were then counterstained with hemotoxylin, and coverslips were sealed with neutral balsam.

After the completion of immunostaining, sections were examined using a Leica DMIRB computerized microscope (Leica, Wetzlar, Germany) with a Leica digital camera DFC 320 attachment (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK). Images were captured and stored for analysis. The SPSS statistical package version 17.0 (SPSS Inc., USA) was used to analyze all data. ANOVA testing was used to determine statistical differences in the data. All results were expressed as mean ± SD; *P*-values below 0.05 were considered statistically significant.

Western blot analysis of HGF and c-Met proteins

Total protein was extracted from six alpaca full-thickness buttock skin samples (3 white and 3 brown) using a protein extraction kit (Beyotime, Beijing, China). The protein concentration was measured using the BCA protein assay kit (CWBIO, Beijing, China). Twenty five μg of denatured protein from each sample were separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% skimmed milk in TBST (150 mM NaCl, 10 mMTris pH 7.0, 0.05% Tween-20), and incubated with primary antibodies (HGF, 1:200 in TBST, bioss, China; c-Met, 1:200 in TBST, bioss, China; β-actin, 1:1000 in TBST, CWBIO, and China) in 5% skimmed milk blocking buffer overnight at 4 °C. Membranes were washed three times for 15 min with TBST, and incubated with goat anti-rabbit secondary antibody (1:10,000, CWBIO, China) for 2 h at RT. The membranes were again washed for 15 min with TBST. The ECL Western Blot Kit (CWBIO, Beijing, China) was used to detect the signal. The intensity of each protein was analyzed using ImageJ Software (National Institutes of Health, USA) and normalized to values obtained for β-actin (Suppl. Table 1). All experiments were performed in triplicates.

qPCR analysis of HGF and c-Met mRNA

Total RNA was isolated from six alpaca full-thickness buttock skin samples (3 white and 3 brown) using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA integrity was evaluated using 1% gel electrophoresis, and the concentration of total RNA was determined using the ND-100 (NanoDrop Technologies, Wilmington, DE, USA). Subsequently, 1 μg DNase-treated RNA of each sample was converted to cDNA using PrimeScript® RT Master Mix (Perfect Real Time) (TaKaRa, Dalian, China). The cDNA was used for the qPCR analysis of mRNA abundance using the gene specific forward and reverse primers (HGF-F, HGF-R, c-Met-F, c-Met-R). β-actin served as a reference gene (primers are listed in Table 1). All reactions were performed in triplicates on the Stratagene Mx3005P qPCR system (Stratagene Agilent, USA). The 25 μl PCR reactions used 12.5 μl SYBR Premix Ex Taq™ II, 0.5 μl forward primer (10 pM), 0.5 μl reverse primer (10 pM), 0.5 μl ROX reference dye, 2.0 μl template, and 9 μl water. The reactions were incubated in a 96-well plate at 95 °C for 10 s, followed by 40 cycles at 95 °C, 52 °C, and 72 °C for 5, 20, and 15 s. Abundance of HGF and c-Met mRNA was normalized relative to the abundance of β-actin mRNA using the comparative threshold cycle method established by Livak (2001).