INTRODUCTION

Avian feathers are derived from skin and play important roles in flying, protection, and communication. There are many different forms, sizes, and colors of feathers among birds. Feather development includes processes such as induction, molting, differentiation, and cell death. Feathers are composed of rachis, branches, and barbules (Widelitz et al., 2003). Several molecular signaling molecules affect feather morphogenesis, including wnts, bone morphogenic proteins (bmps), and noggin (Lin et al., 2006). The bmps enhance the size of the rachis, and noggin increases branching (Yu et al., 2002). Several genes affect feather development; for example, the expression of hex plays an important role in the initiation of feather bud development (Obinata and Akimoto, 2005), and chick delta-1 expression is associated with formation of feather primordia (Viallet et al., 1998).

The chicken is an important avian model system and is a powerful tool for studying development and disease (Brown et al., 2003). In particular, the avian integument provides a model for analyzing skin appendage morphogenesis (Chen and Chuong, 1999). The Chinese Silky (CS) chicken strain, a Chinese local breed, has a “silky” feather type, and the White Leghorn (WL) chicken strain has a sheet-like feather type. The fluffy type of feathers is caused by defective microscopic hooklets and can be found on the whole body of Silky fowl. In our previous study, we located the silkiness locus on chicken chromosome 3 and found no recombination between the marker CAU0006 and the silkiness locus. Marker CAU0006 is located within the sobp gene (Gao, 2006; Gao et al., 2006). Based on these results, we proposed sobp as a candidate gene associated with feather development.

The sobp gene (encoding sine oculis binding protein homolog), also named jxc1, was first identified and studied in mice. Spontaneous mutation of sobp in mice increases the sound threshold, resulting in abnormal hearing. The sobp gene also controls development of the mouse organ of Corti (Chen et al., 2008). In mice, sobp is expressed in many tissues, including the brain, lung, and heart (Chen et al., 2008). In addition, mutated sobp is highly expressed in the brain limbic system and causes intellectual disability (Birk et al., 2010). Other functions of sobp have not been described, and there are no reports of sobp expression or function in other species. The sobp gene has 6 exons, spanning a region of chromosome 3 from 70.35 Mb to 70.47 Mb. Twelve gene records of sobp-like sequences have been recorded in GenBank, including sequences from mouse, rat, human,
dog, chicken, fly, *Xenopus*, horse, chimpanzee, cattle, monkey, and *Monodelphis domestica*.

In this study, we describe the chicken *sobp* gene, including its homologous relationship among several species and its mRNA and protein size. We also report that *sobp* expression is approximately 1-fold higher in the dorsal skin of WL than in the dorsal skin of CS, during all stages of incubation. In addition, *sobp* expression is also 1-fold higher in the feather follicle of WL compared with the feather follicle of CS in adult chickens. The expression of signaling molecules affecting feather development, such as WNT5A, β-CATENIN, and SHH was similar. Our results suggest the differential expression of *sobp* could be the reason for the distinct feather types of WL and CS chickens.

**MATERIALS AND METHODS**

**Experimental Birds**

Thirty WL chicken eggs and 30 CS eggs were incubated and sampled at various developmental times (embryonic day (E) 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, and postnatal day (P) 1). At E1, 2, 3, 4, 5, 6, and 7, whole embryos were collected from 3 chickens (WL chicken and CS chicken) and kept in liquid nitrogen for RNA extraction and real-time PCR. At E8, 10, 12, 14, and 16, dorsal skin was collected from 3 chickens (WL chicken and CS chicken) and kept in liquid nitrogen for RNA extraction and real-time PCR.

**sobp Gene Isolation by Reverse-Transcription PCR**

Total RNA was extracted from tissues (heart, brain, lung, kidney, eye, and skin) from newborn WL and CS using Trizol reagent (Tiagen, Beijing, China). The chicken *sobp* gene was amplified by reverse-transcription (RT) PCR. Approximately 1 μg of total RNA was used in first strand cDNA synthesis (M-MLV reverse transcriptase, Invitrogen), with oligo d(T)18 primers. The primers for *sobp* were 5'-GAAGGCTACG-3' (forward) and reverse 5'-GGGTAGCTTGTGTTT-3' gapdh (reverse). The PCR conditions were denaturation at 94°C for 5 min, 32 cycles of denaturation at 94°C for 30 s, renaturation at 60°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 7 min. The length of the amplified fragment was 441 bp. The *gapdh* gene was used as a control (5'-AGCCAGCTTCTTCTGAGA-3' forward and reverse 5'-TTCCTTGTACACACC-3'). The PCR procedure was the same as for *sobp*.

**Western Blot Analysis**

Antigenic peptides, corresponding to the SOBP amino acid sequence from 504 to 729, were expressed in *Escherichia coli* using vector pET-30a (Novagen, Germany). Polyclonal rabbit anti-chicken SOBP antibodies were generated in our laboratory. To analyze the expression of SOBP, protein was extracted from the skin of WL and CS chickens using cell lysis buffer for Western blot analysis and immunoprecipitation (Beyotime, Shanghai, China). Concentrations were determined using a BCA protein assay kit (Beyotime). Protein (200 μg) was electrophoresed on a 10% SDS-PAGE gel, and the separated proteins were transferred to a PVDF membrane. The membrane was blocked with 5% skim milk powder in TBST overnight at 4°C. The anti-SOBP polyclonal antibody was diluted to 1:1,000, added to the skim milk solution, and incubated for 1 h. Anti-β-actin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted to 1:1,000 and used as the control. After washing with TBST 3 times, the blots were incubated with horseradish peroxidase-conjugated secondary antibody diluted to 1:5,000 (Zhongshan GoldenBridge Biotechnology Co. Ltd., Beijing, China). The proteins were visualized with SuperSignal West Pico (Thermo Scientific, Waltham, MA).

**Analysis of sobp Expression by Quantitative Real-Time PCR**

Using the RNeasy fibrous tissue mini kit (Qiagen, Germany), total RNA was extracted from WL and CS chic embryos at E1 to E7, and total RNA from the skin was separately extracted from chick embryos at E8, E10, E12, E14, and E16. Approximately 300 ng of total RNA was used for first strand cDNA synthesis (M-MLV reverse transcriptase, Invitrogen), using oligo d(T)18 primers. β-actin was used as an internal reference to verify successful reverse transcription and to normalize the amount of cDNA amplified. Quantitative real-time PCR was performed on an ABI 7900HT real-time PCR system machine (Applied Biosystems Inc., Foster City, CA). The cDNA in each sample was amplified 3 times with SYBR Green PCR master mix (Applied Biosystems). The fold expression was determined as 2−ΔΔCt. The primers for β-actin were 5'-GGGGTACTTATTGGCCTAGG-3' (forward) and reverse 5'-TTCCTTGTACACACC-3'. The primers for *sobp* were 5'-CCACCCACATTTCTGTTCTC-3' (forward) and reverse 5'-AGCCGGTGGTAGCTTGTGTTT-3'. Using the software Primer3 (Applied Biosystems), PCR primers were designed to amplify the target cDNA.

**Northern Blot Analysis**

Total RNA was extracted from WL and CS tissues with Trizol reagent (Invitrogen). A total of 30 μg of RNA was fractionated on a denaturing 1% agarose gel containing formaldehyde, transferred to Hybond N+ membrane with the capillary method and Northernmax transfer buffer (Ambion, Austin, TX) and fixed with UV cross-linking. The membrane was probed with 32P-labeled standard DNA that was complementary to.
the target RNA. Template DNA (50 ng) was labeled with [α-32P]dCTP with Klenow DNA polymerase and random primers (Promega, Fitchburg, WI). Prehybridization and hybridization were carried out in ultraseensitive hybridization buffer (Ambion) at 45°C for 24 h in total. After hybridization, membranes were washed at low stringency in 2× saline sodium citrate (SSC), 0.1% SDS at 45°C for 10 min, at medium stringency in 1× SSC, 0.1% SDS at 45°C for 10 min, and at high stringency in 0.5× SSC, 0.1% SDS at 45°C for 10 min. 18s rRNA was used in the Northern blot analysis as an internal RNA loading control. The gray level was calculated with Quantity One software (Bio-Rad, Hercules, CA).

In Situ Hybridization

The sobp hybridization probe was amplified from cDNA by PCR and cloned into the pGEM-T easy vector (Promega). The primers were 5′-AAGCTTCTTG-GCTGGTA TGG TTA TG-3′ (forward) and 5′-GGATCCTCTTCTGCTGGTGGCTTTAT-3′ (reverse). The underlined sequences represent the restriction enzyme sites of HindIII and BamHI. The dorsal skin at P38 was collected and immediately stored in liquid nitrogen. In situ hybridization was performed on 4% paraformaldehyde-fixed 8-μm frozen sections with DIG-labeled sobp probes (Roche, Switzerland). Images were acquired with a Leica 2500 microscope.

Immunohistochemical Analysis

The antibodies used were as follows anti-SOBP (1:50), anti-β-CATENIN (1:50; Abcam), anti-SHH (1:50; Abcam), anti-WNT5A (1:50; Abcam). Secondary antibodies and labeled streptavidin-peroxidase were purchased from Zhongshan Goldbridge Biotechnology Co. Ltd. (Beijing, China). Images were acquired with the Image Plus system (Olympus Corp., Ina, Japan). The expression levels of every targeted gene were calculated using the software Image-Pro Plus (Media Cybernetics, Bethesda, MD).

RESULTS

RT-PCR Analysis of sobp Expression and Differential Expression in Skin by Western Blotting

The expression level and relative mRNA abundance of sobp in tissues were measured. The RT-PCR analysis showed that sobp was expressed in the heart, brain, lung, kidney, eye, and skin (Figure 1B). The sobp gene was expressed at its highest level in the brain, lung, and skin of WL and the heart and brain of CS. However, the expression profile of sobp in other tissues was different between the 2 strains. The level of sobp in skin was higher in WL than in CS (Figure 1B). Using our polyclonal antibody, SOBP was detected in the skin of both WL and CS. In addition, SOBP was differentially expressed in skin between WL and CS chickens. The size of SOBP was approximately 96 kDa.

sobp Expression Levels in Embryos, Skin, and Other Tissues During Development

We investigated the relative expression levels of sobp mRNA in whole embryos, skin, and other tissues at different developmental stages. Expression of sobp transcripts in whole embryos differed between WL and CS. At E3 and E5, sobp expression levels between WL and CS chickens were significantly different (Figure 2A). In skin, sobp expression levels were significantly different at E8, E12, and E14, and sobp was expressed at least approximately 1-fold higher in the skin of WL than in the skin of CS during embryo development (Figure 2B). The distribution of sobp expression and relative mRNA abundance in embryonic tissues were also assessed using Northern blot analysis. The sobp gene was expressed in the heart, brain, lung, kidney, and eye in both WL and CS (Figure 3A). The sobp gene expression was highest in CS heart tissue, and its expression in skin during embryonic development was different between the 2 strains. Expression in WL skin was clearly higher than the expression in CS skin (Figure 3B). Using gray level analysis, the values for WL were almost
1-fold higher than in CS. The size of the sobp mRNA detected in these studies was approximately 5 kb.

**Differential Expression of sobp in Feather Follicles Between WL and CS Chickens**

After the first molting, the covered feathers were obviously different between the 2 types of chicken, whereas those in the primary regimens region were similar. The feather of dorsal skin at P38 was clearly different between the strains (Figure 4A). At later stages, differences in expression were verified with in situ hybridization, and differences in skin were clearly seen (Figure 4B). The sobp gene was localized similarly between WL and CS, and it was expressed in the whole feather follicle, including the stratum corneum, germinal layer, feather pulp, the wall of the feather follicle, and the feather bulb (Figure 4B).

**Immunohistochemistry of Signaling Molecules Associated with Feather Development**

Several signaling molecules are associated with feather development, including sonic hedgehog (shh), wnt5a, and β-catenin (Lin et al., 2006). We located and examined the expression of these molecules in dorsal skin using immunohistochemistry on tissues collected at P38. The tissues sections represent approximately the same section for every gene examined. The SOBP and WNT5A proteins were localized similarly, and were expressed in the whole feather follicle, including the stratum corneum, germinal layer, feather pulp, the wall of the feather follicle, and the feather bulb. The SHH protein is expressed in the stratum corneum, germinal layer and the feather bulb. The β-CATENIN protein is mainly expressed in the wall of feather follicle. The expression level of SOBP was 1-fold higher in feather follicle of WL than in the CS (Figure 5). Although SOBP expression levels differed between strains, the expression levels of WNT5A, β-CATENIN, and SHH did not differ (Figure 5).

**DISCUSSION**

Following the result of genetic mapping, the chicken gene sobp was cloned and characterized. Skin development is initiated at E8 in chickens (Widelitz et al.,...
2003). At E8, E12, E14, sobp was expressed at least approximately 1-fold higher in the skin of WL than in the skin of CS during embryo development and was expressed 1-fold higher in the feather follicle of WL than in CS in the adult chicken.

At the RNA and protein levels, differences in sobp expression were clearly seen. Given that the shape of WL and CS feathers are quite distinct, our results suggest that the differential expression of sobp may be the reason for the distinct feather shapes.

Does the differential expression of sobp affect the expression of other genes? Several signaling molecules are involved in feather morphogenesis, such as WNT5A, SHH, and β-CATENIN. The SHH protein mediates key interactions between the epithelium and the mesenchyme during early feather development (Ting-Berreth and Chuong, 1996). Furthermore, wnt genes have roles in hair follicle development (Chuong et al., 1996; 1998, 1999).

**Figure 4.** Differential sobp expression in skin between White Leghorn chickens and Chinese Silky chickens using in situ hybridization of feather follicles. A) Feather phenotype varieties on the dorsum in White Leghorn and Chinese Silky chickens at P38. B) In situ hybridization of feather follicles in White Leghorn and Chinese Silky chickens. The sobp gene was localized similarly between White Leghorn and Chinese Silky and expressed in the whole feather follicle, including the stratum corneum germinal layer, the feather pulp, the wall of the feather follicle, and the feather bulb. Key: 1 = the wall of the feather follicle; 2 = the stratum corneum and germinal layer; 3 = the feather pulp; and 4 = the feather bulb. The brown-yellow signal is the positive signal.

**Figure 5.** Immunohistochemical analysis of signaling molecules. A) Expressions of SOBP and signaling molecules that affect feather development were analyzed by immunohistochemistry. Key: 1 = the wall of the feather follicle; 2 = the stratum corneum and germinal layer; 3 = the feather pulp; and 4 = the feather bulb. The brown-yellow signal is the positive signal. B) The expression levels of SOBP and signaling molecules that affect feather development were analyzed using the software Image-Pro Plus (Media Cybernetics, Bethesda, MD). The expression of SOBP was significantly different between the 2 strains (* P < 0.05). The expression of SOBP was twice as high in the feather follicle of White Leghorns as in the feather follicle of Chinese Silkies. However, signaling molecules that affect feather development (WNT5A, SHH, and β-CATENIN) do not differ significantly between White Leghorns and Chinese Silkies. The data are the means ± SD from triplicate samples in 3 separate experiments. A statistical t-test was used to compare the expression level between the White Leghorn and Chinese Silky chickens.
Olivera-Martinez et al., 2001; Tanda et al., 1995), and WNT5A is a target of SHH in hair follicles (Reddy et al., 2001). β-CATENIN signaling can initiate feather bud development (Noramly et al., 1999) and is associated with the conversion of part of the avian foot scales into feather buds (Widelitz et al., 2000). In this study, the expression of WNT5A, SHH, and β-CATENIN were examined by immunohistochemistry. However, no differences were observed between the 2 strains tested. Other signaling molecules involved in feather morphogenesis, such as bmps (Harris et al., 2002; Botchkarev and Sharov, 2004) and FGFs (Tao et al., 2002; Mandler and Neubüser, 2004; Song et al., 2004), may interact with sobp directly or indirectly, leading to the distinct feather phenotype.

In a recent study, a genomic region showing significant association with silky feathers was identified on chicken chromosome 3, between the SNP markers rs16287115 and rs14371625, spanning the genome from 64.5 Mb to 69.7 Mb (Dorshorst et al., 2010). The map location of sobp is close to, but outside of, the mapping interval indicated by Dorshorst, which suggests the presence of another gene affecting feather morphology. Whether sobp indeed regulates feather development should be the subject of further studies. For example, functional complementation assays should be performed by knockdown of sobp expression in WL and overexpression of sobp in CS and their effect on feather phenotype recorded.

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REFERENCES


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