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Identification of a feather β-keratin gene exclusively expressed in pennaceous barbule cells of contour feathers in chicken

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ABSTRACT

Feathers are elaborate skin appendages shared by birds and theropod dinosaurs that have hierarchical branching of the rachis, barbs, and barbules. Feather filaments consist of β-keratins encoded by multiple genes, most of which are located in tandem arrays on chromosomes 2, 25, and 27 in chicken. The expansion of the genes is thought to have contributed to feather evolution; however, it is unclear how the individual genes are involved in feather formation. The aim of the present study was to identify feather keratin genes involved in the formation of barbules. Using a combination of microarray analysis, reverse-transcription polymerase chain reaction, and in situ hybridization, we found an uncharacterized keratin gene on chromosome 7 that was expressed specifically in barbule cells in regenerating chicken feathers. We have named the gene *barbule specific keratin 1* (*BlSK1*). The *BlSK1* gene structure was similar to the gene structure of previously characterized feather keratin genes, and consisted of a non-coding leader exon, an intron, and an exon with an open reading frame (ORF). The ORF was predicted to encode a 98 aa long protein, which shared 59% identity with feather keratin B. Orthologs of *BlSK1* were found in the genomes of other avian species, including turkey, duck, zebra finch, and flycatcher, in regions that shared synteny with chromosome 7 of chicken. Interestingly, *BlSK1* was expressed in feather follicles that generated pennaceous barbules but not in follicles that generated plumulaceous barbules. These results suggested that the composition of feather keratins probably varies depending on the structure of the feather filaments and, that individual feather keratin genes may be involved in building different portions and/or types of feathers in chicken.

1. Introduction

Feathers of extant birds are the most complex integument appendages among vertebrates (Prum and Brush, 2003). A contour feather, for example, consists of a central, stiff shaft called the rachis with softer vanes on each side. The vane consists of numerous side branches called barbs, aligned parallel to each other and at an angle to the rachis. The barbs have a similar array of second-level side branches called barbules on each side. These complex branched structures arise from barb ridges in feather follicles (Yu et al., 2004). Variations in the pattern of barb ridge formation within the follicles generate differences in shape and structure within a single feather or among different feather types, which provides birds with diverse functions, such as protection, display, camouflage, heat retention, and flight (Alibardi and Sawyer, 2006; Alibardi and Toni, 2008).

Feathers were long considered the defining anatomical feature of birds. Over the course of the past two decades, however, fossilized soft tissues of non-avian dinosaurs have been discovered in China, which indicated that feathers are not restricted to birds (Clarke, 2013). In fact, the theropod dinosaurs, such as *Archaeopteryx* and *Microraptor*, which are closely related to birds, had pinnate feathers that were structurally similar to the feathers of extant birds (Clarke and Middleton, 2006). Fossil data also indicated that feathers have evolved from simple filaments, and that filament- and feather-bearing species were common in the lineage of dinosaurs including, *Tyrannosaurus rex*, many small

Abbreviations: aa, amino acid(s); AP, alkaline phosphatase; *BlSK1*, *barbule specific keratin 1*; bp, base pair(s); BP, barbule plate; BR, barb ridge; *COL6A2*, *collagen type VI alpha 2*; E2, estradiol-17β; *FKB*, *feather keratin B*; *FTCD*, *formimidoyltransferase cyclodeaminase*; *Gapdh*, *glyceraldehydes-3-phosphate dehydrogenase*; *MCM3AP*, *minichromosome maintenance complex component 3 associated protein*; ORF, open reading frame; P, pulp; *POFUT2*, *protein O-fucosyltransferase 2*; RR, rachidial ridge; RT-PCR, reverse transcription-polymerase chain reaction; 3′RACE, rapid amplification of cDNA 3′ end.

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raptors, and birds (Clarke, 2013). Recent findings suggest that early pinnate feathers played a role in sexual selection (Li et al., 2010, 2012; Chinsamy et al., 2013), but were unable to sustain the aerodynamic forces of the powered flapping flight stroke (Nudds and Dyke, 2010).

The feathers of extant birds are composed of β-keratins that belong to a multigene family whose members are closely related to but distinct from mammalian α-keratins (Rogers, 1984; Presland et al., 1989a,b). In chicken, the feather β-keratin gene subfamily is the most complex of the β-keratin subfamilies and is composed of at least 83 genes, although the exact number of genes remains unclear (Greenwold and Sawyer, 2010). Studies on the molecular evolution of the feather β-keratin genes revealed that they first appeared after the appearance of feathered dinosaurs and subsequently evolved via repeated duplications (Greenwold and Sawyer, 2011, 2013). Gene family expansions have been shown to contribute to the emergence of novel, lineage-specific morphological features (Barten et al., 2001; Pearson et al., 2005; Vandebergh and Bossuyt, 2012). Similarly, the expansion of the feather β-keratin genes is considered not only to have altered the biophysical nature of the feather establishing its role in powered flight, but also to have allowed the great diversity of feathers both in function and form that is seen in extant birds (Greenwold and Sawyer, 2013). The expression of five feather β-keratin genes, *feather keratin A* to *E*, in feather tissues from 14-day old chick embryos (Presland et al., 1989a), and *feather keratin A* in embryonic and adult chicken feathers (Presland et al., 1989b) has been detected by Northern blotting and primer extension analysis, respectively. In situ hybridization has localized *feather keratin B* (*FKB*) mRNA both in rachises, rami, and barbules of chickens (Ng et al., 2012). Furthermore, expressed sequence tag (EST) analysis has revealed the expression of feather β-keratins from all major clades; however, the tissue sources of the chicken EST data are extremely diverse; ovaries, testes, eye, fat, spleen, breast muscle, and various other glands (Greenwold and Sawyer, 2010). Thus, to our knowledge, it remains unclear how the individual genes are involved in feather formation. The molecular dissection of feathers, therefore, is necessary for a better understanding of the development and evolution of feathers.

The feathers of extant birds are a waterproof, breathable, lightweight construction combining thermal isolation, rigidity, and flexibility. These properties are related partly to the ability of barbule hooklets to hold the different parts of a feather together (Kovalev et al., 2014). The evolution of hooklets permitted the evolution of compacted closed and aerodynamic efficient vanes in large asymmetric contour feathers (Alibardi, 2005, 2007). Therefore, in the present study, an attempt was made to identify feather β-keratin genes involved in the formation of barbules as the first step of molecular dissection of feathers.

2. Materials and methods

2.1. Animals

Two-day-old Okayama-Jidori chickens were provided by the Okayama Prefectural Center for Animal Husbandry and Research (Okayama, Japan). The chickens were housed with free access to commercial food and were used in the experiments. Animal procedures were performed in accordance with the guidelines of the Experimental Animal Committee of Okayama University.

2.2. Estradiol-17β (E2) treatment

After plucking feather follicles from the saddle of 5-week-old male Okayama-Jidori chickens, estradiol-17β (E2; Sigma-Aldrich, St. Louis, MO, USA) in a silastic tube (0.062-inch ID, 0.125-inch OD; Dow Corning, Midland, MI, USA) 2 cm long was implanted subcutaneously under etherization. Cholesterol (Wako, Osaka, Japan) was used instead of E2 in the control animals.

2.3. Total RNA preparation

Total RNA was prepared from pooled feather follicles (3 to 5 pieces) of Okayama-Jidori chickens using TRIsure reagent (Bioline, London, UK). To remove co-extracted yellow pigments, which may inhibit cDNA synthesis, total RNA from the feather follicles was subjected to the guanidinium thiocyanate/CsCl gradient method using a TLS-55 rotor and Optima TLX Ultracentrifuge (Beckman Coulter, Brea, CA, USA). After RNA integrity was assessed by 1% agarose gel electrophoresis, and to remove co-extracted genomic DNA, the total RNA was treated with deoxyribonuclease I (Amplification Grade; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and used for the analyses either by microarray, rapid amplification of cDNA 3′ end (3′RACE), or reverse-transcription polymerase chain reaction (RT-PCR).

2.4. RNA pools for microarray analysis

As in other breeds of chicken, distinct sexual dimorphism is seen in the feathers in the saddle of adult Okayama-Jidori chickens. The saddle feathers, ornamental feathers on the back of adult males, are gold with black patches, and are lanceolate and deeply fringed because of the absence of barbules on the distal ends of the feather barbs. In adult females, on the other hand, feathers in the saddle are dull brown and have a solid vane with no fringing and a round-shaped tip (Fig. 1A). In males, the tip of the feathers has barbs with no barbules (Fig. 1B), whereas, in females, the barbs carry an array of side branching of barbules on both sides (Fig. 1C). Juvenile feathers in both sexes are similar to those of adult females and no obvious gender difference has been observed (Oribe et al., 2012). In chickens, the showy plumage of adult males is the neutral or default development state in both sexes, and the production of ovarian estrogen superimposes the development of cryptic plumage in adult females (Oribe et al., 2012). In fact, the administration of estrogen can induce female-type feathers in adult males (Fig. 1A and D). Furthermore, feather follicles can be induced to produce new feathers at almost any time by plucking the feathers from the follicles. Therefore, feather follicles producing juvenile feathers, or adult male or female feathers can be prepared from a single male chicken. This system was considered to be particularly effective in experimental animals with heterogeneous genetic backgrounds, such as chickens. Therefore, we decided to explore barbule-specific keratin using this system. We plucked juvenile feather follicles in the saddle of a 5-week-old male Okayama-Jidori chicken. Then, we plucked the feather follicles of the regenerated adult feathers in the same experimental animal again at 8 weeks of age and implanted subcutaneously silastic tubes filled with estradiol-17β (E2). In the presence of E2, the regenerated feathers showed a female-like shape and pigmentation (Fig. 1A and D). The barred pattern in the feathers (Fig. 1A) was produced by the allele at the sex-linked barred locus. The feather follicles that generated the female-type feathers were plucked when the chicken was 11 weeks old. All the feather follicles used were plucked when the tips of the feathers were developing, and total RNA was extracted for the microarray analysis.

2.5. Microarray analysis

Microarray analysis was carried out using a commercial array service (Agilent Expression Array; Takara Bio, Yokkaichi, Japan) containing a total of 43,803 oligo probes that target chicken genes. Briefly, total RNA from feather follicles was quantified and qualified using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Cy3-labeled cRNA was prepared using 0.5 μg of each total RNA and a Low Input Quick Amp Labeling Kit, one-color (Agilent Technologies). The Cy3-labeled cRNA probes (1.65 μg each) were hybridized to a Chicken (V2) Gene Expression Microarray, 4×44K (Agilent Technologies) and then washed using a Gene Expression Hybridization Kit (Agilent Technologies) and a Gene Expression Wash Buffers Pack (Agilent