Identification of *pennaceous barbule cell factor* (*PBCF*), a novel gene with spatiotemporal expression in barbule cells during feather development

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Abstract

Bird contour feathers exhibit a complex hierarchical structure composed of a rachis, barbs, and barbules, with barbules playing a crucial role in maintaining feather structure and function. Understanding the molecular mechanisms underlying barbule formation is essential for advancing our knowledge of avian biology and evolution. In this study, we identified a novel gene, *pennaceous barbule cell factor* (*PBCF*), using microarray analysis, RT-PCR, and *in situ* hybridization. *PBCF* is expressed in barbule cells adjacent to the ramus during pennaceous barbule formation, where these cells fuse with the ramus to establish the feather's branching structure. *PBCF* expression occurs transiently after melanin pigmentation of the barbule plates but before the expression of *barbule-specific keratin 1* (*BlSK1*). Orthologues of *PBCF*, predicted to be secreted proteins, are conserved across avian species, with potential homologues detected in reptiles, suggesting an evolutionary lineage-specific adaptation. Additionally, *PBCF* is expressed in non-vacuolated notochord cells and the extra-embryonic ectoderm of the yolk sac, hinting at its broader developmental significance. The *PBCF* gene produces two mRNA isoforms via alternative splicing, encoding a secreted protein and a glycophosphatidylinositol (GPI)-anchored membrane-bound protein, indicating functional versatility. These findings suggest that PBCF may be involved as an avian-specific extracellular matrix component in cell adhesion and/or communication, potentially contributing to both feather development and embryogenesis. Further investigation of PBCF's role in feather evolution and its potential functions in other vertebrates could provide new insights into the interplay between development and evolution.

Keywords: feather; barbule; branching; chicken; yolk sac membrane; notochord

Abbreviations:

PBCF, pennaceous barbule cell factor; BlSK1, barbule specific keratin 1; GPI, glycophosphatidylinositol; cRNA; complementary RNA; E2, estradiol-17β; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse-transcription polymerase chain reaction; bp, base pair(s); ORF, open reading frame; PBM, peri-notochordal basement membrane; E2 male, E2-treated male; INHBA, Inhibin Subunit Beta A; GLI3, GLI Family Zinc Finger 3; C7orf25, Chromosome 7 Open Reading Frame 25; PSMA2, Proteasome 20S Subunit Alpha 2; MRPL32, Mitochondrial Ribosomal Protein L32; NC, notochord

1. Introduction

Feathers in birds are highly complex integumentary structures found in vertebrates (Yu et al., 2002; Prum and Brush, 2003; Alibardi, 2017). They serve essential functions such as flight, thermoregulation, camouflage, and display. Structurally, each feather consists of a central rachis with barbs extending symmetrically on either side. Smaller structures called barbules branch in two rows from the ramus of each barb (Yu et al., 2004). In two adjacent barbs, the barbules of the distal barbs (distal barbules), equipped with barbicels, interlock with the barbules of the proximal barb of the neighboring barb (proximal barbules). This interlocking mechanism ensures the structural integrity and functionality of the vane, which is essential for aerodynamic efficiency, waterproofing, and insulation (Prum and Brush, 2003). The presence of barbules is

73 indispensable for these functions and marks a significant evolutionary advancement in
74 modern birds, tracing back to their evolutionary transition from feathered dinosaurs
75 (Prum, 1999; Alibardi, 2005; Alibardi, 2007; Clarke, 2013; Kovalev et al., 2014; Ksepka,
76 2020).

77 Feather development involves specialized processes within the feather follicles (Yu
78 et al., 2004). Barb ridges give rise to barbs which are composed of ramus and barbules,
79 which form through the differentiation of barbule cells into structures called barbule
80 plates (Alibardi and Sawyer, 2006; Alibardi, 2007). These barbule cells are pigmented by
81 melanocytes and undergo keratinization (Watterson, 1942; Alibardi and Toni, 2008;
82 Saranathan and Finet, 2021), forming robust and functional feather structures (Yu et al.,
83 2004). While substantial progress has been made in understanding the molecular
84 mechanisms underlying feather formation, including coloration and sexual dimorphism
85 (Jung et al., 1998; Jiang et al., 1999; Harris et al., 2002; Yu et al., 2002; Widelitz et al.,
86 2003; Yue et al., 2006; Yoshihara et al., 2012; Yue et al., 2012; Alibardi, 2017; Okamura
87 et al., 2019; Widelitz et al., 2019; Li et al., 2021; Saranathan and Finet, 2021; Chen et al.,
88 2024; Nozawa et al., 2024; You et al., 2024), the mechanisms driving barbule formation
89 remain largely unexplored. Recent studies have further investigated the genetic and
90 molecular basis of feather diversity and the patterning and structure of epidermal
91 appendages (Chen et al., 2015; Boer et al., 2017; Ng and Li, 2018; Chang et al., 2019;
92 Terrill and Shultz, 2023), but the specific pathways involved in barbule formation are still
93 not fully understood.

94 Previous studies have identified specific genes involved in barbule differentiation
95 in chicks (Kowata et al., 2014). In this study, we identified a novel gene, *PBCF*, through
96 microarray analysis, RT-PCR, and *in situ* hybridization. *PBCF* exhibits spatiotemporal

expression in basal pennaceous barbule cells during feather development, suggesting its pivotal role in pennaceous barbule formation. By elucidating the expression patterns and potential functions of PBCF, this study sheds light on the genetic mechanisms underlying feather structure development and provides a foundation for exploring the evolutionary processes shaping avian morphology. Furthermore, these findings contribute to a broader understanding of how genetic evolution drives the emergence of novel morphological and functional traits in living organisms.

2. Materials and Methods

2.1. Animals

Two-day-old Okayama-Jidori and Tosa-Jidori chickens were obtained from the Okayama Prefectural Center for Animal Husbandry and Research (Okayama, Japan) and the Japanese Avian Bioresource Project Research Center (Hiroshima University, Higashihiroshima, Japan), respectively. The chickens were housed with free access to commercial chicken food and used for experiments. Okayama-Jidori chickens were utilized for microarray analysis, while Tosa-Jidori chickens were used for reverse-transcription polymerase chain reaction (RT-PCR) and *in situ* hybridization. Fertilized broiler chicken eggs were obtained from a commercial supplier (Fukuda Poultry Breeding Farm, Okayama, Japan), and the embryos were incubated at 37°C for the required number of days before use in experiments. All animal procedures were performed in accordance with the guidelines of the Experimental Animal Committee of Okayama University, and this study was specifically approved by the committee.

2.2. Scanning Electron Microscopy (SEM)

Feathers were thoroughly air-dried and then cut into appropriately sized pieces using scissors. The samples were subsequently coated with gold using a JFC-1200 Fine Coater (JEOL, Tokyo, Japan) and observed under a scanning electron microscope (SEM) JSM-6510LV (JEOL, Tokyo, Japan).

2.3. Estradiol-17β (E2) treatments *in vivo*

To induce regeneration of feather follicles, feathers were plucked from the saddle region of five-week-old male Okayama-Jidori chickens or from eight-week-old male Tosa-Jidori chickens, followed by hormone treatments. Estradiol 17-β (E2; Sigma-Aldrich, St. Louis, MO, USA) was subcutaneously implanted under anesthesia immediately after plucking using a silastic tube (0.062 in. ID × 0.125 in. OD; Dow Corning, Midland, MI, USA). The tube length was 2.5 cm for Okayama-Jidori chickens and 1.0 cm for Tosa-Jidori chickens. Cholesterol (Wako, Osaka, Japan) was used as a control instead of E2 in the control animals.

2.4. Total RNA preparation

Total RNA was extracted from pooled feather follicles (3 to 5 follicles) of Okayama-Jidori chickens or 4 to 6 follicles of Tosa-Jidori chickens using TRIsure reagent (Bioline, London, UK). To eliminate co-extracted yellow pigments, total RNA underwent purification using the guanidinium thiocyanate/CsCl gradient method with a TLS-55 rotor in an Optima TLX Ultracentrifuge (Beckman Coulter, Brea, CA, USA). RNA integrity was assessed by 1% agarose gel electrophoresis, and total RNA was subsequently treated with deoxyribonuclease I (Amplification Grade; Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol to remove co-extracted genomic DNA. The

purified RNA was then used for either microarray analysis or reverse-transcription polymerase chain reaction (RT-PCR). For RNA preparation from embryos or embryonic yolk sack of broiler chickens, TRI Reagent (COSMO BIO, Tokyo, Japan) was used according to the manufacturer's guidelines for RT-PCR analysis.

2.5. Microarray analysis

Microarray analysis was performed using an Agilent Expression Array (Takara Bio, Yokkaichi, Japan) containing 43,803 probes for chicken genes. Total RNA from feather follicles was quantified and assessed for quality using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Cy3-labeled cRNA was prepared from 0.5 μg of total RNA using the Low Input Quick Amp Labeling Kit, one-color (Agilent Technologies). The Cy3-labeled cRNA probes (1.65 μg) were hybridized to a Chicken (V2) Gene Expression Microarray, 4x44K (Agilent Technologies), followed by washing with the Gene Expression Hybridization Kit and Wash Buffers Pack (Agilent Technologies). Arrays were scanned with a G2505C Scanner (Agilent Technologies) following standard protocols, and data were analyzed using GeneSpring GX11 software (Agilent Technologies).

2.6. RT-PCR

Reverse transcription was performed using either the Thermo Script RT-PCR system (Invitrogen, Carlsbad, CA, USA) or the ReverTra Ace qPCR RT Kit (TOYOBO, Shiga, Japan), depending on the experiment. A total of 0.7 to 3 μg of RNA was reverse-transcribed according to the manufacturer's instructions and used for subsequent RT-PCR. RT-PCR was conducted with Platinum Taq DNA Polymerase (Invitrogen) or Tks Gflex

DNA polymerase (Takara Bio, Kusatsu, Japan) using a thermal cycler (Gene Amp PCR System 9700, Applied Biosystems) or Life Eco Thermal Cycler (Nippon Genetics, Aichi, Japan). For reactions using Platinum Taq DNA Polymerase, the PCR conditions were: initial denaturation at 94°C for 2 min, followed by cycling reactions including denaturation at 94°C for 30 s and annealing/extension at 60 °C for 1 min for *BlSK1* mRNA and *GAPDH* mRNA, or at 63 °C for 1 min for *PBCF* mRNA was performed (Fig. 1 and Fig. 2). For reactions using Tks Gflex DNA polymerase, the conditions were: initial denaturation at 94 °C for 1 min, followed by cycling reactions including denaturation for 10 s at 98 °C, annealing for 15 s at 53 °C, and extension for 60 s at 68 °C (Fig. 7 and Fig. 8). All primer sets used in this study, PS1 to PS6, are listed in Table 1.The primer sets used for RT-PCR were as follows: PS2 for *PBCF* mRNA, PS1 for *BlSK1* mRNA, and PS6 for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA. The PCR cycle numbers for *BlSK* and *GAPDH* were 27 and 18, respectively. For *PBCF*, the cycle numbers were 35 (Fig. 1, Fig. 2, Fig. 4, and Fig. 8) and 29 (Fig. 7). A one-tenth aliquot of each PCR reaction was electrophoresed on a 2.0% agarose gel, stained with ethidium bromide, and visualized under ultraviolet illumination. For the identification of alternative splicing of *PBCF* mRNA, RT-PCR was performed using RNA extracted from the yolk sac membrane of 3-day-old broiler chicken embryos, with primer set PS4 and PS5. Tks Gflex DNA Polymerase was used for the reactions. The conditions were as follows: initial denaturation at 94°C for 1 minute, followed by 35 cycles of denaturation at 98°C for 10 seconds, annealing at 53°C for 15 seconds, and extension at 68°C for 60 seconds. The amplified cDNA fragments were purified using the NucleoSpin Gel and PCR Clean-up kit (Takara Bio), and then sequenced. Sequencing was performed with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA,

USA) and an ABI 3500/3500xL Genetic Analyzer (Applied Biosystems). The known transcript corresponding to the reference sequence XM_429784.7 was designated as *PBCF* type 1 mRNA, while a novel transcript generated by alternative splicing and identified in this study was designated as *PBCF* type 2 mRNA. These sequences have been deposited in the DDBJ/GenBank databases under accession numbers LC846667 (*PBCF* type 1 mRNA) and LC846668 (*PBCF* type 2 mRNA), respectively.

2.7. *in situ* hybridization

Feather follicles from E2-treated male Tosa-Jidori chickens, as well as embryos and yolk sacs from broiler chickens, were fixed overnight at room temperature in 4% paraformaldehyde, embedded in O.C.T. Compound (Sakura Finetek Japan, Tokyo, Japan), and frozen in liquid nitrogen. Tissue sections (10 μm thick) were prepared using a cryostat, air-dried, and processed through sequential immersions in PBS-T, proteinase K (Nacalai Tesque, Kyoto, Japan), and 4% paraformaldehyde prior to *in situ* hybridization. A 347 bp *PBCF* cDNA fragment was amplified by RT-PCR using the primer set PS3. DIG-labeled sense and antisense *PBCF* riboprobes were synthesized using a Riboprobe Combination System - SP6/T7 RNA Polymerase (Promega, Madison, WI, USA) according to the manufacturer's instructions. Hybridization was performed with 1 ng/μl DIG-labeled probes in a solution containing yeast tRNA, 50% deionized formamide, and 10% dextran sulfate at 55°C overnight. After hybridization, the slides were washed in formamide-SSC solutions, followed by incubation with anti-DIG-alkaline phosphatase Fab fragments (Roche Diagnostics, Mannheim, Germany) and immunostained using nitro blue tetrazolium chloride and 5-bromo-4-chloro-3'-indoylphosphate (Wako Pure Chemical Industries, Osaka, Japan). Probe preparation and hybridization conditions for *BlSK1* were

described previously (Kowata et al., 2014).

2.8. Bioinformatic analysis

Homologous sequences were searched using BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) against the NCBI non-redundant (nr) protein database. The search employed the amino acid sequence of PBCF (XP_0405191) as the query. Default BLASTp parameters were used, with an expected value (E-value) cutoff of 1e-5 to identify sequences with significant homology. PBCF homologous sequences from species such as the White-throated Tinamou (*Tinamus guttatus*, accession number: KGL83543.1), Mallard (*Anas platyrhynchos*, accession number: EOB04786.1), Chuck-will's-widow (*Antrostomus carolinensis*, accession number: KFZ47189.1), Common Cuckoo (*Cuculus canorus*, accession number: KFO81754.1), Grey Crowned Crane (*Balearica regulorum*, accession number: KFO14632.1), Dalmatian Pelican (*Pelecanus crispus*, accession number: KFQ59038.1), and Barn Owl (*Tyto alba*, accession number: KFV53320.1), along with homologous sequences and mRNA from the Chinese softshell turtle (*Pelodiscus sinensis*), Green anole (*Anolis carolinensis*), and Chinese alligator (*Alligator sinensis*), were retrieved from the NCBI database. The reference IDs for mRNA from the Chinese softshell turtle, Green anole, and Chinese alligator were XM_040663188.1, XM_006124091.2, XM_008112876.3, and XM_006020434.1, respectively. Sequences were aligned using CLUSTALW (http://www.genome.jp/tools/clustalw/) and BioEdit 7.2 software (https://bioedit.software.informer.com/) to identify conserved regions. To compare two sequences across their entire span, the Needleman-Wunsch Global Align Protein Sequences tool from NCBI was used. Synteny analysis was conducted using the

Ensembl Genome Browser (http://asia.ensembl.org/index.html). Signal peptides and their cleavage sites were predicted using SignalP-5.0 (https://services.healthtech.dtu.dk/services/SignalP-5.0/). Protein localization predictions were carried out using PSORT II (https://psort.hgc.jp/) and MULocDeep (https://www.mu-loc.org/) (Jiang et al., 2021; Jiang et al., 2023). Additionally, glycophosphatidylinositol (GPI) anchors were predicted with PSORT II and NetGPI-1.1 (https://services.healthtech.dtu.dk/services/NetGPI-1.1/)(Gíslason et al., 2021).

3. Results and Discussion

3.1. Identification of a novel gene, *pennaceous barbule cell factor* (*PBCF*), by microarray analysis

Sexual dimorphism in the saddle feathers of adult Okayama-Jidori chickens is well-documented (Oribe et al., 2012). In males, these feathers are lanceolate with prominent fringing due to the absence of barbules at the distal ends of the barbs. In contrast, adult female saddle feathers have a solid vane with no fringing and a rounded tip. While male feather tips lack barbules (Fig. 1A; Male), female feathers possess barbules branching from both sides of the barbs (Fig. 1A; Female). The showy plumage of adult males represents the default developmental pathway in both sexes, with ovarian estrogen inducing female-specific plumage (Oribe et al., 2012; You et al., 2024). Additionally, estrogen administration can induce female-like feathers in adult males (Fig. 1A; E2 Male).

Feather plucking stimulates the regeneration of new feathers, enabling the study of feather follicles that produce either male or female feathers from the same male chicken (Kowata et al., 2014). In this study, feather plucking was performed on male

chickens that were later treated with estradiol-17β (E2).This system is particularly useful in studies involving animals with heterogeneous genetic backgrounds, such as chickens. Using this approach, we investigated the genes involved in barbule formation. To comprehensively analyze differences in gene expression between male-type and female-type feathers, we selected microarray analysis as our method. Feather follicles from the saddle region of a 5-week-old male Okayama-Jidori chicken were plucked. Regenerating adult feathers were collected three weeks later, when the chicken was 8 weeks old. At this point, silastic tubes containing E2 were implanted subcutaneously. Feather follicles producing female-type feathers were plucked when the chicken reached 11 weeks of age. All follicles were collected during feather tip development, and total RNA was extracted for microarray analysis.

Comparing gene expression among the sampled feather follicles, we identified an uncharacterized gene, LOC420770 (EntrezGene ID: 420770) localized on chicken chromosome 2, as the most upregulated gene in estrogen-treated male feather follicles generating barbules. This gene, which exhibited a 315.8-fold increase in expression (E2-treated male vs adult male), was named *Pennaceous Barbule Cell Factor* (*PBCF*) in this study based on its expression in barbule cells in the barbule plate, proximal to the barb cells destined to become the ramus during pennaceous barbule formation, as revealed in this study. It should be noted, however, that this finding is based on a single experiment.

3.2. Expression of *PBCF* in feather follicles of contour feathers

To validate the microarray data and compare the expression profiles of *PBCF* with *BlSK1*, a barbule-specific keratin gene previously identified (Kowata et al., 2014), RT-PCR analyses were conducted using RNA from the feather follicles of adult chickens

289 of both sexes, including E2-treated adult males. All follicles were plucked during feather
290 tip development. As shown in Fig. 1B, *PBCF* was expressed at significant levels in
291 follicles developing barbules, consistent with *BlSK1* expression. The observed variability
292 in RT-PCR bands for *PBCF* and *BISK1* among male samples may be attributed to slight
293 differences in the developmental stages of the feathers at the time of sampling. These
294 findings confirm the microarray results and suggest that *PBCF* is likely involved in
295 barbule formation.

296 Contour feathers consist of both pennaceous barbules in the vane and
297 plumulaceous barbules in the fluff (Fig. 2). In pennaceous barbules, hooklets on the distal
298 barbules interlock with the proximal barbules of adjacent barbs, creating closed vanes on
299 either side of the rachis. In contrast, plumulaceous barbules lack hooklets and are uniform
300 in shape, contributing to the feather's fluffy structure (Alibardi and Toni, 2008). To
301 determine whether *PBCF* is involved in the formation of both barbule types, we
302 conducted RT-PCR analysis on RNA extracted from the feather follicles of saddle feathers
303 at different developmental stages: the tip stage (when feather tips are being formed), the
304 vane stage (when barb and barbule structures are developing), and the fluff stage (when
305 the terminal fluffy region is forming) in adult male and female chickens. The results are
306 shown in Fig. 2A and Fig. 2B, respectively. Similar to *BlSK1*, *PBCF* expression was
307 detected only in follicles developing pennaceous barbules, suggesting a specific role for
308 *PBCF* in pennaceous barbule formation.

309 To further confirm the expression of *PBCF* in pennaceous barbule cells, we
310 performed *in situ* hybridization on cross-sections of feather follicles developing
311 pennaceous barbules, using a probe specific for *PBCF* mRNA. This method was chosen
312 because it enables the visualization of the spatial distribution of gene expression within

313 the tissue, allowing us to precisely localize *PBCF* expression to specific cell types, such
314 as barbule cells. As shown in Fig. 3A, the antisense probe specifically stained only the
315 cells in the barbule plate, proximal to the barb cells destined to become the ramus. No
316 staining was observed in the neighboring section treated with the sense probe. Based on
317 these results, we named the gene *LOC420770* as *pennaceous barbule cell factor* (*PBCF*).
318 To clarify the location of *PBCF* mRNA-expressing cells, a schematic diagram of the barb
319 ridge is presented in Fig. 3B. These findings suggest that the *PBCF* gene might play a
320 role in pennaceous barbule formation, particularly in the attachment of barbules to the
321 ramus, leading to barb development.

322 Cellular events in feather organogenesis, including proliferation,
323 differentiation, pigmentation, and maturation of keratinocytes, occur along the proximal–
324 distal axis in feather follicles (Yu et al., 2002; Yu et al., 2004). To gain insight into the
325 role of *PBCF* in feather development, feather follicles were divided into four segments
326 along the proximal–distal axis, and RT-PCR analysis was performed on each segment to
327 localize the expression of *PBCF* and *BlSK1* mRNAs. Representative results are shown in
328 Fig. 4A.

329 We found that *PBCF* was expressed earlier than *BlSK1*, suggesting that *PBCF*
330 may function prior to the keratinization of barbule cells. The difference in expression
331 timing between *PBCF* and *BlSK1* was further confirmed by *in situ* hybridization of cross-
332 sections from different parts of the feather follicles using antisense probes specific to each
333 mRNA (Fig. 4B). In the proximal (basal) barb ridge region of the feather follicles, where
334 melanocytes actively transfer melanin to barbule cells, no positive signals were detected
335 with either probe. In the region of the follicle where pigmentation of barbule cells with
336 melanin had just been finished, only *PBCF*-positive signals were observed, while in the

more distal barb ridge region, only *BlSK1*-positive signals were detected. These findings indicate that *PBCF* is expressed in basal barbule cells during the stage between pigmentation and keratinization. The three-dimensional structure of the barb ridge is crucial for understanding the spatial distribution of gene expression. This can be better understood by referring to Figures 3 and 5 in Alibardi (2017) (Alibardi, 2017) , Figure 10 in Alibardi (2007) (Alibardi, 2007), or Figure 1, S1, and 6 in Chang et al (2019) (Chang et al., 2019). Furthermore, PBCF-positive signals were exclusively observed in basal barbule cells, even in barbule plates lacking black melanin (eumelanin), suggesting that eumelanin is unlikely to obscure the *in situ* hybridization signals. However, we cannot completely rule out the possibility that high levels of eumelanin in other barbule cells might obscure the detection of in situ hybridization signals.

3.3. Possible Orthologs of *PBCF* in Avian Species and Other Animals

PBCF is expressed in barbule cells in a spatiotemporal manner and is likely to play a role in pennaceous barbule formation. Using the BLAST search against the NCBI database with the PBCF amino acid sequence (XP_0405191) as a query, we identified full-length open reading frames (ORFs) or partial ORF sequences in a total of 63 avian species, distributed across various avian classifications. These sequences were frequently annotated as 'hypothetical proteins' or 'uncharacterized proteins', and all of the full-length proteins are predicted to be secretory, as determined by SignalP-5.0, a computer program used for signal peptide and cleavage site prediction. Figure 5 shows examples of species from different classifications, comparing their amino acid sequences. The percentage identity to chicken PBCF in the regions indicated by the upper line ranged from 57% to 71%, suggesting that PBCF may play a conserved role in barbule formation across avian

361 species.

362 Synteny analysis using the Ensembl database identified potential orthologs of
363 *PBCF* in several reptilian species (Fig. 6A). These genes include "protein TsetseEP-like"
364 in the softshell turtle (*Pelodiscus sinensis*), "chondroitin proteoglycan 3" in the green
365 anole (*Anolis carolinensis*), and "testis-expressed protein 29-like" in the Chinese alligator
366 (*Alligator sinensis*). These genes share a similar structure with *PBCF*, consisting of three
367 coding exons and two introns, with the stop codon TAA located at the first codon of the
368 third exon, as inferred from the mRNA sequences available in the NCBI database (Fig.
369 6B). However, the amino acid sequence identity with chicken PBCF was relatively low:
370 36/112 (32%) for the softshell turtle, 40/176 (23%) for the green anole, and 42/137 (31%)
371 for the Chinese alligator. Among the reptilian species, sequence identity ranged from 28%
372 to 38%. Despite the low identity, these proteins are predicted to be secretory, as
373 determined by SignalP-5.0. These findings suggest that *PBCF* may have originated in the
374 Sauropsida lineage and subsequently evolved its specific function in birds. Further
375 phylogenetic analysis of this gene could provide valuable insights into the evolution of
376 feathers.

377

378 **3.4. Expression of *PBCF* in Embryonic Tissues**

379 The embryonic expression of *PBCF* was investigated using RT-PCR and *in*
380 *situ* hybridization to gain insight into its potential role. As shown in Fig. 7A, *PBCF* was
381 significantly expressed in 2- to 4-day-old embryos. *In situ* hybridization localized this
382 expression specifically to the non-vacuolated cells of the notochord (Fig. 7B). In zebrafish,
383 these non-vacuolated cells, which are rich in rough endoplasmic reticulum, are
384 responsible for secreting extracellular matrix proteins that form the thick peri-notochordal

basement membrane (PBM), a structure essential for the notochord's function as the axial skeleton of the embryo (Yamamoto et al., 2010). The similar localization of PBCF in the notochord suggests it may play a comparable role in the formation of the PBM, which is crucial for maintaining the structural integrity of the notochord during early development.

Furthermore, *PBCF* expression was also detected in the yolk sac membrane and extraembryonic ectoderm of 3-day-old embryos by RT-PCR (Fig. 8A) and *in situ* hybridization (Fig. 8B), respectively. The basement membrane in the yolk sac membrane is produced by the extraembryonic ectoderm, suggesting that PBCF may be involved in the production or maintenance of this membrane. Considering that chondroitin proteoglycan 3 in the green anole is a potential orthologue, it is possible that PBCF may also be a type of chondroitin proteoglycan. Chondroitin proteoglycans are known to bind with other extracellular matrix components, such as collagen and elastin, thereby contributing to the stabilization of tissue structures. Additionally, they play a role in regulating intercellular signaling. PBCF may exhibit similar functions. These findings strongly suggest that PBCF is either secreted or located on the cell surface, functioning as a critical component of the extracellular matrix to provide structural support and facilitate cell-cell communication.

3.5. Alternative Splicing and Possible Post-Translational Modifications of PBCF

Using RT-PCR with RNA from the yolk sac membrane of 3-day-old embryos, we detected partial sequences of both the known transcript corresponding to *PBCF* type 1 mRNA and a novel transcript generated by alternative splicing (referred to as *PBCF* type 2 mRNA), which has a different ORF from *PBCF* type 1 mRNA. These sequences identified here are available in the DDBJ/GenBank databases under accession numbers

LC846667 for *PBCF* type 1 mRNA and LC846668 for *PBCF* type 2 mRNA. The structures of both mRNAs and their predicted proteins are schematically presented in Fig. 9A and 9B, respectively.

Type 1 PBCF and type 2 PBCF are predicted to be proteins of 108 and 136 amino acids, respectively, sharing an N-terminal 107 amino acids, including an N-glycosylation site at position 89 and a 20-amino-acid signal peptide predicted by SignalP-5.0, a computer program used for signal peptide and cleavage site prediction. Both PSORT II, a tool for predicting protein localization, and MULocDeep, a sub-cellular and sub-organellar localization prediction tool, predict that type 1 PBCF is a secreted extracellular protein, whereas type 2 PBCF is predicted to be located in the cell membrane. Furthermore, both PSORT II and NetGPI-1.1, a tool for predicting GPI anchors, predict that type 2 PBCF is GPI-anchored, with NetGPI-1.1 identifying the omega-site at position 107.

Based on these computational analyses, type 1 PBCF is synthesized from *PBCF* type 1 mRNA as an N-glycosylated secreted protein. Type 2 PBCF, on the other hand, is initially synthesized from *PBCF* type 2 mRNA as an N-glycosylated precursor protein with a GPI anchor attachment signal at its C-terminal region. After the propeptide is removed, a GPI anchor is potentially added to the protein, possibly at position 107, anchoring it to the cell membrane. As a result, membrane-bound and soluble forms of PBCF, differing by only one amino acid, are generated through alternative splicing and post-translational modifications.

Contactin 2 (TAG-1/SNAP in rodents, axonin-1/SC2 in chickens) is a cell surface protein in the nervous system, existing as both a GPI-anchored membrane-bound form and a soluble form. It plays crucial roles in neuronal development, including cell

adhesion, axon guidance, and synaptic organization, through homophilic interactions (Chataigner et al., 2024). Like Contactin 2, the dual form may enable PBCF to function both at the cell surface and in the extracellular space, contributing not only to its roles in feather development but also to its functions in the notochord and yolk sac membrane.

However, as noted above, sequences corresponding to *PBCF* type 1 mRNA have been reported in many avian species, whereas *PBCF* type 2 mRNA was identified for the first time in this study and has not been previously reported. This suggests that the production of *PBCF* type 2 mRNA through alternative splicing may not be evolutionarily conserved among birds but instead may be restricted to chickens and closely related species. This raises intriguing questions about the evolutionary diversification of PBCF, indicating that type 2 PBCF might have evolved to serve a species-specific function in chickens and related taxa. Comparative analyses across a broader range of avian species will be critical for understanding this potential evolutionary divergence.

Furthermore, the properties of type 1 and type 2 PBCF described in this study are based on computational predictions. The actual biochemical characteristics and functions of these proteins remain to be experimentally validated. Functional studies, including gene knockout experiments and biochemical assays, will be essential to confirm the predicted post-translational modifications and the roles of PBCF in feather development. Broader taxonomic sampling and comparative analyses will also be necessary to determine whether the mechanisms identified here are generalizable across birds. These future studies will provide valuable insights into avian physiology and development, contributing to our understanding of the evolutionary mechanisms underlying feather diversity and adaptation.

Chang et al. (2019) demonstrated that Wnt2b signaling is critical for the

formation of pennaceous barbules, while its absence leads to the formation of plumulaceous barbules (Chang et al., 2019). However, the downstream targets of Wnt2b remain unknown. In our study, we identified *PBCF* as a potential downstream target of Wnt2b, highlighting its possible role in barbule cell differentiation and identity specification.

3.6. Conclusion

This study addresses a fundamental question in developmental biology: how feather structures are precisely formed through genetic regulation. Here, we identified and characterized a novel gene, *PBCF*, which may play a critical role in feather barbule formation, particularly in the development of pennaceous barbules.

Using a feather regeneration system, where male-type saddle feathers lacking barbules and female-type saddle feathers with barbules are regenerated from the same location on an adult male chicken, we observed that *PBCF* is highly expressed in feathers producing barbules. This expression is associated with the formation of barbules following estrogen-induced regeneration. *In situ* hybridization localized *PBCF* expression to basal barbule cells, suggesting its involvement in the attachment of barbules to the ramus. The timing of PBCF expression, which occurs transiently between melanin pigmentation and keratinization, indicates that it may facilitate the transition from pigmentation to keratinization. Beyond feather morphogenesis, *PBCF* was detected in embryonic tissues, including the notochord and yolk sac membrane, implying a broader role in embryonic development and structural integrity. The discovery of alternative splicing and potential post-translational modifications producing both membrane-bound and secreted forms of PBCF provides further insight into its diverse roles in extracellular

processes.

This study provides new insights into the molecular mechanisms underlying feather development and highlights the potential importance of PBCF in broader biological contexts. Future functional analyses, including gene knockout and biochemical studies, will be essential to confirm the roles of PBCF in feather morphogenesis and embryonic development. Comparative studies across avian species are also needed to explore the evolutionary implications of PBCF's dual forms and their contributions to morphological diversity and adaptation.

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Figure Legends

Fig. 1. Expression of *PBCF* mRNA in saddle feather follicles. (A) Representative photos showing the overall structure of saddle feathers and scanning electron micrographs focusing on the tips of saddle feathers, each presented for males, females, and E2-treated males (labeled as "E2 male"). White and black arrowheads indicate rami and barbules, respectively. Scale bar = 100 μm. (B) Representative electrophoretic patterns of RT-PCR products using primer sets specific for *PBCF*, *BlSK*, and *GAPDH*. Three samples are shown in each case. The amplicon sizes for *PBCF*, *BlSK*, and *GAPDH* were 435 bp, 234 bp, and 533 bp, respectively. Left lanes are the 100-bp ladder used as the molecular marker.

Fig. 2. Expression of *PBCF* mRNA in feather follicles during saddle feather development in adult Tosa-Jidori chickens. (A) Expression of *PBCF* mRNA in males. The upper panels show representative scanning electron micrographs of the tip, vane, and fluff portions of saddle feathers. The lower panels show representative electrophoretic patterns of RT-PCR products of RNA isolated from saddle feather follicles at the development stage of the tip, vane, and fluff. Three samples are shown in each case. The amplicon sizes for *PBCF*, *BlSK*, and *GAPDH* were 435 bp, 234 bp, and 533 bp, respectively. Left lanes are the 100-bp ladder used as the molecular marker. (B) Expression of *PBCF* mRNA in females. The upper panels show representative scanning electron micrographs of the tip, vane, and fluff portions of saddle feathers. The lower panels show electrophoretic patterns of RT-PCR products of RNA isolated from saddle feather follicles during the development of the tip, vane, and fluff. Three samples are shown for each stage. The amplicon sizes for *PBCF*, *BlSK*, and *GAPDH* were 435 bp, 234 bp, and 533 bp, respectively. Left lanes are the 100-bp ladder used as the molecular marker.

Fig. 3. *PBCF* mRNA-expressing cells in the saddle feather follicles of adult male Tosa-Jidori chickens implanted with E2. (A) Light micrographs showing representative *in situ* hybridization images of cross-sections using *PBCF* antisense (left panel) and sense (right panel) probes. White arrowheads indicate positive signals. Scale bar = 50 μm. (B) A schematic diagram of the barb ridge corresponding to the area shown in (A).

Fig. 4. Timing of *PBCF* mRNA expression during barbule development in saddle feather follicles of adult male Tosa-Jidori chickens implanted with E2. (A) Representative electrophoretic patterns of RT-PCR products of RNA isolated from various parts of the

feather follicles. Three samples are shown for each part. The amplicon sizes for *PBCF*, *BlSK1*, and *GAPDH* were 435 bp, 234 bp, and 533 bp, respectively. Left lanes are the 100-bp ladder used as the molecular marker. (B) Light micrographs showing representative examples of *in situ* hybridization in cross-sections using *PBCF* antisense (upper panel) and *BlSK1* antisense (lower panel) probes. Arrowheads indicate positive signals. Scale bar = 50 μm.

Fig. 5. Alignment of the amino acid sequences of avian PBCF. Homologous sequences were retrieved from NCBI database. Conserved amino acid residues among these species are indicated by asterisks. The percentage identities compared to chicken PBCF in the upper lined region are also shown. For the species names and accession numbers, please refer to the Materials and Methods section.

Fig. 6. Possible orthologs of PBCF in non-avian animals. (A) Conserved synteny around *PBCF* in the chromosomes of various species. Open boxes represent genes, including *INHBA* (*Inhibin Subunit Beta A*), *GLI3* (*GLI Family Zinc Finger 3*), *C7orf25* (*Chromosome 7 Open Reading Frame 25*), *PSMA2* (*Proteasome 20S Subunit Alpha 2*), and *MRPL32* (*Mitochondrial Ribosomal Protein L32*). Chicken *PBCF* and possible orthologs in the green anole, Chinese soft-shell turtle, and Chinese alligator are indicated as solid boxes. (B) The exon-intron structures of the possible orthologs of *PBCF*. The structures of mRNAs for genes *LOC420770*, *LOC103279167*, *LOC102459790*, and *LOC102377478* are shown. Exons are represented by boxes, with coding regions by shading. For the species manes and accession numbers, please refer to the Materials and Methods section.

688

689 **Fig. 7.** Expression of *PBCF* mRNA in broiler chicken embryos. (A) Representative
690 electrophoretic patterns of RT-PCR products of RNA isolated from whole embryos at
691 days 2 (E2), 3 (E3), 4 (E4), 6 (E6), and 8 (E8). Three samples are shown for each stage.
692 The amplicon sizes for *PBCF* and *GAPDH* were 435 bp and 533 bp, respectively. Left
693 lanes are the 100-bp ladder used as the molecular marker. (B) Light micrographs showing
694 *in situ* hybridization of cross-sections of 3-day-old embryos using *PBCF* antisense (left)
695 and sense (right) probes. Arrowheads indicate the non-vacuolated cells of the notochord.
696 NC; notochord. Scale bar = 50 μm.

697

698 **Fig. 8.** Expression of *PBCF* mRNA in the yolk sac of broiler chicken embryos. (A)
699 Representative electrophoretic patterns of RT-PCR products of RNA isolated from the
700 yolk sac of 3-day-old embryos (E3). Three samples are shown. The amplicon size for
701 *PBCF* was 435 bp. Left lane is the 100-bp ladder used as the molecular marker. NC
702 indicates the product of a negative control PCR performed without template DNA to
703 confirm the absence of contamination. (B) Light micrographs showing *in situ*
704 hybridization of cross-sections of the yolk sac membrane from 3-day-old embryos using
705 *PBCF* antisense (left) and sense (right) probes. Arrowheads indicate the extra-embryonic
706 ectoderm layer. Scale bar = 50 μm.

707

708 **Fig. 9.** Schematic representation of the chicken *PBCF* gene and its gene products. (A)
709 Gene and mRNAs. The gene structure is depicted with exons represented by boxes and
710 coding regions shaded. *PBCF* type 1 mRNA and PBCF type 2 mRNA are produced by
711 alternative splicing. (B) Predicted proteins. The predicted signal peptide cleavage site (20),

712 N-glycosylation site (89), and GPI-modification site (107) are indicated. Type 1 PBCF is
713 predicted to be a secreted form, whereas Type 2 PBCF is predicted to be a GPI-anchored
714 membrane-bound form. Amino acid positions are numbered.
715
716