Origin of Feathers: Feather Beta (β) Keratins Are Expressed in Discrete Epidermal Cell Populations of Embryonic Scutate Scales

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ABSTRACT   The feathers of birds develop from embryonic epidermal lineages that differentiate during outgrowth of the feather germ. Independent cell populations also form an embryonic epidermis on scutate scales, which consists of peridermal layers, a subperiderm, and an alpha stratum. Using an antiserum (anti-FβK) developed to react specifically with the beta (β) keratins of feathers, we find that the feather-type β keratins are expressed in the subperiderm cells of embryonic scutate scales, as well as the barb ridge lineages of the feather. However, unlike the subperiderm of scales, which is lost at hatching, the cells of barb ridges, in conjunction with adjacent cell populations, give rise to the structural elements of the feather. The observation that an embryonic epidermis, consisting of peridermal and subperidermal layers, also characterizes alligator scales (Thompson, 2001. J Anat 198:265–282) suggests that the epidermal populations of the scales and feathers of avian embryos are homologous with those forming the embryonic epidermis of alligators. While the embryonic epidermal populations of archosaurian scales are discarded at hatching, those of the feather germ differentiate into the periderm, sheath, barb ridges, axial plates, barbules, and marginal plates of the embryonic feather filament. We propose that the development of the embryonic feather filament provides a model for the evolution of the first protofeather. Furthermore, we hypothesize that invagination of the epidermal lineages of the feather filament, namely the barb ridges, initiated the formation of the follicle, which then allowed continuous renewal of the feather epidermal lineages, and the evolution of diverse feather forms. *J. Exp. Zool. (Mol. Dev. Evol.) 295B:12–24, 2003.*   © 2003 Wiley-Liss, Inc.

The discoveries of filamentous skin appendages on dinosaurs (Chen et al., '98; Xu et al., '99a,b; Ji et al., 2001) have stimulated considerable interest in the evolutionary origin of feathers (Feduccia, '99; Brush, 2000; Maderson and Homberger, 2000; Chuong et al., 2000; Xu et al., 2001; Prum, 2002). The most recent discovery of skin appendages on a nonavian dromaeosaur supports the view that feather-like appendages evolved before birds and flight (Norell et al., 2002). Since the feathers on today's birds are considered to be one of the most complex of epidermal appendages (Feduccia, '99; Stettenheim, 2000), understanding their mode of development may provide clues about their origin (Gilbert, 2000; Wagner, 2000; Gilbert and Bolker, 2001). One view of the origin of feathers is that they evolved through modifications of reptilian scales (Maderson, '72; Lucas and Stettenheim, '72; Regal, '75; Sengel, '76; Zhang and Zhou, 2000; Jones et al., 2000), while others hypothesize that feathers are novel structures evolving with the first follicles (Prum, '99; Brush, 2000).

Embryonic development is modular and hierarchical in nature, i.e., it progresses by means of discrete morphogenetic fields, organ rudiments, or cell lineages (Gilbert, 2000; Gilbert and Bolker, 2001). Thus, evolution may occur through changes at several levels in these discrete developmental modules (Raff, '66; Hall, '98; Hogan, '99; Gilbert, 2000; Wagner, 2000; Gilbert and Bolker, 2001). Studies of skin development have demonstrated that the amniote epidermis forms discrete suprabasal populations of cells as it becomes stratified during embryogenesis (Wessells, '61; Montagna and Lobitz, '64; Sawyer, '72; Parakkal and Alexander, '72; Sawyer et al., '74a,b; Sengel, '76, '86; Bereiter-Hahn et al., '86; Sawyer et al., '86). For example, the epidermis of certain reptiles displays distinct epidermal strata, which have evolved the capacity to elaborate unique morphologies, structural proteins, and functions (Maderson, '65, '72; Alexander and Parakkal, '69; Sengel, '76; Sawyer and Fallon, '83; Landmann, '86; Carver and Sawyer, '87; Alibardi and Thompson, 2000; Maderson and Alibardi, 2000; Alibardi and Sawyer, 2002). Using in vitro culture, Flaxman et al. ('68) demonstrated that the epidermis of squamates exhibits autonomy with respect to differentiation of its numerous strata or cell populations. In birds, discrete cell populations characterize the epidermis of embryonic scales (Wessells, '61; Fell, '64; Sawyer, '72a,b; Sawyer et al., '74a,b; Sengel, '76, '86; Sawyer and Goetinck, '88; Knapp et al., '93; Barnes and Sawyer, '95) as well as embryonic and adult feathers (Matulionis, '70; Lucas and Stettenheim, '72; Sengel, '76, '86; Haake et al., '84; Sawyer et al., '86). Sengel ('76) points out that the autonomy demonstrated by the squamate epidermis is reminiscent of that seen for the isolated scutate scale epidermis of the chick embryo (Wessells, '64; Dodson, '67).

The first suprabasal layer formed by the embryonic epidermis of reptiles, birds, and mammals is the periderm referred to as the primary (1°) periderm in cases where a secondary (2°) periderm is formed below the 1° periderm. Using a periderm specific monoclonal antibody, Kitamura et al. ('90) demonstrated that the 1° periderm is an independent cell population common to both feathers and scales of birds, but distinctly different from the 2° periderm and subperiderm of scales and the sheath and barb ridges of feathers. Precursor cells of this peridermal lineage, which are recognized at the limb bud stage, separate from the germinative basal lineage and form the continuous peridermal population. The cells of this epidermal lineage proliferate and differentiate autonomously throughout embryogenesis, until they are lost at hatching (Sawyer et al., '74a,b; Tanaka and Kato, '83a,b; Kitamura et al., '90).

In the chick embryo, the subperidermal cell population forms beneath the peridermal populations and, like the peridermal cells, proliferates and differentiates autonomously (Wessells, '61, '64; Fell, '64; Sawyer et al., '74a,b; Sengel, '76, '86; Tanaka and Kato, '83a,b; Tanaka et al., '87). The subperiderm forms in the complete absence of scutate scale development in the scaleless (sc/sc) mutant chicken (Sawyer and Abbott, '72; Sawyer et al., '74b; Sawyer, '79) where it also expresses β keratins (Shames and Sawyer, '86, '87). Furthermore, the subperidermal population forms in the scutate scale epidermis, which results from the experimental recombination of the chorionic ectoderm with the scutate scale dermal ridge (Kato, '69).

The term alpha stratum (AS) refers to the suprabasal cell population, which lies between the subperiderm and the beta stratum of scutate scales, and expresses alpha (α) keratins, but not β keratins (Sawyer et al., '74a,b; Dhouailly, personal communication). The alpha stratum and all the embryonic layers above it are lost at hatching. Numerous studies have demonstrated that the beta stratum, which makes up the plate-like epidermal structure on the dorsal surface of scutate scales, is induced by the dermis of the definitive scale ridge at 12 days of embryogenesis (see review, Sawyer et al., '86).

Recently, Alibardi and Thompson (2001) identified peridermal and subperidermal populations in the scale epidermis from embryonic alligators. This embryonic epidermis of alligator scales is also lost at hatching. Of interest, granules similar to the peridermal granules seen in the 1° and 2° peridermal cells of scutate scales and the peridermal and sheath cells of the feather are also seen in the embryonic epidermis of the alligator (Alibardi and Thompson, 2001).

Table 1 shows a comparison of the terminology used for the epidermal cell populations in the avian scutate scale (Wessells, '61; Sawyer, '72a,b; Sawyer et al., '74a,b) with that used for the feather (Matulionis, '70; Lucas and Stettenheim, '72; Sengel, '76; Haake et al., '84). Although Sengel ('76) presents a figure summarizing the numerous epidermal populations making up the developing scutate scale epidermis, the alpha stratum was not

TABLE 1. Embryonic epidermal cell lineages

CELL[1] LINEAGES	CHICKEN[2] SCALE	CHICKEN[3] FEATHER	Feather-type β Keratin PRESENT
Primary (1°) Periderm	Primary (1°) Periderm	Periderm	Yes
Secondary (2°) Periderm	Secondary (2°) Periderm	Sheath	No
Subperiderm	Subperiderm	Barb Ridges	Yes
Stratum Intermedium	Alpha Stratum	Axial Plate Marginal Plate	No
Squamous Layer	Beta Stratum		No
Stratum Germinativum	Stratum Basale	Stratum Basale	No

[1]Sengel ('76)
[2]Wessells,'61, Sawyer,'72a,b, Sawyer et al., ('74a,b)
[3]Matulionis ('70), Lucas and Stettenheim ('72), Sengel ('76), Haake et al., ('84)
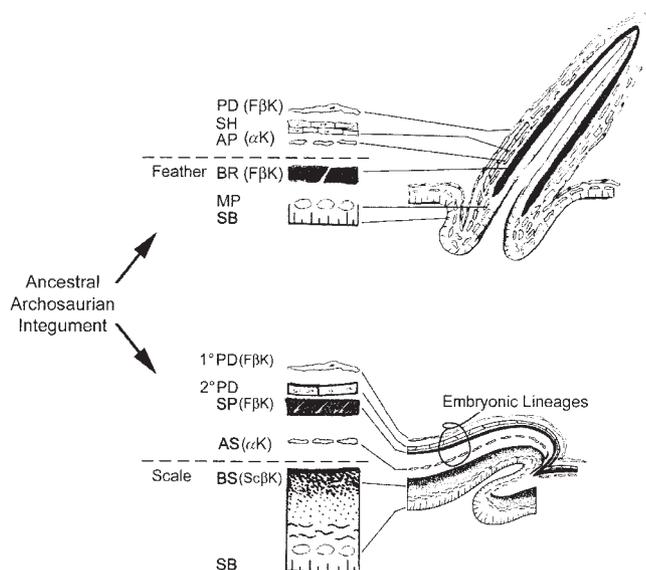
Fig. 1. Schematic representations of an embryonic scutate scale and feather, demonstrating the epidermal lineages of the scale (primary 1° periderm, 1° PD; secondary 2° periderm, 2° PD; subperiderm, SP; and the alpha stratum, AS), and the feather (periderm, PD; sheath, SH; axial plate, AP; barb ridges, BR; and marginal plate, MP). In the scutate scale, the cells of the 1° periderm and subperiderm express the feather-type β keratins (FβK), whereas the cells of the secondary (2°) periderm and alpha stratum (AS) express alpha type keratins (αK). After hatching, the embryonic epidermis of the scale is sloughed off (dotted line) and the beta stratum (BS), expressing scale-type β keratins (ScβK), remains above the stratum basale (SB) and stratum intermedium along the outer surface of the scale. In the feather, the cells of the periderm and barb ridge express the feather-type β keratins (FβK), whereas the cells of the sheath, axial plate, and marginal plate express alpha type keratins (αK). After hatching, only the structural elements resulting from the cells of the barb ridge remain protruding above the surface of the skin.

included at that time. Figure 1 illustrates the arrangement of these epidermal populations during the development of the avian scutate scale and feather.

The molecular mechanisms that underlie the epithelial-mesenchymal interactions, which regulate the patterning and development of integumentary appendages, have been conserved throughout vertebrate evolution (Chuong, '98; Crowe et al., '98; Hogan, '99; Noramly et al., '99; Sharpe, 2001; Fuchs et al., 2001). In reptiles, the great variety of scale types and patterns (Baden and Maderson, '70; Maderson, '72, '85; Parakkal and Alexander, '72; Sengel, '76; '86; Landmann, '86; Bereiter-Hahn et al., '86; Carver, '88; Maderson and Alibardi, 2000) and the patterned expression of the epidermal keratins (Alexander and Parakkal, '69; Baden and Maderson, '70; Sengel, '76, '86; Carver and Sawyer, '87; Sawyer et al., 2000; Alibardi and Sawyer, 2002) by discrete cell populations will undoubtedly be shown to be regulated by the same pattern formation genes and signaling systems as in birds and mammals. In fact, hetero-specific, epidermal-dermal recombinants between reptiles, birds, and mammals demonstrate that the initial signals for forming skin appendages are functional, even between these different amniote classes (Garber et al., '68; Dhouailly, '73, '75; Sengel, '76, '86; Dhouailly et al., '78; Crowe et al.,'98; Dhouailly et al., '98). The signaling pathways regulating cell division, determination, and differentiation appear to be evolutionarily conserved as well (Raff, '96; Chuong, '98; Noramly and Morgan, '98; Crowe

et al., '98; Hogan, '99; Gilbert, 2000; Fuchs et al., 2000).

In birds, numerous studies have shown that the developmental mechanisms responsible for forming the scales and feathers are similar (Dhouailly, '75; Sengel, '76; Dhouailly et al., '78, '98; Zou and Niswander, '96; Kanzler et al., '97; Chuong, '98; Crowe and Niswander, '98). In fact, several experimental manipulations have demonstrated the conversion of presumptive scale epidermis to feathers and presumptive feather epidermis to scales (Rawles, '63; Sengel, '76, '86; Dhouailly et al., '78, '80; Tanaka et al., '83; Song and Sawyer, '96; Zou and Niswander, '96; Kanzler et al., '97; Widelitz et al., 2000). In some experimental tissue recombinants, barb ridge-like structures have been observed forming within the scale epidermis (Fisher and Sawyer, '79) or within the general epidermis without the presence of a feather germ (Brotman, '77a, '77b). In fact, barb ridges form in the arrested feather filaments that develop in experimental epidermal-dermal recombinations between six-day dorsal chick epidermis and 14.5-day dorsal mouse dermis (Dhouailly, '73; Sengel, '76).

As outgrowth of embryonic scales and feathers occurs, discrete epidermal cell populations develop specific morphologies and undergo unique patterns of cytodifferentiation, including the expression of a number of structural proteins, i.e., α keratins, β keratins, and histidine rich proteins (HRP) (Kemp and Rogers, '72; O'Guin and Sawyer, '82; O'Guin et al., '82; O'Guin, '84; Haake et al.,'84; Sawyer et al., '86; Barnes, '93; Barnes and Sawyer, '95; Rogers et al., '98). More than 30 homologous β keratins have been identified in the chicken (Wilton et al., '85; Gregg and Rogers, '86; Presland et al., '89a, '89b; Whitbread et al., '91; Rogers et al., '98). Four distinct subfamilies have been identified, the feather β keratins (FβK), the feather-like β keratins (FlβK), the claw β keratins (ClβK), and the scale β keratins (ScβK). The expression of the histidine rich proteins, believed to be filament-binding proteins, is also closely coordinated with the expression of FβK and FlβK in embryonic feathers (Whitbread et al., '91; Rogers et al., '98). It is believed that all the avian β keratin genes shared a common ancestral gene (Whitbread et al., '91), and that the similarities in the 5′ noncoding and flanking regions of the β keratin subfamilies and the HRP genes are responsible for the ''leaky'' expression of the β keratin subfamilies in the various epidermal appendages (Wilton et al., '85; Presland et al.,

'89a, '89b; Whitbread et al., '91). For example, Presland et al. ('89a) propose that the expression of some ScβK and ClβK by feather epidermis is a remnant of the evolution of feathers from scales.

Antibodies that recognize the entire assemblage of β keratins have been used to localize β keratins in the differentiating epidermal cells of the scales and claws of reptiles and birds, and the beak, feather, lingual nail, and spur of birds (O'Guin and Sawyer, '82; O'Guin et al., '82; O'Guin, '84; Dhouailly and Sawyer, '84; Haake et al., '84; Sawyer et al., '86; Carver and Sawyer, '87; Carver, '88; Shames et al., '89, '91; Barnes, '93; Knapp et al., '93; Mays, '98; Sawyer et al., 2000; Alibardi and Sawyer, 2002). An antiserum that is monospecific for a histidine rich protein (HRP) has been used to demonstrate that HRP is expressed in the barb ridge cells and barbules of the embryonic feather (Barnes and Sawyer, '95). This antiserum demonstrated that HRP expression also occurred in scutate scales, but was restricted to the embryonic epidermis, mainly the cells of the subperiderm. Using molecular probes specific for the scale-type β keratins, Shames and Sawyer ('86, '87) showed that the mRNA for a scale-type β keratin is also expressed by the cells of the subperiderm of scutate scales. Furthermore, data indicate that the feather-type β keratins are expressed in developing scutate scales (Presland et al., '89; Barnes, '93; Sawyer et al., 2000), but their specific tissue location has remained unclear.

In order to specifically localize the expression of the feather-type β keratins in the epidermal cell populations of developing scutate scales, we generated an antiserum against an amino acid sequence conserved in the β keratins, which characterize feathers. This antiserum, anti-FβK, was then used to localize feather-type β keratins in the epidermal populations of embryonic scutate scales and feathers using confocal microscopy. We find that the feather-type β keratins are not only expressed in the epidermal populations of the feather (periderm and barb ridges), but they are also expressed in discrete epidermal strata (periderm and subperiderm) of the embryonic epidermis of developing scutate scales. We discuss this observation in light of the recent discovery of an embryonic epidermis in the scales of alligators (Alibardi and Thompson, 2001) and the expression of feather-type β keratins in alligator embryos (Mays, '98). Furthermore, we present a hypothesis for the origin of the protofeather and the origin of the feather follicle.

METHODS

DNA sequences of feather β keratins

DNA sequences for the Turkey Vulture and Wood Stork (Genbank accessions, AF308826 and AF308827) were obtained using the following methods. Genomic DNA was isolated from the blood of adult birds housed at the Riverbanks Zoological Park, Columbia, SC. Primers for PCR amplification were designed from the 5′ cap (F26, CGCCCTCATCCACKTCTCTT) and 3′ untranslated regions (R972, CTCAACTTGCTTCAGGA-TYAA) of feather β keratin genes, using an alignment (available from the authors) of Genbank accessions: J00847, M37698, X17509, X17510, and X17511. PCR was carried out in 50 μL volumes with final reaction concentrations of: 50 mM KCl, 10 mM Tris-HCl at pH 9, 1% Triton X-100, 1.5 mM MgCl$_2$, 150 μM of each dNTP, 250 μg/mL BSA (Fraction V, Sigma, St. Louis, MO), 0.5 μM of each primer, 1 unit *Taq* DNA polymerase (Promega, Madison, WI), and 50 ng of DNA. Hot Start PCR using Hot Beads (Lumitek, Salt Lake City, UT) and high annealing temperature (50–60°C depending upon species) were employed for all amplifications. PCR products were ligated into pGEM-T vector (Promega) and transformed into XL-10 Gold *E. coli* (Stratagene, La Jolla, CA). Inserts were amplified from bacteria by colony PCR using M13 forward and reverse primers. Sequences were determined directly from colony PCR products using BigDYE terminator chemistry and an ABI 377XL automated sequencer (Applied Biosystems, Foster City, CA) or DYEnamic direct cycle sequencing chemistry (Amersham Life Science, Cleveland, OH) and a LiCor automated sequencer (LiCor, Lincoln, NE). DNA sequences were edited and translated into amino acid sequences using Sequencher 3.1 (Genecodes, Ann Arbor, MI). Amino acid sequences were aligned by visual inspection. Table 2 shows the alignment of amino acid sequences for the feather β keratins and the sequence of 23 amino acids selected for peptide synthesis.

Synthesis of the peptide antigen

The 23-mer, VGSTTSAAVGSILSEEGVPINSG-CONH$_2$ was synthesized on an Applied Biosystems *Pioneer* automated peptide synthesizer. Fmoc-L-amino acids were purchased from *SynPep* (Dublin, CA). Dimethylformamide (DMF), diisopropylethylamine, 1,8-diazabicyclo [5.4.0] undec-7-ene (DBU), and piperidine were purchased from Fisher/ACROS. The Fmoc-PAL-PEG resin and 7-azabenzotrazol-yloxytris phosphonium hexafluorophosphate (PyAOP) were obtained from Applied Biosystems. High performance liquid

TABLE 2. Alignment of amino acid sequences for β keratins was determined directly or inferred from DNA sequences

	Amino Acid Position							
		20	30	40	50	60	70	80
Turkey Vulture	ANSCNEPCVRQCQDSRVVIEPSPVVVTLPGPILSSFPQNTAVGSTTSAAVGSILEEGVPINSG							
Wood Stork	. . . . I . . . . A . . . . . S . .							
Pigeon	. . . . Q . . . . S . . . . C							
Chicken FBK A	. . . . Q . . . . S . . . . S . .							
Chicken FBK B	. . . . Q . . . . S . . . . Q . . . . . SC .							
Chicken FBK D	. . . . . . A . . . . Q . . . . L . . . S . . . . S . .							
Chicken Feather-like	. . . . T . . . . Q . . . . T . . . SA . . . . . . A . . AG . . . . S . .							
Chicken Claw	. D . . . . P . . T . . . . QPAT . . . F . . . . YA . . . . AGVP . . . . .GMGGTFGRGAGF							
Chicken Scale	. D . G . . . . P . . TT . . . Q P . . . . . F . . . . .DSV . . . SG APIF . GSSLGY . GSSLGY							

[1]Sequences correspond (from top to bottom) to accessions: AF308826, AF308827, KRPYF4, X17511, X17510, X17509, X17521, M37698, and X00315 of GenBank or GenPept.
The feather β keratin antiserum (anti-FβK) was generated using a synthetic polypeptide whose sequence was identical to the underlined amino acids of the Turkey Vulture sequence.

chromatography (HPLC) was performed on a Shimadzu 10A instrument, equipped with an SPD-10A UV-Vis detector. Experimental details follow: Peptide synthesis was performed using the *Pioneer's* standard Fmoc protocol on 0.10-mmol scale. Fmoc-PAL-PEG resin functionalized at 0.18 mmol/g was used as the solid support. For each coupling step, 4.0 molar eq. of amino acid were activated with PyAOP and diisopropyl ethylamine. Fmoc de-protection was achieved with a solution of DBU (2%) and piperidine (2%) in DMF. UV-feedback monitoring on the *Pioneer* was used to verify that each synthetic step had gone to completion. *N*-terminal Fmoc de-protection was performed following the last coupling reaction. Cleavage of the peptide from the resin and concomitant side chain de-protection was achieved with 5.0 mL of water/TFA (9:1). The crude peptide was precipitated with ether and centrifuged to furnish a pellet. This material was dissolved in acetic acid and lyophilized. Final purification was obtained by reverse phase HPLC with an Alltech *Econosphere* C-18 column (4.6 × 250 mm). An acetonitrile/water gradient (20–35% over 15 min) with a flow rate of 1.20 mL/min was used to elute the pure peptide (0.15% TFA was used in both solvents). UV monitoring at 220 nm indicated that the major chromatography peak eluted at ca. 13 min. This peak was collected, and its electron impact ionization mass spectrum confirmed a mass peak at 2,131 amu, corresponding to the desired peptide. Note that this peptide is a C-terminal amide.

Preparation of the antiserum against the feather β keratin

The FβK antiserum (anti-FβK) was produced in a male New Zealand White rabbit. The synthetic peptide antigen (VGSTTSAAVGSILSEEGVPIN-SG) was cross-linked to Keyhole Limpet Hemocyanin (KLH) using glutaraldehyde (Zola, '87). Both the primary and secondary injections of dialyzed Freund's emulsified antigen contained 200 mg of the synthetic peptide cross-linked with KLH. Serum was collected 14 days after the second injection. Initial screening of the serum against extracts of feather keratins demonstrated specificity for the feather β keratin. The pre-immune serum gave no reactivity.

The anti-FβK antiserum was diluted 1:5,000 in phosphate buffered saline for immune blotting. A 1:100 dilution of anti-FβK was used in conjunction with fluorescein-tagged anti-rabbit antibodies for confocal microscopy (Shames et al., '89, '91; Knapp et al., '93).

RESULTS

Because previous studies suggested that feather type β keratins were expressed in developing scutate scales (Barnes, '93; Sawyer et al., 2000), we generated an antiserum highly specific for the feather-type β keratins to determine the tissue location of the feather-type β keratins in the embryonic epidermal cell lineages of scutate scales. Using a synthetic polypeptide whose amino acid sequence was conserved in the feather-type β
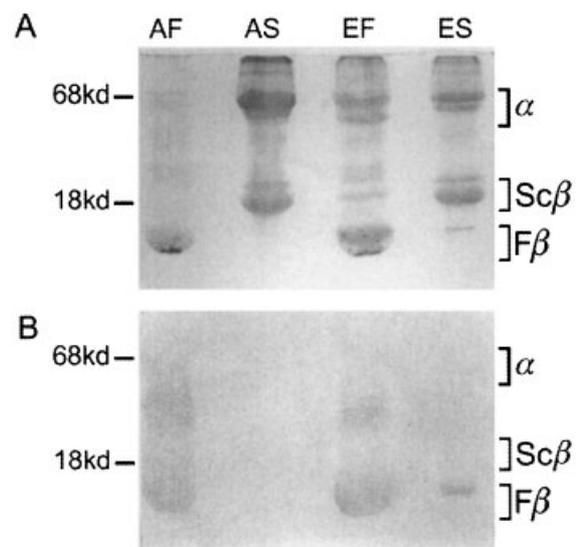
Fig. 2. Reaction of FβK antiserum with epidermal β keratins of adult feather (AF), adult scutate scale (AS), embryonic feather (EF), and embryonic scutate scale (ES). (**A**) The epidermal keratins extracted with Triton × 100/1.5 M KCl were separated by electrophoresis on a 10% poly-acrylamide gel. They were stained with Coomassie Brilliant Blue. The alpha (α) keratins migrate as bands in the range of 40–70 kilodaltons (kd), while the scale-type β keratins (Scβ) migrate to the range of 17–20 kd, and the feather-type β keratins (Fβ) migrate to the range of 10–14 kd (Shames et al., '89, '91). (**B**) The keratins separated in (A) were transferred to a nitrocellulose membrane and incubated overnight with the FβK antiserum. The FβK antiserum was localized with goat anti-rabbit horseradish peroxidase (Shames et al., '89, '91). The feather-type β keratin (Fβ) is present in the adult (AF) and embryonic feathers (EF) as evidenced by the broad band in the molecular weight range of the feather β keratins (Fβ). The bands at higher molecular weight are the result of aggregation known to occur with β keratins (Shames et al., '89, '91). The feather-type β keratin (Fβ) is absent from the adult scale (AS), but is present in the embryonic scale (ES). Notice that the FβK antiserum does not react with the α keratins (α), nor the scale-type β keratins (Scβ) present in both the adult and embryonic scale.

keratins of different birds (French, 2001; Table 2), we generated an antiserum (anti-FβK) in a rabbit (Knapp et al., '93). While the sequence of the synthetic polypeptide is 74–100% similar to the FlβK and FβK, it differs from the same region of ClβK and ScβK by 70% and 80%, respectively (Table 2).

Figure 2 shows that anti-FβK reacts with the feather-type β keratins extracted from adult and embryonic feather, but not with scale-type β keratins (ScβK) extracted from adult scale epidermis. In extracts of embryonic scales, the anti-FβK does not react with the ScβK, but does react with a polypeptide band in the molecular weight range of feather-type β keratins. The ScβK present in the adult and embryonic scale extracts reacts with antisera that recognize the family of beta keratins (Carver, '87; Shames et al., '89, '91; Knapp et al., '93; Mays, '98; Sawyer et al., 2000; Alibardi and Sawyer, 2002).

To localize the feather β keratins in developing feathers and scales, we used the anti-FβK antiserum in conjunction with confocal microscopy of tissue sections (Fig. 3). The fluorescence shows that feather-type β keratin is present in the medullary and cortical cells of the barb ridge and the barbule cells. The peridermal cells also contain feather-type β keratin. It is not found in the cells of the sheath or the stratum basale. These data are consistent with light and electron microscopic studies of early feather development (Matulionis, '70; Lucas and Stettenheim, '72; Sengel, '76; Haake et al., '84).

In early scale formation, the epidermis also consists of a peridermal cell population above the stratum basale (Wessells, '61; Sawyer, '72a,b; Sengel, '76; Tanaka and Kato, '83; Sawyer et al., '86; Sawyer and Goetinck, '88; Sawyer, '90). As scale development progresses, a stratum intermedium forms between this primary (1°) periderm and stratum basale. Again, the stratum intermedium and basale of the developing scale give rise to discrete cell populations, known as the embryonic layers or strata. The three embryonic lineages, designated secondary (2°) periderm, subperiderm, and alpha stratum, along with the primary (1°) periderm, are lost at hatching and do not contribute to the scale of the newly hatched chick. We have now determined that the feather-type β keratins are expressed exclusively in the cells of the 1° periderm and subperiderm of the embryonic scutate scale epidermis (Fig. 3C).

The stratification, proliferation, and cytodifferentiation described for these transient cell popula-
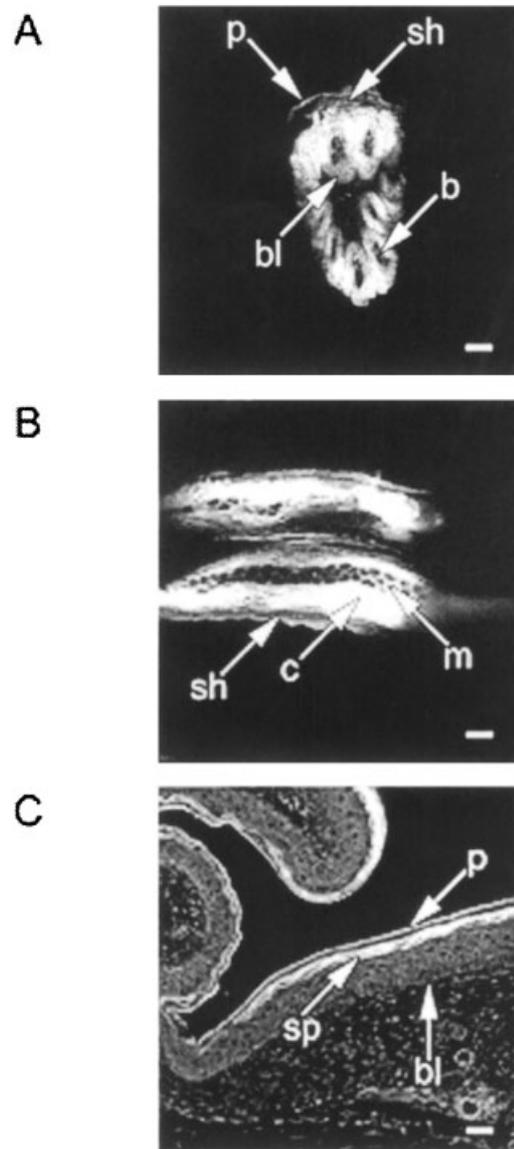
Fig. 3. Confocal microscope image showing localization of feather-type β keratin using FITC tagged anti-rabbit IgG antiserum localizing the anti-FβK in (**A**) a cross section and (**B**) longitudinal section of a 17-day embryonic feather, and (**C**) in a longitudinal section of a 17-day embryonic scutate scale. Abbreviations: b=barb ridge, sh=sheath cells, c=cortical barb ridge cells, m=medullary barb ridge cells, p = 1° periderm, sp=subperiderm, bl=stratum basale region.

tions (Sawyer, '72a,b; Sawyer et al., '74a,b; Sawyer et al., '86; Sawyer and Goetinck, '88; Sawyer, '90) of scutate scales are remarkably similar to those described for the epidermal cell lineages of the feather epidermis (Matulionis, '70; Lucas and Stettenheim, '72; Sengel, '76; Haake et al., '84). The cytodifferentiation of 1° peridermal cells of feathers and scutate scales is the same. They

display peridermal granules, surface microvilli, and a few cytoplasmic filaments. They both express a peridermal specific marker (Kitamura et al., '90), as well as feather-type β keratins. The cells of the 2° peridermal layer of the scale between the 1° peridermal and subperidermal populations do not contain the feather-type β keratins, yet they do possess the peridermal granules, and the large particulate areas that form as the peridermal granules disappear (Matulionis, '70; Sawyer et al., '74a,b). The cytodifferentiation of the 2° peridermal cells appears to be the same as that of the sheath cells of the feather filament.

The subperidermal cells of the developing scale react strongly with the FβK antiserum. These cells become very large and fill with interweaving bundles of 3 nm β keratin filaments. Expansive cell-cell junctional complexes form between the subperidermal cells, resulting in the subperidermal layer becoming a continuous structural element consisting of feather-type β keratins, scale-type β keratins (Shames and Sawyer, '86, '87), and HRPs (Barnes and Sawyer, '95). This cytodifferentiation of the subperidermal cells is very similar to that seen for the barb ridge and barbule cells of the developing feather, which also express feather-type β keratins, scale-type β keratins (Presland et al., '89a), and HRPs (Barnes and Sawyer, '95), and form the continuous structural elements of the feather. Interestingly, a population of cells, which expresses only α keratins and displays peripherally located bundles of alpha keratin filaments (Sawyer et al., '86; Dhouailly, personal communication), forms below the subperiderm. The epidermal cells of this layer, the alpha stratum, differentiate in a manner very similar to the cells of the axial plate of the feather filament (Matulionis, '70; Lucas and Stettenheim, '72). While the axial plate and marginal plate cells function to separate the barbs and barbules of the forming feather filament, the cells of the alpha stratum separate the beta stratum of the scale from the sloughing embryonic strata above. These cell populations are lost around the time of hatching.

DISCUSSION

Did feathers, with their unique structures and specific expression of β keratins, evolve from reptilian scales, or did they evolve as novel structures originating with the first follicle and expressing novel feather keratins? Our studies demonstrate that the feather-type β keratins are not unique to the barbs and barbules of feathers. They are expressed in the peridermal cells of both scutate scales and feathers, and more importantly in the subperiderm of embryonic scutate scales. These observations support our view that the peridermal and subperidermal population of scutate scales are homologous with the cells that give rise to the peridermal and barb ridge lineages of feathers, respectively. Furthermore, comparison of the cytodifferentiation of the 2° periderm of scutate scales and the sheath cells of the feather support their homology, and similar comparison of the cytodifferentiation of the cells of the alpha stratum with that for the axial and marginal plate cells support their homology as well.

We hypothesize that the early formation of the feather germ (day 6.5–7.5), which precedes formation of the definitive scale ridge (day 12) by several days (Sawyer, '72a,b; Sawyer and Abbott, '72; Sengel, '76), allowed epithelial-mesenchymal interactions and their signaling systems to influence the differentiation of the primitive epidermis of the feather germ, thereby establishing the periderm, sheath, barb ridges, and marginal plates of the feather filament. Once established, the invagination of these epidermal lineages stimulated the formation of the follicular structure, including the dermis, which then regulated the renewal of feathers and the generation of their diverse shapes. Clearly, stem cells are present in the follicular epidermis, which are capable of renewing the epidermal lineages of the feather.

At the level of skin appendage, the signaling systems for pattern formation appear to be conserved in the vertebrates (Sharpe, 2001). Formation of epidermal cell populations, capable of undergoing cytodifferentiation independent of each other, appears to be a plesiomorphic feature of the amniote epidermis (Flaxman et al., '68; Sengel, '76). Furthermore, the expression of β keratins appears to be a plesiomorphic feature of amniotes (Alibardi and Sawyer, 2002), while the expression of feather-type β keratins appears to be a plesiomorphic feature of archosaurians (Barnes, '93; Mays, '98; Sawyer et al., 2000). A sequence of 20 amino acids has been isolated from the alligator claw that is homologous with a conserved amino acid sequence in the coding region of the avian scale, claw, and feather-type β keratin subfamilies (Sawyer et al., 2000; French, 2001), and immunological data indicate the presence of the feather-type β keratins in the

epidermis of the alligator (Barnes, '93; Mays, '98; Sawyer et al., 2000). Although immunological data demonstrate that keratins homologous with the β keratin gene family are expressed in chelonians, lepidosaurians, as well as archosaurians (Alibardi and Sawyer, 2002), no gene sequence information is available for these keratins at the present time. The known sequence data on lizard claw keratin indicate that amino acid domains exist that show homology to the β and α keratin families (Inglis et al., '87).

In scutate scales, all the embryonic epidermal cell populations, which are also represented in the developing feather filament, are formed during scale development (Fig. 1). They are lost at hatching so no presumptive feather-like lineages remain in the scale epidermis; only the ScβK containing beta stratum of the mature scale is retained. Of interest, initiation of appendage morphogenesis, by presumptive feather forming dermis, in the presumptive scale epidermis of the chicken foot (prior to 12 days of embryogenesis), results in the development of normal feathers (Rawles, '63; Sengel, '76). In this case, the cell populations, which form in the presumptive scutate scale epidermis, are able to differentiate into the epidermal lineages characteristic of the feather filament. This feather forming potential of the presumptive scutate scale epidermis normally ends as the germinative basal cells respond to new inductive signals from the scutate scale dermis after 12 days of incubation (Rawles, '63; Sawyer and Abbott, '72; Sengel, '76; Dhouailly et al., '78; Sawyer, '79). After 12 days of embryogenesis, the basal cells of the outer scale surface generate the scutate scale beta stratum, while those of the inner scale surface generate an alpha stratum. In the hatched bird, these strata do not differ from a typical stratum corneum, where cornified epidermal cells are continually sloughed from the surface (O'Guin, '83; Sawyer et al., '86). Numerous tissue-recombination studies have demonstrated that the dermis of the embryonic scutate scale acquires its ability to induce the beta stratum of scutate scales around 12 days of incubation (Rawles, '63; Sawyer and Abbott, '72; Sengel, '76, '86; Brotman, '77a,b; Fisher and Sawyer, '79; Dhouailly et al., '79; McAleese and Sawyer, '81, '82).

The similarities in the epidermal cell populations that make up the feather and the embryonic layers of the developing scutate scale (Fig. 1), as well as the embryonic epidermis of the alligator (Alibardi and Thompson, 2001), suggest that the epidermal appendages of crocodilians and birds evolved through the developmental modification of epidermal cell populations, which characterized the integument of ancestral archosaurians. Most likely, these epidermal populations had already evolved the ability to express different members of the α and/or β keratin gene families. Developmental changes in the spatial and temporal expression of genes regulating pattern formation and signaling systems would have created the integumentary structures seen in living archosaurians.

Developing feathers of modern birds first produce an embryonic feather filament with periderm, sheath, barb ridges (with barbules and axial plate cells), and marginal plate cells, prior to making a follicle. The cell lineages at the distal end of the feather filament actually undergo cytodifferentiation and express FβK and HRP prior to the formation of the follicle. Just before invagination of the feather epidermis to begin formation of the feather follicle, epidermal proliferation shifts to the more basal and proximal regions of the feather filament (Lucas and Stettenheim, '72). With follicle formation, germinative activity functions to increase and renew the epidermal populations of the feather, i.e., the sheath, barb ridge, and marginal plate lineages. A similar shift of epidermal proliferative activity to more basal and proximal regions occurs in the late stages of scutate scale development (Sawyer, '72b, '90), yet in this case, epidermal proliferation does not renew the embryonic populations of the scale, but replaces the sloughing cells of the beta stratum. Since cytodifferentiation of the cell populations of the embryonic feather is regulated by the expression of conserved signaling systems before a follicle is established (Zou and Niswander, '96; Crowe and Niswander, '98; Crowe et al., '98; Noramly et al., '99; Chuong et al., 2000; Widelitz et al., 2000; Scaal et al., 2002), perhaps the signaling systems involved in the development and cytodifferentiation of the cell populations of the embryonic feather filament also play a role in the formation of the follicle.

In summary, our comparison of the cytodifferentiation of the embryonic populations of scutate scales and the cytodifferentiation of the epidermal populations of the embryonic feather filament strongly support the view that these populations are homologous. We also suggest that these epidermal lineages are homologous with those of the embryonic epidermis of the alligator (Alibardi and Thompson, 2001) and had their origin in an

ancestral archosaurian. We hypothesize that temporal and spatial changes in the expression of genes regulating development led to the formation of the first protofeathers in the archosaurian integument, which may have consisted of elongated barb ridge-like structures still enclosed in a tapering feather sheath (see Haake et al., '84). At some point, dehiscence of the sheath would have resulted in protofeathers consisting of tufts of barb ridge-like structures composed of feather-type β keratins. As the barb ridges evolved the ability to form barbules, the tufts of barb ridge-like structures would appear as tufts with branching filaments. This sequence of events is very reminiscent of the development of modern feathers prior to the formation of the feather follicle (Matulionis, '70; Lucas and Stettenheim, '72; Sengel, '76, '86; Haake et al., '84). To achieve the structures of modern feathers, especially those of flight feathers, evolution of the follicle was necessary (Lucas and Stettenheim, '72). Because the barb ridges and the other lineages associated with the feather filament invaginate to form the feather follicle, we propose that the conserved signaling systems involved in generating the original epidermal lineages of the feather (such as the barb ridges) were also involved in building the follicle. Once formed, variation in the organization and morphogenesis of the generative cells in the follicle could have resulted in the evolution of the numerous feather forms seen in modern birds.

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