
Avian Integument Provides Multiple Possibilities to Analyse Different Phases of Skin Appendage Morphogenesis

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To analyse the morphogenic events during skin appendage formation, it is important to have an animal model that offers distinct patterns at various stages of development and is accessible to analysis using state of the art technology. The avian integument is such a model. Combining experimental embryologic approaches, organ cultures, and gene transduction technology, we are now able to begin to address the molecular basis of pattern formation, primordium initiation, anterior-posterior axis forma-

tion, proximo-distal axis formation, phenotypic determination, and others. Parallel mechanisms are usually found in feathers and hairs, and the avian integument model has matured to be a major source of new findings in the study of skin appendage morphogenesis. More information on the avian integument model can be found at website <http://www-hsc.usc.edu/cmchuong>. **Key words:** alopecia/Blaschko line/feather/hair. *Journal of Investigative Dermatology Symposium Proceedings* 4:333-337, 1999

THE FEATHER MODEL

In order to manage disease conditions involving skin and skin appendages, we need to understand the molecular and cellular events underlying skin appendage morphogenesis (Chuong, 1998; Oro and Scott, 1998). The avian integument has been used as a major model in developmental biology (Sengel, 1976). Although the avian integument includes feathers, scales, claws, beaks, combs, etc., for the purpose of this review we will focus on the feather. We will first describe the development of the feather, and then compare it with mammalian hair. Although there are differences, both share many common features as skin appendages (**Table I**).

Both feathers and hairs are complex structures that originate from a flat piece of skin. Their formation starts with the interaction between the epidermis and the mesenchyme. The epithelium has to be competent but the signals to initiate an individual primordium from a specific location come from the mesenchyme. The result is the formation of an epithelial placode and a dermal condensation (**Fig 1**). The dermal condensation is at first radially symmetric. Its anterior-posterior (A-P) asymmetry is endowed by the epithelium and with proliferation, the skin appendage elongates along the proximo-distal (P-D) axis. The early stages of feather development, up to the long bud stage, is growth above the skin surface. Later on, the epithelium invaginates into the dermis to form the follicle. The hair, however, initiates its primordium beneath the skin surface. At the follicle stage, both feather and hair are composed of layers of epithelial cells ensheathing a cluster of dermal cells called the dermal papilla. They continue to lengthen when new postmitotic cells are added at the proximal end. Depending on the number of available cells, the rate these cells enter cell

cycles, and the length of the anagen period, their length can be regulated (Wang *et al*, 1999). Both feather and hair can regenerate, called molting in feathers, under the influence of seasons and hormones (Lucas and Stettenheim, 1972).

Feathers serve many purposes for the avian species; soft down feathers keep them warm, colorful feathers allow them to communicate, and strong wing feathers enable them to soar in the sky. These different types of feather are constructed by different types of keratins (Rogers, 1985). There are also many different types of hair found in nature, but they are less diverse structurally.

Feathers are also distinct for their branching structure, which involves up to three levels: the rachis, the major branch; the barbs, the secondary branches; and the barbules, the tertiary branches. The branches are formed when the epithelial sheet, with the basal layer being the innermost layer, invaginates periodically to segregate regions that will either keratinize or apoptose. The death of cells in the marginal plate epithelia creates spaces between barb plate epithelia. A similar fractal like process takes place to form the barbules. In hairs, there is no branch formation. Most conspicuously, feathers have elaborate color patterns. Besides the biochrome pigments that give them the spectrum of chemical colors, they also have structural colors that render iridescences (Gill, 1995). Thus the feather is also a superb model to study how mother nature dresses up in colorful magnificence.

Feather formation involves many fundamental cellular processes that are widely studied by basic scientists (Chuong, 1993; Chuong and Wideltz, 1998). We, the feather biologists, wish to understand how the molecular and cellular events are utilized to construct fascinating structures. But besides catering to the wonders of the curious minds, our research presents relevance to the practical world. Recent studies employing molecular biology techniques have shown that the findings from the feather parallel those from human disease research. One example of such a link is that whereas retrovirus mediated overexpression of SHH causes extra large feather buds (Ting-Berreth and Chuong, 1996b; Morgan *et al*, 1998), the activation of the SHH pathway is involved in Gorlin syndrome and human basal cell carcinoma (Bale *et al*, 1998; Johnson *et al*, 1996; Oro *et al*, 1997; Xie *et al*, 1998).

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Abbreviations: A-P, anterior-posterior; P-D, proximo-distal; SHH, sonic hedgehog.

Table I. Comparison of feather and hair

Events	Feather	Hair
Induction based on epithelia-mesenchymal interactions	Yes. The thickened epithelium is called feather placode	Yes. The thickened epithelium is called hair germs
Appendage primordia	Feather buds protruding out of skin surface	Hair pegs invaginate into dermis
A-P asymmetry in primordia	Yes	Yes
Follicle	Yes. Proliferating epithelium is called feather collar	Yes. Proliferating epithelium is called hair matrix
Within the filament	Pulp filled with mesenchyme tissues transiently	No pulp. Cortex and medulla made of epithelial cells
Branch formation of the filament	Yes, go through differential cell death to form barbs and barbules	No such process
Cycling of appendages	Yes. Called molting	Yes
Color of appendages	Complex pigment patterns, also have iridescences	Solid color based in density and size of melanin granules
Regulation by sex hormones	Yes	Yes, but much less obvious
Regeneration induced by dermal papilla	Yes. Induced by dermal papilla	Yes. Induced by dermal papilla

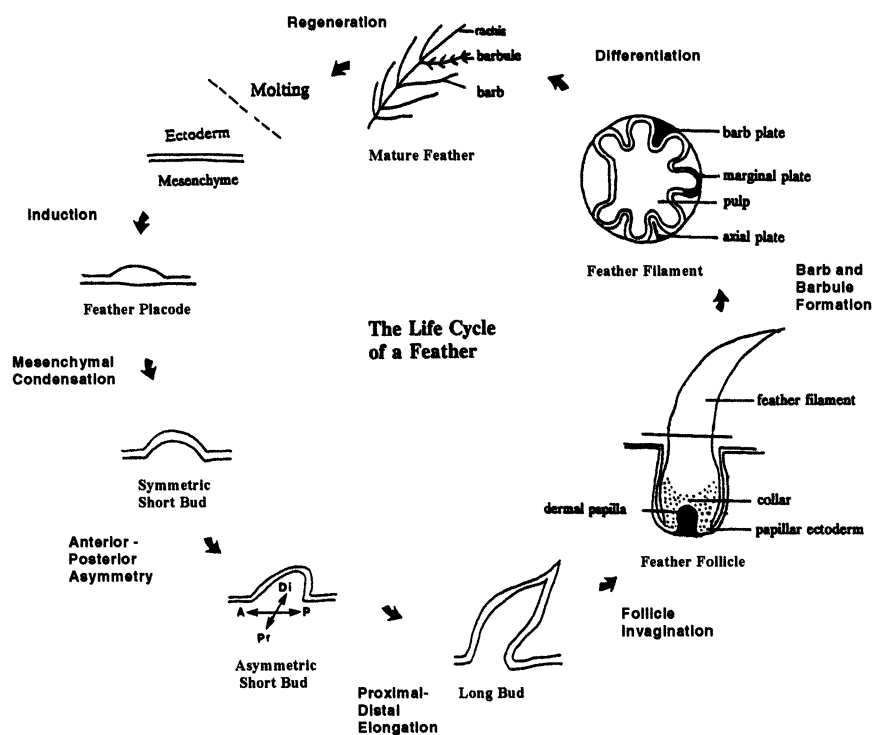


Figure 1. Diagram of the life cycle of a feather. See text for description. Revised from Fig 1 in Chuong (1993).

CELLULAR AND MOLECULAR APPROACHES

Experimental embryology The earliest efforts to learn how feathers or other skin appendages are formed involved manipulating the embryonic tissues with classical experimental procedures, namely transplantation and recombination (Dhouailly, 1973; Sengel, 1976). There are many kinds of skin appendages on different species of organisms (e.g., mouse hair *versus* chick feather) and on different regions of the same organism (e.g., chick dorsal feather *versus* chick midventral apterium *versus* chick foot scales). The early transplantation experiments sought to discover what determines the regional specificity of the various types of skin appendages. Blocks of tissue were dissected from early embryos and transplanted elsewhere in the body to find that the determination factors of the diverse characteristics originate from the paraxial mesoderm very early in embryogenesis (reviewed in Sengel, 1990; Dhouailly *et al.*, 1998).

To discover the roles of epidermis and dermis, skins from different sources were separated into epidermis and dermis and then cross-cultured or recombined. Heterospecific recombinations of lizard dorsal epidermis and chick dorsal dermis resulted in growth

of scale primordia arranged hexagonally (the feather pattern) (Dhouailly, 1975). This result demonstrates that the size and distribution pattern of the primordia are determined by the dermis, the dermal signal "to make an appendage" is recognizable among different classes of vertebrates. The type of skin appendages is usually dictated by the dermis. In heterotopic recombinations between chick midventral apteric epidermis and chick dorsal dermis resulted in growth of feathers. In heterospecific recombination between lizard epidermis and chicken dorsal dermis, the lizard epidermis only knows how to make lizard skin appendages, so upon receiving the same signal, scales are formed.

The epidermis has to have competence to respond. When recombination experiments are done between scaleless epidermis and normal dorsal dermis, no feathers are formed, but the vice versa recombinations of normal dorsal epidermis and scaleless dermis produce feathers. Scaleless chick mutants have no scales and very few feathers. These results not only direct the scaleless gene activity to the epidermis (Brotman, 1977), but also illustrate the importance of epidermis competence. When these similar experiments are performed in mature skin, the results are more complex, depending on the competence and determined state of germinative epidermal

cells (Jahoda and Reynolds, 1993). From these results, we hypothesize that epithelial precursor cells go through stages of differentiation, and they become less flexible and more determined as they progress (Chuong, 1998). To understand skin appendage morphogenesis comprehensively, we must keep in mind that it is a chronologic event, thus studying at different temporal points and integrating those results are important. And although these classical experiments have provided us with much information, more molecular studies are needed. These experiments have shown that there is cross-talk between the two tissues; however, they did not tell us what the molecules are. In recent years, many development-related signaling molecules have been identified and it becomes possible to study the roles of these molecules in skin appendage morphogenesis (Chuong *et al*, 1996). In the following, we summarize some of these approaches.

Searching for the molecular basis To elucidate which molecular signals are involved, one can either assay for the possible role of a known molecule or search for new candidates. Because other more genetically accessible systems have already discovered many interesting genes and of the fact that many genes or genetic pathways have repeatedly been shown to be conserved across species (Noveen *et al*, 1998), we often select genes of interest from these other systems. Many of the genes we studied are homologs to *Drosophila* genes that play important developmental roles. Other sources of interesting molecules are discovered from mouse mutants with skin abnormalities and human genetic diseases involving the skin.

Besides this “borrowed genetics” strategy, it is possible to use nonbiased differential expression screening to search for candidate genes. Under this category, it is possible to use a subtraction library or differential display-like techniques to fish out molecules whose expression is changed before and after a morphogenetic process. With the human genome project approaching its end and new molecular technology being developed, we can expect many more molecules to be studied.

Once we have candidate genes, we need to have chicken clones, either through our own efforts or as generous gifts from other laboratories. The chick homologs are often cloned through reverse transcriptase-polymerase chain reaction and cDNA library screening. With a chick probe, we first analyse their expression at various stages of feather development. We often employ *in situ* hybridization to analyse expression at the RNA level. This can be done on the intact embryo or tissue (whole mount) or on histologic sections (Fig 2A, Nieto *et al*, 1996; Jiang *et al*, 1998). The advantage of whole mount *versus* section *in situ* is that one can obtain a three-dimensional overview of gene expression at different stages because there is a feather growth gradient on the embryo. The *in situ* results tell us not only whether a gene is expressed, but also when in development and where in the tissue it is expressed. With this information, we can then start making hypotheses and performing functional studies. For molecules that are better characterized and against which we have antibodies, immunohistochemical staining can be carried out to detect expression at the protein level. Often a comparison of the expression of two genes is helpful in providing clues to the relationship of their roles. This can be achieved through double staining, such as double *in situ* (Nieto *et al*, 1996; Jiang *et al*, 1998).

Perturbation of molecular function After we discover that a certain gene is expressed in an interesting manner, we wish to know its function. The chick skin is useful for *in vitro* functional studies because there are a variety of culture techniques that allow us to address different issues in skin appendage morphogenesis. A typical chicken skin tissue culture uses chicken dorsal skin from stage 30 embryos. At the beginning, it is a homogeneous piece of skin. After 4 d, many feather buds will have formed and are regularly arranged in a hexagonal pattern (Jiang and Chuong, 1992). This culturing system can be modified so as to reset the developmental process to earlier stages. One modification is called

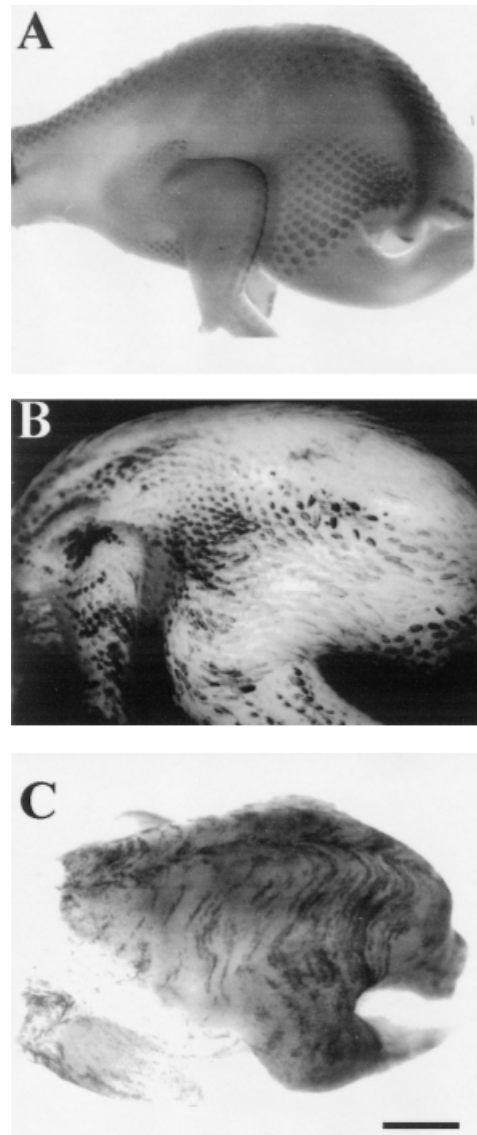


Figure 2. Examples of molecular localization as *in vivo* expression pattern or following experimentation. (A) Non-radioactive whole mount *in situ* hybridization of *Msx-1* to show the overall feather pattern. Alkaline phosphatase is used to develop the color (Chen *et al*, 1997). Lateral view of embryonic day 8 chicken embryo. (B) Demonstration of exogenous gene transduction in embryonic chicken skin using retrovirus. Chicken embryos were injected with RCAS-Alkaline phosphatase virus (Morgan and Fekete, 1996) at day 4 and incubated for seven more days before fixation. The dark patches represent areas with virus infection and expressing alkaline phosphatase gene. (C) “Chicken Blaschko lines.” Chicken embryos were injected with replication defective spleen necrosis virus carrying beta galactosidase between embryonic days 1 and 2 (Chuong *et al*, 1998). The parallel lines represent progenies of the same lineages. Human Blaschko lines have been implicated in congenital and acquired skin disorders (reviewed in Happle, 1985). Scale bar: 3 mm.

recombination, in which the epithelium and the mesenchyme are separated and recombined with rotation. Doing so, we learned that the location of feather buds is determined by the mesenchyme, whereas the orientation is determined by the epithelium. Preexisting epithelial placodes are unstable and will disappear following separation. The mesenchyme will then induce new feather buds from the epithelium (Chuong *et al*, 1996). Another method is called reconstitution. In this method, after the separation of mesenchyme from epithelium, mesenchymal cells are disassociated into single cells and then plated at high density. Then a piece of epithelium is placed above. In this case, the

mesenchyme is set back to the stage when the cells are more equivalent and forced to reorganize, but it retains the ability to form evenly spaced feather buds (Jiang *et al*, 1999).

These three ways of culture allow us to study feather bud morphogenesis at different developmental stages. They also enable us to easily add exogenous agents and study their effects on the developmental process. There are also different ways to administer exogenous factors. To add a factor globally, one simply dilutes the factor into the culturing media. For example, we have added antibodies against adhesion molecules and extracellular matrix molecules to culturing media to perturb cell surface interaction and study the effect of adhesion in various stages of feather development (Jiang and Chuong, 1992; Chuong *et al*, 1994). To test the effect more locally, one can deliver the reagent via a coated bead so a gradient of the tested molecule is formed from the source. This is ideal because in many cases cells sense the difference in relative concentration more effectively than the presence of the molecule in the media. Depending on the nature of the molecule, different types of beads are employed. For example, growth factors such as FGF and TGF β are coated on Affigel blue beads (Widelitz *et al*, 1996; Ting-Berreth and Chuong, 1996a), whereas a pharmacologic reagent such as cAMP is coated on AGI-X8 anion exchange beads (Noveen *et al*, 1995).

To perturb molecular function at the genetic level, it is possible to use virus mediated gene therapy-like technology to cause misexpression. One can infect cultures *in vitro* or embryos *in vivo*. For *in vitro* experiments, one can culture the skin explants in the presence of virus. For *in vivo* infection, one can inject virus into the amniotic cavity or into the early limb buds, which will later develop into wings or legs covered with feathers or scales (**Fig 2B**). The infected tissues or embryos are incubated for a length of time before assaying for changes in the size, number, and spacing of feather primordia, orientation of feather buds, and phenotypic transformations such as the conversion of feathers into scales. The timing of the injection should be noted for consideration when one analyses the result because the same virus administered at different developmental stages can produce different phenotypes (Morgan *et al*, 1998). When sonic hedgehog is overexpressed prior to stage 22 (Hamburger and Hamilton, 1951), there are disorganized ectodermal growths and an overall inhibition of feather buds; at stages 22 and 23, large feather buds formed; and after stage 23, there is no apparent defect. These differences suggest the dynamic nature of feather morphogenesis and remind us of the importance of timing and competence in developmental studies.

Genes do not work in isolation. In most cellular processes, many genes and molecular pathways are involved. Thus to determine the role of a pathway, evidence from both overactivation and inhibition is helpful. To activate a pathway, overexpression of a gene or its constitutive active form is used. For inhibition, one can overexpress a dominant negative mutant form or an antagonist. Sometimes antisense is also used. Thus far, most of the viral transduction experiments used an avian Rous Sarcoma virus derived vector called RCAS (Replication-Competent Avian Sarcoma Virus) (Morgan and Fekete, 1996). Another virus of choice is the adenovirus (Leber *et al*, 1996). We have more experience using RCAS; however, preliminary experiments have shown that adenovirus can also infect chicken skin cells without perturbing feather development (Wang *et al*, unpublished data).

The downstream effects of activating a molecular pathway are cellular processes. We have observations and evidence that processes such as cell proliferation, adhesion, migration, differentiation, and apoptosis occur in feather morphogenesis. To study cell proliferation, we have used BrdU labeling (Chen *et al*, 1997). For cell migration, we can use Dil labeling or defective virus carrying reporter genes to track cells (Chuong *et al*, 1996). Cells at different stages and locations can be injected and traced for their distribution at different stages of development. An example is using spleen necrosis virus carrying LacZ (**Fig 2C**; Mikawa *et al*, 1991). It is fascinating to observe that the labeled avian epidermal cell

distribution resembles those of human Blaschko lines (Chuong *et al*, 1998). Many skin diseases have been shown to present along this linear distribution (reviewed in Happle, 1985). To study differentiation, we can examine expression of feather-specific keratin markers (Rogers, 1985). And for examining apoptosis, we can do Tunnel assays and various apoptosis molecular markers.

Several examples studying signaling molecules and adhesion molecules in early stages of feather morphogenesis are summarized in Widelitz and Chuong (1999).

Strengths and weaknesses of the avian integument model As has been stated above, the avian skin has many strengths as a research model, particularly the accessibility of experimental embryologic manipulations. Another advantage is the presence of a wide spectrum of apparently different sizes and shapes of feathers in one single bird. Whereas human beings also have these body regional differences and mouse hairs have various types (Sundberg, 1994), they are not easy research models to study region-specific appendage size/shape or sexual dimorphism.

This model also has its weaknesses, however. One of them being that data on genetics and integument of chickens are not as complete as those on mice (Sundberg *et al*, 1998) and humans. Whilst data on the human genome project and mouse are rapidly accumulating, the chicken genome project has started but is lagging behind (Burt *et al*, 1995). On the other hand, there are mutations affecting chicken integument appendages such as *scaleless* and *wingless*. Another potential source of data that has not been exploited is the large collections of chicken or bird variants and breeds raised by poultry fanciers (Smyth, 1990; Simes, 1990). They show a wide spectrum of appendage shapes and pigment patterns ranging from the bizarre to the beautiful, but many of them are controlled by single genes! They also show how molecular processes can be pushed to the extreme to create morphology that can only be imagined in Nature. This is a treasure box yet to be opened.

The ability to produce transgenic and knock-out mice is still the most powerful aspect of the mouse model. Although attempts were made (Ono *et al*, 1994), the technology to produce transgenic birds is not yet here. For now, it is possible to take a "pseudo-genetic approach" using viral vectors such as those used in gene therapy. The introduction of active or dominant negative genes to early chicken epithelial precursor cells has contributed to new understanding of skin development; however, the application and interpretation are still limited by the incomplete transduction and we hope future work from the research community will make transgenic chicken available.

Another practical issue is that the background information on avian skin cell biology requires more work. For example, the identification of the equivalent of bulge stem cells (Cotsarelis *et al*, 1990) in feather follicles, or the expression mapping of keratin markers (Rogers, 1985), needs to be further pursued. This should be possible once more investigators use the avian integument model. More information on the avian integument model can be found at website <http://www-hsc.usc.edu/~cmchuong>. More information on the chicken genome can be found at the US Poultry Gene Mapping website <http://www.poultry.mph.msu.edu>, and the Roslin Institute ChickMap website <http://www.ri.bbsrc.ac.uk/chickmap>.

CONCLUSION

Skin appendage formation is a complex morphogenic phenomenon. Although many biomedical researches are carried out in the hope of enhancing human health and curing diseases, many unexpected clues and novel findings have come from basic developmental biology studies. Among the many examples, avian embryology is one of the most powerful models. It is also advantageous to compare and contrast the role of signaling molecules in different model systems so that we can appreciate the conservation and variation of developmental pathways. The study of avian integument, in conjunction with transgenic mouse

studies (Sundberg *et al*, 1998) and human diseases such as ectodermal dysplasia (Slavkin *et al*, 1998), will bring our knowledge of skin appendage morphogenesis to a new level in the next decade.

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