

## The Making of a Feather: Homeoproteins, Retinoids and Adhesion Molecules

Cheng-Ming Chuong

### Summary

We have been using feather development as a model for understanding the molecular basis of pattern formation and to explore the roles of homeoproteins, retinoids and adhesion molecules in this process. Two kinds of homeobox (Hox) protein gradients in the skin have been identified: a 'microgradient' within a single feather bud and a 'macrogradient' across the feather tract. The asynchronous alignment of different Hox macrogradients establishes a unique repertoire of Hox expression patterns in skin appendages within the integument, designated here as the 'Hox codes of skin appendages'. It is hypothesized that these Hox codes contribute to the phenotypic determination of skin appendages. High doses of retinoic acid cause a morphological transformation between feather and scale, while low doses of retinoic acid cause an alteration of the axial orientation of skin appendages. We have tested the ability of molecules directly involved in the feather formation process to mediate the action of the Hox codes, and surmise that adhesion molecules are potential candidates. Using specific Fabs to suppress the activity of adhesion molecules, we have found that L-CAM is involved in the formation of the hexagonal pattern, N-CAM is involved in mediating dermal condensations, tenascin is involved in feather bud growth and elongation, and integrin  $\beta$ -1 is essential for epithelial-mesenchymal interactions. More work is in progress to fully understand the molecular pathways regulating the feather formation process.

### Introduction

Induction and morphogenesis involve complex processes that lead to ordered arrangements of cell groups and differentially expressed genes at specific sites. Cellular processes in morphogenesis have been identified, including cell proliferation, cell migration, cell recognition, cell differentiation and cell death. Regulated coordination of these processes leads to the formation of organs with specific phenotypes and orientations. The molecular basis of these processes and their regulation are just beginning to be understood.

To analyze the molecular mechanisms of morphogenesis, it is essential to have a model system in which most, if not all, of the above cellular processes take place in a manner that can be tested. We have chosen feather morphogenesis as

such a system for the following reasons. (1) Feathers are branched structures containing three levels of complexity and are positioned in hexagonal patterns on a two-dimensional plane<sup>(1)</sup>. The geometric patterns help to amplify subtle changes which are not easily observed in other models. (2) Because feathers develop in a growth gradient over time, starting along the midline of the back and spreading distally in the spinal tract, a series of developmental stages can be studied in one histological section. (3) Large numbers of feather buds (approximately 20,000 are present on one bird<sup>(2)</sup>) facilitate biochemical analyses. (4) *In vitro* culture systems<sup>(3)</sup> are available in which a flat explant can form many feather buds. (5) The accessibility of chicken embryos *in ovo* makes transgene expression and transplantation/recombination experiments possible. (6) The presence of non-lethal skin appendage mutants and special strains<sup>(4)</sup> permit some genetic studies, although sophisticated approaches such as those in mouse genetics still await further development in avian genetics.

The mechanism of feather pattern formation was first explored extensively two to three decades ago. The major experimental approaches involved epithelial-mesenchymal recombination and transplantation of embryonic skins. The results showed that skin appendages derive from complex interactions between the epithelium and the mesenchyme, that the major phenotype-determining forces appear to be in the mesenchyme, and that these signals are so fundamental to development that they can cross-talk between tissues from different species. Readers who are interested in these classical studies will find them well summarized in a book by Senegal<sup>(5)</sup>. The number of phenomenological studies, however, subsequently decreased since there was no means of analyzing the molecular basis of the changes seen.

In the past decade, many new categories of molecules including adhesion molecules, homeobox genes (Hox), oncogenes, etc, have been identified. Many of these molecules have been shown to play pivotal roles in embryonic development, including skin development. For example, cellular oncogenes such as *c-myc*, *c-myb* and *c-ets-1* are expressed during the growth phase of skin appendages<sup>(6,7)</sup>. Growth factors such as BMP-4, a member of the TGF- $\beta$  superfamily, are present in early whisker placodes<sup>(8)</sup>, and PDGF is found in the dermal condensations and feather collar epithelia<sup>(9)</sup>. Some of these findings are summarized in Table 1, which includes the relevant references. These advances suggest that the time is ripe for us to re-examine skin appendage morphogenesis with these molecules in mind. Among these molecules, my laboratory has focused on the roles of homeoproteins and adhesion molecules, and in this review I will summarize our progress in recent years. I have used the title 'The Making of a Feather', with deliberate reference to the book 'The Making of a Fly' in which Lawrence<sup>(30)</sup> elegantly summarizes our new understanding in the pattern formation of *Drosophila*. The subtitle 'Homeoproteins, Retinoids and Adhesion Molecules' refers to the specific molecular subjects of this review and should not be taken to imply that these molecules are enough to make a feather. The title does point to the direction which we hope the field will take and also to the hope that our understanding

of the molecular cascade involved in the making of a feather will someday be equivalent to our current understanding of the making of a fly.

### The Life Cycle of a Feather

The process of feather formation in the chicken embryo is briefly summarized in Fig. 1A. However, for a detailed description, the reader should refer to Lucas and Stettenheim<sup>(1)</sup> and Sengel<sup>(5)</sup>. Feathers start as a flat sheet of ectoderm. The underlying mesoderm acquires inductive properties and interacts with the ectoderm, initiating the differentiation of the epithelial feather placode. The placode epithelium undergoes rapid cell proliferation, while the mesenchymal cells beneath it increase by cell recruitment, as well as by cell proliferation. Thus the growing feather bud has both ectodermal and mesodermal components. The anterior of the feather bud is designated as the side of the bud that forms an obtuse angle to the body surface, and the feather axis is defined as the anterior-posterior axis of the feather bud (A ↔ P in Fig. 1A). The whole bud invaginates into the skin to form the feather follicle, with the mesodermal core forming the dermal papilla and the feather pulp and the bud epithelium becoming the collar and the feather filament. The collar is the region where new epithelial cells are added to the growing feather. Therefore, in the feather filament, the tip is more mature than the base. The epithelium sheet invaginates and fuses, forming the alternating marginal plates and barb plates. The fused marginal plate cells are later committed to cell death; their elimination subsequently generates a distinct space between barb plates. Most of the barb-plate epithelium becomes keratinized and forms the feather barbs themselves, becoming the primary branched structure. Another cycle of cell death recapitulates a similar process with axial plates forming within the barb plates. Later, axial plate cells also die, generating spaces within barbs, and thus creating the secondary branched structures (barbules). The mature

adult feathers then go through several molting cycles in which feather filaments are detached. New induction occurs between the dermal papilla and neighboring epithelium (papillar ectoderm) to regenerate a new feather. Thus the life cycle of a feather is completed and renewed. It should also be mentioned that feathers on the body surface are distributed in specific regions called 'tracts'. Feathers within a tract develop independently from feathers in other tracts and with different time tables<sup>(31)</sup>.

The culture model described in this review is illustrated in Fig. 1B and C. Stage-33 chicken embryonic dorsal skin is dissected and placed on an organ culture dish. The skin explant is cultured for 4–6 days in DMEM/2% fetal calf serum. During this period, dermal condensations form elongated feather buds<sup>(10)</sup>. This culture also demonstrates the macropattern and micropattern of feathers<sup>(5)</sup>. The term 'macropattern' refers to the position of feather germs within a feather tract, and in the specimen (spinal tract) shown in Fig. 1C, the feather germs are hexagonally arranged. 'Micropattern' refers to the appearance of a single feather including phenotype (eg. down feather versus flight feather) and orientation (Fig. 1C, note all feather buds in this figure point towards 3 o'clock).

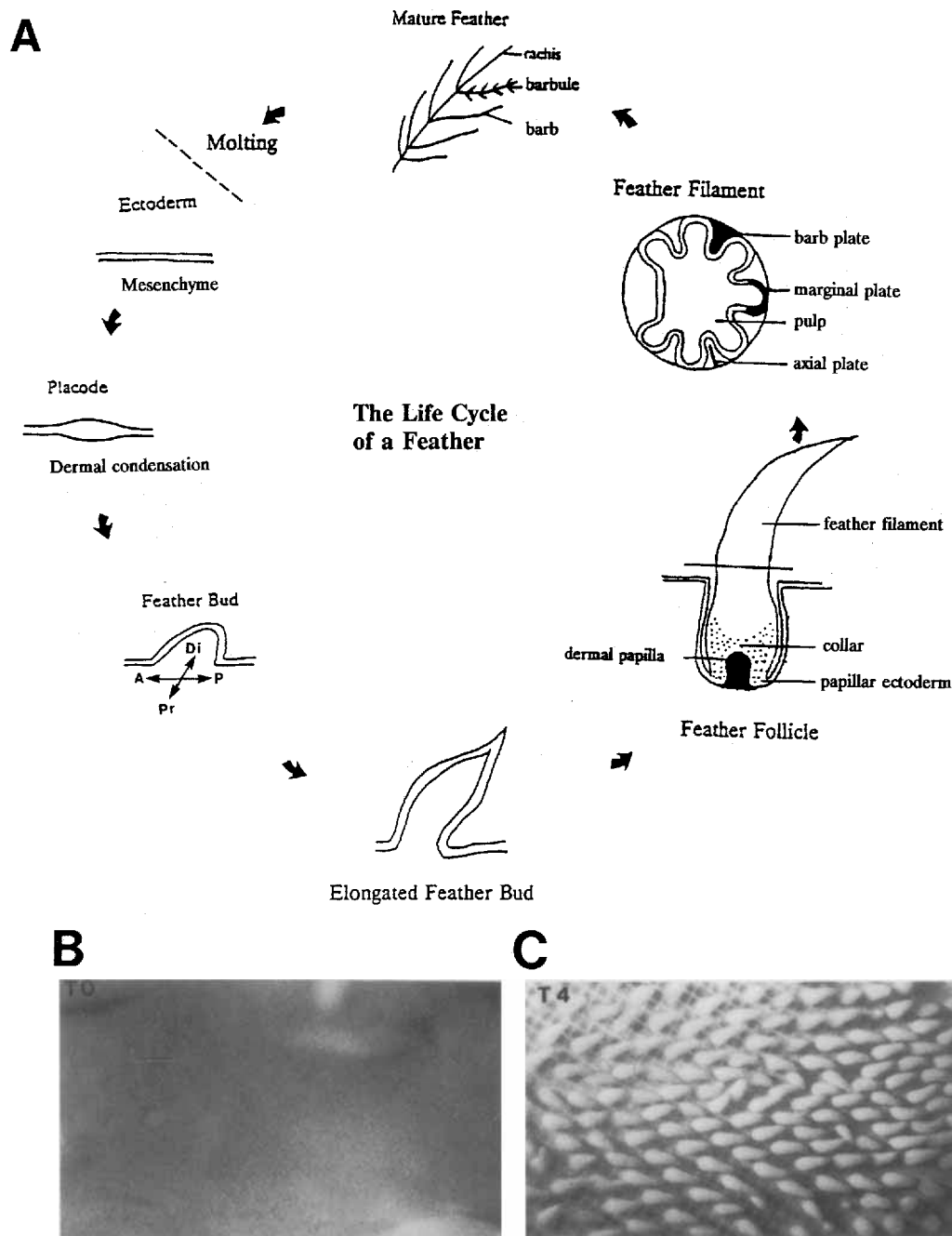
### Position-specific Expression Patterns of Homeoproteins Are Found in Different Skin Appendages

Despite the common features in feather development, feathers show great morphological diversity even on the same bird. The morphological pattern of feathers differs from region to region. One of the key mysteries to be solved is how these regional differences in patterning are set up in the first place. What is the molecular nature of the 'epigenetic factors' residing in feather mesenchyme that determines feather phenotypes?

A group of homeobox (Hox) genes have recently been identified in both invertebrates and vertebrates. They were

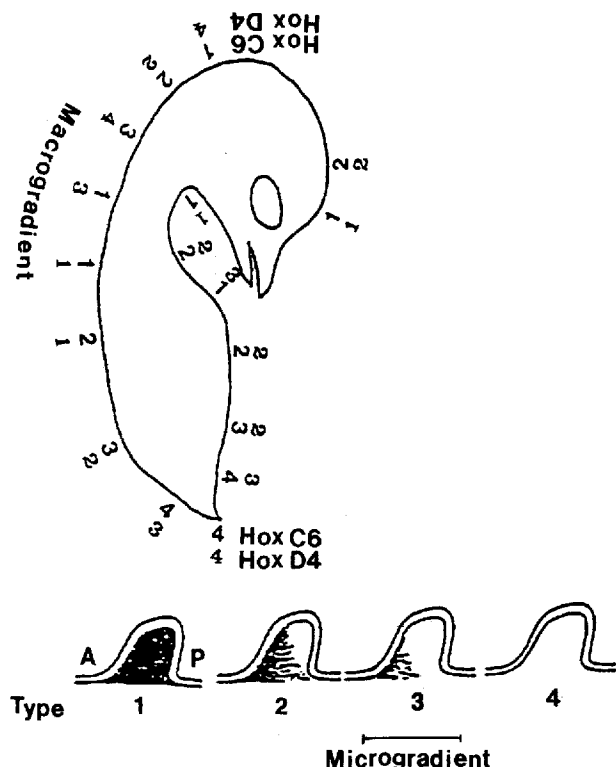
**Table 1.** Summary of molecules known to be involved in the morphogenesis of skin appendages (mainly feather, some from hair)

Morphogenetic processes	Molecules involved	References
Initiation	Growth factors, CAMs	BMP 4 <sup>(8)</sup> , tenascin <sup>(10)</sup>
Axial specification and Phenotypic determination	Hox genes, Retinoids	XIHbox 1, Hox 4.2 <sup>(11)</sup> , Hox 3.1 <sup>(12)</sup> RA <sup>(13,14)</sup>
Mesenchymal condensation and Dermal papilla	Matrix molecules CAMs	Collagen, fibronectin <sup>(15)</sup> ; proteoglycan <sup>(16,17)</sup> ; basement membrane <sup>(18)</sup> N-CAM, L-CAM <sup>(19,20)</sup> ; cadherin, epimorphin <sup>(21)</sup> ; integrin, tenascin <sup>(10,23)</sup>
Cell proliferation	Oncogenes, Growth factors	myc <sup>(6)</sup> , myb, ets-1 <sup>(7)</sup> EGF, FGF <sup>(24,25)</sup> ; BMP 2 <sup>(26)</sup> ; TGF-β (our unpublished data)
Cyto-differentiation	Keratins	Different keratins <sup>(27,28,29)</sup>
Cell death	Adhesion molecules	N-CAM <sup>(20)</sup>



**Fig. 1.** (A) Illustration of the life cycle of a feather. Read counter-clockwise starting from Ectoderm. After induction occurs between ectoderm and mesenchyme, the two components become feather placode and dermal condensation respectively. They grow together to form feather buds. Feather buds has an anterior-posterior (A-P) polarity and this A-P axis is referred as the 'feather axis'. Elongated feather buds then invaginate into the skin to form feather follicle in which the mesodermal components become the dermal papilla while the epidermal components form the papillar ectoderm (reservoir for future feathers), the collar (major sites of cell proliferation) and the feather filament (feather proper). Cross section of the feather filament at the indicated level showed that the originally smooth epithelium goes through invagination and cell death (cell in marginal and axial plates), and finally the branched structure of feather (by cells derived from barb plates) forms<sup>(1)</sup>. When the feather is worn out, it detaches at the level between collar and papillar ectoderm. A new feather cycle begins when the regenerating papillar ectoderm interacts with dermal papilla and neighboring mesoderm components<sup>(58)</sup>. This is called feather molting. A, anterior; Di, distal; P, posterior; Pr, proximal. A ↔ P defines the A-P axis of feather bud. Not to scale. (B,C) Feather explant culture model. B, Dorsal skin from Hamburger and Hamilton<sup>(59)</sup> stage-33 chicken embryo was dissected and placed on an organ culture filter. The explant was cultured in the presence of DMEM/2% fetal calf serum and 5% CO<sub>2</sub>. C, Elongated feather buds formed after four days in culture. Note that the feather germs are arranged in a hexagonal pattern and each feather bud points towards the posterior (3 o'clock direction in all figures of this paper).

originally discovered in fruit flies with homeotic mutations, in which the phenotypes of one body part are transformed into the other<sup>(32)</sup>. Cloning of several genes responsible for homeotic mutations has resulted in the finding that the products of these genes are transcription factors sharing the so called homeobox motif; these genes have been termed homeobox (Hox) genes<sup>(33)</sup>. Vertebrate Hox genes were later identified by DNA homology and were shown to have position-specific expression patterns along the body axis<sup>(34)</sup> as well as the limb axis<sup>(35)</sup>. The specific combination of Hox expression patterns in a specific region, termed the 'Hox code', is considered to determine the identity of that specific



**Fig. 2.** Position-specific homeoprotein expression pattern in skin appendages: a preliminary mapping of Hox code of skin appendages. Between full expression (Type 1) and non-expression (Type 4), there are different degrees of homeoprotein gradient within the feather bud which we defined as microgradient, some passing the midline of the feather bud (Type 2), and some not passing the midline of the bud (Type 3). These four types are schematically shown at the bottom of the figure. These types are arbitrary and in reality, there appears to be a continuous spectrum of gradients. At the feather tract level (Top), there are macro homeoprotein gradients which are reflected by the gradual shift from Type 1 to Type 4. The numbers along the surface of the chicken embryo refer to the types shown at the bottom of the figure. The numbers nearest the embryo surface refer to Hox C6 (or XIHBox 1, Hox 3.3, see ref. 39 for correspondence of nomenclature), and the second sequence of numbers refers to those patterns for Hox D4 (Hox 4.2). Note that the out-of-phase alignment of macrogradient for Hox C6 and D4 (shown here), and potentially other Hoxes (not shown) make a large repertoire of Hox codes possible. A, anterior; P, posterior of the feather bud. Orientation of number also indicates orientation of the microgradient.

region<sup>(36,37)</sup>. An alteration of the Hox expression pattern causes an alteration of identity in vertebra<sup>(36)</sup>, hindbrain rhombomeres and their derivatives<sup>(37)</sup>, or limb digits<sup>(38)</sup>.

With the remarkable roles of Hox genes in pattern formation demonstrated in other systems, we hypothesized that Hox genes were also involved in the phenotypic determination of skin appendages. We began to test this hypothesis by examining the homeoprotein distribution in developing feather buds. Using antibody to Hox C6 (raised against XIH-Box 1 fusion protein which is most similar to Hox 3.3, or Hox C6 in the new nomenclature<sup>(39)</sup>), we indeed observed an anterior-posterior gradient of homeoproteins in the feather buds<sup>(11)</sup>. I call this a 'microgradient' to parallel the 'micropattern' defined by Sengel<sup>(5)</sup>. Because of the different feather tracts, the axis of a feather bud is not always in parallel with that of the body axis. When there is a discrepancy, the axis of the microgradient is always in agreement with the feather axis, not the body axis. When early feather germs are transplanted or rotated, the homeoprotein gradient axis is still in accord with the final feather bud axis (our unpublished data). Thus we hypothesize that the microgradient of homeoproteins in a skin appendage is involved in establishing the anterior-posterior axis of that skin appendage. The repetitive presence of this Hox microgradient in feather buds (thousands of times, using the average figure of 20,000 feathers per bird) suggests that homeoproteins may be fundamentally involved in setting up the axes of cell fields, including the body axis, limb axes or skin appendage axes.

Interestingly, further examination showed different degrees of homeoprotein gradients in different feather buds and that these patterns are body position-specific (Fig. 2 and ref. 40). Tentatively, we defined four types of homeoprotein expression patterns, with Type 1 representing the highest amount of homeoproteins (see legend to Fig. 2). Examination of the distribution of Hox C6 antigen showed a gradual transition from Type 1 to Type 4 along the feather bud tract, reflecting a gradual decrease in Hox C6 expression levels. I will call this alteration along the feather tract 'macrogradient', again in parallel with Sengel's macropattern nomenclature. When antibody to Hox D4 (previously Hox 4.2) was used, we observed a similar microgradient with four expression types but, to our surprise, the macrogradient was out of phase with that of the Hox C6 macrogradient (Fig. 2). It can be seen that the asynchronous macrogradient expression pattern of different Hox genes can easily lead to a large repertoire of possible combinations. Currently, in my laboratory, we are carrying out a comprehensive mapping of the different Hox genes in different feather buds. The overall trend is towards a staggered, overlapping macrogradient of homeoproteins. This leads to a specific combination of Hox expression patterns for each feather bud, which we term the 'Hox code of skin appendages'.

The Hox code of skin appendages is more elaborate than the Hox code along the body axis. In the body axis, Hox expression has its anterior and posterior boundary along the entire body. In feather buds, the microgradient ranges from the anterior to the posterior bud repetitively. Thus, in terms of the levels of Hox gradient, an individual feather field is equivalent to a body field. The asynchronous macrogradient leads to unique Hox codes for skin appendages. Just to get an

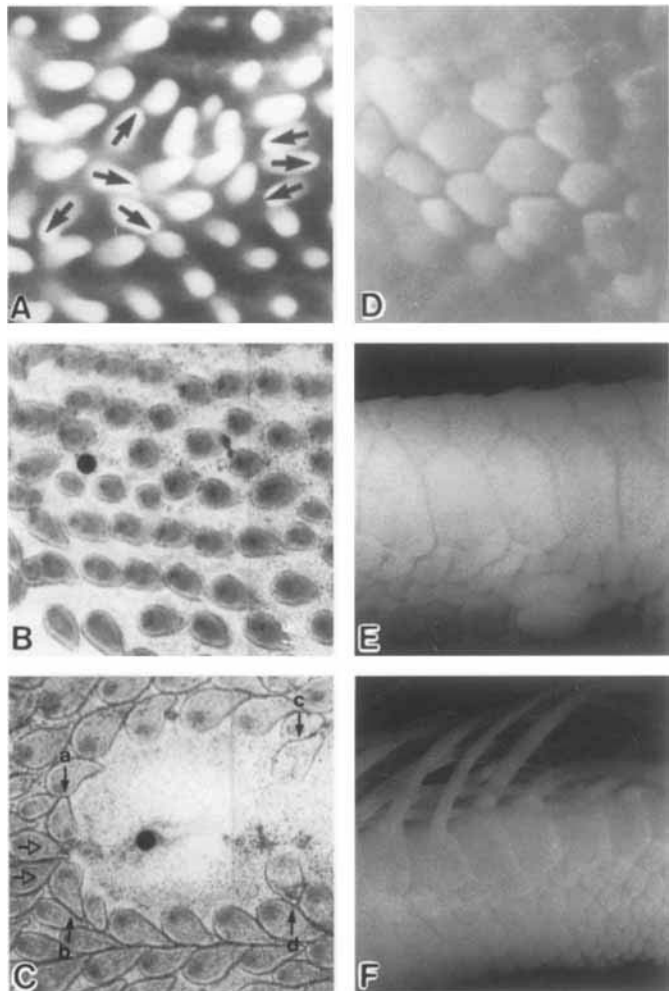
idea of the potentials, if we tentatively use the four types of homeoprotein expression patterns in feather buds (Fig. 2), and 38 for the number of Hox genes which have been identified in mouse and human<sup>(39)</sup>, there will be approximately  $4^{38}$  or  $7 \times 10^{22}$  possible codes! Because different types of skin appendages are distributed in a position-specific fashion along the body surface, we have formulated the working hypothesis that the Hox code of skin appendages is involved in determining the phenotypes of skin appendages.

It is possible that, during organogenesis, elaboration of the basic theme of the Hox gradient (such as that seen in the body axis) may occur and be built upon the original gradients to create more variations. One possible variation is that, in the limb bud, the shift of the Hox-4 axis to be nearly perpendicular to the Hox-1 axis generates a two-dimensional Hox code for different digits<sup>(35)</sup>. In the skin, the asynchronous and repetitive expression pattern of homeoproteins makes a large repertoire of Hox codes for skin appendages possible (as estimated above), without the burden of making new genes. Of course not all Hox genes may be expressed in feather buds and the codes may have some redundancy in phenotypic specificity. This developmental mechanism may have made it much easier during evolution to generate many feather varieties among the 8,800 species of birds<sup>(2)</sup>.

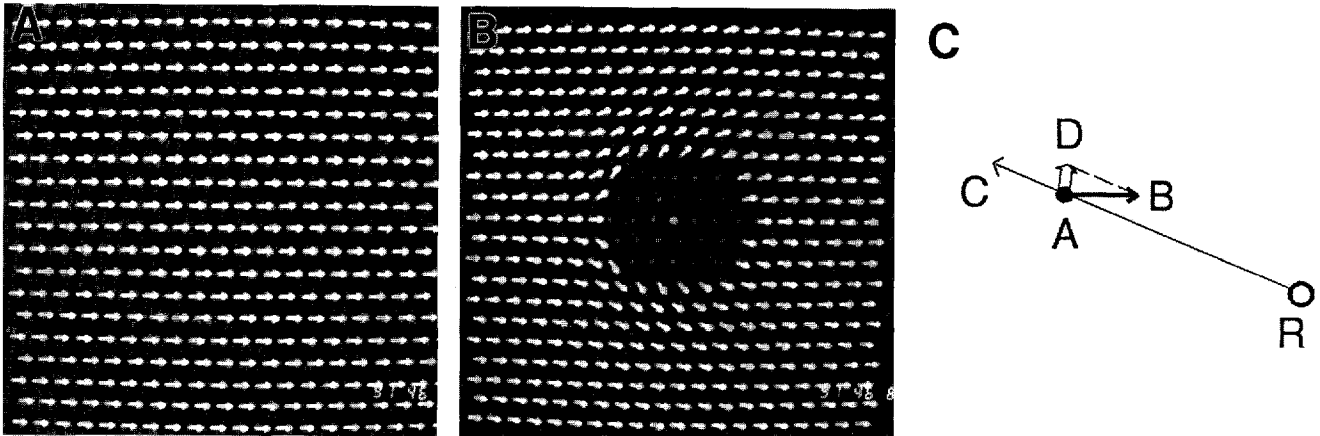
### Retinoic Acid Gradient Can Modulate the Phenotype and Orientation of a Feather in a Concentration-dependent Way

To test the above hypotheses, we set out to alter Hox expression patterns in skin appendages. Unfortunately, transgenic bird technology is not yet mature. One effective alternative is to perturb the Hox code by retroviral gene introduction, which has been shown to alter digit identity in the limb bud<sup>(38)</sup>. We have successfully introduced a foreign gene (*E. coli*  $\beta$ -galactosidase) into feather buds<sup>(41)</sup>, and the introduction of other genes is in progress. In the meantime, retinoic acid (RA) has been shown to alter Hox gene expression in a concentration-dependent way<sup>(42)</sup> and to be capable of altering spinal vertebrae Hox codes and phenotypes<sup>(36)</sup>. Thus we attempted to alter Hox codes by introducing different concentrations of RA to the skin explant culture.

We began by adding RA to the media of explant cultures. At lower concentrations (1–1.6  $\mu$ M), we observed an increasing randomness of feather bud orientation (compare Figs 1C and 3A). To further analyze this intriguing phenomenon, we placed a RA-soaked anion exchange bead on the feather explant to form a RA gradient. In earlier studies, a RA bead was implanted in the anterior limb bud, leading to mirror image duplication of the A-P limb axes<sup>(43)</sup> which have been shown to exhibit concomitant duplication of Hox-4 codes<sup>(44,45)</sup>. In the skin explant, a radial zone of inhibition, with small round buds in the periphery, was observed. Most interestingly, a rim of disoriented buds around the inhibitory zone was also noted (Fig. 3B and C; ref. 14). The new axes of the disoriented buds appeared to be determined by a combination of the original feather axis-determining force and a new axial force pointing centrifugally away from the RA source. A simulated computer model using the vectorial sum



**Fig. 3.** Homeotic transformation of skin appendages by RA. (A) Alteration of feather bud axial orientation by RA in the medium. Stage-33 skin explant was cultured with 1.5  $\mu$ M RA in medium for 6 days. The feather buds showed random orientation (arrows). (B,C) Alteration of feather bud axial orientation by a local RA gradient. Anion exchange beads (dark circles) were soaked with DMSO (B) or 3.3  $\mu$ M 13-*cis* RA (C), placed on stage-31 skin explant, and cultured for 6 days. The feather buds formed in the presence of DMSO, retinol or retinal did not alter feather orientation. Feather buds formed in the presence of RA beads showed an altered feather orientation to the original (open arrows) and the degree of axial alteration depended on their positions relative to the RA bead. The feathers anterior to the bead turn almost 90° (a and b in C). Another bead (not shown) sits to the right of c and d in C. (D) Transformation of feather bud into scale-like structures. Stage-33 skin explants were cultured with 2.5  $\mu$ M RA for 6 days. (E,F) Transformation of scales into feathery scales. E-9 chicken embryos treated with DMSO (E) or 100  $\mu$ g RA (approximately 0.3  $\mu$ mole) on the scale primordia showed feathery scales 2 days later (F). The final RA concentration of the *in ovo* experiment in which RA was dripped onto the scratched chorio-allantoic membrane is hard to estimate. *In vitro* experiments in which scale explants were cultured in the presence of RA also showed a small feather-like structure, but never as well developed as in *in ovo* experiments. E, normal appearance of metatarsal scales. F, feather grown out from the scales. *In vitro* scale culture gave similar, but less dramatic results. Arrows, orientation of feather buds.

**Box 1.** Computer model showing alteration of skin appendage axes by a point source of 'morphogen'.

In the photographs, each arrow represents a hexagonally arranged skin appendage axis. (A) Control. (B) Experiment. A bead with 'morphogen' capable of modulating the axis orientation is placed in the center of the skin appendage field. The altered angle of each new feather is calculated using the formula below, as illustrated in (C).

$$|\vec{AC}| = K \frac{[R]}{|\vec{RA}|^n}$$

where R=source of morphogen capable of modulating feather axis, in this case, RA.

[R]=concentration of morphogen in the source. In this case, it reflects the amount of RA adsorbed on the anion exchange bead AG1-X8.

A=the location of a certain feather germ.

$\vec{AB}$  = the original feather axis-forming force, shown as bold arrow.

$\vec{RC}$  = a hypothetical new modulating axial-forming force pointing centrifugally away from R, shown as thin arrow.

$\vec{AC}$  = the axis modulating force acting on feather germ A.

$|\vec{RA}|$  = distance between morphogen source and a feather germ.

n=power with which the strength of morphogen decreases. In our program, one can designate any number. In the figure shown, we assumed that RA diffuses within the feather explant plane and assigned a value of 2.

K=constant.

Inhibitory zone: we assigned a value for RA higher than that at which all feather germs are inhibited.

It is obvious that  $\vec{BD} = \vec{AC}$ . We then performed the vector sum  $\vec{AB} + \vec{BD} = \vec{AD}$ .

$\vec{AD}$  shown by the open arrow, is the new axis of the disorientated bud.

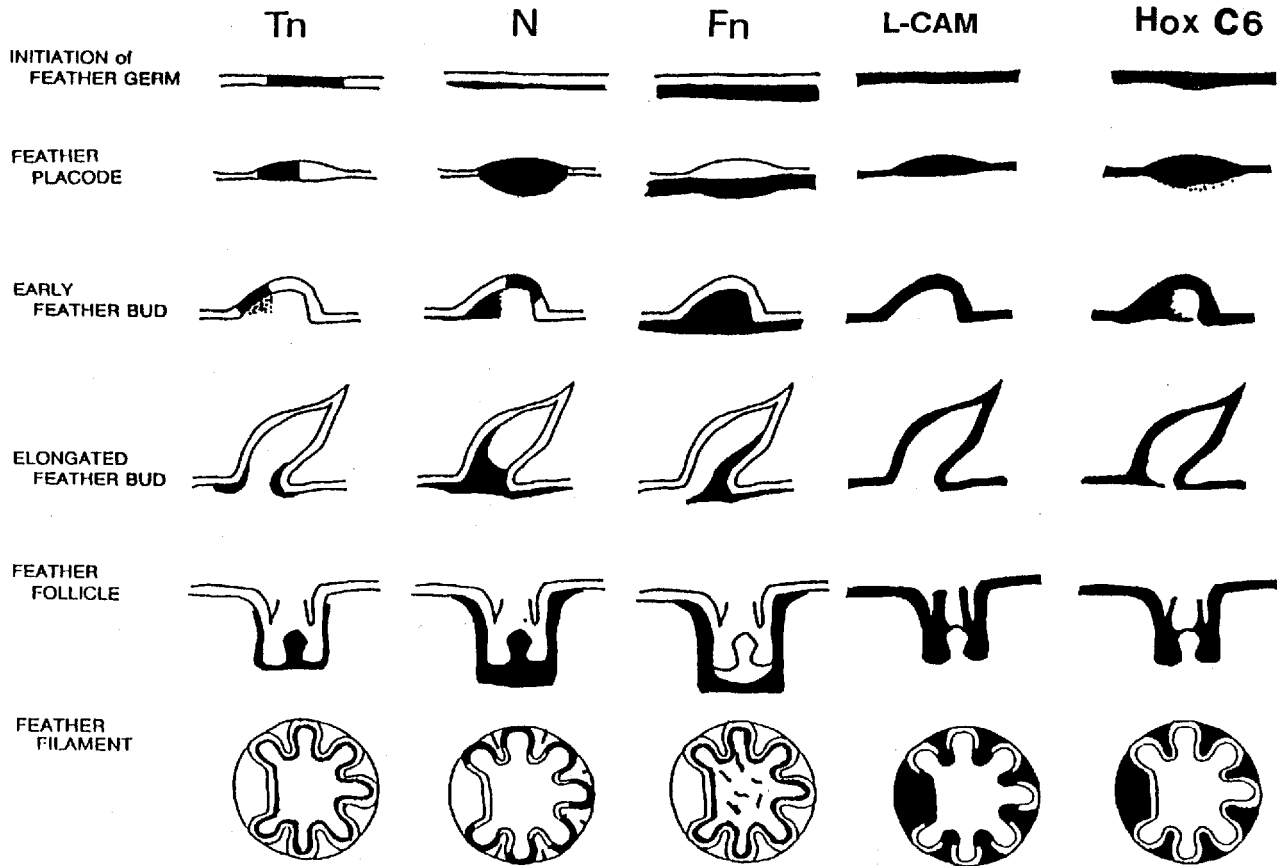
Using this program, we can place a RA bead at any position on a plane of hypothetical feather germs arranged in a hexagonal pattern. The result of the computer generated image is amazingly similar to the experimental results shown in Fig. 3C.

of the endogenous and the RA-induced feather axial determination forces is shown in Box 1 and the result is very similar to the experimental result (compare with Fig. 3C). In the orientation-altered buds, the axis of the homeoprotein microgradient is parallel to the final feather bud axis. These results imply that an RA gradient can reverse the orientation of the micro homeoprotein gradient and hence alter the orientation of feather axis.

At higher RA concentrations (2–2.5  $\mu\text{M}$ ), we observed transformations of feather buds into a scale-like morphology (Fig. 3D)<sup>(14)</sup>. In a complementary way, treatment of scale primordia led to the formation of feathery scales (Fig. 3E,F)<sup>(46, 47)</sup>. In mouse, hair follicles have been shown to be transformed into glands by vitamin A<sup>(48)</sup>. We have defined this type of change as the 'homeotic transformation of skin appendages', which means that a skin appendage is replaced with a different skin appendage phenotype normally found elsewhere on the body surface. The definition derives from

the term homeotic mutation<sup>(32)</sup> and we use 'transformation' to cover both genetic and non-genetic causes. Besides chemically induced homeotic transformation, some genetically based homeotic transformations of skin appendages, such as the feathery scale, are also observed in genetic variants of chicken and pigeons<sup>(49)</sup>.

Results from our laboratory and from others are consistent with the hypothesis that skin appendages are formed in two stages: firstly, a decision to make a skin appendage, followed by a specification stage during which phenotypes are determined<sup>(13)</sup>. When a high enough concentration of RA is added at the specification stage, the phenotype of the skin appendage is altered. We hypothesize that this homeotic transformation is mediated through disruption of Hox codes of skin appendages. Exogenous RA is likely to alter Hox expression patterns because transcription of different Hox genes has been shown to be RA-concentration dependent<sup>(42)</sup>. When the RA-transformed, scale-like feather buds were sec-



**Fig. 4.** Schematic summary of the expression pattern of adhesion molecules in feather morphogenesis. Tenascin (Tn), N-CAM (N), fibronectin (Fn) and L-CAM. Expression of Hox C6 is also summarized for comparison. The black color indicates the presence of the particular adhesion molecule. Please refer to Fig. 1A for identification of structures. See text for explanation.

tioned, the anterior localized expression patterns of Hox C6 and Hox D4 were found to be diffusely distributed<sup>(14)</sup> and were similar to the Hox expression pattern in normal scales (manuscript in preparation). These results are consistent with the hypothesis that Hox codes of skin appendages are involved in determining the phenotypes of skin appendages.

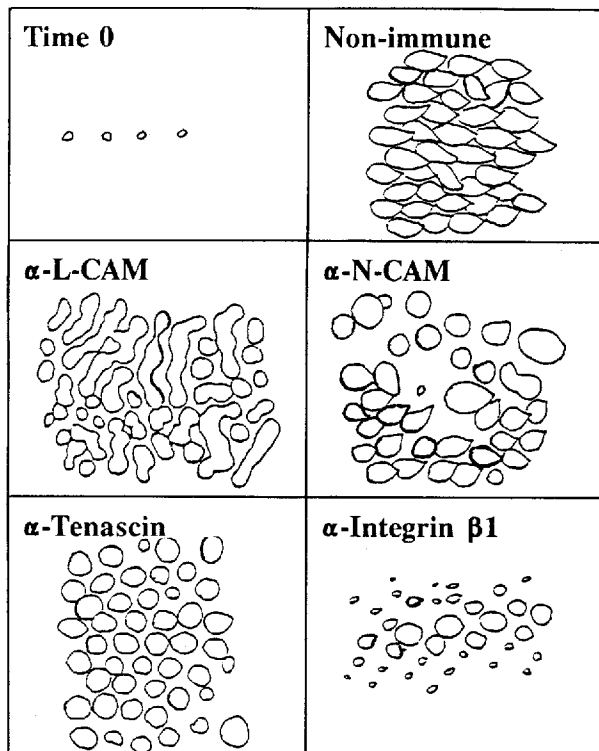
Although we have shown that RA can modulate the phenotypes and axes of skin appendages, we do not know whether RA is indeed the physiological substance mediating these processes. Both beta and gamma RA receptors have been identified in the skin<sup>(50,51,52)</sup>. We have identified endogenous retinoids in chicken skin by HPLC, which showed both qualitative and quantitative changes in the elution profiles during development from stage 31 to 37 (our unpublished data). The presence of retinoic acid receptors and the dynamic retinoid profiles during development strongly suggest that retinoid is an endogenous morphogen.

#### Adhesion Molecules Mediate Hexagonal Pattern Formation, Dermal Condensation and Feather Elongation

Suppose Hox genes, which are transcription factors, indeed

determine the phenotypes of skin appendages. What then are the downstream molecules that mediate the morphogenetic process? During the formation of a feather, cells change their positions relative to one another and new cell interactions occur, in a sense to 'mold' the shape of a feather. These interactions are modulated at the cell surface and involve adhesion molecules and their receptors. Therefore cell adhesion molecules are good candidates for molecules involved in 'morphoregulation'<sup>(53)</sup>. We have been studying the roles played by N-CAM, L-CAM, tenascin, fibronectin and integrin during feather development<sup>(10,19,20)</sup>, and our results to date are summarized below.

The expression patterns of adhesion molecules are spatially and temporally specific, and highly dynamic (Fig. 4). Here, I will mention just some dramatic examples, but for a detailed description, please refer to the original papers<sup>(10,19,20)</sup>. To study induction, we have searched for the earliest molecules that are specifically expressed in the placode. Tenascin is expressed in a periodic way corresponding to the presumptive feather placode, thus suggesting that tenascin may play an important role in placode development<sup>(10)</sup>. The feather placode has previously been defined by morphology and no molecular heterogeneity has been described. Using antibodies to



**Fig. 5.** Perturbation of feather morphogenesis with antibodies to adhesion molecules. Tracings from explant cultures treated with antibodies to adhesion molecules. Non-immune, non-immune Fab;  $\alpha$ -L-CAM, anti-L-CAM;  $\alpha$ -N-CAM, anti-N-CAM;  $\alpha$ -Tenascin, anti-tenascin;  $\alpha$ -Integrin- $\beta$ 1, anti-integrin  $\beta$ -1. Please see text for explanation and refer to Jiang and Chuong<sup>(10)</sup> for original data.

adhesion molecules, we now can define at least three domains of placode epithelium: anterior domain, positive for tenascin only; distal domain, positive for N-CAM only; and posterior domain, negative for both adhesion molecules but positive for fibroblast growth factor receptor<sup>(25)</sup>. In the feather bud mesoderm, N-CAM, tenascin and Hox C6 are present in the anterior bud with partial overlap of their expression gradients, while fibronectin is expressed in the posterior feather bud. In the feather filament epithelium, the alternate epithelial stripes which define the cell death zones are N-CAM positive<sup>(20)</sup>. Thus the spaces between feather branches are full of N-CAM during development. Therefore, there appear to be cellular groups which work as units of morphogenesis during development. The borders of these cell groups are defined by adhesion molecules, and at least some of them are coupled by gap junctions<sup>(54)</sup> which may facilitate the sharing of signal molecules and differentiation fates.

The potential roles of these adhesion molecules in feather development are currently being tested by us. We have initially explored their function by suppressing the activities of adhesion molecules in feather explant cultures (Fig. 1B). Antibodies (Fab fragments) to L-CAM (E-cadherin), N-CAM, tenascin and integrin were added to the media and the results analyzed. The results were distinctly different aborted pat-

terns, suggesting that different adhesion molecules play different roles in the morphogenetic process (Fig. 5). With anti-L-CAM, the dermal condensation fused to become horizontal stripes<sup>(55)</sup>. Anti-N-CAM was characterized by unevenly sized dermal condensations and a distorted hexagonal pattern. Anti-tenascin led to round-shaped feather buds similar to those in younger cultures, presumably due to inhibition of feather bud growth. Anti-integrin  $\beta$ -1 subunit and anti-fibronectin caused the separation of epithelium and mesenchyme, hence preventing further epithelial-mesenchymal interactions and feather development<sup>(10)</sup>. The results confirm the hypothesis that adhesion molecules are involved in the different morphogenetic stages of feather development. Alteration of any of these steps by modulating the amount or specific sites of adhesion molecules can produce different shapes of skin appendages, and may be the 'tools' to realize the phenotypes. It is interesting to note that some Hox genes can act on the 5' regulatory region of the N-CAM and tenascin genes<sup>(56,57)</sup>. However, more studies are essential to define further the connections between adhesion molecules and Hox genes.

### Conclusions

We have reviewed our current knowledge of the involvement of homeoproteins, retinoids and adhesion molecules in the formation of feather buds. Future directions of research will include further testing of the Hox code of skin appendages hypothesis and determination of how adhesion molecules interact to form a feather. One powerful approach is to alter gene expression through retroviral gene expression<sup>(38)</sup>. We have had initial success in introducing an unrelated gene into feathers without interfering with feather growth<sup>(41)</sup>. Work is now in progress to introduce exogenous Hox genes and adhesion molecules into feather buds.

The making of a feather includes many morphogenetic processes such as the initiation of the bud, the formation of the hexagonal pattern, the condensations of mesenchymal cells and the elongation of feather buds (Table 1). Some of the many molecules that have been shown to be expressed or functionally involved are included in the table. From the data summarized in Table 1, it will be obvious that the molecules discussed here are only a fraction of those involved. We hope that someday we can determine how all these molecules work together to make a feather, a structure so delicate and precise, yet which seems to contain the key to some fundamental principles of morphogenesis. We hope that the understanding obtained will contribute to vertebrate morphogenesis in general.

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Cheng-Ming Chuong is at the Department of Pathology, HMR 204, 2011 Zonal Ave, School of Medicine, University of Southern California, Los Angeles, CA 90033, USA