Adhesion Molecules and Homeoproteins in the Phenotypic Determination of Skin Appendages

Cheng-Ming Chuong, Randall B. Widelitz, and Ting-Xin Jiang
Department of Pathology, School of Medicine, University of Southern California, Los Angeles, California, U.S.A.

We examined the roles of adhesion molecules and homeoproteins in the morphogenesis of skin appendages using feather as a model. The expression pattern of these molecules in different stages of feather development were very dynamic. For example, neural cell adhesion molecules are present first in the dermal condensations, then in distal bud epithelium, then in the dermal papilla, and finally in the marginal and axial plates. Tenascin is present first in the placode, then in the anterior bud epithelium and mesoderm, and then in the dermal papilla. The expression patterns suggest that the adhesion molecules are involved in forming the boundary of cell groups that interact to form skin appendages. Antibody perturbation of embryonic skin-expant cultures showed that liver cell adhesion molecules are involved in establishing the hexagonal pattern, neural cell adhesion molecules are involved in the formation of dermal condensations, tenascin appears to be involved in the growth of feather buds, and integrin is essential for epithelial-mesenchymal interactions. Using antibodies to X1Hbox 1 (similar to Hox 3.3 or C6) and Hox 4.2 (or D4), we showed that there is a homeoprotein gradient within the feather buds, and that the expression pattern is position-specific. It is hypothesized that Hox codes, derived from the combined expression pattern of homeoproteins, determine the phenotypes and orientation of skin appendages. Experiments using retinoids in the media or retinoid-soaked beads to create a local retinoid gradient are consistent with this hypothesis. As demonstrated here, feather development provides an excellent opportunity to analyze the molecular cascade of skin-appendage morphogenesis.

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The integument forms the barrier between the body and the environment and allows the creation of a stable internal environment. Skin appendages derive from dermal and epidermal components that transform a piece of flat skin into a unique structural entity such as the hair, nail, claw, horn, gland, or feather. In addition to the essential protective function, the evolution of repeated skin appendages has led to a variety of new functions across animal species including temperature maintenance, movement, sexual display, defense, aggression, secretion, etc. To be able to manage conditions in which skin and skin appendages are damaged, it is important to know the fundamental mechanism and molecules involved in the development of skin appendages.

We have been approaching skin appendages from a developmental point of view. We have adopted the development of the chicken feather as our major model for the following reasons. i) The fundamental processes of hair and feather formation are similar. ii) The availability of a feather bud culture system facilitates a variety of experiments including temperature maintenance, movement, sexual display, defense, aggression, secretion, etc. To be able to manage conditions in which skin and skin appendages are damaged, it is important to know the fundamental mechanism and molecules involved in the development of skin appendages.

In recent years, many new molecules have been identified. These include cell adhesion molecules (reviewed in [5,6]), homeobox genes (reviewed in [7]), oncogenes, growth factors (reviewed in [8]), keratin subtypes [9,10], etc. These new developments have led to exciting progress in hair or, more broadly, in skin appendage research. In this article, we will review the roles of adhesion molecules and homeoproteins in feather development and the working hypotheses derived from these results.

ROLES OF ADHESION MOLECULES IN SKIN-APPENDAGE MORPHOGENESIS

In the feather placode stage, the most dramatic staining patterns observed are the periodic patches of NCAM in each dermal condensation [11] and the presence of tenascin over each perspective epithelial placode [2]. These patterns emerge before the morphologic changes in the placode epithelium; thus these molecules are likely to be involved in the early stages of placode development (Fig 1a,b) and appear to precede the expression of bone morphogenetic protein-4 (BMP-4), a transforming growth factor beta family member, found in early mouse whisker placodes after epithelial elongation [12]. In the feather bud stage, another level of cellular heterogeneity is defined. At least three cellular domains can be identified from the apparent homogeneous feather bud epithelium. The anterior domain is positive for tenascin but negative for NCAM, the distal domain is positive for NCAM but negative for tenascin, and the posterior domain is lacking both (Fig 2, Fig 1c,d). L-CAM is present in all three domains [11]. Feather bud mesoderm, which appears homogeneously in phase-contrast images, can also be sub-divided into different cellular domains according to different adhesion molecule expression patterns. NCAM and tenascin are enriched in the anterior mesoderm [2] and fibronectin is enriched in the posterior mesoderm [13]. Integrin beta 1 is widely distributed throughout the bud mesoderm [2]. As the feather bud continues to grow and
Figure 1. Adhesion molecules in skin appendage morphogenesis. The distribution of NCAM and tenascin was determined by immuno-localization with alkaline phosphatase-conjugated secondary antibodies. At the feather-placode stage, NCAM is present in the dermis and enriched in the dermal condensations (a), whereas tenascin is expressed in the placode epithelium in a periodic way (b). At the feather bud stage NCAM was found in the anterior mesoderm and the distal epithelium (c), whereas tenascin was found in the anterior epithelium with some speckled staining in the anterior mesoderm (d). Arrowheads, epithelial border. When the feather follicle forms, L-CAM is present throughout the epithelia (e), N-CAM is present in the dermal papilla and marginal and axial plates of the feather filament epithelia (f, note the periodic staining pattern that determines the branch pattern of feather) (f). Tenascin is also in the dermal papilla and beneath the feather sheath (g). Hair has a similar expression pattern and the N-CAM positive dermal papilla in an adult mouse hair is shown in h. The staining in panels e, f, and h, are from immunofluorescence. Bar, 100 μm.

Invaginate to form the feather follicle, both NCAM and tenascin become enriched at the base of the follicle, in the dermal papillae (Fig 1f,g). NCAM expression was also observed in the placode and dermal papilla of mouse hair ([14], Fig 1h). In the adult, the hair shaft is maintained as a cylindrical structure, whereas the feather filament epithelium becomes a branched structure created by the differentiation of two interleaved cell groups: the first group keratinizes to form the barb plates and the second group dies to leave space between keratinized bars and barbules (marginal and axial plates). L-CAM is present on all the filament epithelia whereas NCAM is present on those cell groups that are destined to die ([15], Fig 1e). Thus there appear to be distinct cellular domains expressing different adhesion molecules and, in many cases, adhesion molecules appear earlier than recognizable cellular changes.

From this and other studies, we hypothesize that cell groups linked by adhesion molecules work as units for morphogenesis. The
Figure 2. Perturbation of feather morphogenesis with antibodies to adhesion molecules. Dorsal skin from stage 33 chicken embryo was dissected and cultured on an organ culture filter for 4 d in DMEM/2% fetal bovine serum with 0.2 mg/ml of designated Fab'. (I, day 0 culture. b, day 4 control culture. c, day 4 culture in the presence of anti-L-CAM. d, anti-N-CAM. e, anti-tenascin. f, anti-integrin beta 1. Arrow, midline of the spinal tract (the skin over the spinal cord region). Arrowheads, small buds; star, missing buds. Bar, 100 µm.

The role of the adhesion molecules is to define the border of cell domains. Cells within the border may be coupled by gap junctions or other mechanisms to share the same signals and differentiation fates. Tissue interactions are based on an interplay of cell groups rather than single cells. Therefore, 1) the expression of adhesion molecules in cell groups should be transient, 2) similar molecules may be used in different cell groups, and 3) a disruption of the adhesion molecules will lead to alterations in the size of cell groups or stall their further development. In epithelia, we indeed observed that the transient expression of NCAM segregates epithelia into different cell domains destined for different functions. This has been seen in the development of the feather placode, otic placode [16], and lens placode. In mesenchyme, we observed the transient expression of NCAM in dermal (bud mesoderm, dermal papilla), kidney tubule [17], precartilage [18], and osteogenic nodule [19] condensations.

To further test this hypothesis in skin appendage formation, we used embryonic chicken dorsal skin explant cultures (H and H stage 32) and tested the effect of antibodies to various adhesion molecules. In this culture system, feather germs develop from a homogeneous explant sheet into discrete elongated feather buds arranged in a hexagonal pattern (Fig 2a,b). With anti-L-CAM (E-cadherin), the hexagonal pattern was lost and the dermal condensations fused into horizontal stripes [20]. Inhibition of proteoglycan synthesis was shown to have a pattern similar to that caused by anti-L-CAM [21]. In mouse, anti-N and P cadherin were shown to inhibit whisker formation in explant cultures [3]. Disruption of NCAM led to an uneven segregation of dermal condensations and the culture was characterized by big, small, and missing buds with a distorted hexagonal pattern ([2], Fig 2f). Buds cultured in the presence of anti-tenascin stopped growing and became small and round, similar to young buds ([2], Fig 2e). Anti-integrin beta 1 subunit antibodies produced the most complete inhibition of feather development; the feather germs remained at the dermal condensation stage. Microscopic examination of these cultures showed a separation of the epithelium and mesenchyme, hence inhibiting epithelial-mesenchymal interactions ([2], Fig 2f). Anti-fibronectin had a similar but less severe effect than anti-integrin. The distinctly different perturbed phenotypes of skin appendages suggest that these adhesion molecules are involved in different stages of feather morphogenesis. Restricted gap junction communications in cell domains have also been observed in early feather buds [Serras F, Fraser S, Chuong CM: Mechanisms of skin morphogenesis. III. Patterns of gap junctional communication studied by micromanipulation of Lucifer Yellow (submitted) and hair follicles [22]. Alteration of gap junction communication in cell groups disrupted by anti-adhesion molecules is currently under investigation.

Figure 3. Homeoprotein gradient in feather buds. Top, four expression homeoprotein patterns are found in developing feather buds: pattern 1, buds with homeoprotein throughout the mesoderm; pattern 2, buds with a homeoprotein gradient passing the midline; pattern 3, buds with a homeoprotein gradient not passing the midline; and pattern 4, buds lacking homeoproteins. A, anterior; P, posterior. Middle, position-specific homeoprotein expression pattern in skin appendages. Bottom, hypothesis showing that the phenotype of a skin appendage is a function (f) of the Hox codes, and a representation analogous to that of a mathematical matrix function is shown.
protein gradient within the feather buds is involved in setting up the anterior-posterior axis of skin appendages. Further examination showed another surprising finding: different feather buds have different homeoprotein expression patterns. These patterns can be classified into four categories: category 1, buds expressing Hox C6 throughout the mesoderm; category 2, buds in which the homeoprotein gradient passes the midline; category 3, buds with a homeoprotein gradient not passing the midline; and category 4, buds not expressing Hox C6 (Fig 3, top). For some homeoproteins, such as C6, there are position-specific expression patterns. Another homeoprotein, Hox 4.2 of D4 [26a], also is expressed in four patterns, but is out of phase from the patterns observed for C6 (Fig 3, middle). If we define the combination of Hox expression patterns (categories 1-4) as "Hox codes of skin appendages," and we account for about 40 Hox genes distributed in four chromosome clusters; we can see the potential repertoire of Hox codes is enormous (40). In mice, it also was shown that there is a position-specific Hox expression pattern in the dermal papilla [29] suggesting the existence of similar skin domain-specific Hox expression patterns in both avians and vertebrates. This is different from the "Hox codes of vertebratae" in that vertebral homeoproteins are either present or absent rather than expressed over variable regions within a gradient.

Because different types of skin appendages are distributed on the body surface, and the Hox codes are position specific, we hypothesize that Hox codes act to determine the phenotypes of skin appendages just as they determine the appendage phenotypes in Drosophila [30]. The hypothesis has two components: the combined Hox codes determine the phenotype of a skin appendage (e.g., flight feather, down feather, scale, whisker, glands, etc), and the orientation of the Hox gradient determines the anterior-posterior axis of the skin appendage. The Hox code can be reset by altering the retinoid metabolism enzymes or the expression levels of retinoid receptors. Subtle alterations in homeoproteins can then lead to a large variety of distinct skin appendage phenotypes.

If this hypothesis is correct, the following corollaries should be tested. 1) To describe Hox codes in skin appendages completely and to correlate them with different shapes of skin appendages. 2) To perturb Hox codes and analyze the effect upon phenotypes and axes of skin appendages. There are two general ways to do this. One is to use retinoic acid (RA) because it has been shown to alter Hox expression patterns both in vitro [31] and in vivo [32]. The other is to ectopically express Hox genes using molecular biologic methods such as retroviral gene transfer [35]. We are currently working on this approach. For RA, we recently showed that low concentrations of RA randomized feather bud orientations whereas high concentrations of RA transformed feather buds into scale-like skin appendages ([34], Fig 4A-D). In contrast, treatment of scale primordia led to the formation of feathery scales ([35,36], our unpublished data).

In mouse, it has been shown that vitamin A transformed hair follicles into glands [37]. Seeding of these scale-like skin appendages showed that the anterior localization of NCAM and C6 had become much more diffusely distributed (Fig 4E-H). Sections in vivo indeed have more diffuse distribution patterns of C6 and Hox D4 (manuscript in preparation).

The alteration of the feather axis is intriguing. To more precisely define the alterations of orientation, we presented a RA gradient by a RA-soaked anion exchange bead [38]. Immediately next to the bead, there was a zone of inhibition. Interestingly, at the rim of the inhibitory zone, feather buds were disoriented and the new axis 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 (Fig 5A,B). A computer model was written to simulate this process. The new orientation was defined by the vectorial sum of the endogenous axial determination force and the exogenous axial modulatory force diffused from the RA bead. The prediction was very similar to that of the experimental result (Fig 5C,D). These results suggest that RA and the homeoproteins are directly or indirectly involved in feather bud axis determination.

**SUMMARY**

Morphogenesis of skin appendages is one of the major models of classical embryology. These investigations have demonstrated that skin appendages derive from complex interactions between the epi-
RA, which can modulate the homeoprotein expression pattern, can stage in setting the phenotypes and orientation of skin appendages. To further analyze the determination stage, homeoproteins appear to play a role in the determination of skin appendage axes. Adhesion molecules appear to be involved in recruiting cells and modulating feather axis. A, the location of a skin appendage. 3, the original feather axis forming force, shown in arrow. A, shown in open arrow, is the new axis of the disorientated bud. It is obvious 3 = z.

Vector sum \( \overrightarrow{AC} = \overrightarrow{AB} + \overrightarrow{BD} \). The strength of \( \overrightarrow{AC} \) is calculated according to the formula. [R], concentration of morphogen in the bead. [RA], distance between morphogen source and a feather germ. n, power with which the strength of morphogen decrease. In this figure, \( n = 2 \), as in the case of diffusion. K, constant. Inhibitory zone: we assigned a morphogen value over 21.

Figure 5. Modulation of the orientation of feather buds by a local RA gradient. Anion exchange beads soaked in retinoids were placed on top of stage 31 skin and cultured for 6 d. A, control beads soaked in dimethylsulfoxide. B, beads soaked in 2 mg/ml 13 cis-RA. Note the degree of axial alteration of the buds depend on their positions relative to that of the bead. The ones anterior (left) to the beads turn almost 90°. C, computer model showing alteration of skin appendage axes by a point source of "morphogen." In the drawing, each arrow represents a hexagonally arranged skin appendage axis. D, a hypothetical bead secreting "morphogen" capable of modulating the axis orientation is placed in the center of the skin appendage field. The way the altered angle of each skin appendage is calculated is illustrated in the bottom panel. The computer-generated image (D) is amazingly similar to the experimental results (B). R, source of morphogen capable of modulating feather axis. A, the location of a skin appendage. AB, the original feather axis forming force, shown in arrow. AC, the axis modulating force, pointing radially away from R, acts on A. AD, shown in open arrow, is the new axis of the disoriented bud. It is obvious \( \overrightarrow{BD} = \overrightarrow{AC} \). Vector sum \( \overrightarrow{AD} = \overrightarrow{AB} + \overrightarrow{BD} \). The strength of \( \overrightarrow{AC} \) is calculated according to the formula. [R], concentration of morphogen in the bead. [RA], distance between morphogen source and a feather germ. n, power with which the strength of morphogen decrease. In this figure, \( n = 2 \), as in the case of diffusion. K, constant. Inhibitory zone: we assigned a morphogen value over which all feather germs are inhibited.

molecular mechanism of skin appendage morphogenesis, future directions include direct alteration of specific genes using retroviral gene transfer [55].

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