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Molecular Histology in Skin Appendage Morphogenesis

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ABSTRACT Classical histological studies have demonstrated the cellular organization of skin appendages and helped us appreciate the intricate structures and function of skin appendages. At this juncture, questions can be directed to determine how these cellular organizations are achieved. How do cells rearrange themselves to form the complex cyto-architecture of skin appendages? What are the molecular bases of the morphogenesis and histogenesis of skin appendages? Recently, many new molecules expressed in a spatial and temporal specific manner during the formation of skin appendages were identified by molecular biological approaches. In this review, novel molecular techniques that are useful in skin appendage research are discussed. The distribution of exemplary molecules from different categories including growth factors, intracellular signaling molecules, homeobox genes, adhesion molecules, and extracellular matrix molecules are summarized in a diagram using feather and hair as models. We hope that these results will serve as the ground work for completing the molecular mapping of skin appendages which will refine and re-define our understanding of the developmental process beyond relying on morphological criteria. We also hope that the listed protocols will help those who are interested in this venture. This new molecular histology of skin appendages is the foundation for forming new hypotheses on how molecules are mechanistically involved in skin appendage development and for designing experiments to test them. This may also lead to the modulation of healing and regeneration processes in future treatment modalities. *Microsc. Res. Tech. 38:00–00, 1997.* © 1997 Wiley-Liss, Inc.

INTRODUCTION

Histology, which demonstrates the cellular organization of tissues, has helped us to appreciate the structures and function of skin appendages. Skin appendages derive from interactions of the epidermis and dermis within the skin. Elaboration of defined domains of the skin leads to the formation of new skin appendages with different functions. In general, there are two categories of skin appendages, characterized by either protrusion out of or invagination into the body surface. The skin appendages that protrude out of the body surface are hair, feather, scale, nail, claw, etc. They provide a variety of functions to individuals, ranging from environmental protection to ritual mating displays. The skin appendages that invaginate are the sebaceous gland, sweat gland, mammary gland, etc. They provide specialized physiological functions to individuals, ranging from environmental adaptation to child rearing.

How are the complex cyto-architecture of these skin appendages achieved? Recent progress in molecular biology has allowed us to identify many new molecules which are important in cell interactions and embryonic development. These molecules can be categorized as growth factors, homeobox genes, cell adhesion molecules, extracellular matrix molecules, etc (Chuong, 1993; Chuong et al., 1993; Chuong et al., 1996). Using immunocytochemistry, in situ hybridization and other novel molecular biology techniques, it is now possible to study the spatial and temporal distribution of these molecules during the morphogenesis of skin appendages. The objective of this article is to introduce new techniques used in the molecular histology of skin appendages and to summarize recent findings in feather and hair development as useful references. These findings will set the ground work for us to explore the mechanism(s) underlying the processes of development, cycling, and regeneration in skin appendage morphogenesis. We also discuss the fundamental implications that these molecular studies have for development and evolution of skin appendages.

MATERIALS AND METHODS

Molecular Histology Methods for Skin Appendage Research

Identifying mRNA Expression

Whole mount in situ hybridization with non radioactive probe. This method permits a three dimensional overview of the distribution of specific mRNAs. This

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replaces the tedious work of three dimensional reconstruction from section staining using an image analysis program. Furthermore, in situ hybridization probes can easily be generated using PCR technology (Erlich, 1989). In addition, it is easy for us who study skin to obtain a piece of appropriately sized skin (eg., up to $10 \times 5 \times 1$ mm) for this purpose. We think this technique will play a major role in the molecular mapping of skin development in the future.

In our laboratory, we use a protocol based on Sasaki and Hogan (1993). Briefly, chicken embryo skins dissected in RNase free phosphate-buffered saline (PBS) are fixed in 4% paraformaldehyde for 2 hours or at 4°C overnight. Tissues are dehydrated, rehydrated, bleached, and treated with proteinase K. Samples then are subjected to secondary fixation in 0.2% glutaraldehyde in 4% paraformaldehyde/PBS before hybridization at 70°C overnight in buffer (50% formamide, $5 \times$ SSC, 1% SDS, 50 µg/ml heparin, 50 µg/ml tRNA) containing 2 µg/ml digoxigenin-labeled riboprobes. After washing and RNase A treatment (50 µg/ml) the tissues are incubated with anti-digoxigenin Fab′ conjugated to alkaline phosphatase (Boehringer Mannheim). Alkaline phosphatase is detected with 4.5 µl/ml NBT with 3.5 µl/ml BCIP (Promega) following standard protocols. Samples are dehydrated, rehydrated, cleared, and mounted. If desired, the block can be sectioned to identify more specifically the distribution of the probes and cell types. For this purpose, the samples are dehydrated through methanol for 10 mins, isopropanol for 15 minutes, and tetrahydronaphthalene for two 15 minutes washes. The samples are then washed three times in wax at 60° for 20 minutes each wash. The wax is then allowed to set. Sections can be cut (7–20 µm), dried onto slides and then de-waxed with Histoclear (Wilkinson, 1992). An example using probes against Msx-2 homeobox gene is shown in Fig. 1A. When necessary, double staining in situ can be achieved using the above alkaline phosphatase method together with biotin-nucleotide labeled probes followed by avidin-conjugated peroxidase (O'Neill and Bier, 1994). Alternatively, to visualize the probes with a fluorescence microscope, anti-digoxigenin Fab′ conjugated with rhodamine and avidin conjugated with fluorescein can be used.

Two color non-radioactive section in situ hybridization. Two different transcripts can be detected on the same section using digoxygenin and fluorescein labeled riboprobes. The probe used to detect the more strongly expressed gene is labeled with fluorescein. The fluorescein labeled probe is detected first with an anti-fluorescein antibody coupled to alkaline phosphatase using fast Red as a substrate. After finishing the first detection, this antibody is removed with an acid glycine buffer (100 mM glycine-HCl buffer, pH 2.2, 0.1% Tween-20) and then the digoxygenin labeled probe is detected with an anti-digoxygenin antibody coupled again to alkaline phosphatase using BM purple (Boehringer Mannheim, Indianapolis, IN).

Section in situ hybridization with radioactive probes. This has been a standard method and is more sensitive than whole mount in situ hybridization. ^{35}S labeled probes allow detection with very high sensitivity. In our lab, we use the protocol of Schreiber et al. (1991). Tissues are frozen without fixation, sectioned to 10 µm on a cryostat, mounted on slides and then fixed with 4% paraformaldehyde. Subsequently, they are placed in 0.1 M triethanolamine, pH 8.0 and 0.25% acetic anhydride for 10 minutes. Tissues are then dehydrated and hybridized in buffer (50% formamide, $4 \times$ SSC, $5 \times$ Denhardt's, 1% SDS, 10% dextran sulfate, 250 µg/ml tRNA, 25 mg/ml poly A, 25 mg/ml poly C, and 0.1 M DTT) containing 0.3 ng/µl in vitro transcribed RNA probes for 3 hours to overnight at 50°C. Sections are washed and unbound probe is removed by RNase A treatment. The slides are then dehydrated and exposed to emulsion (Kodak, Rochester, NY). The temperature should be adjusted for the probes used; longer probes and those with higher GC content require higher temperatures. An example using Msx-2 is shown in Fig. 1B which can also be compared with the results of whole mount in situ hybridizations (Fig. 1A).

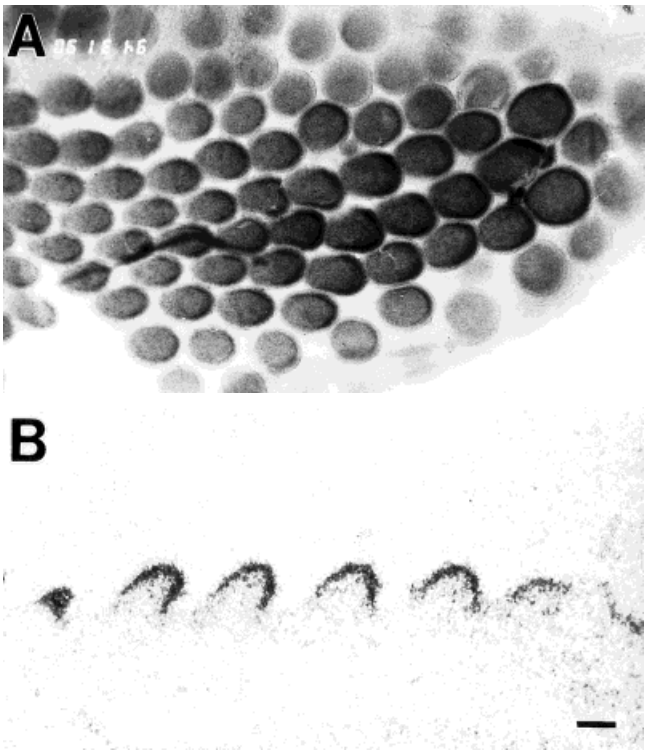

Fig. 1. Examples of localization of mRNA. Whole mount (A) and frozen section (B) in situ hybridization of developing chicken skin using an antisense probe to Msx-2 genes. Panel A uses alkaline phosphatase conjugated anti-digoxigenin antibodies for detection. Panel B uses ^{35}S-UTP and autoradiography for detection. Note Msx-2 is specifically expressed in the feather bud epithelium, but not the inter-bud regions. Size bar, 250 µm.

Molecular expression determined by promoter-reporter constructs and transgenic mice. Transgenic mice provide an in vivo environment for the analysis of gene regulation. Since expression patterns in the skin are easily observed, even researchers who are not studying skin will note interesting staining patterns. However, some unknown factors may influence the cis acting elements and the expression patterns of new genes should be verified with in situ hybridization. In this technique, the cis acting elements of a specific promoter are used to drive the expression of a reporter gene, such as β-galactosidase. The results are visualized by X-gal

staining of whole mount embryos or skin. This technique was used to demonstrate the body position specific expression pattern of Hox 3.1 in mouse skin (Bieberich et al., 1991) and the unique keratin expression pattern in mouse hair (Byrne et al., 1994).

Identifying Protein Expression

Whole mount immunohistochemistry. Whole mount staining can show the three dimensional distribution of specific proteins. We have set up this procedure according to Dent et al., 1989. Briefly, skins were fixed in 20% DMSO/80% methanol, bleached in hydrogen peroxide, washed, then incubated with antibodies. This is followed by secondary antibodies coupled with alkaline phosphatase. After color development, tissues are cleared in benzyl benzoate:benzyl alcohol. Endogenous alkaline phosphatase activity is inhibited by levamisole. To increase penetration of antibodies, Fab forms of first or secondary antibodies can be used. Again this provides a three dimensional view without laboring through computerized three dimensional reconstruction. It should be noted that the distribution of proteins and mRNA can be different.

Section immunohistochemistry. Staining of microtome sections offers the ability to observe the expression pattern within a plane of tissue, unobscured by the surrounding tissue. The distribution can be visualized by immuno-fluorescence or immuno-enzyme. Double staining techniques can be used to compare the distribution of two molecules. These techniques have been used to demonstrate molecular expression in feather buds (Chuong and Edelman, 1985 and Chuong et al., 1990). An example of tenascin immunofluorescence is shown in Fig. 2A.

Identifying Post-translational Modifications. The function of a protein is not simply dependent on its concentration. Rather, protein function is usually regulated by post-translational modifications which can be detected by a variety of simple histological techniques. Two examples in which this modification can be detected by histologic techniques are shown below.

Glycosylation. Surface protein glycosylation can alter the affinity of cells for one another and hence, modify cell-cell interactions required for tissue morphogenesis. One way to detect post-translational glycosylations is to use the specific sugar binding properties of different lectins, such as biotinylated Con A, wheat germ agglutinin, or peanut agglutinin, followed by fluorescent avidin (Vector) (Chuong et al., 1991).

Phosphorylation. Phosphorylation at Tyr or Ser/Thr residues can modify protein activity. Many receptors have tyrosine kinase activity which upon binding to their ligand can phosphorylate intracellular proteins on their tyrosine residues. Phosphotyrosine can be detected in methanol fixed fresh frozen sections by immunofluorescence using anti-p-Tyr antibodies (Zymed). An example is shown in Fig. 2B. Some transcription factors such as CREB are regulated by phosphorylation on Ser/Thr residues. Phosphorylation of Ser/Thr on CREB can be detected by a specially prepared antibody that recognizes only the phosphorylated epitope (Ginty et al., 1993).

Cellular Behavior. To study the dynamic aspect of histology, it is important to analyze the behavior of cells. Several novel approaches originally used in other fields are mature and can be applied to skin research.

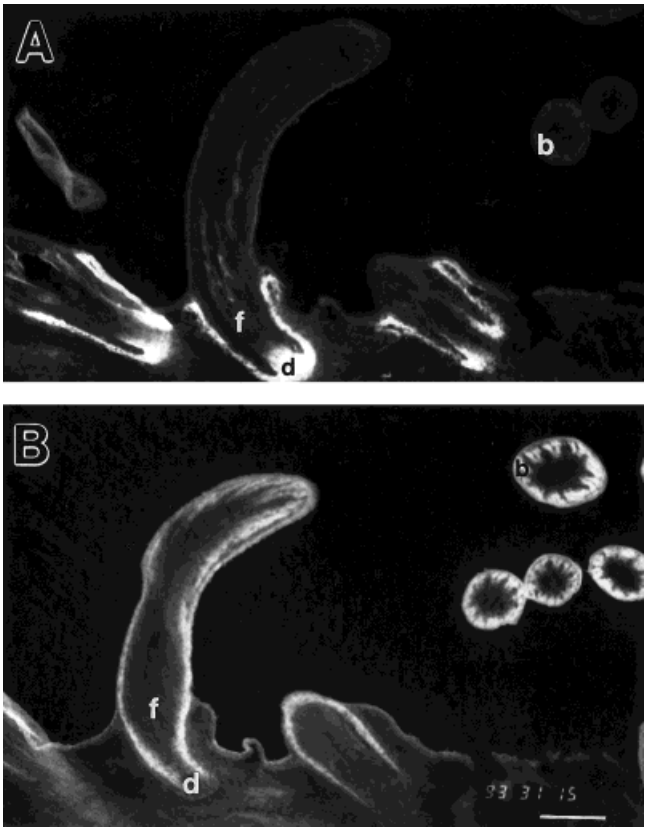

Fig. 2. Examples of localization of proteins and post-translational modification of proteins. Frozen sections of embryonic day 15 chicken skin are stained with antibodies to tenascin-C (A) and phosphorylated tyrosine (B) visualized by fluorescent conjugated secondary antibodies. Note the intricate structure of the feather follicle. Tenascin-C is expressed distinctly in the dermal papilla and feather follicle sheath. Phosphorylated tyr is in the epithelium. Staining of adjacent sections is particularly valuable because it allows for comparison of the distribution of different molecules. b, barb ridge; d, dermal papilla; f, follicle. Size bar, 200 μm.

Tracing Cell Lineage. Classical lineage studies were performed using carbon particles (Spratt, 1955). Several new molecular methods offer new approaches.

Retroviral lineage tracing. Replication defective retroviral vectors offer the ability to trace lineage over an extended period of time since the virus is only passed on to daughter cells and is not diluted in subsequent generations (Fekete and Cepko, 1993; Galileo et al., 1990). Plasmids encoding replication defective retroviral vectors are transfected into packaging cell lines by lipofection (GIBCO). Cells expressing the viral Neo^r marker are selected for resistance to G418. Viral containing media are filter sterilized, concentrated by centrifugation and stored as frozen stock. Viral titers are assessed by infecting susceptible cells with different dilutions of the virus. A limited tissue area is transfected by microinjection of the retrovirus (Widelitz and Chuong, 1992). Detection of the viral infection is vector dependent. Those expressing β-galactosidase can be detected by X-gal histochemistry. Briefly, tissues are incubated in detection buffer (0.5 mg/ml 5-Bromo-4-

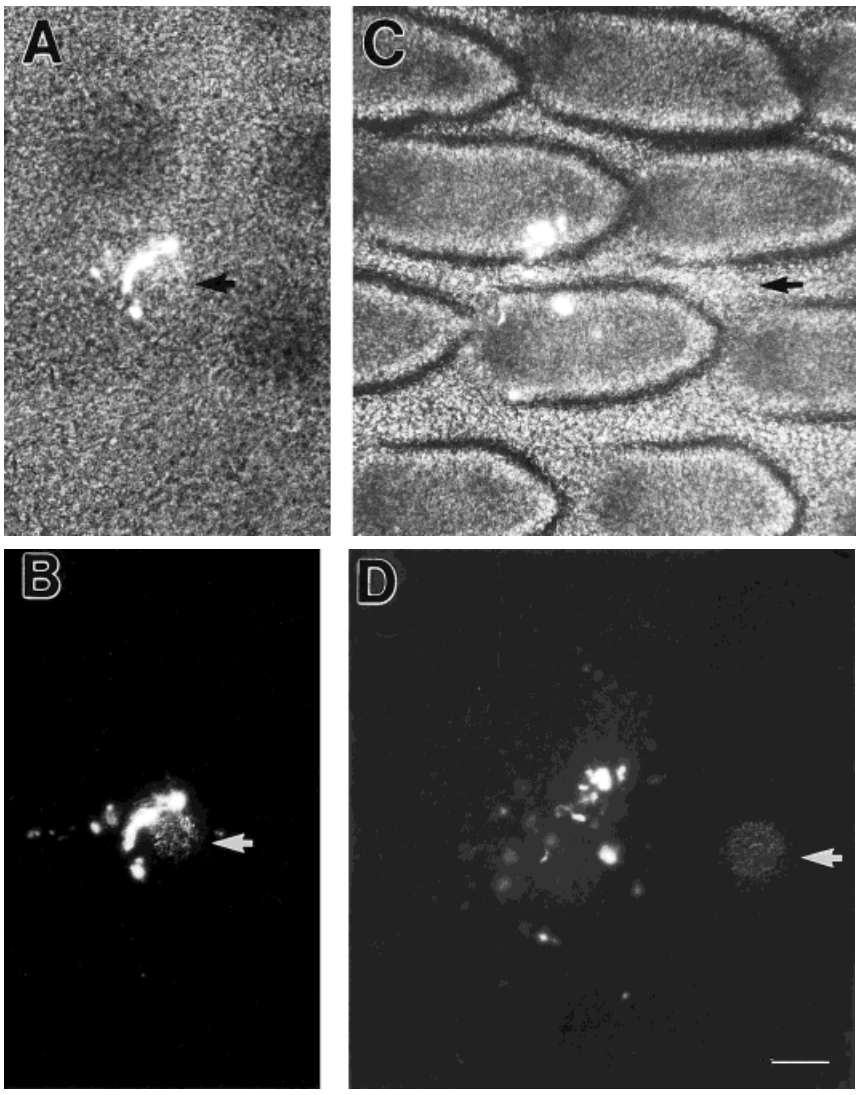

Fig. 3. Examples of monitoring dynamic cellular behavior in a skin explant. Embryonic day 7 skin explants were microinjected with Di I and photographed with bright field (A, C) and fluorescent (B and D) microscopy at the beginning of culture (A, B) and after 3 days in culture (C, D). The site of injection is labeled with an arrow. Note the anterior movement of the fluorescent cells during the three day interval relative to the injection site (indicated by the arrow in each panel). Labeled cells have migrated approximately 500–800 μm. Cells in these inter-condensation regions are incorporated in different feather buds anterior to the injection site. Size bar, 250 μm.

Chloro-3-Indolyl-β-D-galactopyranoside (X-gal), 25 mM potassium ferrocyanide, 25 mM potassium ferricyanide, 2 mM $MgCl_2$, 1 mM spermidine, 0.02% Nonidet P-40, and 0.01% sodium deoxycholate) made in PBS until a blue color develops.

To trace labeled cells under living conditions, β-galactosidase can also be detected with the fluorescent substrate, ImaGene (Molecular Probes, Westerfield et al., 1992). In the later case, skins are immersed in 20 μM ImaGene substrate for 30 minutes, rinsed, and viewed with fluorescence microscopy using a SIT-66 videocamera (Dage MTI, Michigan City, IN). A major disadvantage to retroviral lineage tracing, is that the location of the initial infected cells cannot be precisely identified.

Di I and Di O tracing. Lineage can also be examined by injecting the fluorescent, lipophilic, carbocyanine dyes, Di I and Di O, which stably integrate into cell membranes (Honig and Hume, 1989). This has been used frequently in developmental neurobiology, but can be applied to skin appendage research. These dyes offer several advantages: the precise identity of the labeled cells can be established, the dyes do not spread to neighboring cells and are therefore confined to labeled cells and descendants, they exhibit no toxic side effects, and they can be detected in living tissues, fixed whole-mounts or histological sections. By using different labeling moieties, neighboring lineages can be distinguished from one another within an individual organism. The disadvantage to dye labeling is that the dye is diluted by mitotic cell divisions and can only be followed for a limited time period. An example showing the dispersion of injected cells in cultured skin explants is shown in Fig. 3.

Tracing Proliferating Cells. Cell proliferation also plays a major role in the formation of histological structures. Proliferating cells in tissues can be detected by the incorporation of ^{3}H thymidine or bromodeoxyuridine (BrdU). Skin explants can be labeled in vitro by adding ^{3}H thymidine (10 μCi/ml) to the culture media. Skins are then fixed, sectioned, and dipped in NTB2 emulsion (Kodak) for autoradiography. Alternatively, labeling with BrdU offers the ability to view the proliferating cells using whole mount staining. Cells are incubated with BrdU for 1 h and fixed in 2% paraformaldehyde. After washing with PBS the cellular DNA is denatured in 1.5 M HCl for 30 minutes and neutralized with PBS for 1 hour. The BrdU labeled DNA is stained with monoclonal anti-BrdU antibody (Boehringer Mannheim), followed by biotinylated horse anti-mouse antibody, and then streptavidin conjugated to alkaline phosphatase which is detected with the NBT/BCIP substrate.

Tracing Apoptotic Cells. Programmed cell death plays a role in forming the space between feather barbs and is also involved in hair cycling. Apoptosis results in DNA fragmentation and the histological distribution of apoptotic cells can be determined by in situ labeling (Wijsman et al., 1993; Gold et al., 1993). Briefly, paraffin tissues are washed in xylene to remove the paraffin, rehydrated, and protease treated. The tissues are then labeled with deoxynucleotides in the presence of biotin-11-dUTP or digoxigenin-11-dUTP. Labeled nuclei are detected with avidin or anti-digoxigenin antibodies. Labeled apoptotic cells can be distinguished from necrotic cells by histological examination. Alternatively, since DNA fragmentation occurs hours prior to cell death in apoptotic cells, necrotic cells can be photolabeled with ethidium monoazide which will be excluded by living cells but intercalates into the DNA of necrotic cells.

RESULTS

Although skin appendages have distinct morphologies, they all follow common developmental processes. All skin appendages arise from the result of epithelial-mesenchymal interactions. Mesenchymal tissues become competent to form specific skin appendages very early in embryonic development, usually before any morphological changes in either epithelia or mesenchyma can be detected. We know mesenchyma have such an ability through recombination experiments (Sengel, 1976). Following this initial interaction, a domain of epithelial cells is defined which sometimes takes on a specialized placodal morphology. These epithelial cells together with the adjacent mesenchyme form a "skin appendage field." This is followed by proliferation and evagination (for those outward skin appendages) or invagination (for glands) of the epithelial sheet. Condensed mesenchymal cells surround the active growing region of epithelial cells. Axial orientation, shape, and size are then determined for each kind of skin appendage. Finally, in the skin appendage anlage, epithelial cells differentiate into specialized products and a skin appendage is formed.

In the following section, we will show the molecular histology of some exemplary molecules during skin appendage morphogenesis using feather and hair as models. One major difference between feather and hair germs is that, feather germs protrude out of the body surface to form the "bud," while hair germs invaginate into the body to form the "hair peg." However, the similarities are obvious when we compare their molecular histology during development. Nails form in a very similar fashion, and the structure appears like a giant follicle. Horns, have been considered to be analogous to a bundle of hair follicles arranged in a huge concentric ring (Chapman, 1986). The common ground underlying these different skin appendages can best be appreciated when their molecular processes are compared.

We also hope that the study of the molecular histology of skin appendages can produce a more precise definition of the morphogenetic process. The staging shown in Figs. 4–10 is defined according to morphological features. In the past, without molecular markers, it was not possible to define developmental stages according to molecular processes. This produces controversies which we think are not necessary. For example, it was often asked, does the epithelial placode or the dermal condensation appear first? Classical histology tells us that placodes can be seen before dermal condensations can be recognized. This was done by histological sections. However, even with careful sectioning, it probably takes more than a hundred condensed mesenchymal cells to form a recognizable structure. If these techniques are improved so initial dermal condensations can be detected as a small cellular aggregate (less than 10 cells), this early dermal condensation may precede the formation of the epithelial placode. In addition, if these early dermal condensation cells exhibit different adhesive properties or cellular behaviors than the rest of the mesenchymal cells, they may reflect differences in molecular expression. If we can identify these molecules, these will be the real initial events in the formation of feather germs. It is obvious that many molecular events precede morphological changes, the problem is to identify them. We will have to constantly revise the molecular description as more advanced and more sensitive detection methods become available.

Molecular Histology In Feather Morphogenesis

Since feather buds on the body surface develop at different paces, it is not informative to say that this feather bud is from a certain age chicken embryo. Defining developmental stages according to the feather itself will be more appropriate. However, staging of feather bud development has not been clearly defined. We take the liberty to suggest a staging system using morphological criteria and describe them in the figure legend of Fig. 4. In the following sections, we map the molecular expression patterns on to these stages.

Growth Factors and Receptors (Fig. 4)

Shh (Sonic hedgehog). Expression begins as a dot in the center of the early epithelial placode. At the short feather bud stage, expression is at the distal feather tip. By the long feather bud stage, expression has expanded to the distal tip plus the posterior epithelium. In the feather follicle, expression is in the marginal plate epithelium (Ting-Berreth and Chuong, 1996a).

TGF β (TGF β 1.2, 2 and 3). The distribution is uniform in the epithelium and in the placode mesen-

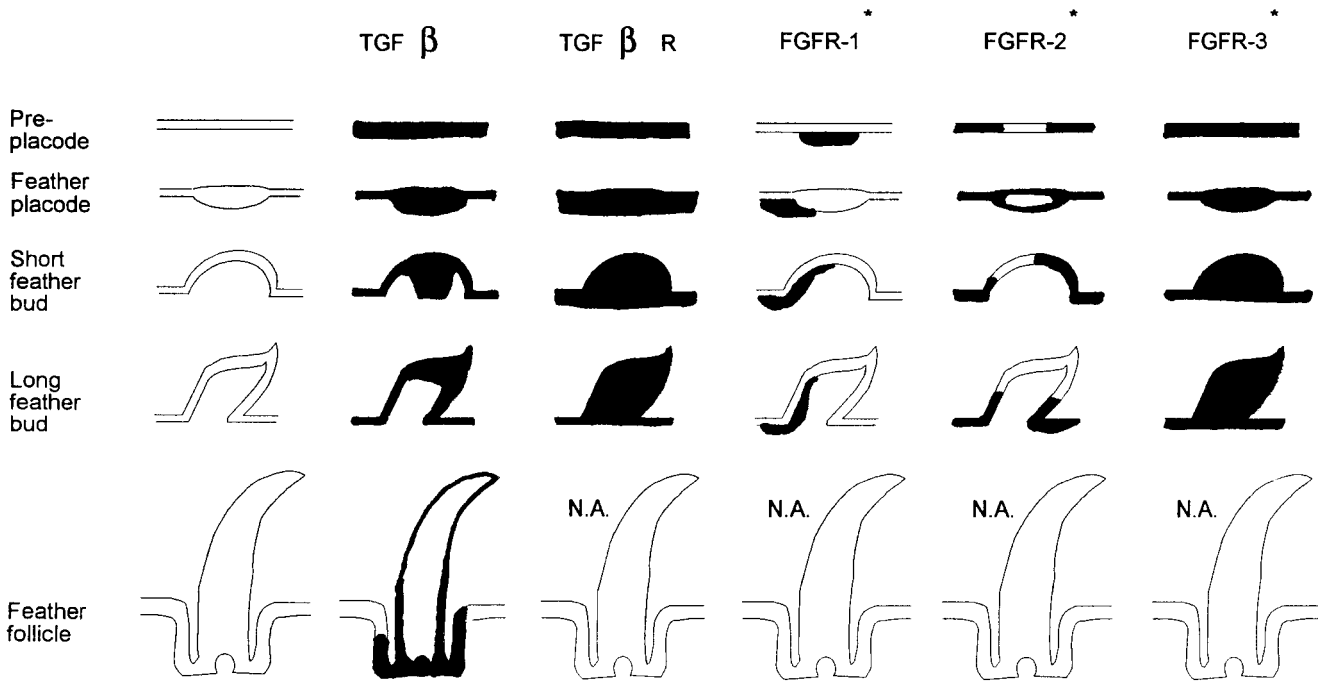

Fig. 4. Schematic representation of the distribution of growth factors and their receptors including Shh, TGF β (shown is 1.2, 2, and 3), TGF β R (TGF β receptor, shown is type 2), FGFR-1, FGFR-2, and FGFR-3 during different stages of feather morphogenesis. Transcript localization determined by in situ hybridization are indicated by an asterisk (*). Those without an asterisk are proteins detected by antibodies. N. A., not available.

The first column shows different stages of feather development and is described here, based on our own observations and those from previous scholars (Chuong, 1993; Sengel, 1976; Lucas and Stettenheim, 1972).

Pre-placode feather stage. Pre-placode mesenchymal tissue has the ability to form skin appendages, but has not yet induced individual, recognizable morphological features such as a placode. Although no morphological differences can be detected, transplantation experiments demonstrate that early mesenchymal tissues are already specified toward their developmental fates and that they can send "dermal signals" to the epithelium to form the epithelial placode. Morphologically, a homogeneous layer of dense dermis (about 2.5 nuclei/μm^3) (Sengel, 1976; Lucas and Stettenheim, 1972) composed of spindle shaped cells forms beneath the ectoderm. In the trunk, these cells derive from loosely arranged mesenchymal cells migrating in from the dermatome. In the dorsal skin, dense dermis first forms along the midline then spreads bilaterally.

Feather placode stage. This stage is defined as the period from the beginning of epithelial cell elongation to the time the feather germ starts to bulge out from the body surface. The cells within the placode will undergo different growth control regulation than cells found in the "inter-appendage" epithelia. Placodal cells eventually give rise to the skin appendages themselves by elongating from a cuboidal to a columnar shape. The non-placode epithelial cells remain to form the smooth skin surface.

Short feather bud stage. This stage is defined as the period from the formation of the feather bulge to the time when the length (or height) of the feather bud equals the base of the feather bud. This is the time dermal cells actively condense beneath the placodal epithelia. Dermal condensations probably result from both centripetal cell migration and cell proliferation. It was shown that there is about a 24 hr period when cell proliferation in the center of the condensation stops. This is followed by active cell proliferation and the rapid increase of mesenchymal cells inside the feather germ (to reach about 5.2 nuclei/μm^3) (Sengel, 1976). This stage can be further divided into two stages marked by the asymmetric appearance in the latter half of this stage. This stage is equivalent to the hair peg stage.

Long feather bud stage. This is defined as the period from the time that the length of the feather bud grows longer than the base to the time when the invagination of the feather base begins. In this stage, epithelial cells proliferate rapidly and soon the epithelial cells outnumber the mesenchymal cells. The active growing points appear to be on the distal end of the bud first, but soon relocate to the flanking region of the feather buds, near the junction of feather buds and skin surface. High cellular oncogene activity is observed in these regions. New cells are generated and inserted here. The major feature of this stage is the enlargement of feather buds and production of epithelial cells which form the feather itself. This stage is equivalent to the bulbous hair peg stage.

Feather follicle stage. This is defined to cover the period from the time the invagination of the feather base begins till the mature follicle forms. As feather buds elongate, the buds "sink" into the dermis. The epithelia flanking the feather buds invaginate to wrap around the feather germ and form the outer and inner feather sheath. Together they form the feather follicle in which the dermal papilla cells are at the base. Stem cells migrate or are pushed downward from the outer sheath. Induction occurs between epithelial cells adjacent to the side of the dermal papilla and the dermal papilla. These epithelial cells are named papillar ectoderm and are equivalent to the "germinal epithelium" of the hair follicle (Reynolds and Jahoda, 1991). Above the dermal papilla, new epithelial cells are inserted and this region is named the "collar," equivalent to the matrix of the hair. This renewal ability allows the molting cycle of skin appendages and the ability to regenerate following injury.

chyme at the placode stage. In the feather bud stages, the distribution becomes more limited to the feather germ region, and the expression decreases remarkably in the interbud mesenchyme. Expression is particularly strong at the dermal-epidermal junction (Ting-Berreth and Chuong, 1996b). The example shown here applies an antibody that recognizes TGF β 1.2, 2 and 3 (R & D).

TGF β receptor. Type 2 expression is uniformly distributed in the epithelium and mesenchyme of preplacode and placode stage feather buds. In the early feather bud stages, distribution remains uniformly distributed between bud regions and interbud regions. It is not until the late long feather bud stage that TGF β receptors become more enriched in the feather germ

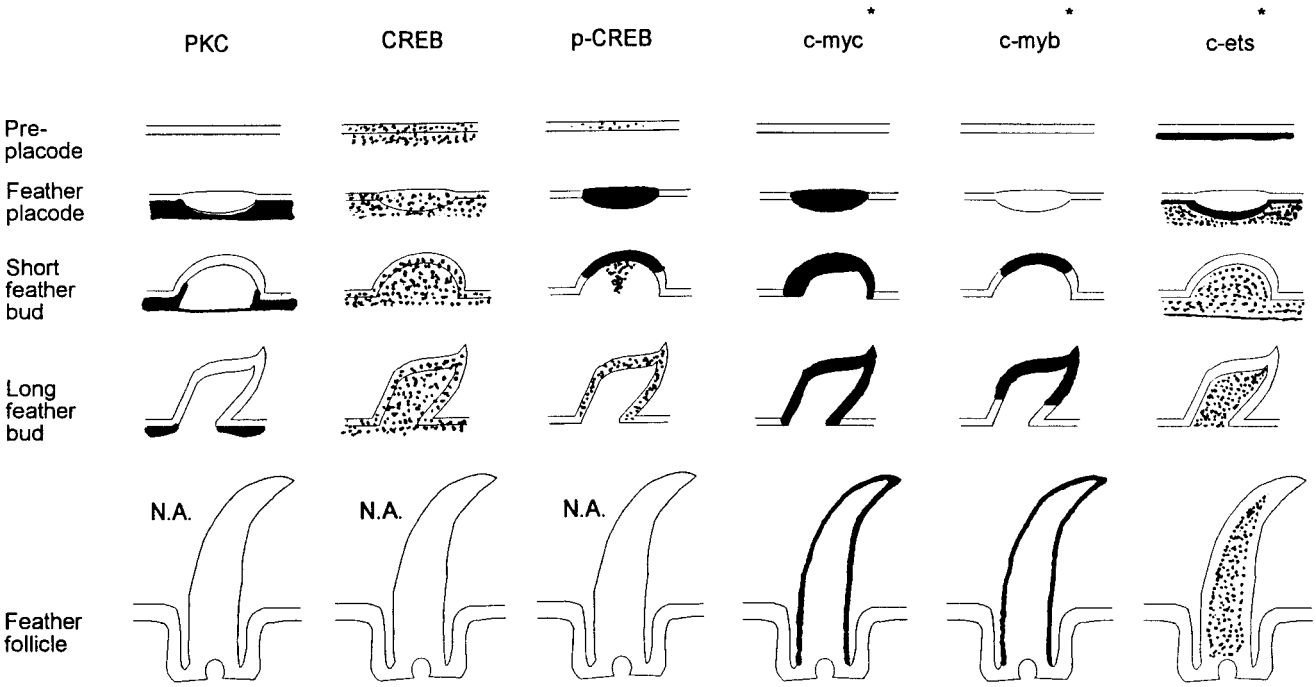

Fig. 5. Schematic representation of the distribution of intracellular signaling molecules including PKC, CREB, p-CREB, c-myc, c-myb, and c-ets during feather morphogenesis. Transcript localization determined by in situ hybridization are indicated by an asterisk (*). Others are done by immunocytochemistry. N. A., not available.

regions. Thus it appears that the TGF β ligand exerts more specificity than its receptor in feather development (Ting-Berreth and Chuong, 1996b). TGF β receptors type 1–3 have been identified. The example shown here applies an antibody that recognizes the type 2 TGF β receptor (UBI).

FGF Receptors. Type 1 is mainly seen in the mesenchyme beneath the putative placode at the pre-placode stage. Expression then is found in the anterior mesenchyme of the developing placode and remains there until the feather bud stage (Noji et al., 1993; Patstone et al., 1993). Type 2 is expressed in a periodic fashion within the epithelia at the pre-placode stage. In the short bud stage, it is present in the interbud epithelia but is also seen in the posterior feather bud epithelium. In the long feather bud stage, it is in the interbud epithelia and the epithelia flanking the feather bud (Noji et al., 1993). In contrast Patstone et al., 1993 report that type 2 was predominantly expressed in the anterior epithelium, but was visible throughout the bud and interbud regions. Type 3 was found ubiquitously distributed over the epithelia and mesenchyme in all stages (Noji et al., 1993). Patstone et al., 1993 found it at the apex of the feather and in the flanking epithelium and mesenchyme, but not throughout the epithelium and mesenchyme. Differences in sensitivity between their methodologies may lead to these discrepancies.

Intracellular Signaling Molecules (Fig. 5)

PKC (Protein kinase C). Using a pan-PKC antibody, PKC is absent in the pre-placode stage and is first expressed in a thick mesenchymal layer when feathers are about to form. At the site of placode formation, PKC diminished, suggesting that the disappearance of PKC permits the formation of skin appendages. Our pharmacological study proved this point (Noveen et al., 1995b).

CREB (c-AMP responsive element binding protein). Using antibodies to this transcription factor (Ginty et al., 1993), the distribution is shown to be ubiquitously distributed in the epithelia and mesenchyme in all stages (Noveen et al., 1995b).

p-CREB. This antibody reacts with the phosphorylated epitope of CREB which is its active form (Ginty et al., 1993). In the pre-placode stage, phosphorylation begins to be detected in the putative placode region. Phosphorylation then intensifies in both epithelium and mesenchyme, but is limited to the feather placode and bud region. In the long bud stage, most of the staining is in the bud epithelia (Noveen et al., 1995b).

c-myc. Transcripts were not detected until the placode stage, where they were located in the epithelium. They are enriched in the feather bud region compared to the interbud region. The epithelial expression remains through the feather follicle stage (Desbiens et al., 1991).

c-myb. Transcripts begin to appear in the short bud stage, when the mRNA is localized to the epidermis of the distal bud. The region of expression increases in the long feather bud stage and the follicle stage to include most of the feather epithelium. The interbud epithelium remains negative (Desbiens et al., 1991).

c-ets. Transcripts were mainly expressed in the mesenchyme. The expression is high in mesenchyme beneath the feather placode and in the feather bud, although there is also some expression in the interbud

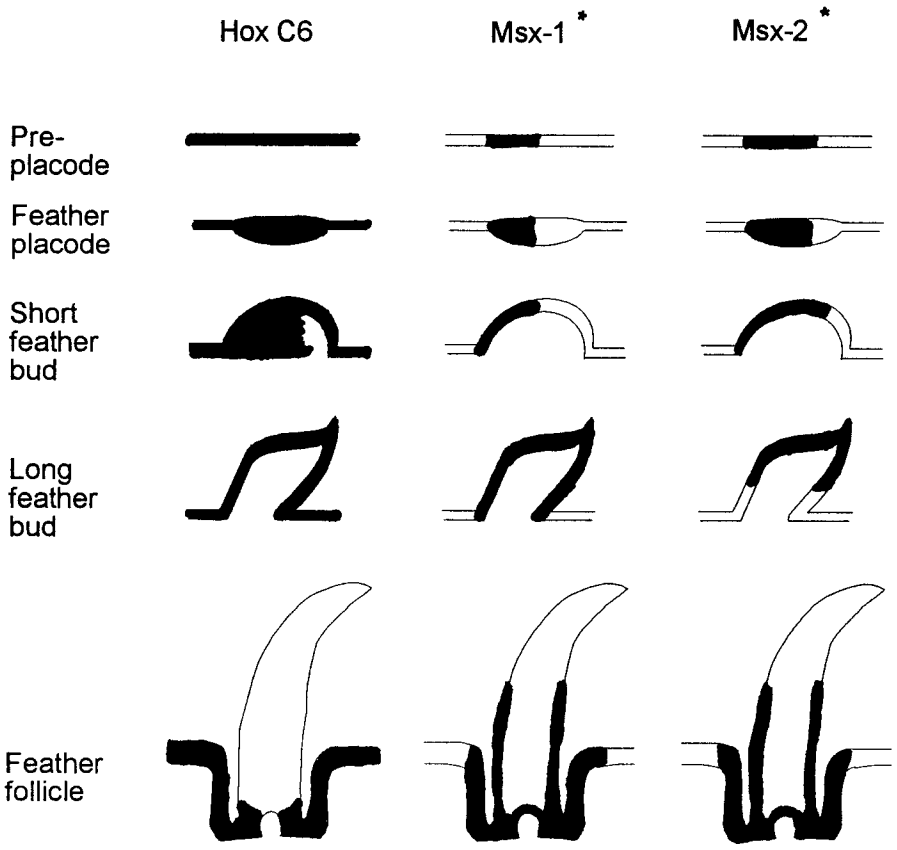

Fig. 6. Schematic representation of the distribution of homeobox proteins and hox genes including Hox C6, Msx-1 and Msx-2 during different stages of feather morphogenesis. Transcript localization determined by in situ hybridization are indicated by an asterisk (*). Hox C6 is done by immunocytochemistry.

mesenchyme. In the follicle stage ets is mainly in the feather pulp. ets is also expressed in the endothelial cells (Desbiens et al., 1991).

Homeobox Genes (Fig. 6)

Hox genes. Using Hox C6 as an example, distribution in the epithelium is uniform from the pre-placode stage to the long bud stage. At the follicle stage, Hox C6 is in the feather collar region and disappears in the more differentiated distal feather filament epithelium. The interesting expression pattern is in the mesenchyme. At the short feather bud stage, there is a transient Hox C6 expression pattern which forms an anterior-posterior Hox gradient across the feather bud and a second, more interesting expression pattern which forms a gradient across the anterior-posterior body axis (Chuong et al., 1990). In the dorsal trunk, feather buds at the cervical level express Hox C6 throughout the bud mesenchyme, at the thoracic level, feather buds express Hox C6 as a gradient, and at the caudal level, feather buds do not express Hox C6. Hox D4 has a similar position specific expression pattern which is out of phase with the Hox C6 gradient (Chuong, 1991). A Hox code hypothesis was proposed to suggest that these Hox expression patterns are used to determine feather phenotypes (Chuong et al., 1993).

Msx genes. Both Msx-1 and Msx-2 transcripts were found specifically in the placode epithelia, but not the interplacode epithelia. In the small feather bud, they are asymmetrically expressed in the anterior feather bud epithelium. In the long feather bud stage, expression covers the distal feather bud epithelium and the asymmetry is lost. In the follicle stage, Msx genes are expressed mainly in the collar region. The expression of Msx-1 and Msx-2 are similar but non-identical and there is no observed body position specific expression pattern (Noveen et al., 1995a).

Adhesion Molecules (Fig. 7)

L-CAM/E-cadherin. This calcium dependent adhesion molecule is expressed in all of the epidermis at different stages. Staining intensifies in the placode region (Chuong and Edelman, 1985).

N-CAM. This immunoglobulin superfamily adhesion molecule is expressed in both epithelium and mesenchyme. At the pre-placode stage, N-CAM was expressed homogeneously in the mesenchymal cells immediately beneath the epithelium. As the placode forms, expression becomes limited to dermal condensations. At the short feather bud stage, N-CAM is asymmetrically expressed in the anterior bud. At the follicle stage,

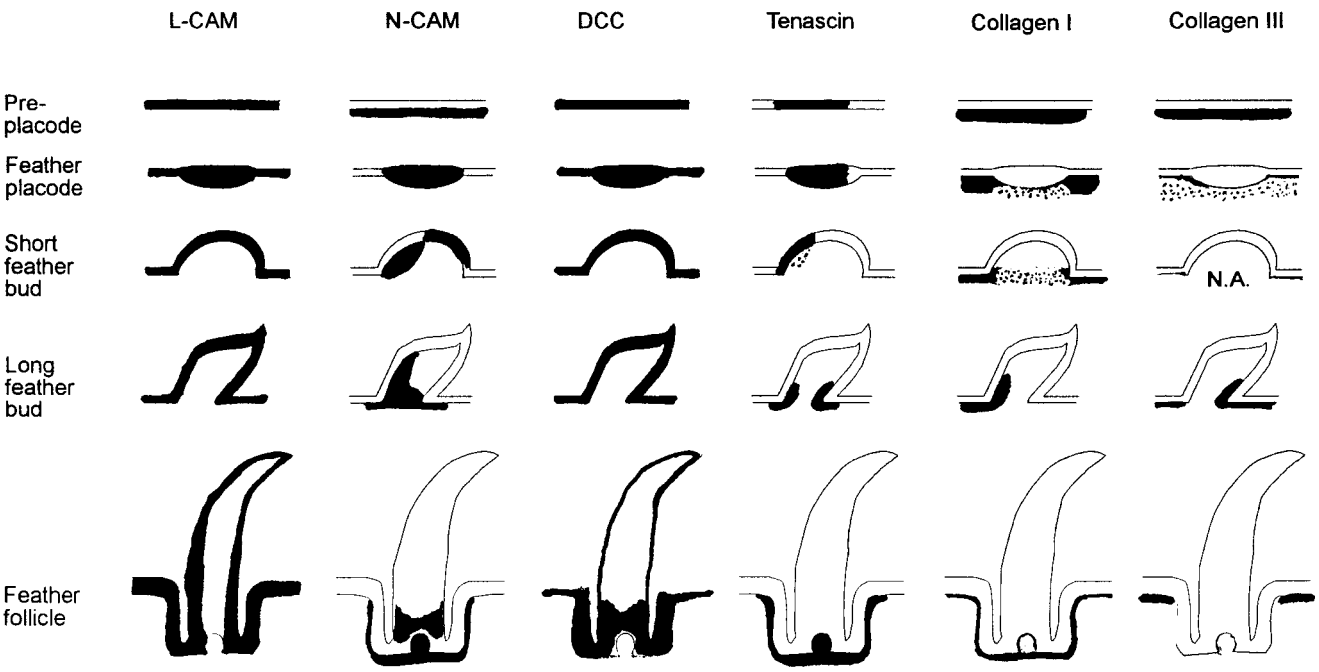

Fig. 7. Schematic representation of the distribution of adhesion and extracellular matrix molecules including L-CAM, N-CAM, DCC, Tenascin, Collagen I and Collagen III during feather morphogenesis. Done by immuno-cytochemistry. See text for references.

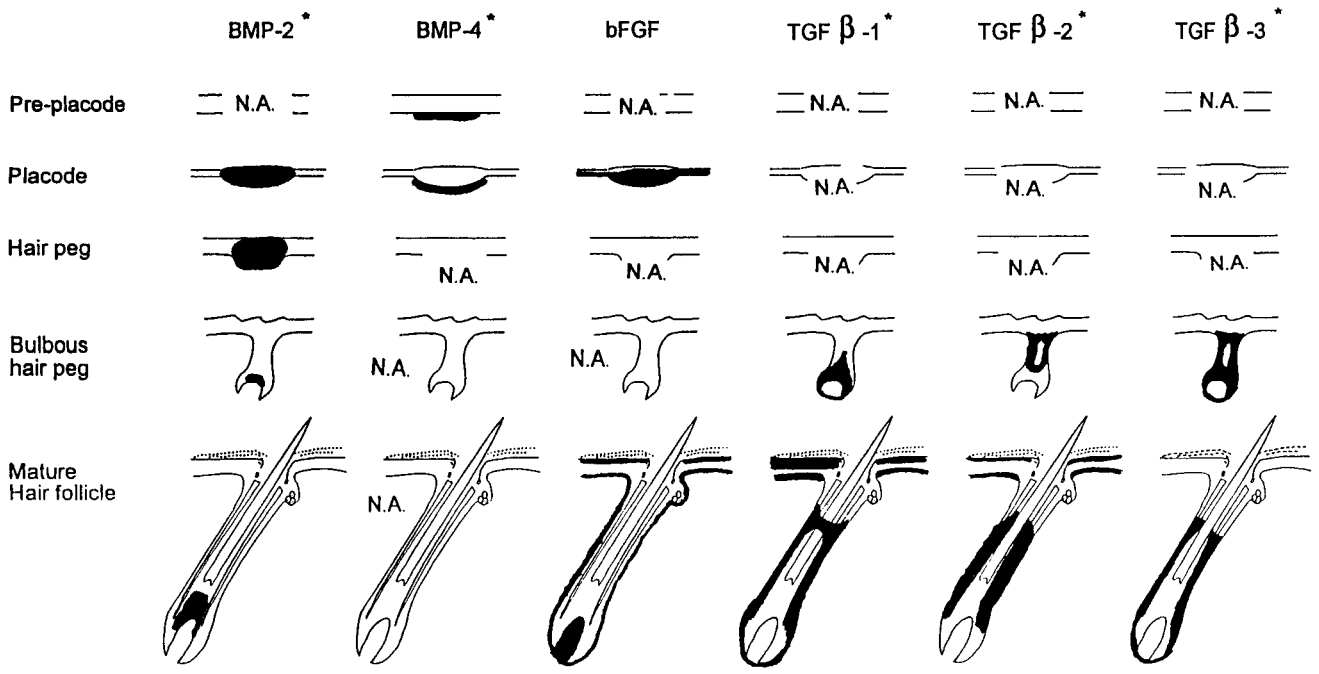

Fig. 8. Schematic representation of the distribution of growth factors and receptors including BMP-2, BMP-4, bFGF (basic FGF, or FGF 2), TGF β-1, TGF β-2, and TGF β-3 during hair morphogenesis. The staging is based on Davidson and Hardy, 1952 and Hardy, 1992. Transcript localization determined by in situ hybridization are indicated by an asterisk (*).

N-CAM is enriched in the dermal papilla and in the mesenchyme surrounding the feather sheath (Chuong and Edelman, 1985). NCAM is also expressed in the epithelium in a dynamic fashion. It is seen in the placode epithelium, distal feather bud epithelium and the collar epithelium (Jiang and Chuong, 1992; Chuong and Edelman, 1985).

DCC (a gene deleted in Colorectal Carcinoma). This is a gene deleted in chromosome 18q of colon cancer and the gene turns out to be homologous to NCAM (Fearon

et al., 1990). DCC is also expressed uniformly in the skin epithelium. In the mature follicle, expression is enriched in the collar epithelium and the follicle epithelium. In the interbud epithelium, DCC is enriched in the basal layer (Chuong et al., 1994).

Extracellular Matrix (Fig. 7)

Tenascin (Tn-C). In the early stage, tenascin is expressed in the placode epithelium. In the small feather bud stage, tenascin becomes enhanced in the anterior bud epithelium, then disappears from the epithelium. Tenascin begins to appear in the anterior bud mesenchyme during the short bud stage. At the long bud stage, tenascin appears in both flanking mesenchymal regions of the feather buds. At the follicle stage, tenascin is enriched in the dermal papilla and the dense mesenchyme surrounding the follicle (Jiang and Chuong, 1992).

Collagen. Type I is expressed at the epidermal-dermal junction in the pre-placode stage. Staining becomes more intense in the interbud regions during the placode and short feather stages. By the long feather stage, collagen type I is present in the anterior feather bud mesenchyme. Expression is in the dermis at the base of the feather and in the dermal papilla at the follicle stage. Type III first appears at the epidermal-dermal junction and is more intense in the interbud region during the placode stage. Staining is mainly in the interbud mesenchyme during the short feather bud stage and then in the posterior bud mesenchyme during the long feather stage. Only the interbud mesenchyme expresses collagen type III at the follicle stage (Mauger et al., 1982).

Differentiation Products

Keratin. Feather keratin is not as well characterized as hair keratin. However, α keratin and β keratin have been identified. Overall α keratin is expressed in the skin, and β keratin is expressed in the differentiated regions of skin appendages including mature feather filaments and scales (Haake et al., 1984).

Molecular Histology in Hair Morphogenesis

For comparison with the molecular histology of feathers and for use as a reference, we have compiled the following information.

Growth Factors and Receptors (Fig. 8)

BMP-2. Transcripts are found in the placode epidermis during the early developmental stage. In the bulbous hair peg stage, it is in the matrix region. In the mature follicle, BMP-2 is found in the precortex region (Lyons et al., 1990).

BMP-4. Transcripts are found specifically in the mesenchyme beneath the placode during early hair morphogenesis (Jones et al., 1991).

FGF. FGF-1 (or acidic FGF) is expressed in towards the tip of the inner root sheath (du Cros et al., 1993). FGF-2 (or basic FGF) is expressed in the extracellular compartment of the stratum granulosum, in the mesenchyme at the epidermal-dermal junction of the mature hair follicle of mouse skin and in the dermal papilla (Gonzalez et al., 1990; du Cros et al., 1993). FGF-5 is expressed in the outer hair sheath and has been implicated in the regulation of the hair cycle. The FGF 5 gene is the locus for the long hair, Angora mouse (Hebert et al., 1994). FGF-7 is expressed in the dermal papilla during early and middle anaphase (Rosenquist and Martin, 1996). FGF-3, -4, -6, -8, and -9 are not detected in the hair follicle.

FGF receptor. In the late anagen phase of the first hair cycle, FGFR 1 is expressed in the dermal papilla, FGFR 2 is in the matrix cells near the dermal papilla, FGFR 3 and 4 are expressed at the periphery of the hair bulb, and FGFR 4 is also expressed in the inner and outer root sheath (Rosenquist and Martin, 1996).

TGFα. Targeted disruption of TGFα in transgenic mice produces disoriented hair follicles and wavy hair resembling the wa-1 mutant phenotype (Luetteke et al., 1993; Mann et al., 1993). TGFα functions through the EGF receptor. A second mutation producing wavy hair, wa-2, is caused by a reduced tyrosine kinase activity from the EGF receptor (Luetteke et al., 1994). These findings demonstrate the importance of the TGFα pathway to hair development.

TGFβ. In the mouse skin, TGFβ-1 is expressed in the inner root sheath of the bulbous hair peg and in the dermal sheath in the mature follicle. TGFβ-2 is expressed in the outer root sheath of the bulbous hair peg and in the dermal sheath in the mature follicle. TGFβ-3 is expressed in both the inner and outer root sheath of the bulbous hair peg and in the dermal sheath in the mature follicle (Lyons et al., 1990).

Intracellular Signaling Molecules

PKC (protein kinase C). The pattern in mouse skin is similar to chicken skin with expression enriched in the interfollicular mesenchyme (our unpublished results).

bcl-2. Expression was regulated by the hair cycle. In the epithelium of the bulb, basal cells of the outer root sheath, and the bulge, staining was observed during anagen, but was diminished in catagen and absent in telogen. Staining within the dermal papilla was present at each phase of the hair cycle (Stenn et al., 1994).

Homeobox Genes

Hox genes. In skin, several Hox genes have been shown to be expressed in a dynamic fashion (Detmer et al., 1993; Rieger et al., 1994). In the hair, expression of Hox 3.1 in the dermal papilla is dependent on the body position: the highest expression levels are present in the posterior skin (Bieberich et al., 1991). We also observed body position specific expression of Hox C6 and D4 in the whisker dermal papilla.

Adhesion Molecules (Fig. 9)

E-cadherin. As determined in developing human skin, E-cadherin is in the suprabasal epidermal cells at the pre-placode stage and at the intermediate level epidermal cells at the hair peg stage. Expression is strong in the epidermis and in the core of the bulbous hair peg (Kaplan and Holbrook, 1994).

I-CAM. It is intermittently expressed in the periderm at the pre-placode stage. In addition to the periderm staining, expression is also seen in the outer root cells of the hair peg (Kaplan and Holbrook, 1994).

N-CAM. It is expressed weakly and homogeneously throughout the mesenchyme in the pre-placode stage. At the hair peg stage, staining diminishes in the inter-appendage regions but becomes higher in the mesenchyme surrounding the peg. In the bulbous hair peg stage and follicle stage, expression is intense in the

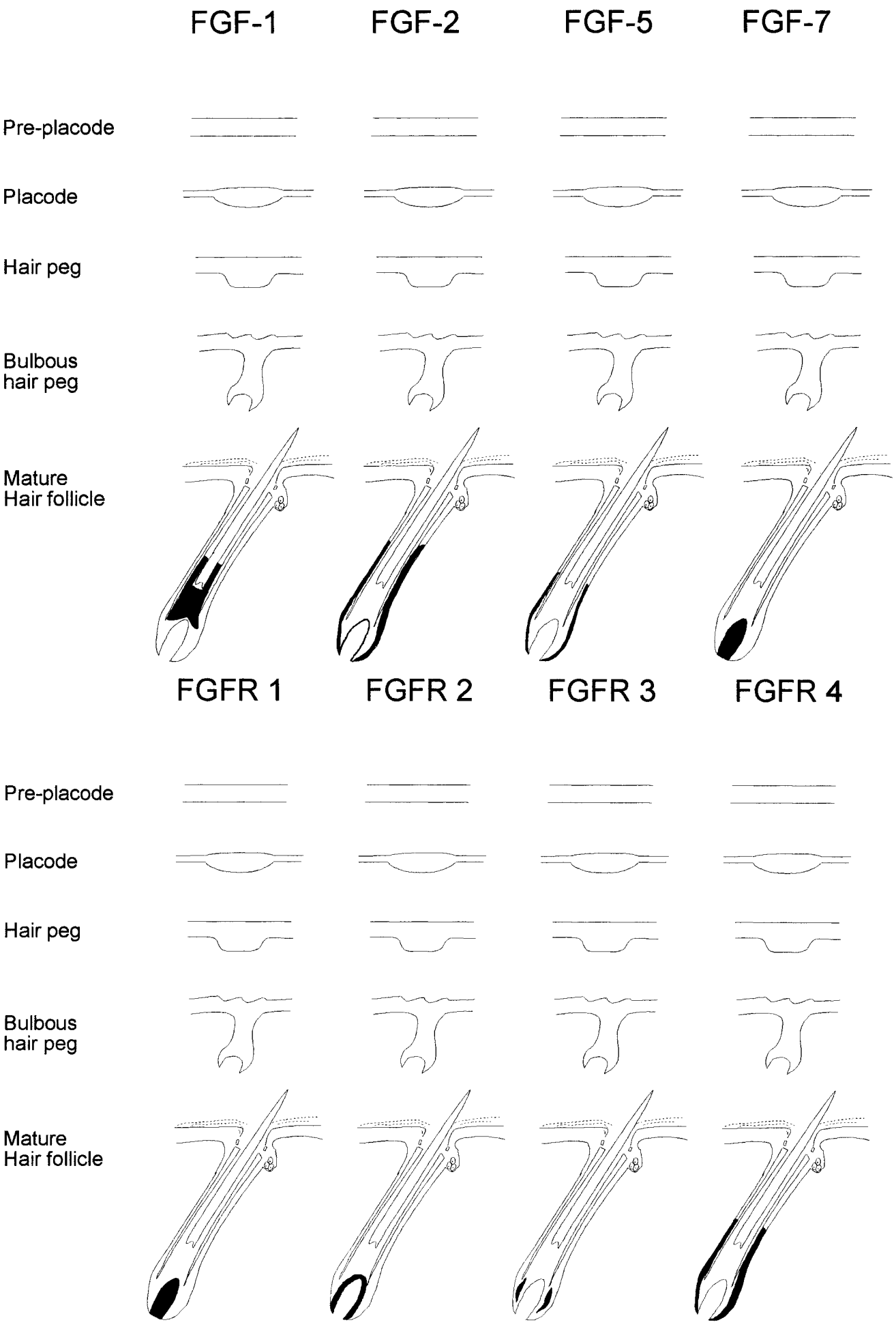
FGF-1
FGF-2
FGF-5
FGF-7
Pre-placode
Placode
Hair peg
Bulbous hair peg
Mature Hair follicle
FGFR 1
FGFR 2
FGFR 3
FGFR 4
Pre-placode
Placode
Hair peg
Bulbous hair peg
Mature Hair follicle

Fig. 9.

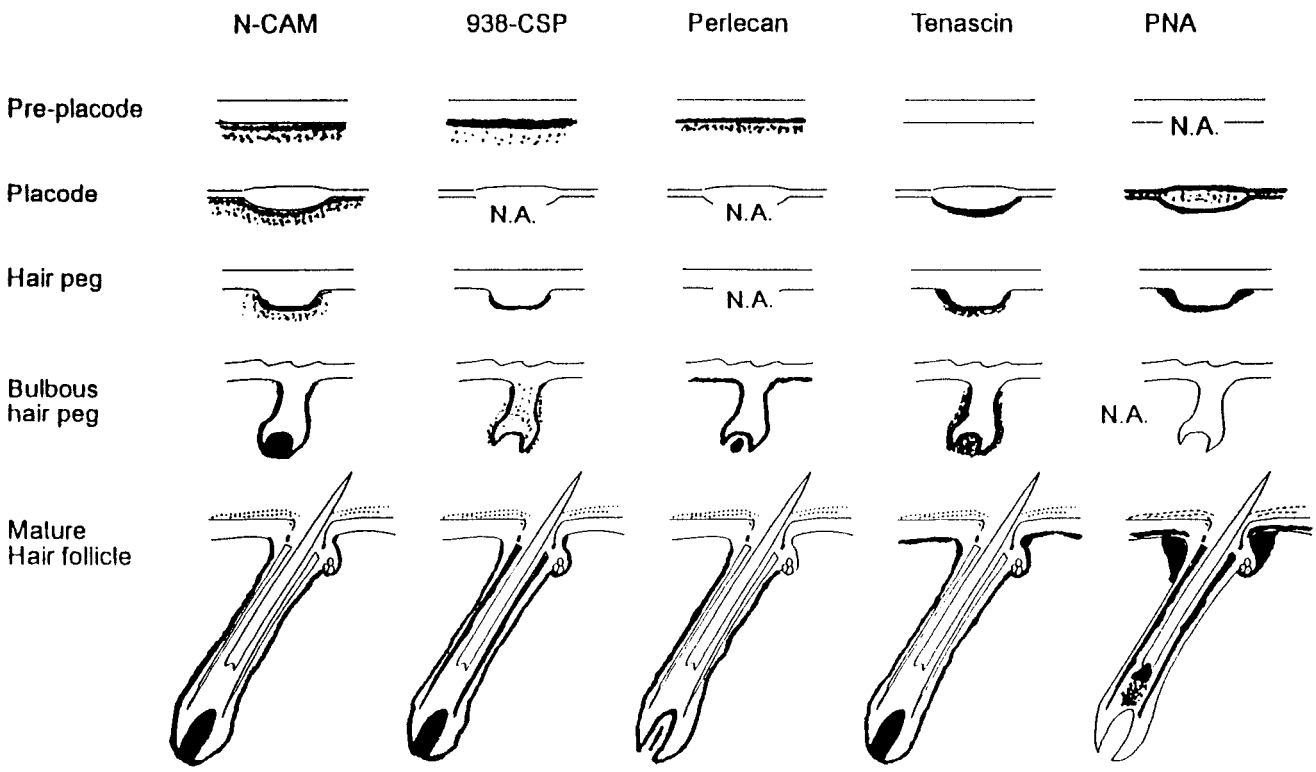

Fig. 10. Schematic representation of the distribution of adhesion molecules, extracellular matrix molecules and lectins including N-CAM, 938-CSP (Chondroitin sulfate proteoglycan), Perlecan, Tenascin, and PNA (peanut agglutinin) during hair morphogenesis.

dermal papilla (Chuong et al., 1991; Kaplan and Holbrook, 1994).

Extracellular Matrix (Fig. 9)

Chondroitin sulfate proteoglycan. One example 938-CSP is shown. Expression is dispersed through the pre-placode epidermis and dermis and is concentrated at the epidermal-dermal junction. Staining diminishes in the inter-appendage regions at the hair peg stage but remains in the peg region. Staining is mainly in the dermis in the mature follicle (Kaplan and Holbrook, 1994).

Collagen-type I and type III. They are present in the dermal sheath and dermal papilla of the mature human hair follicle (Messenger et al., 1991).

Perlecan. It is present in the epidermal-dermal junction at the pre-placode hair stage. Staining is greatly enhanced by the bulbous hair peg stage and is also apparent in the dermal papilla in the mature follicle of human hair (Kaplan and Holbrook, 1994).

Tenascin. Expression first appears in the dermis at the epidermal-dermal junction during the placode stage. Expression diminishes in the inter-appendage regions through the hair peg stage. Here, staining also is positive in the dermal papilla. By the mature hair follicle stage, staining is still positive in the dermal papilla, and is also present in the inter-appendage regions at the epidermal-dermal junction in human and mouse hair (Kaplan and Holbrook, 1994; Chuong et al., 1991).

Differentiation Products. The major differentiation products are keratin and its associated proteins. These form the end point of the formation of hair. Readers interested in this topic should be referred to references such as Lynch et al., 1986; Lane et al., 1991.

Modification of Cell Surface Molecules (Fig. 9)

Peanut agglutinin (PNA). Glycoproteins containing PNA binding sites are found in the superficial layers of the mesenchyme and the apical surface of the epithelium in the hair placode. In the hair follicle, it is found in the hair matrix, the inner root sheath, and the basal lamina of the outer root sheath. It is also highly expressed at the unique site of hair follicle invagination (Chuong et al., 1991).

DISCUSSION

The above summary is meant to present useful references for those who are interested in the molecular mechanism of skin appendage morphogenesis. We can appreciate that skin appendages with different appearances arise through the use of similar molecules. In fact, by comparing the molecules at work during skin appendage formation with the molecules involved in primary induction, we can also see that similar groups

Fig. 9. Schematic representation of the distribution of FGF and FGFR transcripts in the hair follicle. Transcript localization was determined by in situ hybridization (Rosenquist and Martin, 1996).

of molecules are used repetitively. Thus secondary induction, which produces organs such as skin appendages through tissue interactions, is a variation of the fundamental theme of the primary induction. The induction allows secondary fields for organogenesis to be set on top of the original embryonic field. It is through this principle of "field on top of field" that organisms can build various organs to cope with the complex and constantly changing environment during evolution.

Skin is the interface between the organism and the environment. The need to cope with the environment is acute and there is a need for successful species to be able to generate integuments with different phenotypes rapidly. It was proposed that the formation of skin appendages can be divided into two stages (Dhouailly, 1973; Hardy, 1992). In the first stage, an epithelial placode is induced from which a skin appendage can form. The phenotypes of this skin appendage are defined in the second stage and can be altered if perturbed early enough. We hypothesize that the phenotypes of skin appendages depend on the body position-specific skin homeobox codes (Chuong, 1993). We suggested that the unique expression pattern of the homeobox genes (Scott, 1992) in a skin appendage domain determines the phenotype of that skin appendage (Chuong et al., 1993). The combination of these transcription factors lead to the differential expression of other molecules such as adhesion molecules or cyclins which then contribute to the formation of different skin appendage phenotypes. This mechanism for skin appendage diversity is more versatile than dependence upon an appendage specific gene, such as a "hair specific gene" and allows the rapid generation of a large variety of skin appendages available for natural selection in a constantly changing environment during evolution.

Genetic variation within a species also influences skin appendage phenotypes. For example, there are nearly one hundred mouse mutants with altered skin appendages (Sundberg, 1994; Sundberg and King, 1996). Molecular and pathological research of these mice will provide insight into the molecular defects underlying skin appendage formation. Furthermore, as transgenic mice are generated to test gene function, possible molecular links to skin, and skin appendage formation are observed as unexpected abnormalities in the integument. This is because defects of the skin are obvious and usually non-lethal. One dramatic example is the discovery that FGF 5 is present in the hair sheath. Mutation of FGF 5 leads to an elongation of the hair cycle at anagen phase and is responsible for the long hair of *Angora* mice (Hebert et al., 1994). This unexpected finding not only tells us that FGF 5 is involved physiologically in regulating hair growth, but it also sheds a fresh understanding on the function of FGF 5.

Studying the molecular mechanism of normal and abnormal skin appendages relies heavily on first establishing their molecular histology. The intricate molecular patterns will inspire us to hypothesize how these molecules are involved and will help us to design experiments to test these hypotheses. For example, following the expression patterns of relevant molecules, we tested the roles of Shh, TGFβ and FGFs and were able to show that they promote the formation of feather buds (Ting-Berreth and Chuong, 1996 a,b; Widelitz et al 1996. Finally, as gene therapy advances, gene delivery through a skin appendage may be advantageous. The molecular histology of skin appendages will also be useful to determine the distribution of RNA and protein and will aid in designing tissue specific gene delivery using tissue specific promoters (Vassar et al., 1989; McNab et al., 1990).

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