Early Events in Skin Appendage Formation: Induction of Epithelial Placodes and Condensation of Dermal Mesenchyme

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The formation of skin appendages represents a morphogenetic process through which a homogeneous system is converted into a patterned system. We have pursued molecules involved in the early placode induction and mesenchymal condensation stages of this process. We found that intracellular and extracellular signaling molecules collaborate to position the location of feather primordia and initiate mesenchymal condensations mediated by adhesion molecules. During the inductive stage, cells interact in a fashion best described by a reaction-diffusion mechanism. Thus in early feather morphogenesis, low level adhesion molecules drive cell interactions. The interactions were modulated by extracellular signaling molecules, which eventually increase the level of signaling molecules at sites of feather initiation and subsequently the level of adhesion molecules (Jiang *et al*, 1999a). These physico-chemical events lead to the formation of dermal condensations and epithelial placodes at sites of feather primordia, thus achieving the earliest and most fundamental events of skin appendage formation: induction. *Key words: adhesion molecules/BMP/embryonic induction/feather/FGF/hair/mesenchymal condensation/reaction diffusion/SHH/β-catenin. Journal of Investigative Dermatology Symposium Proceedings 4:302–306, 1999*

During the development of skin appendages, the flat putative skin is topologically transformed into different types of skin appendages, which are basically variations of a common theme (Chuong, 1998; Chuong and Noveen, 1999). The feather has been a good model for skin appendage formation (Chen and Choung, 1999). From the flat presumptive skin hexagonally arranged feather buds protrude out of the skin surface (**Fig 1**). During this process, through cell interactions and cell migration cells become part of the feather primordia or interprimordia region (Widelitz *et al*, 1997; Chuong and Widelitz, 1998; Dhouailly *et al*, 1998). Recently we have reviewed the developmental processes of different skin appendages (hair, feather, gland, nail, etc.) and have deduced the developmental stages shared by most skin appendages as follows: (i) induction stage (forming skin appendage primordia); (ii) morphogenesis stage (molding the primiordia into a particular shape); (iii) differentiation stage (differentiating the developing organ anlage into specific physical characteristics with specialized keratins or chemical properties); (iv) cycling (opportunities for shedding and renewal of appendages, modulatable by physiologic conditions) (Wu-Kuo and Chuong, 1999). In this mini review, we will focus only on the induction stages of feather morphogenesis (**Fig 2**). We will review how adhesion molecules and signaling molecules (**Table I**) work to achieve this early morphogenetic process (**Fig 2**).

ADHESION MOLECULES

We started this research by studying the roles of adhesion molecules, initially neural cell molecules (NCAM), during mesenchymal condensation. In addition to being expressed in the nervous system, we found that NCAM was expressed in several inductive sites of epithelial–mesenchymal interactions (Chuong and Edelman, 1985), including dermal condensations, precartilage condensations, kidney tubule condensations, etc. We used antibodies, in the form of Fab fragments, to perturb the process of mesenchymal condensation formation. Skin explant cultures were grown in which cells form dermal condensations in 2 d and feather buds in 4 d. With this as an assay, we demonstrated that antibodies to L-CAM can block the segregation of dermal condensations (Gallin *et al*, 1986). Antibody to DCC, another epithelial adhesion molecule, also led to the failure of dermal condensation formation, suggesting that intact epidermal cell interactions are important for epithelial–mesenchymal interactions (Chuong *et al*, 1994). Treatment of explants with antibodies to NCAM led to uneven sized dermal condensations. Antibodies to fibronectin and integrin β1 led to the separation of epithelium and mesenchyme, whereas antibodies to tenascin C arrested feather bud development (Jiang and Chuong, 1992). From these studies, we can appreciate the importance of cell re-arrangement and the roles of adhesion molecules in the formation of feather primordia (Chuong *et al*, 1998). To further understand the morphogenetic process, we began to search for upstream molecules that direct the expression of these adhesion molecules.

INTRACELLULAR SIGNALING MOLECULES

We took two approaches to examine the involvement of signaling pathways in early feather development (**Table I**). One is to explore the involvement of intracellular signaling molecules and the other is to explore the roles of extracellular signaling molecules. We screened several agonists and antagonists to intracellular signaling

molecules, and found the most interesting results by perturbing protein kinase A (PKA) and protein kinase C (PKC). We used antibodies that could differentiate between phosphorylated CREB and nonphosphorylated CREB. Surprisingly, we found p-CREB expressed in feather primordia at the time feathers were forming. Furthermore, cAMP added to the explant culture promoted the formation of feather domains and the fusion of feather buds. In contrast, antibodies to pan-PKC were used to show that PKC was initially widely distributed in the dermis before feather primordia

formed. When feather primordia started to form, PKC immunor-eactivity disappeared from feather regions. When the PKC agonist phorbol ester was added to the culture, fewer and smaller feather buds formed. These results led us to suggest that a net increase of PKA signaling will favor feather primordia formation, whereas a net increase of PKC activity will favor interprimordia formation (Noveen *et al*, 1995a); however, the PKA or PKC activity of a cell reflects an integration of extracellular signals resulting from cell interactions. We need to search for these extracellular signals.
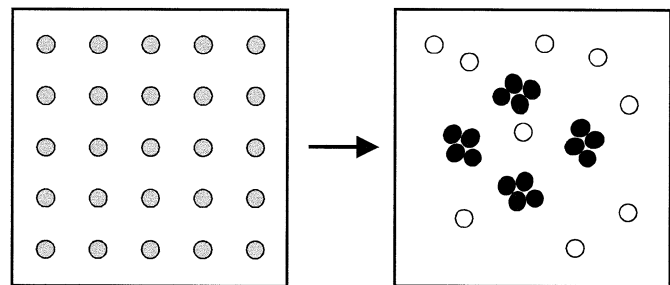
We also examined homeobox transcription factors. Msx-1 and Msx-2 were shown to be expressed in the placode epithelia specifically. At the feather bud stage, they were expressed in the anterior and distal placode. cAMP, a protein kinase A agonist, enhanced dermal condensation formation but suppressed feather bud elongation in the short bud stage. cAMP added to feather explants at this stage suppressed Msx expression, suggesting that Msx genes are associated with the growth of short feather buds (Noveen *et al*, 1995b). In our recent Msx-2 over-expressing transgenic mice, the hair follicles have reduced hair matrix size and varied hair length, implying the involvement of the proximo–distal axis. Whereas Msx genes were expressed in all feather buds, some Hox genes were expressed in restricted regions of the body (Chuong *et al*, 1990; Jiang *et al*, 1999b; Wang *et al*, 1999). The prevalence of a unique set of transcription factors in specific body regions may set different competence in these cells, so they respond differently when receiving the same extracellular inducing molecules.

Figure 1. Schematic drawing representing the early stages of feather primordia formation. The circles represents single cells. Gray represents the initial equivalent state. The square represents the feather field. Black circles represent dermal cells in feather primordia. White circles represent cells in interprimordial regions.

Figure 2. Flow chart representing major processes in successive stages of feather development. The left column represents the four major developmental stages common to skin appendages. Four common stages of skin appendage development are shown: (i) induction; (ii) morphogenesis; (iii) differentiation; and (iv) cycling (Wu-Kuo and Chuong, 1999). The right column shows events more specific to cell and molecular changes.
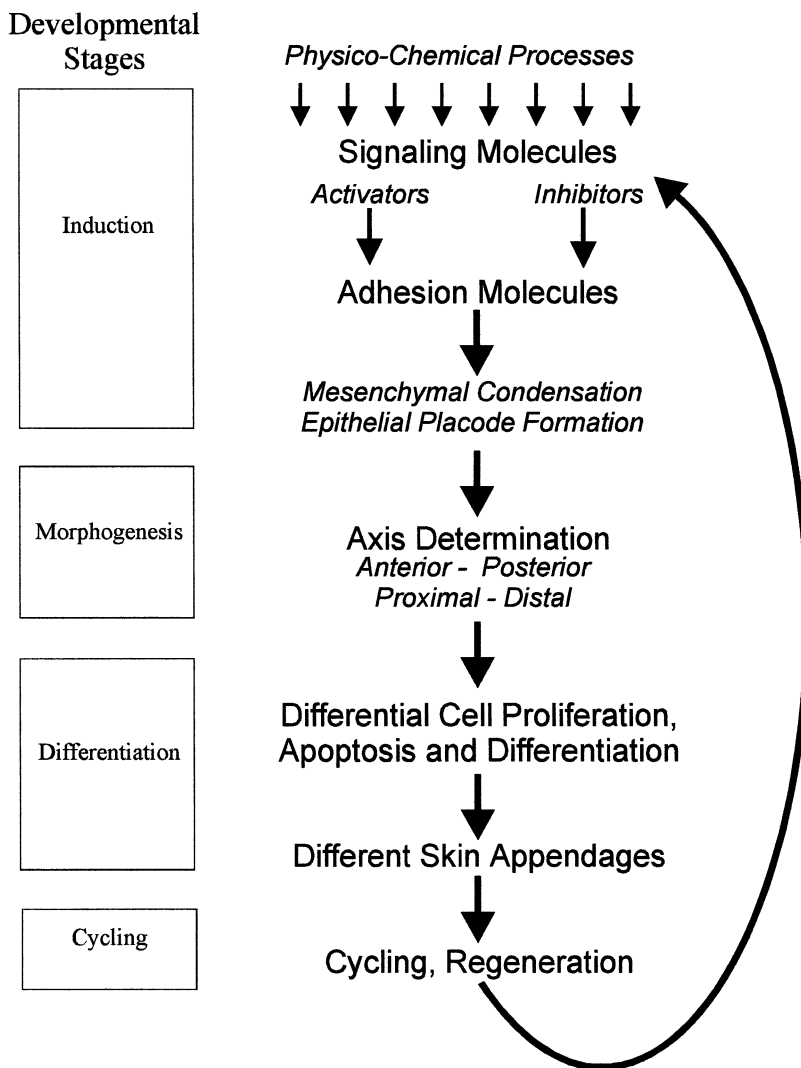
β-catenin also has been shown to play an important role in skin appendage morphogenesis. β-catenin interacts with cadherin, APC, and Lefs (or TCF) and integrates these signals (Polakis, 1999). β-catenin is normally targeted for ubiquitin-dependent degradation by APC. Wnt signaling or mutations in β-catenin or APC block this pathway and stabilize β-catenin. β-catenin can then translocate to the nucleus, associate with Lefs/TCFs to activate transcription of genes involved in growth control, apoptosis, and cell migration (Behrens *et al*, 1996). This pathway has been shown to regulate the growth of skin appendages. Transgenic mice expressing a constitutively active form of β-catenin produce ectopic hairs with unregulated growth control (Gat *et al*, 1998). Transgenic mice over-expressing Lef-1 from an AP1 promoter induced the growth of hairs from oral gum regions (Zhou *et al*, 1995). Mice lacking Lef-1 expression do not form whiskers (Kratochwil *et al*, 1996) or hairs (van Genderen *et al*, 1994). The differential expression of Lef/TCF family members may also contribute to regional specificity of skin appendages.

EXTRACELLULAR SIGNALING MOLECULES

We screened many extracellular signaling molecules (Ting-Berreth and Chuong, 1996a). One of them, TGF-β2, enhanced dermal condensations. This effect was more apparent when the growth factors were delivered in a localized form via beads than when added to the culture media, suggesting that cells may sense signaling molecule concentration differences. Because TGF-β2 was expressed in the placode epithelium, we hypothesized that TGF-β2 may be one of the molecules secreted by the placode to induce dermal condensations. To test this possibility, we peeled off all epidermis and treated the naked mesenchyme with TGF-β2 coated beads. Two days later, we then replaced the epithelium. The region exposed to TGF-β2 reformed feather buds, whereas regions not exposed just became chicken embryo fibroblasts (Ting-Berreth and Chuong, 1996a). This implies that if we can provide an appropriate environment during the wound healing process, we may be able to endow granulation tissue cells with some more regeneration capacities. This task will not be easy. Wound healing differs significantly between embryos and adults. Embryos are capable of scarless wound healing but adult wound healing invokes scarring. The expression of homeobox genes, cytokines, and extracellular matrix also differ between fetal and adult skin (Mackool *et al*, 1998; Stelnicki *et al*, 1998). It is likely that these differences lead to intrinsic differences in fetal and adult skin. The fact that scarring in the fetus begins at the time when the skin appendage inductive ability starts to decrease is consistent with this notion. If we can learn more about the developmental biology of skin appendages and are able to modulate the involved signaling molecules, we may be in a better position to guide wound repair and/or regeneration more effectively.

Table I. Major pathways in early skin appendage development

Signaling molecules
  FGF pathway: *FGF-1, -2, -4, KGF, FGFR, etc.*
  BMP pathway: *TGF β, BMP-2, -4, noggin, follistatin, BMPR, Smads, etc.*
  Protein kinases: *PKA, PKC*
  Hedgehog pathway: *Shh, patched, Gli, Smo, etc.*
  Wnt pathway: *Wnt-3, -5a, -7a, Lef/TCF, Frizzled, β-catenin, Dvl, etc.*
  Notch pathway: *Serrate, Delta, Notch, Fringe, etc.*
  Homeobox pathway: *Msx, Hox, Dlx, engrailed, etc.*
Adhesion molecules
  Immunoglobulin family pathway: *NCAM, DCC, ICAM*
  Cadherin pathway: *N-, P-, E-, cadherin, α-, β-, γ-catenin*
  Integrin pathway: *α, β subunits*
  Extracellular matrix molecules: *fibronectin, laminin, collagen, tenascin, proteoglycan, etc.*

In Drosophila, another signaling molecule, Hedgehog (reviewed in Ingham, 1998), is known to be upstream of dpp, a homolog of bone morphogenetic protein (BMP) and TGF-β. Because of this relationship, we tried to see if sonic hedgehog (SHH) was upstream of TGF-β2 in the skin. We found SHH was expressed in the placode epithelium. We then added ectopic SHH either from beads coated with recombinant SHH or from RCAS retrovirus mediated SHH misexpression. Explants treated with ectopic SHH formed enlarged feather buds, sometimes with abnormal orientations (Ting-Berreth and Chuong, 1996b). Similar results were obtained by Morgan's group (Morgan *et al*, 1998) and they further showed that the effect was time dependent. SHH expression started from the center of the feather primordia. So what upstream molecule(s) directed SHH to this position?

Time course studies using a synchronized epithelium-mesenchyme recombinant showed that FGF 4 and BMP2, 4 were expressed earlier than SHH (Chuong *et al*, 1996). Fibroblast growth factors (FGF) were tested and shown to promote feather bud formation. This was true for FGF 1, 2, and 4. When the beads were placed on the lateral feather bud region containing buds in early developmental stages, FGF induced many new small feather buds. When placed on the midline skin containing older feather buds, FGF caused buds to fuse. When placed on an abdominal apteric region, feather buds were induced. FGF receptors are known to be expressed in feather bud regions, and it was also shown that FGF could induce feather buds from the skin of scaleless chicken mutants (Song *et al*, 1996). Using scaleless skin, FGF were shown to serve as permissive factors to allow skin appendages, including feathers and scales, to form (Dhouailly *et al*, 1998).

Not all signaling factors enhance feather bud formation. BMP works as a negative regulator. Local delivery of BMP suppressed feather formation around the bead. Adding BMP in the media led to increased interprimordia spacing and the reduction of numbers of feather primordia (Jung *et al*, 1998). *In vivo*, BMP was also shown to suppress feather bud formation, and noggin, a BMP antagonist, could promote feather bud formation (Noramly and Morgan, 1998). The Notch/Delta pathway could also be involved in lateral inhibition in setting up feather patterns or subsequent partitioning (Crowe *et al*, 1998; Viallet *et al*, 1998) of feather primordia into anterior and posterior domains (Chen *et al*, 1997).

Some of these molecules produced similar phenotypes. For example, suppression of the BMP receptor induced feather formation in regions normally forming scales and shared a similar phenotype to that produced by over expression of Delta1 (Zou and Niswander, 1996; Crowe and Niswander, 1998) or retinoic acid (Dhouailly *et al*, 1980). This suggests that there is cross talk among these different molecular pathways. Figuring out the relationship between these signaling molecules and adhesion molecules will be a major research direction in the next period.

When feather primordia are nearly formed, they are radially symmetric. Soon they acquire anterior–posterior polarity dictated by the epithelia. The anterior–posterior axis of feather buds is regulated, at least in part, by Wnt-7a (Widelitz *et al*, 1999). Wnt-7a was initially diffusely expressed through the feather tract. Prior to morphologic changes associated with feather formation, Wnt-7a became restricted to regions that went on to form feathers and disappeared from the interbud regions. Later, before the anterior–posterior axis was formed, Wnt-7a expression was further restricted to the posterior feather bud epithelium. Ectopic expression of Wnt-7a caused the feather buds to expand posterior and middle region characteristics. These buds did not elongate to form feather filaments, but became plateau-like flat skin appendages.

PHYSICO-CHEMICAL PROCESSES

Suppose that a molecular cascade directed patterning of the primordia. What molecule might set up the morphogenetic process? If we approach this problem by trying to identify a transcription factor, X, whose distribution precedes feather morphogenesis, but will initiate the morphogenetic cascade, then

who sets up X? One could continue to search for upstream molecules ad infinitum. Another possibility is not to look for one particular molecular determinant, but to take a step back and look at the physico-chemical property of the whole cell, which represents the integration of all the surface molecules and signaling molecules expressed. The sum of these generic processes can lead to the formation of unique patterns and shapes (Newman and Comper, 1990; Newman, 1998).

One of the major physico-chemical processes that can lead to periodic patterning is based on the reaction diffusion mechanism (Turing, 1952). If a reaction diffusion process takes place in the initial stages of feather formation, then all the cells should be equivalent from the start and have an equal opportunity to enter feather bud or interbud forming regions. Competition between regional levels of activator and inhibitor morphogens should underlie the basis for the patterns. The patterning process should reflect the gradual amplification of the initially random oscillations in cell aggregation formation through lateral inhibition and self re-enforcement. We have data suggesting that, at early times in skin morphogenesis, all the cells have an equal probability to become either a feather bud or interbuds. Inhibitors (BMP) and activators (FGF, Shh, noggin), both expressed in the feather primordia, interact according to the prediction, whereas some other signals such as Notch, Delta, Serrate, and others may also be involved in modulating the activators and inhibitors (Crowe *et al*, 1998; Viallet *et al*, 1998). The data suggest the involvement of a reaction diffusion process among other mechanisms (Jung and Chuong, 1998; Jung *et al*, 1998; Jiang *et al*, 1999a). Thus, the most upstream event that sets up the periodic patterns of skin appendages is not a specific molecular code or molecular address. Rather, periodic patterning is established through physico-chemical processes. The physical-chemical property of the cell is the integration of all the molecules expressed by a cell and its local environment. We developed a model for the initial formation of feather primordia and the induction of periodically arranged skin appendage primordia from a homogenous state (**Fig 1**). A more detailed description and discussion of the model can be seen in Jiang *et al* (1999a).

CONCLUSIONS

In summary, we started our search for molecules involved in feather primordia formation by focusing on the roles of adhesion molecules in mesenchymal condensation. To study the regulators of the adhesion molecules, we explored the involvement of intracellular and extracellular signaling molecules. Some molecules promoted feather bud formation and some suppressed feather bud formation. Upstream to these signaling molecules, there seem to be physico-chemical interactions among the cells. The process of feather patterning involves a reaction-diffusion mechanism. A series of experiments have led us to propose the major molecular/cellular cascades (**Fig 2**) functioning in an integrated model (Jiang *et al*, 1999a). There are many rules that we still have to learn. As cells seem to have an innate ability to self-organize into periodic patterns, if we can set up the signals properly, during the management of wound regeneration, we may be able to let competent cells take their own course to regenerate skin and skin appendages (Chuong *et al*, 1998; Chuong, 1998).

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