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Sonic hedgehog signaling pathway in vertebrate epithelial appendage morphogenesis: perspectives in development and evolution

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Abstract

Vertebrate epithelial appendages are elaborate topological transformations of flat epithelia into complex organs that either protrude out of external (integument) and internal (oral cavity, gut) epithelia, or invaginate into the surrounding mesenchyme. Although they have specific structures and diverse functions, most epithelial appendages share similar developmental stages, including induction, morphogenesis, differentiation and cycling. The roles of the SHH pathway are analyzed in exemplary organs including feather, hair, tooth, tongue papilla, lung and foregut. SHH is not essential for induction and differentiation, but is involved heavily in morphogenetic processes including cell proliferation (size regulation), branching morphogenesis, mesenchymal condensation, fate determination (segmentation), polarizing activities and so on. Through differential activation of these processes by SHH in a spatiotemporal-specific fashion, organs of different shape and size are laid down. During evolution, new links of developmental pathways may occur and novel forms of epithelial appendages may emerge, upon which evolutionary selections can act. Sites of major variations have progressed from the body plan to the limb plan to the epithelial appendage plan. With its powerful morphogenetic activities, the SHH pathway would likely continue to play a major role in the evolution of novel epithelial appendages.

Keywords

Evolution; development; skin appendages; morphogenesis; size

Introduction

Sonic hedgehog (SHH) is a fundamental morphogenesis pathway. Its members and regulation have been extensively reviewed elsewhere (see [1] and Toftgard, this issue). The fundamental importance of SHH in organogenesis was demonstrated in mis-expression and knockout mice studies which showed its role in patterning the brain, spinal cord, axial

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skeleton, limbs and epithelial-mesenchymal interactions [2–5]. The functions of SHH in neural development, limb bud development and human diseases are described by Patten and Placzek, Capdevila and Johnson and Toftgard, respectively, in this issue. In this article, we focus on its morphogenetic activities in epithelial development.

Epithelial organs are defined as those organs in which epithelial-derived cells become the major component of the organ following epithelial-mesenchymal interactions. This is in contrast to the limb in which patterning of the mesenchymal component (e.g. cartilage, muscle, tendon) is the major morphogenetic activity following epithelial-mesenchymal interactions. Since epithelial organs are derivatives of the epithelia, we also call them epithelial appendages [6]. In this paper, we will focus on the roles of SHH in epithelial appendage morphogenesis.

Concept of epithelial appendages: distinct epithelial organs are variations on a common theme

Epithelia form the interface between organisms and their environment. External epithelia produce skin that provides animals with specialized structures and functions to interact with the environment and adapt to new niches. This need for new niches drives the diversification for epithelial appendages during evolution, producing feathers, hair, scales, claws, horns, sweat glands and mammary glands (fig. 1A, [6, 7]). These skin appendages provide special functions including barrier and protection, thermoregulation, communication, hunting, defense, flight and secretion. Similarly, for the internal epithelia, formation of a variety of appendages allows animals to feed on different diets. The oral mucosa diversifies to form teeth, tongue papilla, salivary glands and so on. The gut epithelium elaborates to form lungs, various stomach chambers, small and large intestines, the liver, pancreas and so on.

Although the end products of different epithelial organs appear to be different, they share similar molecular, cellular and developmental processes, and the distinct epithelial organs are indeed variations of a common theme (fig. 1B). Epithelial appendages can be viewed as topological transformations of the originally flat epithelia into specialized epithelial derivatives that can either evaginate (e.g. the feather, lung) or invaginate (e.g. the tooth, gland). Here, the prototypical developmental processes of these epithelial appendages are discussed, followed by the functions of SHH in these processes.

Induction stage

Morphologically, different epithelial appendages can originate from a piece of epithelium. Through induction, the homogeneous epithelium segregates into primordial and interprimordial regions. For induction to occur, both the epithelium and mesenchyme have to be in competent states [8]. The end result is the epithelial appendage primordium, which is composed of an epithelial placode and a subjacent mesenchymal condensation. At this stage, epithelial appendage phenotypes are not stable and it is possible to interconvert appendage phenotypes [9].

Morphogenesis stage

During this stage, the primordium is molded into a particular shape of organ anlage (fig. 1B). It acquires anterior-posterior and proximal-distal polarities and will either grow out or invaginate into the mesenchyme. In some cases, it develops follicular structures; in other cases, it forms branches to increase the functional area of the epithelial appendages.

Differentiation stage

Cyto-differentiation occurs and different organs produce specific gene products such as keratin, enamel, enzymes and surfactants. Differentiation may be uncoupled from morphogenesis and can occur in epithelial tissues with seemingly inappropriate morphogenetic structures.

Cycling

Some epithelial appendages have the ability to renew themselves either periodically, such as hair and teeth, or continuously, such as the intestinal villi. The regenerative potential depends on the availability of appropriate stem cells in the adult tissues and the ability of these cells to reenter the morphogenesis stage.

In the following section, several exemplary organs are examined to discuss the roles of SHH in these various morphogenetic stages. SHH, though not essential for the induction and differentiation stages, is a major player in the morphogenesis stage.

SHH in epithelial organ morphogenesis

The importance of SHH in epithelial morphogenesis has been shown by its expression in several regions of epithelial-mesenchymal interactions [3, 10]. The functions of SHH in external epithelia (feather, hair), the oral cavity (teeth, tongue papilla) and internal epithelia (lung, gut) exemplify its morphogenetic activities.

Feather

The feather is the most elaborate skin appendage. The topological transformation of the flat epithelium to three-dimensional complex feather structures is shown in figure 2A [11]. Through interaction with the mesenchyme, the homogeneous feather field epithelium is transformed into the periodically arranged feather primodia. Through the positive and negative feedback of activators [e.g. fibroblast growth factors (FGFs), SHH] and inhibitors [e.g. bone morphogenetic proteins (BMPs)], periodically arranged domains of competent epithelia gradually emerge to become individual primordia [12]. While cell adhesiveness is the driving force [8], the strength of SHH pathway activity may modulate the survival and consolidation of dermal condensations. Subsequently, feather buds elongate to form feather filaments, a process that requires cell proliferation. Later, feather filaments undergo another level of branching morphogenesis. The basal layer, which is on the inner side of the stratified feather filament epithelium, starts to invaginate and segregate into alternating barb plate and marginal plate epithelia. The barb plate epithelia keratinize to form barbs per se,

but the marginal plate epithelia undergo programmed cell death to become the space between barb branches (fig. 2A).

In the inductive stage, though not the earliest molecule, *Shh* is expressed in the epithelial placode (fig. 3A, [13]). Since separation of the epithelium and mesenchyme eventually results in the disappearance of *Shh* and the reappearance of *Shh* in new locations above the dermal condensations, *Shh* expression is mesenchyme dependent [14]. Originally expressed in the center of the epithelial placode, *Shh* then becomes distal-posteriorly localized. Overexpression of SHH by retroviral vectors or recombinant SHH protein causes the formation of extra-large feather buds and expanded feather domains (fig. 3D, [12, 13, 15]). Patched (Ptc), a SHH receptor, is present predominantly in the underlying mesenchyme, but also in the epithelium. Further condensation of mesenchymal cells and increased cell proliferations apparently mediate the effect of SHH on feather growth (fig. 2B). Mis-expression of SHH was further analyzed by forced expression at different time [15]. When *Shh* was transduced prior to the time of feather induction, it caused disorganized epidermal proliferation. At the time of feather induction, SHH caused feather bud formation. After feather induction, SHH had little or no morphologic effect. These results demonstrate that skin has to be competent to respond to SHH signaling, and the response can vary depending on specific regions and times. Thus, the appropriate morphogenetic response of a tissue is not just defined by a single molecule, but also the availability of other members of the pathway and the interaction with other molecular machinery (fig. 2B, [7]).

Later, in feather filament morphogenesis, the SHH signaling pathway is recruited again for establishing periodic pattening in the filament epithelia. When the basal layer is transformed into alternating barb plate and marginal plate epithelia, *Shh* is periodically expressed in the prospective marginal plate epithelia, suggesting a role in establishing the branching pattern of feather barbs (fig. 3B, [13]). The marginal plate epithelia later express neural cell adhesion molecule (NCAM) and apoptose [16, 17]. This activity is reminiscent of the patterning role of hedgehog in the body segments of *Drosophila* embryos [18]. Subsequently, the SHH pathway may be linked to cell adhesion for consolidation of the marginal plate epithelia and to the apoptosis molecular machinery (fig. 2B).

Shh is also expressed in other skin appendages. However, they are expressed in different patterns, which have different morphogenetic consequences. For example, although *Shh* is expressed in the scale of the foot, the expression is weak and diffuse (fig. 3C). The result is the plateaulike morphology of the scale, in contrast to focal expression pattern of *Shh* in feather buds (fig. 3A) which lead to the filament like morphology of the feather.

Hair

Hair development goes through a series of stages from hair germs to hair pegs, which then form hair follicles. In mature skin, appendages such as feathers and hairs undergo cycling, being shed and regenerated. The major phases are anagen, catagen and telogen [19]. During hair development, *Shh* is expressed in the hair placode epithelia. In the follicle, it is localized in the matrix epithelia. Studies on the skin of *Shh* knockout mice have illustrated that hair germs, though able to form without SHH, are growth arrested and cannot elongate into the

dermis to form hair pegs [20, 21]. The ability of dermal condensations to form in *Shh* null mice implies that SHH is not the only molecule that can elicit dermal condensation formation. However, the abnormal size of the dermal papilla implies that SHH is physiologically required for the epithelial-mesenchymal interaction. Since the *Shh* null mutation is embryonic lethal, skin was grafted to nude mice for further analyses. Some abnormally shaped large follicles, able to express hair-specific keratins, formed close to the epidermis. The results suggest that SHH, though not essential for the induction and differentiation of hairs, is required for appropriate hair follicle morphogenesis.

Cancer geneticists studying Gorlin syndrome, a genetic basal cell carcinoma, have recently determined that Ptc is mutated in such patients (reviewed in [22] and Toftgard, this issue). Overexpression of *Shh* in the basal layer, using the Keratin 14 promoter in transgenic mice, causes the formation of basal cell carcinoma and abnormally large hair follicles [23]. Subsequent work has shown that genes within the SHH pathway, including *Smoothened* (*Smo*), *Gli*, *Ptc* and *Shh* itself, are frequently mutated in basal cell carcinoma [24, 25]. Epidermal cells expressing SHH were resistant to p21-induced growth arrest [26]. Thus, in skin appendage morphogenesis, the SHH pathway appears to drive epidermal hyperplasia and morphogenesis, and appropriate coordination with other signaling pathways is essential for the successful formation of the hair as a miniepithelial organ.

In the hair cycle, the important check points are at the end of anagen as it becomes catagen, and at the initiation of anagen from telogen. Using adenoviral vectors, overexpression of SHH was recently shown to enhance the formation of anagen hair follicles [27]. Though this may have promising significance in practical applications, it does not establish SHH as a physiological molecular switch for telogen-anagen transition. As discussed, SHH in skin appendage development is not the earliest molecule to initiate appendage induction, but is essential for morphogenetic activity, including cell proliferation. Overexpression of SHH in telogen follicles may bypass the requirement for the physiological anagen initiators which eventually enhance *Shh* expression.

Tooth

Tooth morphogenesis is regulated by epithelial-mesenchymal interactions and shares a similar molecular signaling network with other epithelial appendages [28]. In tooth, the epithelial signaling centers function in three stages of morphogenesis [29]. The initiation of tooth germ, the formation of crown base and the formation of each cusp. At E 10–11, initiation of mouse molar tooth development becomes morphologically evident when the dental epithelium thickens locally. At the bud stages (E 12–13), the dental epithelium invaginates into the underlying neural crest-derived mesenchymal cells. They become committed dental follicle cells and dental papilla mesenchyme. During the cap stage (E 14–15), the dental epithelium forms a cap enclosing the dental papilla mesenchyme and starts to develop into a distinct, transient morphological structure, the primary enamel knot. The primary enamel knot is morphologically distinct from the rest of the tooth epithelium. The cells are loosely packed and less columnar than the rest of the inner enamel epithelium. Absence of proliferation within the enamel knot and an increase in apoptosis result in the disappearance of the primary enamel knot by E 15. However, the proliferation in the

adjacent epithelium leads to the formation of crow base. The secondary enamel knots then develop, which are clearly visible at E18 and specify the position of individual cusps [30]. Using experimental tissue recombinations, it was shown that tooth development is directed by the dental epithelium before the early bud stage (E 12), and afterwards the dental mesenchyme possesses the capability to conduct the development of an epithelium towards a dental phenotype [31]. Thus, the formation of the enamel knot may be guided by the dental mesenchyme, which is consistent with the recent finding that mesenchyme could provide the patterning information [32].

At E 11.5, *Shh* expression is localized to the epithelial thickening of the tooth germs. *Ptc* expression is restricted to the mesenchyme underlying the tooth thickenings. At E 13, *Shh* is expressed at the tip of the budding epithelium, which then forms the primary enamel knot. *Ptc* and *Gli1* expressions are localized in the dental papilla and in regions of the dental epithelium. At E 14, the expression of *Shh* continues in the primary enamel knot, which is now a morphologically discernible cluster of nondividing epithelial cells. The enamel knot does not incorporate BrdU, in contrast to surrounding epithelium and mesenchyme cells [33]. At E 15–16, *Shh* reappears in the secondary enamel knots. The differential proliferation created by the primary and secondary enamel knots leads to an uneven and complex tooth shape, namely the crown base and individual cusp, respectively.

Recently, *Shh* signaling was shown to involve functional redundancy of downstream *Gli1* genes, and signaling to the dental mesenchyme could be a similar molecular cascade to that found in the developing limb bud [34]. Another study shows that Msx1 works as a component of the *Shh* signaling pathway that leads to *Ptc* induction [35]. Moreover, *Msx1* might function as a competence factor for *Shh* signaling in early tooth development. *Shh* is secreted by the dental epithelium and induces the expression of the transcription factor *Gli1* and *Ptc* in the mesenchyme [34]. In the absence of Msx1, *Shh* was able to induce *Gli1* but failed to induce *Ptc* in the dental mesenchyme [36]. In addition, *Shh, Bmp-2* and *Wnt10b* have been shown to have similar expression patterns in the epithelium between E 11 and E 12, but each has distinct molecular actions on the dental mesenchyme with specific targets: *Ptc* and *Gli1* for *Shh, Lef1* for *Wnt10b* and *Bmp-2* for *Msx1* in the dental mesenchyme [37]. In mice lacking Msx-1, Lef-1 or platelet-derived growth factor (PDGF) receptor α subunit, tooth morphogenesis is arrested prior to the shift from the bud to the cap stage [38–40].

These results demonstrated the crucial role of *Shh* in tooth morphogenesis and also showed that during early tooth development, many of the similar fundamental signaling pathways are used, but linked in different ways that lead to variations unique to the dental epithelium and mesenchyme. This principle is further elucidated when the development of the different-shaped molars from mouse and vole were compared [29]. While their cusps are of different size and shape, the signaling molecular cascade used in forming enamel knots, including *Shh*, are the same. Thus for a particular epithelial morphogenetic process (in this case, the formation of cusp), new genes are not necessarily required [29]. New morphology can be elaborated and evolved by regulation or rearranging the existing morphogenesis process modules.

Tongue papilla

It is interesting to appreciate that similar signaling processes take place in the formation of small epithelial appendages such as tongue papillae. In mammals, there are four different types of tongue papillae on the dorsal surface of the tongue: the fungiform, circumvallate, foliate and filiform papillae. Although taste buds are found in many regions of the oral cavity, pharynx and larynx, 75% of the total number of taste buds are associated with these lingual papillae [41, 42]. Recent studies of the tongue in developing mouse embryos have shown that *Shh*, *Ptc*, *Gli1*, *Bmp-2*, *Bmp-4* and *Fgf-8* transcripts are expressed within the epithelia of the tongue primordia, indicating that signaling molecules are of fundamental importance in papillae morphogenesis [43, 44]. At E 10.5, *Shh* is expressed in the lateral swellings of the dorsal tongue epithelium, but not in the median sulcus. At E 11–13, *Shh* expression is altered from occupying the entire tongue region to becoming restricted to individual spots. Similarly, *Ptc*, *Gli1*, *Bmp-2* and *Bmp-4* are expressed in the dorsal epithelium and share similar punctate patterns at regular intervals which are often symmetrically located in rows at both sides of the median sulcus. At E 14, small eminencies on the anterior part of the tongue are distinguished as the first morphological indications of fungiform papillae through a dissecting microscope [45]. *Shh* transcripts continue to be expressed in the developing fungiform papillae in discrete spot patterns at E 14–15.

Functional analysis of SHH in tongue papillae has not been reported. However, SHH may be involved in the morphogenesis of tongue papillae. The expression pattern of *Shh* in the developing tongue papillae corresponds with brain-derived neurotrophic factor (BDNF) at E 15.5. BDNF is an early marker for differentiation of the papillae epithelium where *Shh* is expressed. It might be interesting to test whether SHH serves as a competence factor in tongue papillae to form taste receptor cells within the core of individual papillae and then recruit the nerve endings later to act as sensory receptors. If tongue papillae contain taste bud precursors, nerve-independent SHH signaling may arguably be involved in the patterning of taste buds. Recent studies have demonstrated that taste papillae (buds) induction is likely independent of innervation. Instead, the ability of the oropharyngeal epithelium to generate taste buds is an intrinsic feature that is acquired very early in embryonic development [46]. The signaling molecules that have been implicated in morphogenetic regulation of numerous other epithelial appendages are also associated with the initiation of tongue papillae formation. The specific mechanisms regarding how the initially uniform expression of the signaling molecules becomes restricted to the sites of forming papillae, how *Shh* and other signaling molecules influence the papillae cell morphology, and how they are involved in the subsequent establishment of innervation patterns remain to be elucidated.

Lung and foregut

During mammalian lung development (reviewed in [47]), the trachea and esophagus develop from the foregut endoderm. In the mouse, at E 9.5, the tracheal diverticulum begins to evaginate from the foregut. By E 11.5, the trachea is separated from the esophagus and situated ventrally as a tube. Main stem and lobar bronchi form, followed by dichotomous branching which encompasses the pseudoglandular (E 10–16) and canalicular (E 16–27)

stages. During the saccular (E 17 to birth) and alveolar (P5–30) stages, the terminal buds dilate and form saclike structures which later become the alveoli. All these processes depend on the interaction between the epithelium and adjacent mesenchyme. Failure of this interaction in early development leads to incomplete separation and the formation of tracheo-esophageal fistula and other defects, as seen in many human congenital anomalies.

Shh is expressed ventrally in the foregut endoderm, the precursor of the lung epithelium [48]. At E 11.5, *Shh* is expressed at low levels throughout the epithelium. Later, *Shh* is expressed at high levels in the distal branching regions of terminal buds [49]. *Shh, BMP 4* and transforming growth factor-β1 (*TGF-β1*) form a molecular circuitry with FGFs to regulate patterning and growth of the lung buds [50]. Overexpression of *Shh*, driven by the surfactant protein-C (SP-C) enhancer/promoter in transgenic mice, demonstrated that SHH, secreted by the epithelia at the branching points during lung branching morphogenesis, seems to cause the adjacent mesenchyme to proliferate [51]. Hypercellularity, apparently due to increased proliferation, was observed in both the epithelium and mesenchyme. Epithelial tubules expanded, but the mesenchyme around the tubules was much thicker, branching was disrupted and no functional alveoli formed.

The role of SHH in foregut development was demonstrated in *Shh* knockout mice [48, 52]. By E 12.5, the trachea and esophagus are normally separated, but in *Shh* null mutants, the trachea and esophagus do not separate appropriately. The mutants manifested esophageal atresia, stenosis and tracheo-esophageal fistula, and sometimes the lungs just form rudimentary sacs. In the hypoplastic lobes, detachment of bronchial mesenchymal cells from endodermal epithelia and severely reduced branching are observed. At the cellular level (fig. 2B), these phenotypes can be attributed to reduced cell proliferation and mesenchymal cell condensation. The separation of the esophagus and trachea may require similar apoptotic processes that create spaces between branches. Though cyto-differentiation is disrupted in the *Shh* null mutant mice, some normal proximo- distal differentiation of airway epithelium does occur.

The *Gli* genes (*Gli1*, *Gli2* and *Gli3*) are zinc-finger transcription factors which function downstream of SHH [53]. *Gli2* and *Gli3* are present during the pseudoglandular stage of lung development, but their levels decrease near birth [54]. In *Gli2*-null mutants, stenosis in the trachea and esophagus and hypoplasia and lobulation defects in the lungs were observed. A gradual decrease of *Gli3* gene dosage resulted in esophageal atresia, tracheo-esophageal fistula and severe lung anomalies. *Gli2* and *Gli3* double knockout mutants lacked lungs, trachea and esophagi, as well as other organs derived from the foregut endoderm, including the thymus, stomach and liver [53].

Therefore, as in the hair, the SHH pathway appears to be essential, not for the initiation of lung primordia formation or proximo-distal cyto-differentiation, but for the morphogenetic separation between the trachea and esophagus and subsequent branching and growth of lung buds. Disruption of SHH and its signaling components may be responsible for some of the foregut-related defects in humans.

SHH in the development and evolution of epithelia and epithelial appendages

What kinds of epithelial morphogenetic activities does the SHH signaling pathway elicit? What does SHH do, and what does SHH not do? Several morphogenetic processes are crucial for the formation of epithelial organs. First, induction breaks the homogeneity of the epithelia, and through tissue interactions, sets aside domains within epithelia for the formation of epithelial appendages. Induction does not seem to require SHH. Regulated cell proliferation controls the ultimate size of the feather primordia. The importance of the SHH pathway, acting in both paracrine and autocrine manners, to this process is illustrated in the elongation of both feather buds and hair pegs and in tumors caused by imbalanced SHH pathway activity. The regulation of organ size is also illustrated by the function of Indian hedgehog (IHH) on long-bone elongation [55]. Analogous processes may exist in epithelial morphogenesis. Overexpression of patched driven by metallothionein promoter in transgenic mice led to an overall decrease of animal sizes [56]. SHH may also modulate size by regulating cell rearrangement and migration, as during mesenchymal condensation in several organs. Ectopic expression of *Shh*, resulting in excess production of basal cells but not suprabasal cells, suggests that SHH is not directly involved in epidermal stratification, but may perturb this process by causing arrest in cell cycle [26]. Based on branching morphogenesis, both lung branching and tooth cusp formation may be analogous processes. *Shh* transcripts are specifically expressed in the epithelia of lung terminal buds or the enamel knots of the tooth, and SHH proteins are secreted into the adjacent mesenchyme and epithelial cells to stimulate cell proliferation. Thus, a precisely placed SHH-expressing domain can cause position-specific cell proliferation and create specific shapes. A different strategy for forming branches is to sculpt out spaces by programmed cell death, as illustrated in feather filament morphogenesis. In this case, SHH is involved in periodic patterning of the epithelia (fig. 3B, [13, 18]). As demonstrated in the zone of polarizing activity (ZPA) of the limb bud [2; Capdevila and Johnson, this issue] and in the spinal cord, by the floor plate which can specify ventral fate ([57–59]; Patten and Placzek, this issue), SHH also functions in establishing polarizing activity. In the feather and hair, *Shh* becomes restrictively located to the distal and posterior ends of the buds, and may be involved in initiating anteriorposterior asymmetry. The ability of hair and lung epithelial cells to differentiate in the *Shh* null mutant demonstrates that the SHH signaling pathway, though required for several different stages in epithelial morphogenesis, is apparently not essential for cyto-differentiation.

If activating SHH at a specific site has such a profound consequence in morphogenesis, how is the SHH pathway regulated? In feather morphogenesis, *Shh* is expressed in a hexagonal pattern. However, other events initiate *Shh* expression at that specific location and time. Using an in vitro reconstitution system, it was recently shown that all cells in the feather field initially have an equal probability of becoming primordial or interprimordial cells. The periodic patterning in feathers appears to be determined by competitive equilibrium processes that involve reaction diffusion [8, 12]. Using the feather model, it was shown that molecules involved in induction can be expressed in a restrictive or de novo mode, with the restrictive mode ones being more upstream. But how do the restrictive mode molecules

interact and lead to the activation of *Shh* promoter to initiate Shh expression? Whereas FGF4 and Wnt 7a have been shown to be required for maintaining *Shh* expression in the ZPA [60], the molecular basis of *Shh* induction remains to be elucidated. Since the shapes and sizes of epithelial appendages can be altered depending on the times and locations of SHH activation, regulation of SHH is of critical importance. Retinoic acid has been shown to convert scales into feathers during their morphogenetic stages [9]. Whereas both feather and scale primordia express *Shh*, expression in the scales is weaker and more diffuse. Some of the retinoic acid effects may be mediated through modulation of SHH levels. Some rapidly growing epithelial appendages undoubtedly express large amounts of *Shh*. The promoter of *Shh* has just started to be characterized [61], and further work in this direction would certainly bring a major breakthrough.

Since the Cambrian age 530 million years ago, many body plans have existed [62]. At the present time, the appearance of new body plans would likely be embryonic lethal. An organism can be viewed as an assembly of developmental pathways, and the latest developmental pathway forms the interface with the environment. When changes occur too upstream in development, they are frequently lethal since it is difficult to accommodate the already complex organisms. When changes occur in tissues arising later in development or added at the end of developmental pathways (e.g. epithelial appendages), they are less likely to be lethal and therefore able to produce new variations. These late products in development also directly interact with the environment and are acted on by evolutionary forces. In evolutionary history, this interface has progressed from variations in the body plan to the limb plan [63], and to the epithelial appendage plan [7]. By ‘adding on’ more appendages, organisms have become more complex in evolution and development. Indeed, in the chicken the body plan is set up at embryonic day 1, the limb at embryonic day 3 and feathers at embryonic day 6. During evolution, new links of developmental pathways may occur and novel forms of epithelial appendages may emerge, upon which evolutionary selections can act. For example, the development of proximal-distal axes from scale-like short skin appendages led to the formation of elongated skin appendages (feathers and hair) and the consolidation of warm-blooded animals (Aves and Mammals). Another recruitment of SHH and related pathways in the later epoch of feather morphogenesis converted feather filaments into branched barbs and barbules, allowing birds to soar in the sky for more than 100 million years. With its powerful morphogenetic activities, the SHH pathway will most likely continue to play a major role in the evolution of novel epithelial appendages in the future.

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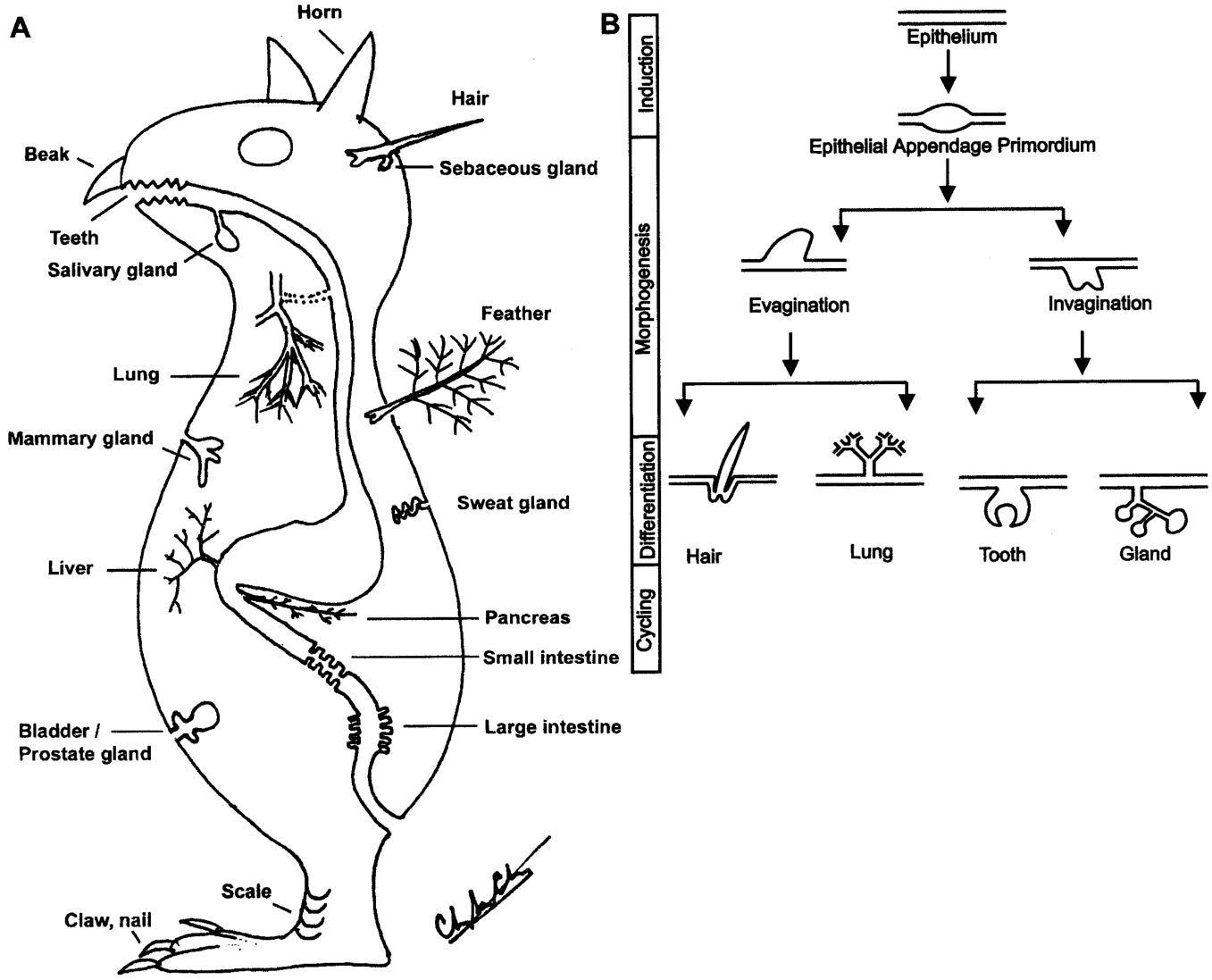

Figure 1.

(*A*) Epithelial appendages are variations on a common theme. They are all results of epithelial-mesenchymal interactions. The epithelia can be ectoderm or endoderm. The results can protrude out or invaginate in from the surface. Adopted from [6]. (*B*) Epithelial appendages share four similar developmental stages. The stages are induction, morphogenesis, differentiation and cycling. In the early stages, the message is simply ‘to make an appendage’ and appendage phenotypes may be interconverted.

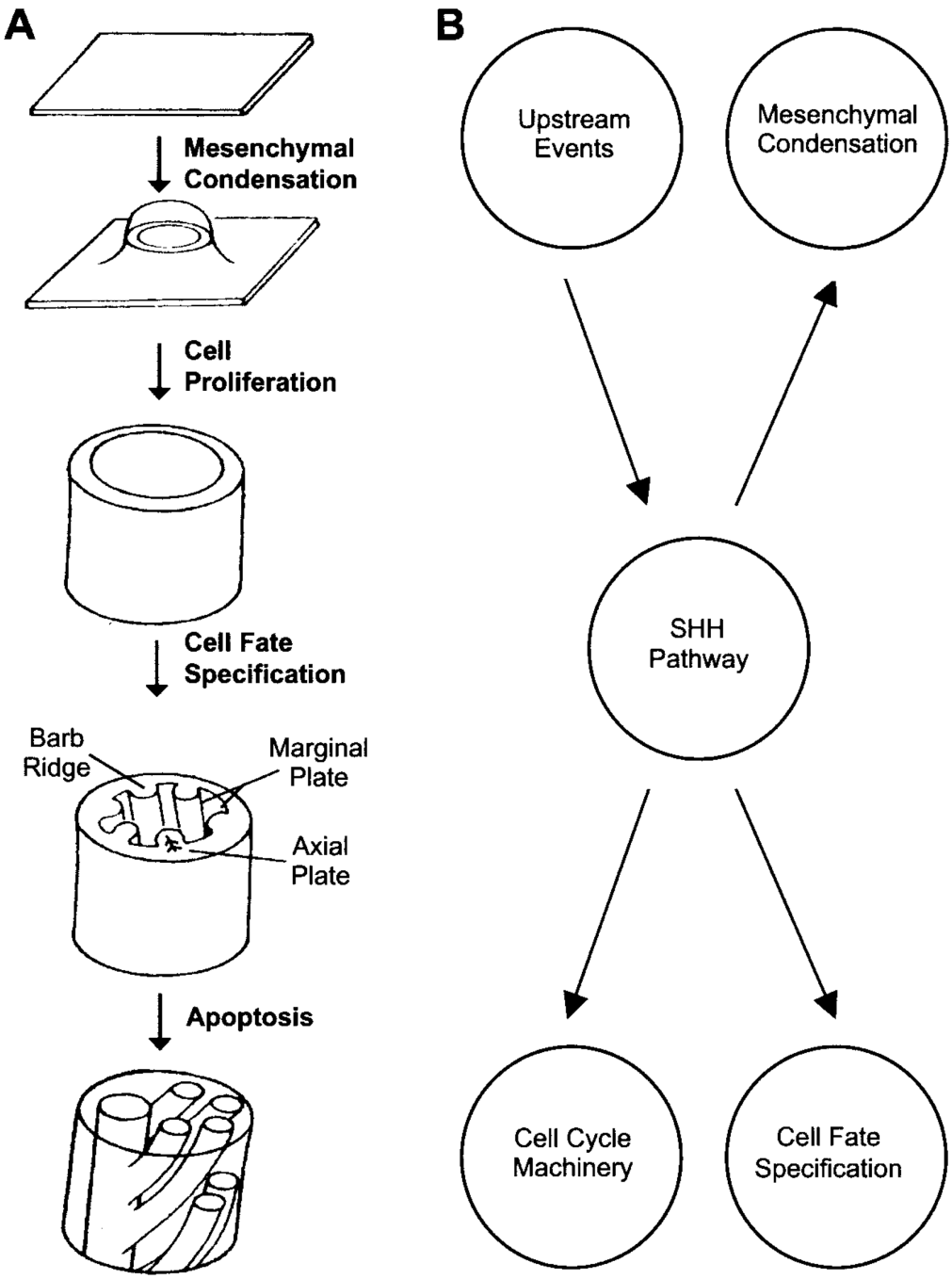

Figure 2.

(*A*) Topological transformation of a flat epithelium into three-dimensional feathers. Planar epidermis, short feather buds, elongated feather buds, invaginated feather filament epithelia and the mature branched structure are depicted. Between these stages are morphogenetic processes that transform their morphology. SHH may be involved in mesenchymal condensation, cell proliferation and apoptosis. Another round of apoptosis led to the formation of barbules (not shown). Panel A is adopted from Chuong and Edelman [16, 17]. (*B*) Connection of the SHH pathway with other molecular machineries endow novel

morphogenetic activities. SHH is involved in many morphogenetic processes such as size regulation, branching morphogenesis, mesenchymal condensation, fate determination and so on. These are achieved through linking to molecular machineries involved in cell proliferation, apoptosis, migration, differentiation and so on.

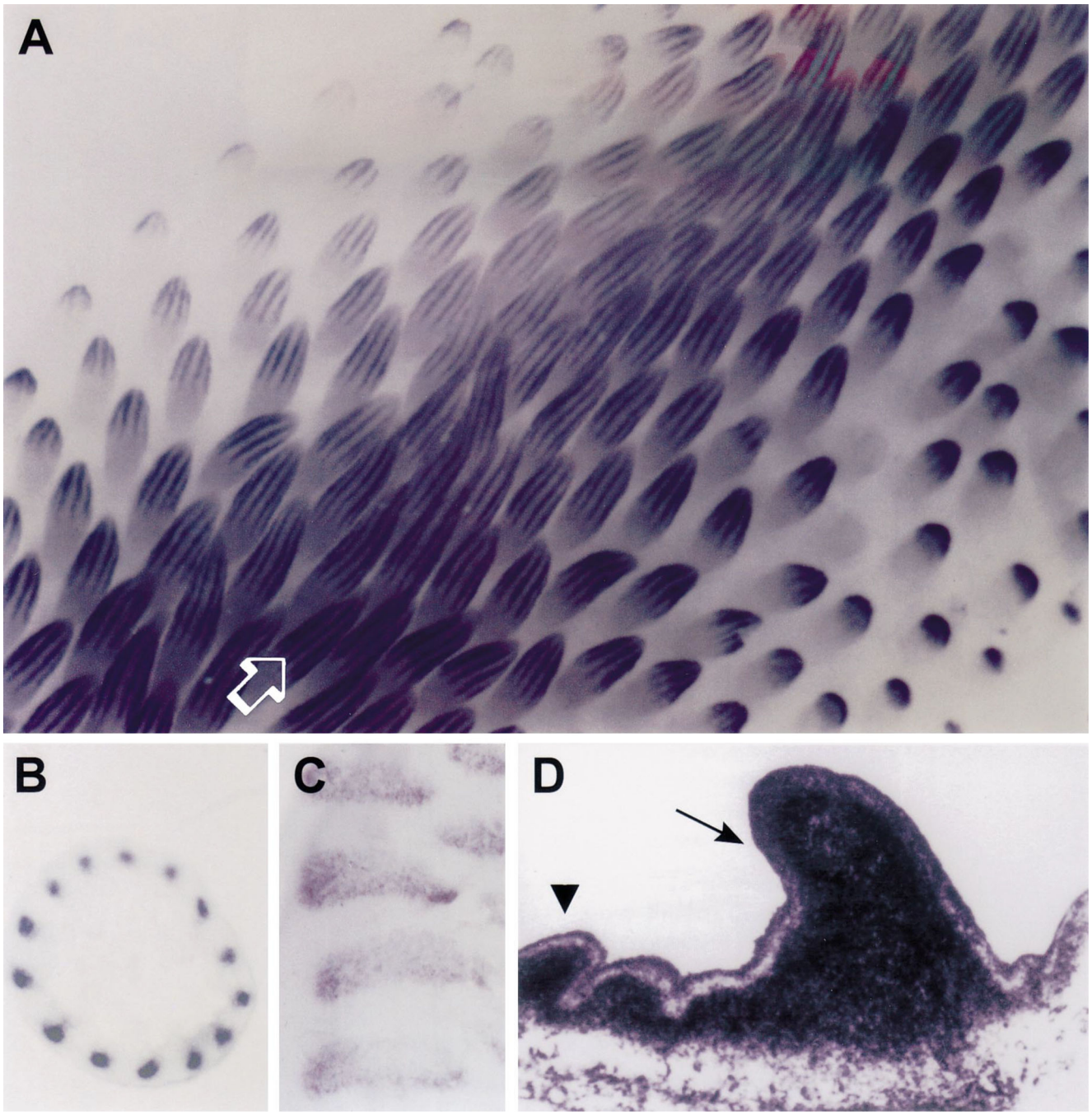

Figure 3.

Expression and function of SHH in feather morphogenesis. (*A*) Whole mount in situ hybridization of *Shh* in embryonic chicken dorsal skin. The midline (blank arrow) has more mature skin, and the lateral part has younger feather primordia. *Shh* is first expressed as a dot in the center of feather primordia, then distal feather buds, then longitudinal stripes (marginal plate epithelia) along feather filaments. (*B*) Cross-sections of a feather filament showing the periodically arranged *Shh* positive marginal plates. (*C*) *Shh* is also expressed in scales, but the expression pattern is weaker and diffused, unlike the focused expression of

Shh in feathers. (*D*) Overexpression of SHH mediated by the RCAS retrovirus make the size of feather buds much larger. Arrowheads, normal-sized feather buds. Arrow, giant feather buds. Panels A, B, D are adopted from Ting-Berreth and Chuong, 1996 [13].