

β -catenin signaling can initiate feather bud development

Selina Noramly^{1,2}, Allison Freeman¹ and Bruce A. Morgan^{1,*}

¹Cutaneous Biology Research Center, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA 02129, USA

²Graduate School of Medical Sciences, Cornell University, New York, NY 10021, USA

*Author for correspondence (e-mail: bruce.morgan@cbr2.mgh.harvard.edu)

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SUMMARY

Intercellular signaling by a subset of Wnts is mediated by stabilization of cytoplasmic β -catenin and its translocation to the nucleus. Immunolocalization of β -catenin in developing chick skin reveals that this signaling pathway is active in a dynamic pattern from the earliest stages of feather bud development. Forced activation of this pathway by expression of a stabilized β -catenin in the ectoderm results in the ectopic formation of feather buds. This construct is sufficient to induce bud formation since it does so both within presumptive feather tracts and in normally featherless regions where tract-specific signals are absent. It is also insensitive to the lateral inhibition that mediates the normal spacing of buds and can induce ectopic buds in

interfollicular skin. However, additional patterning signals cooperate with this pathway to regulate gene expression within domains of stabilized β -catenin expression. Localized activation of this pathway within the bud as it develops is required for normal morphogenesis and ectopic activation of the pathway leads to abnormally oriented buds and growths on the feather filaments. These results suggest that activation of the β -catenin pathway initiates follicle development in embryonic skin and plays important roles in the subsequent morphogenesis of the bud.

Key words: Feather bud, β -catenin, Wnt, Pattern formation, Ectoderm, Chick

INTRODUCTION

The feather tract provides an ideal system to dissect the interactions between cells generating pattern in a competent field. The coordinated subdivision of ectoderm and dermis into follicular and interfollicular fates, as well as subsequent patterning within individual buds, is achieved in part by opposing influences of intercellular signaling molecules expressed in the forming tract (Song et al., 1996; Crowe et al., 1998; Jung et al., 1998; Noramly and Morgan, 1998). Buds form in a number of discrete tracts (pterylae) in reproducible position and sequence within the embryo separated by featherless (apteric) regions. Tract development begins with formation of the dense dermis, a 2- to 3-fold increase in cell density in the mesenchyme underlying the presumptive pterylae. This dense dermis forms throughout the tract and no morphological or molecular distinction between presumptive follicular and interfollicular fates is observable at this stage. Tissue recombination experiments have identified a primary inductive signal that originates in the dense dermis and causes the first morphological distinction between the follicle and surrounding interfollicular skin: the formation of a placode of columnar cells in the epidermis (reviewed in Sengel, 1976; Dhouailly et al., 1998). This epidermal placode in turn signals to the dermis and induces formation of the dermal condensation which acquires its own inductive properties. Reciprocal interactions between these two signaling centers cause the subsequent outgrowth and patterning of the bud. Within each tract, a single row of buds is formed first and

sequential rows of buds are added subsequently as the inductive signal propagates through the tract. As a result, a tract contains buds at successive stages of development arranged in an ordered array. The formation of individual buds and their position relative to each other in a tract are intimately linked. The position of buds within a row, and the spacing between rows is mediated in part by lateral inhibition from previously formed buds (Davidson, 1983; Jung et al., 1998; Noramly and Morgan, 1998). Although the sources of inductive signals and the role of lateral inhibition have been established by tissue recombination experiments, the mechanisms by which pattern is generated remain ill defined. For example, it is unclear whether the inductive signal from the dermis that initiates placode formation is localized beneath the prospective bud, or whether a more generalized inductive signal results in a localized response in the ectoderm as a result of patterning interactions within this layer. To better understand this process, it is necessary to identify the signals that mediate these inductive interactions. Only then can we assess the relative contributions played by localized expression of inductive signals in the dermis and spatial restrictions in responsiveness in the ectoderm to specify the position of the ectodermal placodes.

Several of the molecules that mediate inductive signaling during feather tract formation have been identified, including members of the Bone Morphogenetic Protein (BMP), Fibroblast Growth Factor (FGF), Hedgehog and Notch/Delta families (Song et al., 1996; Ting-Berretth and Chuong, 1996; Crowe et al., 1998; Jung et al., 1998; Morgan et al., 1998;

Noramly and Morgan, 1998). BMP-2 is expressed in both the epidermal placode and the dermal condensation as they form and is an inhibitor of bud formation that contributes to the spacing between initiating buds as well as the subsequent patterning within each individual feather (Jung et al., 1998; Noramly and Morgan, 1998). Experiments manipulating BMP activity in the skin suggest that in addition to the primary inductive signal from the dermis, a secondary inductive signal is generated in the nascent placode itself and contributes to the specification of this structure (Noramly and Morgan, 1998). BMP-2 serves to inhibit the response to the primary inducer as it spreads through the tract and thereby mediates the spacing of bud initiation sites. It also antagonizes the activity of the secondary inducer which acts more locally to recruit surrounding cells to a nascent bud (Noramly and Morgan, 1998).

The Notch receptors and their ligands in the Delta and Serrate families are also expressed in the developing bud (Chen et al., 1997; Crowe et al., 1998). This expression is initially evenly distributed between presumptive follicular and interfollicular regions during the earliest stages of tract formation and becomes localized to the bud ectoderm (Notch-1) and dermis (Delta-1) or interbud (Notch-2) as they are formed. Blocking this localization by the forced expression of Delta-1 in the feather tracts can lead to inhibition of feather formation (Crowe et al., 1998; Viallet et al., 1998). Although it has been suggested that Notch signaling might initiate tract patterning, it remains unclear whether the localization of Notch signaling begins this process or is actually a response to previous signals that initiate bud formation. Although localization of Notch signaling is necessary for bud development to proceed, it may serve to augment and refine responses initially directed by diffusible signals.

Molecules which may serve as the positive inductive signals in this process have also been identified. FGFs can activate feather formation in cultured explants of skin from the scaleless mutant chick (Song et al., 1996). This mutant has a defect in the ectoderm which prevents the formation of most feathers and arrests feather tract development at an early stage (Goetinck and Abbott, 1963). Exogenous FGF can also promote feather bud formation in wild-type skin (Widelitz et al., 1996). Although FGF-2 and -4 are expressed in the epidermal placode (Nohno et al., 1995; Song et al., 1996; Jung et al., 1998), their expression has not been detected prior to placode formation. These observations suggest that either or both of these proteins may serve as a secondary inducer expressed in the placode, but they are unlikely to be involved in the initial induction of this structure.

Forced expression of Shh in the ectoderm can also promote the formation of feather buds (Ting-Berth and Chuong, 1996; Morgan et al., 1998). Conflicting conclusions about its role in feather bud initiation have been proposed. We find that, in most feather buds, Shh is not expressed until after both the dermal condensation and ectodermal placode have formed and conclude that it is not required for the initial specification of feather placodes although it does serve as a secondary inducer within the bud by signaling to both the ectodermal and dermal components (Morgan et al., 1998). In contrast, others have reported that Shh is expressed prior to placode initiation and may be involved in the initiation of bud formation (Jung et al., 1998). Loss of function experiments required to resolve this

issue have not been performed in chick skin. However, since the inductive signals which initiate cutaneous appendage formation are conserved between birds and mammals (Garber and Moscona, 1964; Garber et al., 1968; Dhouailly, 1973; Dhouailly et al., 1998), the fact that hair follicle morphogenesis initiates normally in the skin of mouse embryos lacking Shh supports the conclusion that Shh is only required at later stages of cutaneous appendage development (St.-Jacques et al., 1999; Chiang et al., 1999).

In an effort to identify a primary inductive signal in tract development, we have begun to examine the role of Wnt signaling. Members of the Wnt family have been implicated in the signaling between epithelium and mesenchymal tissue in organs such as the tooth, limb bud, lung, mammary gland and the kidney (Parr and McMahon, 1995; Riddle et al., 1995; Yang and Niswander, 1995; Bellusci et al., 1996; Thesleff and Sharpe, 1997). Several Wnts are expressed early in feather tract development and could play a role in bud morphogenesis. *Wnt7a* is expressed in the epidermal placodes and *Wnt11* transcripts are localized to the dermis (Tanda et al., 1995; Chuong et al., 1996; Morgan et al., 1998; Noramly and Morgan, 1998). Additional Wnt genes are also expressed in the forming feather tract (B. A. M., unpublished data).

Wnt signals are transduced across the cell membrane by the frizzled family of transmembrane receptors (reviewed by Moon et al., 1997a,b; Gumbiner 1998) and several members of this family are expressed in chick skin at the time that feather tract development begins (B. A. M., unpublished data). For a subset of frizzled receptors, an important effect of receptor activation is the stabilization of free cytoplasmic β -catenin levels. In the absence of Wnt signaling, β -catenin is associated with adhesion complexes in the cell membrane and plays a role in intercellular adhesion. Free β -catenin in the cytoplasm is rapidly phosphorylated and thereby targeted for degradation by the ubiquitin-proteasome pathway (Aberle et al., 1997; Orford et al., 1997). However, upon engagement of a subset of frizzled receptors by their cognate ligands, this phosphorylation and associated degradation of β -catenin is inhibited. Cytoplasmic β -catenin then accumulates and translocates to the nucleus, where it can bind to transcription factors of the T-cell factor/lymphoid enhancer binding factor-1 (TCF/Lef-1) family and modulate gene expression (reviewed by Clevers and van de Wetering, 1997). The other subset of frizzled receptors signals through an alternative pathway that appears to involve phosphatidylinositol and calcium signal transduction cascades (Slusarski et al., 1997).

The fact that early inductive events in cutaneous appendage formation are conserved between mice and chicks suggests that experiments implicating β -catenin signaling pathway in hair development also support a role for Wnt signaling in feather tract formation. Mice lacking Lef-1 have fewer hair follicles and lack whiskers (van Genderen et al., 1994). Lef-1 acts in both tissue layers during formation of epithelial appendages. Dermis lacking Lef-1 cannot generate whiskers when recombined with wild-type epidermis (Kratowchwil et al., 1996), while overexpression of Lef-1 in the epidermis leads to abnormal hair follicle formation as well (Zhou et al., 1995). Although Lef-1 may act independently of the Wnt signal transduction cascade (Hsu et al., 1998), complementary experiments with mutant forms of β -catenin suggest these effects on hair development reflect interference with the β -

catenin signaling pathway (Gat et al., 1998). The phosphorylation sites that target β -catenin for degradation are contained in the N-terminal region of the protein and mutation or removal of these phosphorylation sites is sufficient to stabilize the protein and activate this signaling pathway in the absence of Wnt receptor engagement (Funayama et al., 1995; Orsulic and Peifer, 1996). Expression of such a 'stabilized' β -catenin throughout the developing epidermis using a keratin-14 expression cassette did not discernibly affect embryonic skin development (Gat et al., 1998). Both hair follicles and interfollicular skin are histologically normal at birth in these transgenic animals. However, concomitant with the first hair cycle, new hair follicles were induced in interfollicular skin. Additional defects arose within the 'normal' hair follicles as well. These observations demonstrate that the β -catenin signaling pathway can have potent effects on the development of post-natal skin, but they also indicate that widespread activation of this pathway in the epidermis has little effect on the morphogenesis of skin in the mouse embryo.

In the work reported here, we examine the role of β -catenin signaling in the skin of the experimentally accessible chicken embryo. The change in the stability and subcellular localization of β -catenin can be monitored by immunohistochemical techniques to chronicle the activity of this pathway during development of a tissue (Schneider et al., 1996). We find that this signaling pathway is activated in a dynamic pattern throughout skin development from the onset of tract patterning through the generation of polarized buds. This pattern suggested roles for β -catenin signaling in placode formation, intra-bud patterning, and polarized outgrowth of the bud. To evaluate these proposed roles, we forced the expression of a mutant form of β -catenin which serves to activate this pathway using a retrovirus to generate patches of expression in developing skin (Funayama et al., 1995; Capdevila et al., 1998). In contrast to the analogous experiment in the mouse, expression of this mutant protein in embryonic ectoderm is sufficient to induce the ectodermal gene expression normally associated with early placode formation. These induced placodes can recruit underlying dermis to form feather buds. Furthermore, the asymmetric activation of this pathway during the normal progress of bud development is required for polarized outgrowth as ectopic activation within a bud can disrupt this process. We propose that β -catenin signaling is a positive activator of feather formation and that endogenous Wnt signals may regulate its activity and localization to initiate dense dermis formation and placode development as well as to regulate polarized outgrowth of the bud.

MATERIALS AND METHODS

Immunohistochemistry

White Leghorn eggs (SPAFAS) were incubated at 37.63°C. Skin spanning the dorsal pterygiae and femoral tracts was dissected in cold PBS and fixed overnight (4% paraformaldehyde, 4°C). After dehydration through graded sucrose and infiltration with OCT the skin was quick frozen, and alternating 8 μ m sections were processed for immunohistochemistry or in situ RNA transcript detection. Endogenous β -catenin was detected with a monoclonal antibody (15b8, Sigma) diluted 1:500 in PBST followed by secondary detection with fluorescein-conjugated anti-mouse IgG. Sections were counterstained with 7-amino-actinomycin D or TOPRO 3 and images

were captured on a Leica confocal microscope employing sequential scanning to eliminate bleedthrough between channels. In situ hybridization was as described by Morgan et al. (1998). Infected cells were detected with a polyclonal antisera to the viral p27 protein (SPAFAS), or with a monoclonal raised against the HA epitope tag (Babco).

Forced expression of stabilized β -catenin

The RCAS- β -catenin retrovirus was the generous gift of C. Tabin. Stocks of viral inoculum were generated as described by Morgan and Fekete (1996). A stock of 2×10^8 infectious units/ml was injected and embryos harvested as described by Noramly and Morgan (1998). The expression of viral transcripts alone was examined in a total of 31 embryos. Of these, 13 were harvested at day 6 of incubation, 9 at day 7, 6 at day 8 and 3 at day 9. Representative examples are shown in Fig. 2C-F. Prior to day 8, no spatial restriction in infection is observed. However, preferential infection of the ectoderm of the feather bud primordia compared to interfollicular skin is observed after the dermal condensation has formed and the bud rudiment has begun to protrude from the ectoderm. Infection is also common in the apteric ectoderm at these stages. This pattern is observed with control RCAS viruses and apparently arises in part from transcriptional silencing of integrated viral genomes and increased resistance to reinfection as interfollicular ectoderm differentiates. Since this is a replication competent retrovirus, silencing is normally accompanied by rapid reinfection with virus produced by adjacent cells as the block to superinfection is lost.

A total of 16 day-12 and day-13 embryos were examined for gross phenotypic effects. To characterize the effect of exogenous activated β -catenin on endogenous gene expression, whole-mount in situ analysis was performed on a total of 123 embryos. Of these 41 were examined for *BMP-2*, 28 for *Shh*, 15 for *BMP-4*, 19 for *Wnt7a*, 12 for *Lunatic fringe* and 8 for *TCF-1*. To correlate the effects on gene expression with the presence of stabilized β -catenin, these embryos were subsequently analysed for the presence of viral transcripts. To compare the extent of the expression of ectopic *Shh* with induced *BMP-2* expression, sequential detections of *BMP-2* and *Shh* were performed. 22 embryos were examined for *BMP-2* transcripts using a digoxigenin-labeled probe and for *Shh* transcripts using a fluorescein-labeled probe; 22 embryos were examined with *Shh* digoxigenin-labeled probe first and then *BMP-2* fluorescein-labeled probe. Control double detections of *BMP-2* (seven embryos) and *Shh* (two embryos) were also carried out. An additional 72 embryos were analyzed with feather bud markers not shown in this study and the results of these in situ hybridizations were consistent with those described in this paper. Probe templates were as described by Noramly et al. (1996); Song et al. (1996); Laufer et al. (1997); Morgan et al. (1998); Noramly and Morgan (1998). The TCF-1 probe was derived from nt 630-1400 of the chTCF-1 plasmid (Gastrop et al., 1992). Six embryos were sectioned after whole-mount in situ hybridization as described by Noramly and Morgan (1998) to examine the morphology of the induced placodes. Representative samples were also sectioned to confirm the localization of induced gene expression to the ectoderm or dermis. Finally, virus was detected immunohistochemically on sections from an additional 8 embryos to confirm conclusions based on whole-mount analysis.

RESULTS

Localization of β -catenin protein in the developing feather

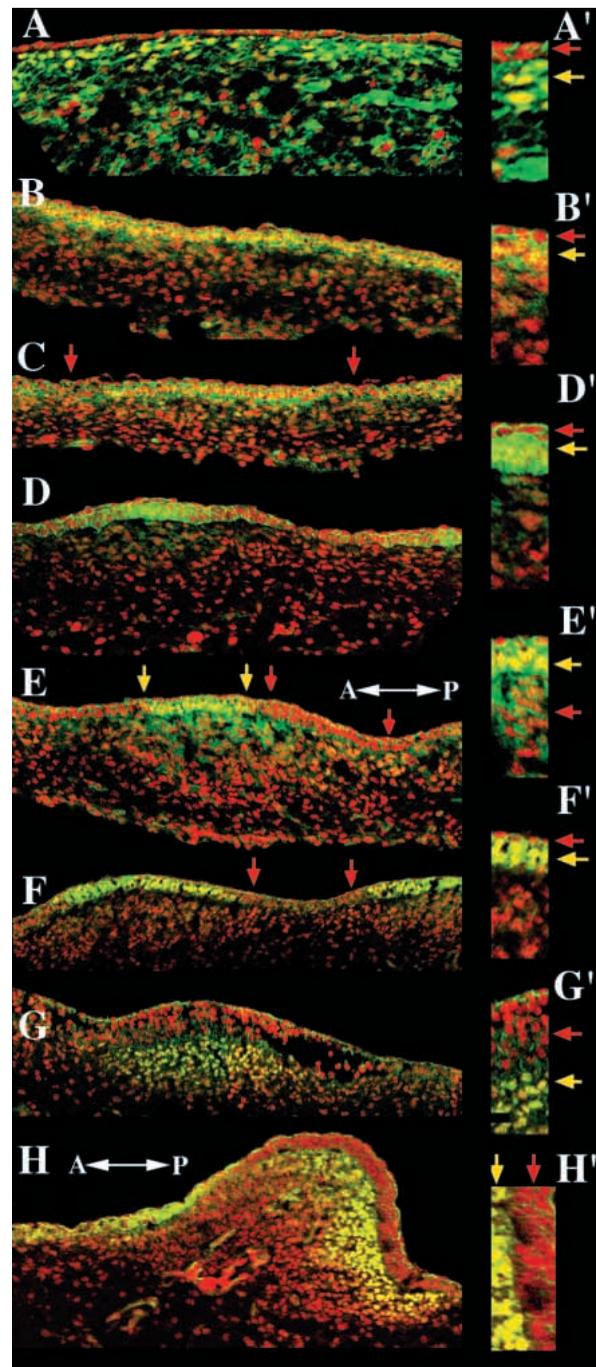
As a first step towards dissecting the role of Wnt signaling in feather tract initiation, we examined the localization of endogenous β -catenin in the skin during tract formation to determine when and where nuclear localization of the protein

indicated that this signaling pathway was active. The dorsal feather tracts initiate at the dorsal midline and spread laterally. At day 6 of incubation, skin development has not begun in lateral regions and the ectoderm is a simple epithelium overlying loosely packed mesenchyme. At this stage, β -catenin staining is prominent at the ectodermal cell surface but is not observed in the cytoplasm or nucleus of cells in either layer (data not shown). In mediolateral regions, the ectoderm consists of a uniform layer of cuboidal cells which give rise to the epidermis overlain by a layer of smaller cells, the periderm, which will be sloughed at later stages in development. Underlying this region, mesenchymal cells have begun to accumulate beneath the ectoderm to form the dense dermis. Nuclear β -catenin accumulation is now observed in the dermal cells adjacent to the ectoderm throughout the forming dense dermis (Fig. 1A). Staining is brightest in cells immediately adjacent to the epidermis and decreases rapidly over the distance of several cell diameters from the surface, while loose mesenchyme beneath this layer does not exhibit nuclear β -catenin accumulation (Fig. 1A'). The nuclear localization of β -catenin throughout the dense dermis is transient and fades rapidly as development proceeds.

A day later in development, dense dermis formation has progressed through the tract and bud formation has begun in more medial regions. Nuclear and cytoplasmic β -catenin is

observed in a broad swath through the lateral ectoderm and no discernible difference in β -catenin levels distinguishes between presumptive placode and interfollicular fates (Fig. 1B). Staining is limited to the basal layer, no nuclear staining is observed in the periderm (Fig. 1B'). In more medial regions, cytoplasmic and nuclear β -catenin staining increases in the forming placode and is abolished in the ectoderm that adopts interfollicular fates (Fig. 1C). When the cells of the placode have adopted a columnar morphology, nuclear β -catenin staining is bright in this region and absent in interfollicular ectoderm (Fig. 1D). As the dermal condensation forms, cytoplasmic staining of β -catenin is observed but no nuclear accumulation is detected (Fig. 1D,E). When the initially

Fig. 1. β -catenin signaling during feather tract development. Skin at successive stages of development was stained with antisera to β -catenin and the nuclear dye 7AAD. Cytoplasmic and cell membrane β -catenin appears green, nuclei appear red unless coincident staining with β -catenin renders them yellow. Higher magnification panels at right aid distinction between the periderm, ectoderm, and dermis. At day 6.5 of incubation, dense dermis is forming in the lateral flank region (A). β -catenin is detected at the cell surface in the ectoderm, (A', red arrow) but is not found in the nuclei. β -catenin is also detected in the cytoplasm of loose mesenchyme below the forming dense dermis but is absent from most nuclei. In contrast, the dermal cells adjacent to the ectoderm show strong nuclear staining (yellow) for β -catenin (yellow arrow). This nuclear staining in the dermis is transient and is largely absent from a corresponding region a day later in development (B). At this stage nuclear β -catenin is observed in the basal layer of the ectoderm in a broad swath that encompasses both presumptive placode and interplacodal cells (B', yellow arrow). No nuclear β -catenin is observed in the overlying periderm (red arrow). Nuclear β -catenin is then lost from the interfollicular regions as the placode is specified (C, vertical arrows) and becomes localized to the placodal epidermis (D, D'). As the bud develops an A/P axis, strong nuclear β -catenin staining is observed in the ectoderm in a section through the anterior half (E,F), while dermal staining is observed in sections through the posterior of the bud (E,G). (E) Sections through the A/P axis of the bud (anterior = left) reveal a sharp boundary between the region of nuclear β -catenin in the anterior placode (yellow arrows) and posterior domain which lacks nuclear β -catenin. Note the accumulation of β -catenin in the cytoplasm in the anterior dermal condensation (E' red arrow) and nuclear accumulation of β -catenin in the posterior dermal condensation (E, underlying the vertical red arrows). Sections through the anterior (F) and posterior (G) of a bud along the mediolateral axis reveal strong nuclear staining in the ectoderm and dermis respectively, while the pathway is inactive in immediately adjacent cells in the dermis (F') and ectoderm (G'). At the late bud stage, strong nuclear β -catenin staining is seen in the posterior dermis and a restricted region of ectoderm at the anterior of the bud (H). Despite strong staining in the posterior dermis (H' yellow arrow), no signaling is observed in the adjacent ectoderm (H' red arrow).



symmetrical bud rudiment develops a clearly defined A/P axis, nuclear β -catenin is observed in the anterior of the placode (Fig. 1E,F), while in the dermis, high levels of nuclear β -catenin are observed in the posterior of the bud (Fig. 1E,G). This pattern persists through intermediate bud stages (Fig. 1H).

This pattern of nuclear β -catenin localization suggests a role for the pathway in several steps of tract morphogenesis. Formation of dense dermis is an intrinsic property of dermal cells in the presumptive tract (reviewed in Dhouailly et al., 1998) and this process may involve signaling mediated by the β -catenin pathway within this layer. Alternatively, as the distribution of staining suggests, β -catenin signaling in the dermis may be a response to a signal from the ectoderm which is induced during this step. The subsequent pattern of pathway activation in the ectoderm resembles the expression pattern of several genes (including *BMP-2* and *-7*, *Lunatic fringe* and *Wnt7a*), which are first observed at low levels throughout the ectoderm above the dense dermis, and are then upregulated in the placodes as they form (Noramly and Morgan, 1998 and data not shown). This temporal and spatial correlation is consistent with a role for the β -catenin pathway in mediating the primary inductive signal from the dermis which initiates patterning in the ectoderm. The segregation of β -catenin signaling to the anterior of the placode and the posterior of the dermal condensation suggests roles in the oriented outgrowth of the bud at later stages as well. Since β -catenin accumulates in the cytoplasm of cells in the dermal condensation as it forms but is not observed in the nucleus, the transcriptional activity of this signaling pathway is apparently not required to initiate dermal condensation. However, the subsequent translocation of β -catenin to the nucleus in cells located in the posterior half of the dermal condensation implies that an additional layer of regulation may co-operate with this pathway to impose A/P patterning on the bud and orchestrate its directed outgrowth.

Forced expression of β -catenin leads to activation of the feather bud pathway

To test directly the importance of localized activation of this

signaling pathway in feather development, the expression of a stabilized form of β -catenin was forced in the developing chick epidermis using the RCASBP(A) replication competent retroviral vector (Petropoulos and Hughes, 1991; Capdevila et al., 1998). Localized infection of the ectoderm was employed to alter β -catenin signaling in this layer at the onset of feather development. The localization and spread of the virus during patterning of the feather field was determined by detecting viral transcripts in embryos at day 6 to day 9 of incubation (Fig. 2C-F). Small patches of viral transcripts are first detected in the chick ectoderm at day 6 of incubation, several days after infection. At this stage, development of the feather tracts has not yet begun. The first feather placodes appear at day 7 of incubation and the patches of virus are slightly larger due to clonal expansion of infected cells as well as spread of infection to adjacent cells. By day 8 of incubation, the feather fields are well-established and small areas of the feather field and apteric regions (areas between defined feather tracts in the embryo) are

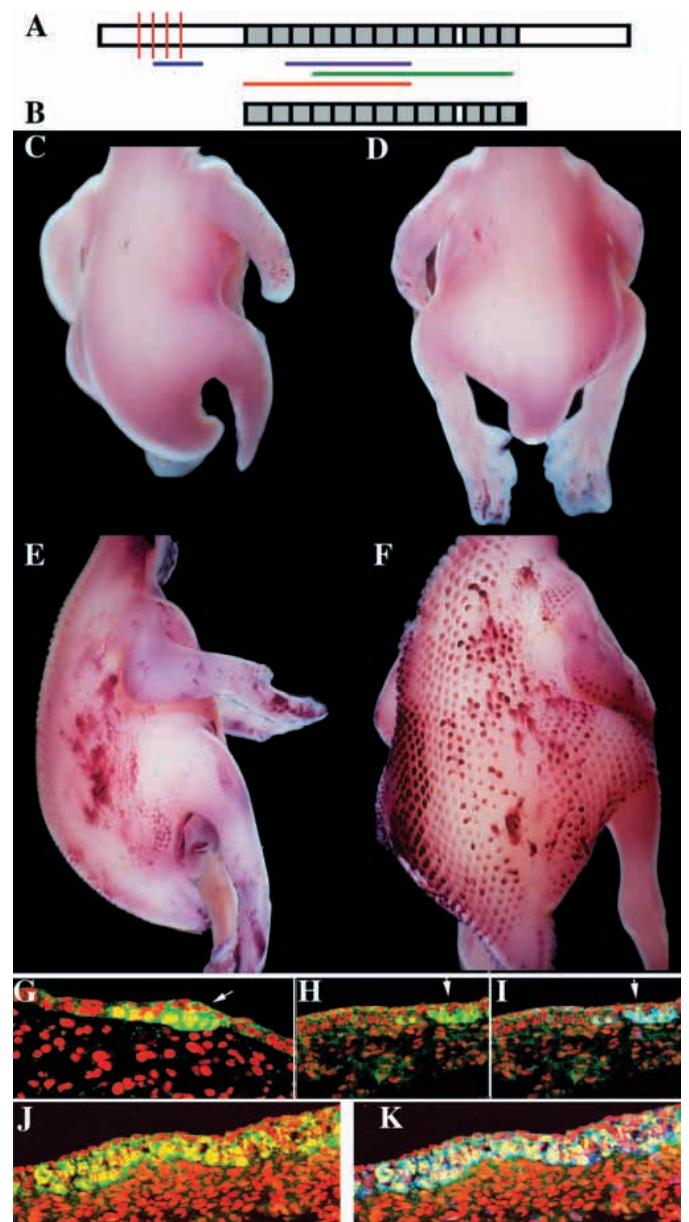


Fig. 2. Infection of chick epidermis by a retroviral vector containing a truncated β -catenin insert. (A) Full length β -catenin includes 13 imperfect armadillo repeats (shown by gray boxes) flanked by N- and C-termini of 130 and 100 amino acids respectively. The bars below this diagram indicate the binding domains for α -catenin (blue), TCF (purple), cadherin (green) and APC (red). Red vertical lines depict residues that are phosphorylated and act as a signal for degradation by the ubiquitin-proteasome pathway. (B) The truncated β -catenin used to activate this pathway comprises the armadillo repeats with an epitope tag indicated by the black box. To track the progress of infection by the retrovirus containing the truncated β -catenin insert, embryos were harvested at 24 hour intervals beginning at 3 days after injection of the viral construct to 6 days after the injection of virus (C-F). Viral transcripts (brown) were detected by whole-mount in situ hybridization. (G) Immunolocalization of the exogenous β -catenin (green) in a focus of infection in the apteric ectoderm of a day-8 embryo shows β -catenin in the cytoplasm and nucleus of the basal epidermis. β -catenin accumulates in the cytoplasm of the periderm but is not observed in the nucleus (arrow). Endogenous β -catenin also accumulates in the cytoplasm and nucleus of infected cells. Nuclear stain (red) and endogenous β -catenin (green) are shown in H and J, while infection is revealed in blue on the same sections (I,K) during an early response (H,I) and as a dermal condensation is forming (J,K).

infected with the β -catenin virus. Through day 8, no spatial restrictions to infection are observed. At day 9 of development, infection can be quite extensive with many buds infected, in particular those that were formed most recently and were located at the edges of the tracts. By this stage, viral gene expression is observed preferentially in feather buds when compared to interfollicular skin. This bias may be explained by preferential silencing of viral gene expression, resistance to reinfection after silencing, or both (see Materials and Methods). The pattern and extent of infection seen in embryos inoculated with the β -catenin virus is similar to that observed in embryos inoculated with control viruses (data not shown). This suggests that no toxicity or growth disadvantage is caused by expression of the truncated β -catenin protein during these stages of development. Sections through the areas of infection showed that the expression of viral transcripts was confined to the ectodermal layer in most instances prior to day 9 of incubation ($n=14$ embryos, greater than 10 foci of infection per embryo; see Figs 2G-K, 7C). When infection was detected in the dermis, these areas were excluded from the analysis reported below.

Patterning of the dorsal and femoral tracts is largely complete by day 13 of incubation although additional development of the buds continues. At this stage, most of the feather buds are long thin filaments evenly dispersed in the smooth epidermis of interfollicular skin (Fig. 3A,E,G). The β -catenin-infected embryos showed ectopic cutaneous appendages in both the feather fields and normally apteric regions (Fig. 3D,H). These were observed as an increase in the number and density of buds found both in the skin between the tracts which is normally sparsely populated with feather buds, and in the mid-ventral apterium where no feathers are formed in uninfected embryos. Ectopic buds were also formed in the interfollicular regions of the feather tracts as well. The size, shape and orientation of the ectopic appendages varied

considerably. In addition, many of the buds that formed in apparently normal positions in the tracts of the injected embryos were thicker than normal, had defects in polarity (Fig. 3F) and/or had abnormal growths on the filaments themselves (Fig. 6E). The disorganized appearance of the feather tracts in infected embryos suggests that either the normal sequential addition of buds was perturbed, that growth rates within individual buds were altered, or both. The pattern of endogenous β -catenin staining is consistent with roles in both bud initiation and subsequent bud outgrowth.

Feather bud initiation

The effect of β -catenin stabilization in bud initiation was examined further by assessing the gene expression changes associated with placode formation in infected embryos as the tracts begin to form. During normal development, several genes including *BMP-2*, *BMP-7*, *Lunatic fringe* and *Wnt7a* are expressed at low levels in the tract ectoderm after dense dermis formation and are subsequently upregulated in the ectodermal placode (Crowe et al., 1998; Jung et al., 1998; Morgan et al., 1998; Noramly and Morgan, 1998). Among these, *BMP-2* is the earliest marker identified to date whose localized expression presages the formation of the placode (Noramly and Morgan, 1998). As the placode is formed, *Lunatic fringe* and *BMP-7* are also upregulated while *Cek-3* expression is repressed (Patstone et al., 1993; Crowe et al., 1998; Noramly and Morgan, 1998). Ectopic expression of *Lunatic fringe*, *BMP-2* and *BMP-7* is caused by infection of the ectoderm with the β -catenin virus, while *Cek-3* is suppressed in infected regions (Figs 5D, 7B,E and data not shown). In addition, the infected ectoderm assumes the columnar morphology of epidermal placode cells (Figs 4H, 7C-E and data not shown). These gene expression responses can be observed both within presumptive tract ectoderm prior to the onset of placode formation in the area, in interfollicular

Fig. 3. Forced expression of β -catenin induces the formation of ectopic cutaneous appendages and abnormally shaped feathers. Control (A) and β -catenin virus-infected embryos (B) were harvested at day 13 of incubation to examine feather morphology. (C) The apteric region between the back and thigh feather tracts in a day-13 control embryo normally forms fewer feathers than within a tract. The β -catenin virus-infected embryos show ectopic cutaneous appendages in the apteria (D). The feathers in the femoral tract of a control embryo (E) are long and thin compared to a similar region in an injected embryo (F). Here, the feathers are misshapen and are oriented randomly. (G) The arrangement of feathers in a regular hexagonal pattern is readily observed after the feathers are clipped close to the skin surface. (H) In an injected embryo, disruptions to the regular pattern are seen in the formation of additional appendages between more normal buds (vertical arrowheads) as well as the formation of fused or large growths (horizontal arrowhead).

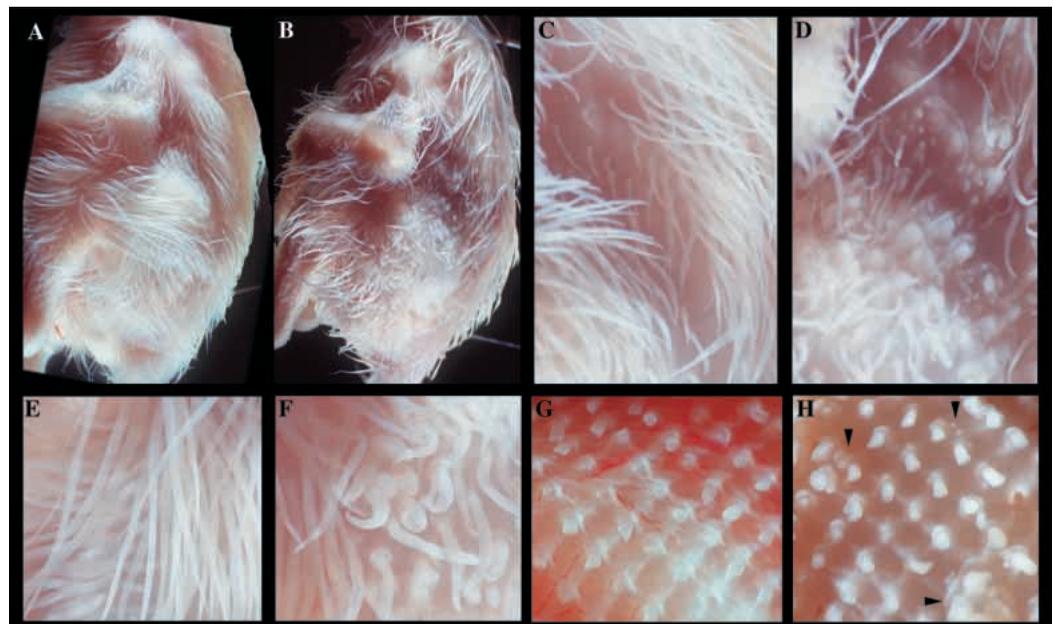
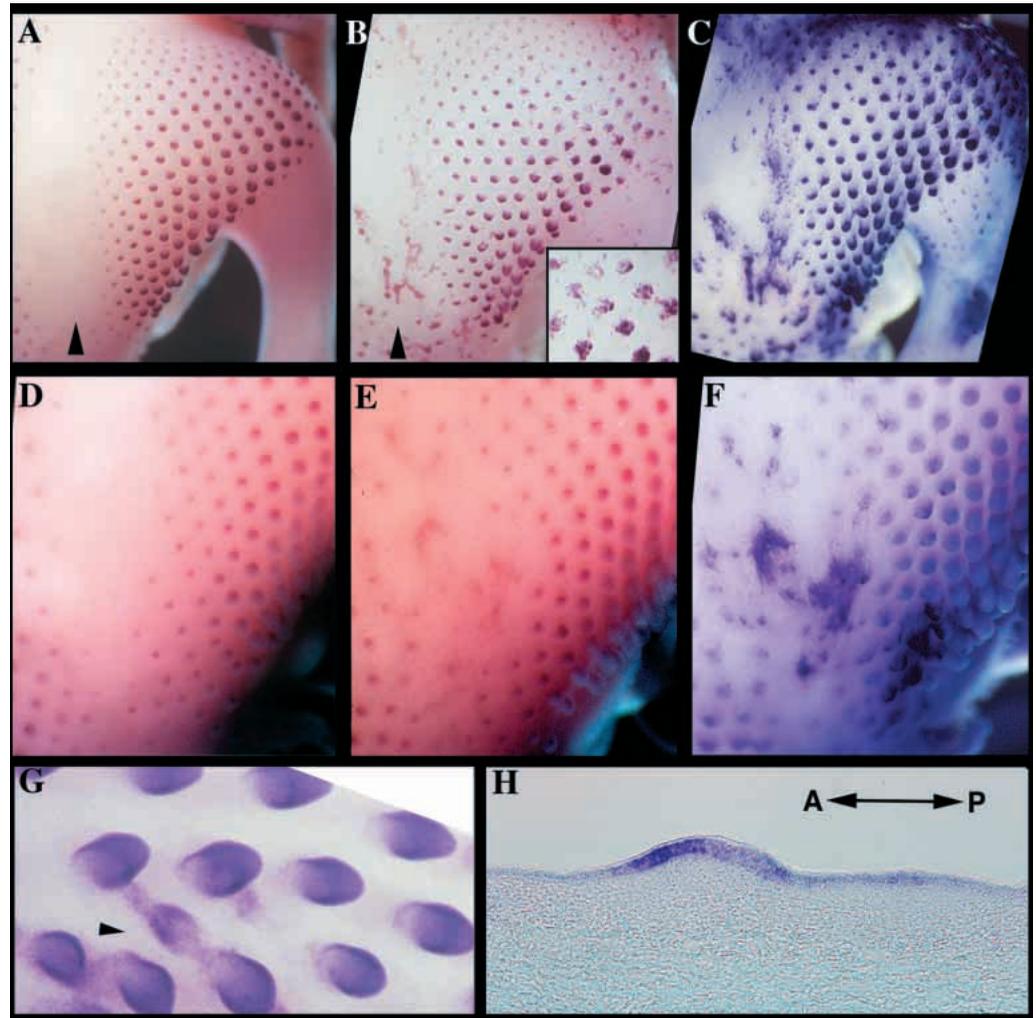


Fig. 4. Activation of placodal and dermal condensation markers. The expression of *Shh* in control (A) and β -catenin virus-infected (B) femoral tracts at day 9 of incubation. The expression of *Shh* is confined to the epidermal placodes and distal portions of the ectoderm in the older feather buds and is absent from the apteric region between the femoral and dorsal tracts (A, arrowhead). β -catenin virus-infected embryos showed areas of ectopic *Shh* expression both in the normally apteric region (B, arrowhead) and within the tract. The inset shows the femoral tract at higher magnification to reveal ectopic expression in interfollicular spaces. These areas of ectopic *Shh* expression coincide with the presence of viral infection as evidenced by subsequent detection of viral transcripts (C). The expression of *BMP-4* in control (D) and β -catenin virus-infected (E) embryos at day 9 of incubation. *BMP-4* expression is normally confined to the dermal condensation of the forming bud and is restricted to the anterior mesenchyme as the bud grows outwards (D). β -catenin virus-infected embryos (E) showed precocious expression of *BMP-4* both within the tract and apteric regions. Subsequent detection of viral transcripts confirmed infection of overlying ectoderm (F). Localization of induced *BMP-4* expression to the dermis was confirmed by sectioning specimens which had been analyzed for *BMP-4* alone (not shown). (G) Forced expression of β -catenin in interfollicular skin causes the formation of ectopic buds revealed by *BMP-2* expression. The arrowhead indicates the plane of section shown in (H) which confirms this bud has the stratified ectodermal placode and dermal condensation of a normal bud.



skin after the onset of bud formation, and in the presumptive apteric regions.

A second step in bud development is the induction of a dermal condensation by signals from the placode. The placodes induced by expression of truncated β -catenin in the ectoderm are functional and recruit the underlying dermis to form a bud. *BMP-4*, *BMP-7*, and *Serrate-1* are expressed in the forming dermal condensation but not in uninduced or interfollicular dermis (Chen et al., 1997; Crowe et al., 1998; Morgan et al., 1998; Noramly and Morgan, 1998). These markers and TCF-1 are induced in dermis underlying infected ectoderm as well (Figs 4E-F, 8C and data not shown). The level of transcripts for the FGF receptor *Cek-1*, normally suppressed in interfollicular skin (Song et al., 1996; Noramly and Morgan, 1998), is maintained beneath infected ectoderm as well (data not shown). This response is observed both within the tracts and in the normally apteric regions. Induced placodes are also capable of recruiting dermis in interfollicular skin. As shown in Fig. 4G and H, these supernumerary buds are comprised of the stratified ectoderm

and associated dermal outgrowth seen during normal bud development (see also Fig. 2J).

Effects of β -catenin on the A/P polarity of the developing bud

During normal development, β -catenin signaling becomes localized to the anterior ectoderm and posterior dermis of the bud (Fig. 1F-H). Disruption of this pattern by infection with the β -catenin virus interferes with A/P patterning in the bud and polarity was abnormal in many feathers in the β -catenin injected embryos. Some were pointing in the wrong direction (Fig. 5B), while others were curled (Fig. 3F). By the stage shown in Figure 5B, infection has frequently spread to the dermis and we cannot discern whether change in β -catenin signaling in the ectoderm alone is sufficient to alter polarity or whether forced activation in the dermis is also required. However, at earlier stages the virus is confined to the ectoderm and it is possible to assess A/P patterning with molecular markers before morphological asymmetry is observed. Expression of genes in the Notch pathway are useful for this

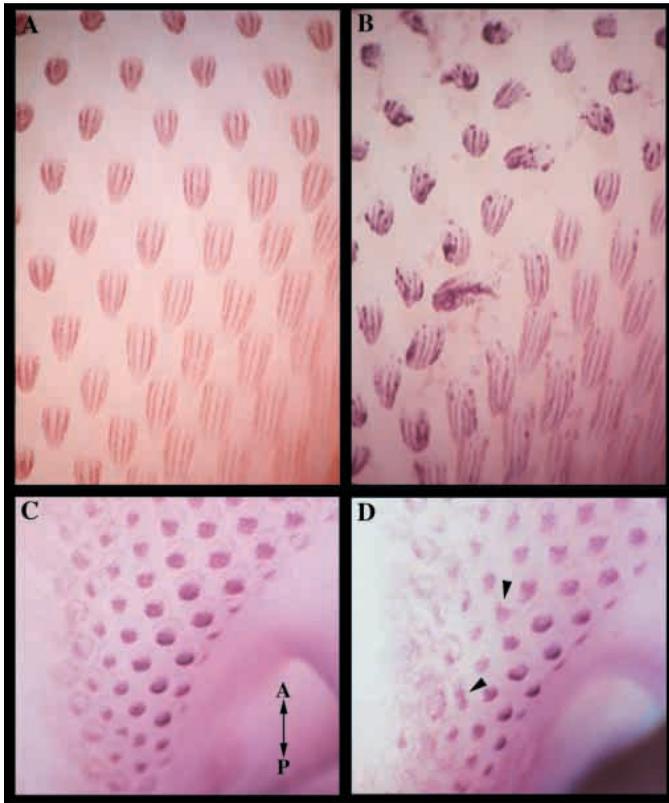


Fig. 5. Effects of exogenous β -catenin on the orientation of forming buds. (A) At day 10 of incubation, feather buds point in a posterior direction revealed by the stripes of *Shh* expression which run along the proximal/distal axis of the feather bud. (B) In β -catenin-infected embryos of a similar age, many buds are abnormally oriented. *Lunatic fringe* expression in control (C) and β -catenin-infected (D) day-8 embryos. The expression of *Lunatic fringe* is dynamic during the earliest stages of bud formation and then becomes confined the posterior quadrant of the developing bud (C). In the β -catenin-infected embryos, disruptions to the normal expression of *Lunatic fringe* are seen (D, arrowheads). The boundaries of *Lunatic fringe* expression are indistinct and abnormally oriented.

development (Chen et al., 1997). *Serrate-2* and *Delta-1* become restricted to the posterior of the bud in the ectoderm and dermis respectively such that the border of expression of these genes is normally parallel to the medio-lateral axis. Physical manipulations which alter bud polarity lead to corresponding changes in *Delta* and *Serrate* gene expression and confirm their utility as markers of polarity (Chen et al., 1997). As shown in Figure 5D, the dynamic but orderly pattern of *Lunatic fringe* gene expression is disrupted in the tracts of infected embryos at early stages of bud morphogenesis. The border of *Delta* and *Serrate* gene expression is often abnormally oriented or curved in infected buds as well (data not shown). Thus A/P patterning is disrupted at early stages of bud development when the normal pattern of β -catenin signaling in the ectoderm is disrupted by forced expression of truncated β -catenin.

purpose. The Fringe genes encode proteins which modulate Notch signaling (reviewed by Irvine and Vogt, 1997) and dynamic changes in *Lunatic fringe* expression are among the earliest identified indicators of A/P patterning in the bud (Noramly and Morgan, 1998). The expression of the Notch receptors and their ligands *Delta* and *Serrate* are also early indicators of anterior-posterior patterning during bud

Intrafollicular patterning

Wnt7a is also asymmetrically expressed in the placode as it develops. It is normally repressed in most of the placode and then expressed in the posterior bud ectoderm after *Lunatic fringe* is localized to this region (S. N. and B. A. M., unpublished data). The domains of *Wnt7a* and β -catenin nuclear localization are complementary at this stage (Fig. 6A,B), suggesting the

Fig. 6. Intrafollicular patterning within β -catenin-induced appendages. (A) *Wnt7a* mRNA (purple) becomes localized to the posterior of the ectodermal placode as the bud begins to grow outwards. (B) In an adjacent section through the feather bud, nuclear β -catenin protein is localized in a complementary pattern in the anterior ectodermal placode. (C) Circles of *Wnt7a* expression are induced by ectopic β -catenin expression in the developing chick skin (arrowheads). (D) Subsequent detection for β -catenin virus localization shows that it is the areas within the *Wnt7a* expression that are infected by the β -catenin virus. (E) Day-13 embryos were often seen to have misshapen feathers, often with ectopic bumps along the filament. (F) *Shh* expression is seen in ectopic bumps on β -catenin-infected feathers (arrowhead) in a day-9 embryo. G shows the same feathers after subsequent detection for viral transcripts.

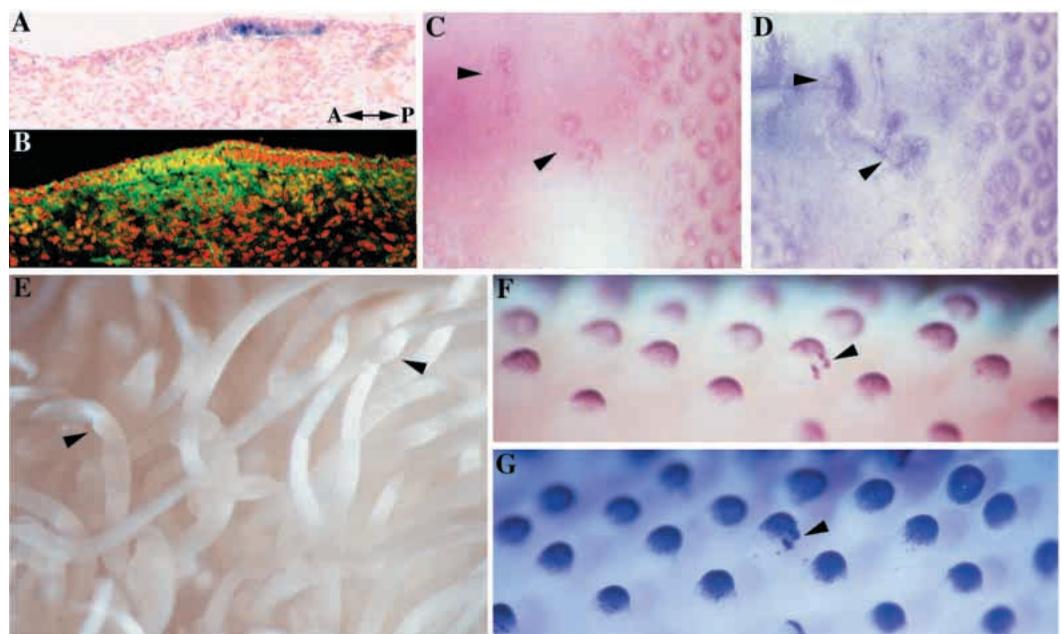
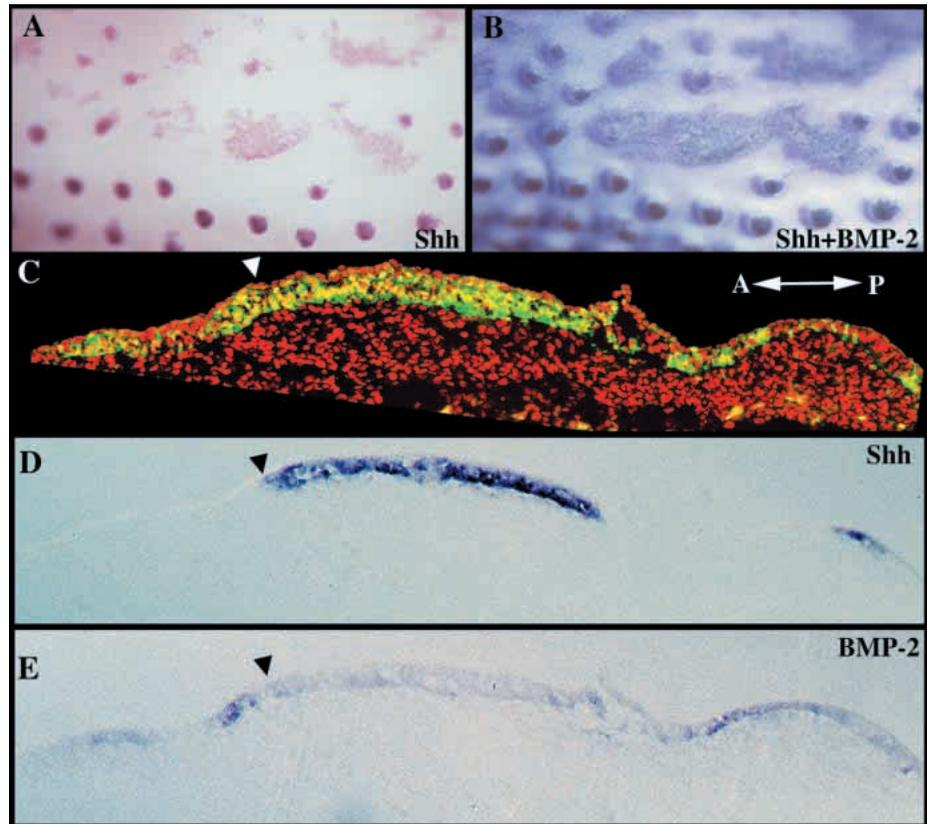


Fig. 7. Regulated activation of *BMP-2* and *Shh* transcripts in β -catenin-infected embryos. (A) Whole-mount in situ hybridization of a day 9 β -catenin-infected embryo to detect the presence of *Shh* transcripts. Widespread ectopic expression of *Shh* in the tract can be seen. (B) Subsequent analysis of the embryo for *BMP-2* transcripts showed them to be more extensive than the ectopic *Shh* expression and upregulated in the anterior of induced bud-like areas. C-E show serial sections through such an induced bud area. (C) The first section was analysed for the presence of the truncated β -catenin construct with an antibody to the HA epitope tag (green) while nuclei were counterstained with TOPRO3 (red). (D,E) In situ hybridization of *Shh* and *BMP-2* transcripts respectively by. (C) Ectopic β -catenin protein is seen throughout the induced bud area. (D) *Shh* expression is excluded from the most anterior region of placode (the region to the left of the arrowhead). (E) *BMP-2* transcripts are seen throughout the induced epidermal placode and are upregulated along the anterior border of the bud (the region to the left of the arrowhead).



possibility that β -catenin signaling represses *Wnt7a* expression within the placode. Consistent with this observation, *Wnt7a* is not expressed in placodes induced by expression of stabilized β -catenin in apteric ectoderm but is induced in the surrounding cells (Fig. 6C,D). A similar pattern of expression surrounding induced placodes is also observed for the *Cek-3*, another gene repressed in the placode as it forms (not shown).

In addition to polarity defects, small spots of infection in the bud ectoderm lead to abnormal growths on the feather filaments (Fig. 6E). These growths can appear over much of the proximal-distal extent of the forming filament and suggest that β -catenin signaling may play important roles in patterning within the bud at later stages. The ectopic growths express *Shh* at levels similar to those observed at the distal tip of normal buds (Fig. 6F,G). It has been suggested that *Shh*-expressing cells play an organizing role at this stage in feather development (Nohno et al., 1995; Noramly and Morgan, 1998). Consistent with this proposal, these abnormal outgrowths are composed of infected (and *Shh* expressing) cells surrounded by apparently uninfected cells. However, we cannot rule out the possibility that previous exogenous gene expression followed by viral inactivation caused the aberrant development of these cells.

During normal feather bud development, *Shh* is expressed initially in a subset of the *BMP-2*-expressing cells (Morgan et al., 1998 and data not shown). As development proceeds, *BMP-2* expression becomes restricted to cells anterior of the *Shh*-expressing cells, which are in turn anterior to cells expressing *Wnt7a* (Morgan et al., 1998). Expression of activated β -catenin in the ectoderm induces both *BMP-2* and *Shh* expression. In early stages of infection, the expression of these two genes appears co-extensive. Whole-mount in situ detection of *Shh* in

infected embryos at day 8 of incubation revealed multiple spots of ectopic gene expression. After subsequent detection with *BMP-2*, no additional spots of ectopic expression were observed ($n=9$ embryos). In a similar fashion sequential detection of *Shh* after *BMP-2* failed to reveal spots of ectopic expression of *Shh* which did not express *BMP-2* ($n=12$ embryos). However, at later stages of development (day 9,10), differential expression of *Shh* and *BMP-2* was observed in large patches of infected ectoderm ($n=22$ embryos, see Fig. 7). Detection of exogenous β -catenin and *BMP-2* and *Shh* transcripts on serial sections through patches of infection in the normally apteric region confirmed that although β -catenin was expressed throughout the induced placode, *Shh* and *BMP-2* expression were not uniformly expressed through the region (Fig. 7C-E). These observations suggest that additional patterning influence can be integrated with the β -catenin pathway downstream of stabilized β -catenin to regulate gene expression within the placode.

DISCUSSION

The pattern of β -catenin signaling during tract morphogenesis and the effects of forced activation of this pathway at different times and places during that process demonstrate successive roles for β -catenin signaling in tract morphogenesis. These roles will be discussed in chronological order during tract formation.

β -catenin signaling and dense dermis formation

The localization of β -catenin to the nucleus of cells adjacent

to the ectoderm as dense dermis forms suggests a role for the pathway in stimulating this step. The graded distribution declining from the surface implies that either the signal activating this pathway emanates from the ectoderm, or that cells which activate this pathway at higher levels migrate beneath the ectoderm. The dermis being the source for this signal is supported by the fact that formation of dense dermis is a property intrinsic to the mesenchyme which is established prior to this stage in development (Saunders and Gasseling, 1957; Saunders, 1958; Mauger, 1972; Dhouailly et al., 1998). However, it is possible that the presumptive dense dermis induces the expression of a Wnt in overlying ectoderm which then signals back to the underlying dermis and accounts for the observed pattern of β -catenin signaling.

Formation of the placode

The first observed subdivision of the tract into presumptive follicular and interfollicular fates occurs in the ectoderm as the ectodermal placode is formed. This could reflect the activity of a similarly localized inductive signal in the dermis. However, we have postulated that the initial inductive signal from the dermis is only generally localized in a broad band, and that patterning in the ectoderm limits the response to this signal and specifies the position and extent of the nascent placodes (Noramly and Morgan, 1998). This model is based in part on the observation that the expression of several genes is first induced in tract ectoderm in a broad band and then restricted to the nascent placode in a fashion similar to the pattern of nuclear β -catenin localization reported here. It is also based on the observation that BMP-2 is an inhibitor of placodal gene responses which is necessary to mediate the spacing of buds. Expression of BMP-2 in the nascent placode could regulate formation of adjacent placodes by inhibiting the response to a broadly expressed inducer in surrounding cells.

Forced activation of the β -catenin signaling pathway in the ectoderm is sufficient to induce ectopic placodes in either presumptive tracts or apteric regions. Although it is possible that infection of the ectoderm recapitulates the entire process of tract formation and acts indirectly by inducing a dense dermis, we find no evidence to support this model. In the apteric regions, ectopic placodal gene expression and morphological changes are observed in the absence of detectable changes in either β -catenin localization or cell density in the dermis (Fig. 2G,H and data not shown). The detected changes in gene expression in the dermis (e.g. *TCF-1*, *BMP-4*) occur after the induction of placodal gene expression and are associated with formation of the dermal condensation (not shown). Thus we conclude that forced activation of the β -catenin pathway in the ectoderm induces expression of a battery of placodal genes directly and circumvents the requirement for dense dermis to generate the signal that normally activates this pathway in the epidermis. We further infer that signaling through β -catenin normally initiates this step in tract development. If so, the early expression of these genes in a diffuse pattern implies that the signal that normally activates this pathway is not initially localized to bud primordia. Since the same response is observed when stabilized β -catenin is expressed in the ectoderm of normally apteric regions, activation of this pathway is sufficient for placode induction and does not require the cooperation of some other signal normally expressed specifically in the forming tracts.

BMP-2 is among the genes induced by the activation of the β -catenin pathway in the ectoderm and we assume a secondary activator of bud development, possibly an FGF, is induced as well. During normal development, the secondary activator would reinforce β -catenin signaling and inhibit response to BMP-2 locally, while BMP-2 acts to block response to the inductive signal from the dermis in the surrounding cells leading to localized formation of a placode (Jung et al., 1998; Noramly and Morgan, 1998). Since nuclear β -catenin staining becomes restricted to the placode, BMP-2 must block the response to inducer upstream of β -catenin in the signal transduction cascade. The fact that forced stabilization of β -catenin can override lateral inhibition and elicit a placodal response in interfollicular ectoderm further suggests that lateral inhibition acts upstream of this step. Lateral inhibition by BMP-2 could occur either by restricting the expression of the inducing signal in the underlying dermis, limiting responsiveness to this signal in the overlying ectoderm, or both. Resolution of this question will be facilitated by the identification of the inducing signal from the dermis.

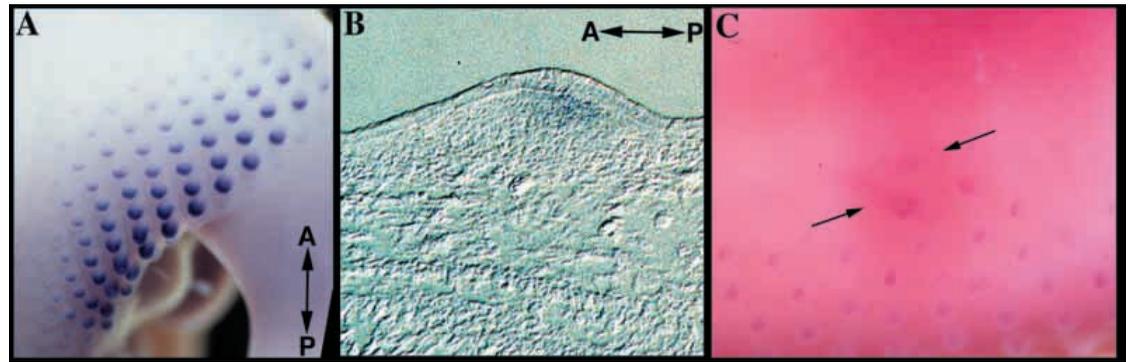
The identity of the signal that activates the β -catenin pathway during early tract development remains unknown. Although other factors may activate the pathway (Papkoff and Aikawa, 1998), a member of the Wnt family is a strong candidate for this signal. In addition to the ability of many Wnts to activate the β -catenin signaling pathway, the cooperative interaction with FGFs (secondary inducer) postulated above and the antagonistic interaction with BMP-2 demonstrated for this primary inductive signal are both characteristics shown by Wnts in other developmental contexts (Yang and Niswander, 1995; Jiang and Struhl, 1996; Penton and Hoffmann, 1996; McGrew et al., 1997; LaBonne and Bronner-Fraser, 1998). Conserved interactions between these pathways may be the foundation of a basic patterning subroutine in embryonic development.

Formation of the dermal condensation

It is noteworthy that when induced and maintained by forced activation of the β -catenin pathway, an ectodermal placode can recruit dermal cells in apteric regions to form a bud. When ectoderm containing early ectodermal placodes from forming tract is recombined with apteric dermis, buds are not formed (reviewed in Sengel, 1976). However, as demonstrated here, this reflects the failure of the apteric dermis to maintain the placode, rather than its inability to respond to it.

Stabilization of cytoplasmic β -catenin occurs during the formation of the dermal condensation, but nuclear localization is not observed until later in development when β -catenin is translocated to the nucleus in the posterior half of the dermal condensation. Virally expressed β -catenin also fails to accumulate in the nucleus of the periderm when it is readily observed in the nucleus of the underlying epidermis (Fig. 2G). This suggests that additional signals may regulate the activity of the pathway, either by promoting nuclear accumulation or inhibiting it. Recent observations in *Drosophila* also suggest that regulated nuclear accumulation of stabilized armadillo (β -catenin) adds an additional level of control to signaling through this pathway (Wu and Cohen, 1999; Cox et al., 1999). One change in dermal cells that participate in the condensation which could promote the nuclear accumulation of β -catenin is increased expression of TCF. Although β -catenin can

Fig. 8. TCF-1 expression in the developing feather tract. (A) The expression of TCF-1 in the femoral tract of a control day-9 embryo. (B) A section through the developing feather tract reveals the expression of TCF-1 is localized to the posterior dermis of the buds with low levels also present in the anterior ectoderm. (C) Ectopic expression of TCF-1 (arrows) is observed in the apteric region of an embryo harvested at day 9 of incubation after infection with the β -catenin virus.



apparently translocate to the nucleus without binding to TCF, its detectable accumulation in the nucleus may depend on interaction with these proteins (Hsu et al., 1998; Cox et al., 1999). TCF-1 expression is increased as the dermal condensation develops and the distribution of transcripts shows an A/P restriction similar to that seen for nuclear β -catenin (Fig. 8). An alternative explanation of this co-localization is that TCF-1 expression is induced by β -catenin signaling. We do not detect abnormal TCF-1 expression in the ectoderm at day 8 of incubation when other ectodermal placode markers like BMP-2 are readily detected. By day 9, ectopic expression of TCF-1 is observed (Fig. 8C), but this lag suggests that this induction is an indirect effect of infection.

Integrating signals during patterning of the bud

After induction of the placode and dermal condensation, localized signaling through the β -catenin pathway contributes to patterning within the bud and coordinated outgrowth of the ectodermal and dermal components of the bud. Alteration of the pattern of β -catenin signaling leads to abnormally oriented buds as well as growths on the feather filaments. During the earlier steps of bud specification, we cannot distinguish whether the localization of β -catenin signaling reflects local expression of activating ligands or restriction in responsiveness of cells to more broadly expressed ligands. However, as anterior/posterior distinctions arise in the bud, the sharp boundary between cells with strong nuclear β -catenin staining in the anterior placode and those with cell surface staining in the posterior of the placode is unlikely to be caused by local expression of ligand. In addition, cells in the dermal and ectodermal layers with strong or absent nuclear β -catenin signaling are juxtaposed in both the anterior and posterior of the developing bud (e.g. Fig. 1H). Although this could reflect the inability of Wnts to signal between layers, spatial restrictions in the ability to respond to Wnts are more likely to explain these observations. Patterning of the bud entails integration of several signals, some of which may act in part by modulating responsiveness to Wnts. The anterior-posterior border of β -catenin signaling in the ectoderm correlates with the boundary of *Lunatic fringe* and *Serrate-2* expression. The ability of Notch to modulate Wnt signaling in other systems suggests that the localization of these modulators of Notch activity may restrict responsiveness to Wnts along the A/P axis. Since forced activation of the β -catenin pathway can alter the expression of *Lunatic fringe* and *Serrate-1* and *-2*, feedback

between these pathways may contribute to the normal establishment of the A/P axis of the bud. It appears that the integration of signaling to modulate responsiveness acts upstream of β -catenin since most activities of the stabilized construct do not appear restricted within the bud. The exception to that observation is the segregation of *Shh* and *BMP-2* expression within an induced placode. Although stabilized β -catenin is expressed throughout the induced placode, *BMP-2* and *Shh* are differentially expressed across its extent at later stages of development. It is possible that the activation of one of these genes is an indirect effect of β -catenin signaling, but we detected no indication of a lag between *BMP-2* and *Shh* induction. Thus it seems likely that other patterning influences combine with the β -catenin pathway to regulate *BMP-2* and *Shh* expression. This signal integration must occur downstream of β -catenin stabilization in the pathway.

Comparison with experiments in the mouse

Several of the phenotypes caused by forced activation of the β -catenin signaling pathway during feather tract development are similar to those observed after a similar β -catenin construct was expressed in the basal epidermis of transgenic mice using a keratin-14 promoter/enhancer expression cassette (Gat et al., 1998). In both cases, cutaneous appendages showed polarity defects and abnormal growths within individual follicles. In addition, ectopic follicles were induced by activation of the β -catenin pathway in both cases. However, a significant difference between these experiments was the fact that no effects were observed in embryonic mouse skin and ectopic hair follicles were not induced in interfollicular skin until the first hair cycle. This led the authors to conclude that a second factor, in conjunction with stabilized β -catenin, was required for the induction of hair follicles. The failure to influence embryonic skin development could then be interpreted either as the absence of this second signal, or as the failure to activate the pathway early enough in development to influence skin patterning.

Several differences between these two sets of experiments could explain the different outcomes. One trivial explanation for this difference is that mouse and chick induce follicles by different mechanisms. However, tissue recombination experiments suggest that these early signaling events are conserved across classes and this explanation is unlikely (Garber and Moscona, 1964; Garber et al., 1968; Dhouailly,

1973, 1975). The two constructs employed to express stabilized β -catenin are also not identical. While both lack the N-terminal domain of β -catenin, ours also lacked the C-terminal domain which is retained in the mouse experiment. Analogous constructs with or without the C-terminal domain behave in a similar fashion in forced expression assays in *Xenopus* and *Drosophila* (Funayama, et al., 1995; Orsulic and Peifer, 1996). The C-terminal domain contains a transcriptional transactivation domain which is required in transfection assays (van de Wetering et al., 1997) and the construct used in the chick presumably acts because it stabilizes the endogenous β -catenin protein (see Fig. 2H,J). Thus the mouse construct would be expected to be more potent, although it is possible that the C-terminal domain confers an additional layer of regulation in some developmental contexts and thereby limits the ability to promote follicle development.

It was also speculated that the keratin-14 expression cassette failed to induce ectopic hair follicles during embryonic development because it was not activated to high levels until too late in skin development. Although expression was not characterized, this cassette is relatively weak. Forced expression in interfollicular chick embryo skin at day 9.5 of incubation leads to induction of ectopic placodes. By the equivalent stage of mouse skin development, morphogenesis of interfollicular skin has progressed to the point that the keratin-14 expression cassette would be 'highly' active, but this level may be insufficient. Implicit in this explanation is the assumption that after a specific stage when levels are adequate, a 'co-activator' required to initiate folliculogenesis in conjunction with activated β -catenin is no longer available in interfollicular skin until provided during the first hair cycle. However, in our experiments, the induction of follicles in apteric regions and in interfollicular skin reveals no such requirement for a co-activator in chick skin. Thus it is possible that a 'co-activator' actually serves to increase expression of the transgene during the hair cycle rather than to co-operate with the transgene product.

Activation of the β -catenin pathway is more uniformly distributed within a region of developing epidermis in the transgenic mouse, while in our experiments the pathway is activated in small groups of cells. During normal development in the chick, the pathway is weakly activated throughout the epidermis and then locally upregulated as placodes form. Differential activation between placodal cells and their neighbors may be required to allow further development along the placodal fate. Further experiments will be required to determine if stabilized β -catenin expressed in analogous fashion can initiate hair follicle formation in embryonic mouse skin.

Conclusions

These experiments demonstrate that the β -catenin signaling pathway is active during early patterning of the feather tract, and that localized activation of this pathway is sufficient to generate an ectodermal placode which can then recruit underlying dermis to form a bud. Signaling through the β -catenin pathway plays several key roles in tract development, and interactions of this pathway with BMP, FGF and Notch signaling are likely to explain much of early tract patterning. These interactions appear to represent a conserved developmental subroutine fundamental to the formation of

many structures in the embryo (reviewed in Hogan, 1999). With the identification of the signals that activate this pathway during tract development, it will be possible to further dissect the mechanisms of pattern formation in the feather field.

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