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Wnt-7a in feather morphogenesis: involvement of anterior-posterior asymmetry and proximal-distal elongation demonstrated with an in vitro reconstitution model

Randall B. Widelitz*, Ting-Xin Jiang*, Chia-Wei Janet Chen, N. Susan Stott, Han-Sung Jung‡ and Cheng-Ming Chuong§

Department of Pathology, School of Medicine, University of Southern California, Los Angeles, CA 90033, USA
*These two authors made equal contributions
‡Present address: Developmental Biology Programme, Institute of Biotechnology, Biocentre 1, University of Helsinki, Finland
§Author for correspondence (e-mail: cmchuong@zygote.hsc.usc.edu)

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SUMMARY

How do vertebrate epithelial appendages form from the flat epithelia? Following the formation of feather placodes, the previously radially symmetrical primordia become anterior-posterior (A-P) asymmetrical and develop a proximo-distal (P-D) axis. Analysis of the molecular heterogeneity revealed a surprising parallel of molecular profiles in the A-P feather buds and the ventral-dorsal (V-D) *Drosophila* appendage imaginal discs. The functional significance was tested with an in vitro feather reconstitution model. Wnt-7a expression initiated all over the feather tract epithelium, intensifying as it became restricted first to the primordia domain, then to an accentuated ring pattern within the primordia border, and finally to the posterior bud. In contrast, sonic hedgehog expression was induced later as a dot within the primordia. RCAS was used to overexpress Wnt-7a in reconstituted feather explants derived from stage 29 dorsal skin to further test its function in feather formation. Control skin formed normal elongated, slender buds with A-P orientation, but Wnt-7a overexpression led to plateau-like skin appendages lacking an A-P axis. Feathers in the Wnt-7a overexpressing skin also had inhibited elongation of the P-D axes. This was not due to a lack of cell proliferation, which actually was increased although randomly distributed. While morphogenesis was perturbed, differentiation proceeded as indicated by the formation of barb ridges. Wnt-7a buds have reduced expression of anterior (Tenascin) bud markers. Middle (*Notch-1*) and posterior bud markers including *Delta-1* and *Serrate-1* were diffusely expressed. The results showed that ectopic Wnt-7a expression enhanced properties characteristic of the middle and posterior feather buds and suggest that P-D elongation of vertebrate skin appendages requires balanced interactions between the anterior and posterior buds.

Key words: Skin appendage, Feather, Hair, Induction, Wnt, Notch, Anterior-posterior axis, Proximal-distal axis, Placode.

INTRODUCTION

Epithelial appendages including feathers, scales, hair, claws, teeth, etc are induced and shaped through epithelial-mesenchymal interactions. Although they each have specialized shapes and functions, these appendages share some common signaling molecular cascades that regulate different cell behaviors such as cell adhesion, cell migration, cell proliferation and cell differentiation (Chuong, 1998). The appendages can be molded into a specific shape by adding or guiding cells to a particular region. Because of the complex yet distinct structure of feathers and the accessibility to experimental manipulation of feather explants (Lucas and Stettenheim, 1972; Sengel and Mauger, 1976; Chuong, 1993), we have been using feather morphogenesis to study the induction, formation and differentiation of epithelial appendages (Chuong et al., 1996; Widelitz et al., 1997). Feather development can be described as occurring over several stages. The first stage is induction, leading to the formation of epithelial placodes and dermal condensations. The primordia then enter the short bud stage, beginning as radially symmetrical structures, and finishing as long buds with anterior-posterior (A-P) and proximal-distal (P-D) asymmetries. Between these two stages, the buds develop a large degree of molecular heterogeneity, essentially marking three feather bud domains. The anterior domain expresses *Msx-1*, *Msx-2*, tenascin and some Hox genes. The posterior domain expresses FGFR-2, *Wnt-7a*, *Delta-1* and *Serrate-1* (Chuong et al., 1990; Noji et al., 1993; Noveen et al., 1995b; Chen et al., 1997; Crowe et al., 1998; Viallet et al., 1998). The middle domain, between the anterior and posterior buds, expresses *Notch-1* (Chen et al., 1997), NCAM (Chuong and Edelman, 1985) and *Shh* (Ting-Berreth and Chuong, 1996b).

The molecular heterogeneity is likely to be a prelude to future structural and functional complexities of the developing placode. For instance, adhesion molecules may modulate the

shape of skin appendages by molding the shape of dermal condensations, which are the anlage of future feather buds. Adhesion molecules are regulated by signaling molecules as we have shown; ectopic TGFβ and SHH expression can lead to the formation of large dermal condensations by enhancing NCAM expression (Ting-Berreth and Chuong, 1996a,b). FGFs can work with SHH to induce new feather buds (Widelitz et al., 1996; Jung et al., 1998). As the feather primordia form, cell proliferation ceases for about 24 hours within the dermal condensations (Wessels, 1965). Proliferation first reappears as a concentric ring (Noveen et al., 1995b) becoming differentially distributed to the posterior buds (Desbiens et al., 1992; Chen et al., 1997). This is partially similar to the expression pattern of *Notch* pathway genes (Chen et al., 1997), and is also very similar to the *Wnt-7a* expression pattern in feather development (Chuong et al., 1996 and this paper).

How might the expression of Wnt-7a regulate cell proliferation? Wnts and wg are homologous secreted signaling molecules (reviewed in Moon et al., 1997; Dickinson and McMahon, 1992; Nusse and Varmus, 1992) involved in axis determination in *Drosophila* and in vertebrates (McMahon and Moon, 1989; Campbell et al., 1993). wg is expressed in the Drosophila ventral leg disc and regulates the expression of decapentaplegic (Dpp) in the dorsal cells (Brook and Cohen, 1996; Jiang and Struhl, 1996). These molecules interact to form the limb and to give it dorsal/ventral (D-V) orientation (Couso et al., 1993). The junction of wg- and Dpp-expressing cells later become the most distal portion of the limb (Brook and Cohen, 1996; Jiang and Struhl, 1996; Lecuit and Cohen, 1997). In the wing, wg expression in the D-V boundary is regulated by Serrate signaling through Notch. Ectopic activation of Notch is sufficient to drive the expression of wg and to form ectopic wing margins (Diaz-Benjumea and Cohen, 1995). Several wg homologs have been identified in vertebrates. Our preliminary investigations demonstrated that Wnt-7a was the predominant *Wnt* gene expressed in developing skin (data not shown). Wnt-7a is expressed in the dorsal vertebrate limb ectoderm (Parr et al., 1993). Knockout mice with a targeted disruption of the *Wnt-7a* gene become ventralized showing that Wnt-7a is involved in establishing the D-V axis (Parr and McMahon, 1995). Retroviral mediated ectopic expression of Wnt-7a in the chicken limb bud led to the expression of Lmx (a LIM homeodomain transcription factor) and the formation of double dorsal limbs (Riddle et al., 1995; Vogel et al., 1995). Suppression of transcription from Lmx by forming a chimeric protein containing the DNA binding domain of Lmx and the repressor domain of engrailed decreased the dorsal characteristics of limbs (Rodriguez-Esteban et al., 1997). Legless mutant mice localize Wnt-7a properly, but fail to limit the expression of Lmx-1b to the dorsal ectoderm as seen in non-mutant mice (Loomis et al., 1998) and subsequently fail to form an AER (Bell et al., 1998). However, ectopic Wnt-7a expression failed to effect AER formation in chicken embryos (Kengaku et al., 1998). Rather, Wnt-3a functioning through β-catenin and Lef-1 produced the formation of an ectopic AER (Kengaku et al., 1998). Thus *wg*/*Wnt* genes are involved in embryonic axis determination in a number of different contexts. While the axes may initiate independently (Zeller and Duboule, 1997), the proper continued development of each axis may be linked.

In this study, we explore the functional significance of feather placode molecular heterogeneity by focusing on Wnt-7a. We overexpress Wnt-7a in feather morphogenesis using a novel procedure to transduce RCAS-mediated genes into feather explant cultures. The results suggest that Wnt-7a overexpression leads to expansion of domains showing characteristics of the middle and posterior buds. As a result, the transformed appendages fail to elongate, even though they can proliferate and differentiate to form barb ridges. We also showed that the distribution of molecules in the Notch pathway are altered in Wnt-7a transformed buds. These results imply that appropriate interactions among the anterior and posterior domains of the placode are essential for the continuous morphogenesis of epithelial appendages.

MATERIALS AND METHODS

Materials

Pathogen-free chicken embryos were obtained from SPAFAS (Preston, CT) and staged according to Hamburger and Hamilton (H&H) (1951).

In situ hybridization

Transcripts in developing chicken skin or cultured explants were detected by in situ hybridization as described for *Shh* (Ting-Berreth and Chuong, 1996b). A 2 kb *Wnt-7a* fragment (Dealy et al., 1993) was labeled with digoxigenin and used as a probe. Positive signals were then detected by immunostaining for the presence of digoxigenin (Boehringer Mannheim, Indianapolis, IN). Probes for *Notch-1* and *Delta-1* were from Myat et al. (1996). The probe for *RCAS polymerase* was from Dr Niswander (Crowe et al., 1998).

Retroviral transduction of reconstituted feather explants

Stage-31 chicken embryo dorsal skins were dissected in HBSS and incubated in 2× calcium/magnesium-free saline with 0.25% EDTA on ice for 10 minutes. The epithelium and mesenchyme were carefully separated using watchmaker's forceps. The mesenchyme was incubated in 0.1% collagenase/trypsin for 10 minutes at 37°C to prepare a single cell suspension. Cells were pelleted by centrifugation and resuspended in viral-containing medium for 3 hours. Reconstituted skin explants were made by plating mesenchymal cells at high density on tissue culture inserts and then placing a piece of intact epithelium on top of the mesenchyme. The reconstituted skin explants were grown in DMEM medium with 10% fetal calf serum. Medium was placed both in the outside well and the inner chamber. A thin layer of the medium was left in the inner chamber, to keep the explant moist and to provide an air-liquid interface. The explant cultures were incubated at 37°C in a humidified 5% carbon dioxide and 95% air incubator. The medium was changed every 2 days.

RCAS-Wnt-7a retrovirus (Yang and Niswander, 1995) was prepared following the method of Morgan and Fekete (1996). Briefly, chicken embryo fibroblasts were transfected with the *RCAS-Wnt-7a* plasmid using lipofectamine (Gibco BRL, Gaithersburg, MD). Cells were cultured in DMEM containing 10% fetal bovine serum and were kept in the logarithmic phase of growth. When cells achieved 70% confluence, the culture medium was exchanged for fresh medium. Medium containing the retrovirus was collected and titered by staining for the viral GAG product and for the expression of the exogenous gene. Virus-containing medium was incubated with separated epithelium or mesenchymal cells for 2 hours at 0°C. Following plating, the reconstituted feather buds continued to be cultured with virus-containing medium.

RT-PCR

RT-PCR of *RCAS* and *RCAS-Wnt-7a* infected reconstituted skin explants cultured for 2 days were carried out using an RNeasy mini kit (Qiagen, Santa Clarita, CA) followed by reverse transcription reactions. The primers used for detection of RCAS mouse *Wnt-7a* PCR were-GGC GGT AGC TGG GAC GTG (Cla12NCO sense) and-CAG CCA CTG GCC TGA GGG (antisense).

Immunochemistry

Immunochemistry of skin sections were performed as described (Jiang and Chuong, 1992). Monoclonal antibody to proliferating cell nuclear antigen (PCNA) was clone PC 10 (DAKO, Denmark). Rabbit polyclonal antibody to c-tenascin was from Jiang and Chuong (1992).

RESULTS

Expression of *Wnt-7a* during feather development

To determine the localization of *Wnt-7a* during feather morphogenesis, we performed in situ hybridization on developing chicken embryos using a 2 kb 3′ fragment as a probe (Dealy et al., 1993). Dorsal views of embryos are shown in Fig. 1A-D, and lateral views are in Fig. 1A′-D′. Chicken skin is divided into regions with feathers (tracts) and lacking feathers (apteric regions) (Lucas and Stettenheim, 1972). Different tracts develop at different rates and propagate in different directions (see arrows in Fig. 1B) (Linsenmayer, 1972; Mayerson and Fallon, 1985). Here whole-mount in situ staining of *Wnt-7a* in the spinal tract, humeral tract, femoral tracts and caudal tracts are shown (Fig. 1). At stage 26, *Wnt-7a* was mostly negative on the body surface, with faint staining initiating at the primary row of the humeral and femoral tracts (Fig. 1A,A′). At stage 29, *Wnt-7a* appeared in the midline of the posterior spinal tract, which split into two parallel stripes toward the anterior regions of the tract. Five spots with more intense staining were present at the posterior end, marking the latent feather buds of the first row (Fig. 1B). At the humeral and femoral tract, staining first appeared all over in the whole tract field (Fig. 1B′; for high power view see Fig. 2G), with strong intensity toward the primary row region. By stage 31, three rows of feather buds were at the posterior end of the spinal tract while only two rows were in the anterior regions (Fig. 1C). In the femoral tract, *Wnt-7a* became restrictively expressed only in the periodically arranged feather primordia (Fig. 1C′). As development proceeded, five rows of buds had developed by stage 33 (Fig. 1D). In the humeral and femoral tracts, similar processes occurred except that the direction of propagation was predominantly toward the midline (Fig. 1C′,D′).

The staining pattern in individual primordia also appeared to change progressively from a uniform distribution, to a circular pattern, to localization within the posterior bud. Feather polarity is shown schematically in Fig. 2. The dynamic *Wnt-7a* distribution pattern prompted us to examine this phenomenon with a higher power view (Fig. 2A-F). The dorsal view shows that *Wnt-7a*-positive round primordia first emerged from the relatively homogeneous smear at stage 29 (with a gradient toward the initiation point, Fig. 2A,G). *Wnt-7a* expression then became restricted toward the border of the primordial domain by stage 31 (right portion of Fig. 2A). As feather bud development continued to progress (Fig. 2A-C, feathers in the midline are more mature than those along the lateral edge), *Wnt-7a* became enriched in the posterior bud by stage 37. From the lateral section, *Wnt-7a* can be seen to be in the placode (Fig. 2D), then in the posterior feather epithelium (Fig. 2E). Following the formation of the feather filament, the staining for *Wnt-7a* became weak and diffuse (Fig. 2F).

Comparison of *Wnt-7a* expression with *Shh*

Wnt-7a appeared to be expressed regionally within the feather field and then became restricted to the feather primordium regions (Fig. 2G,H). Lateral to the currently forming feather bud row, *Wnt-7a* was always expressed as a smear. We compared the expression pattern of *Wnt-7a* with *Shh*, another gene important for feather morphogenesis. *Shh* was positive in the primary row of the spinal tract as a continuous stripe along the A/P axis (Jung et al., 1998). It seems that the mechanism underlying the formation of the primary row and the lateral rows may be different, since the stripe of SHH we reported in Jung et al. (1997) is only seen in the primary row. In subsequent lateral rows, SHH appears directly in the central region of the formed feather buds. The ‘SHH stripe’ can be seen in the primary row of the femoral tract. In contrast, *Wnt-7a* is intially expressed as a two-dimensional smear (not a linear stripe) before buds form. In the lateral rows, *Shh* appeared later than *Wnt-7a* and its distribution was not as widespread as that of *Wnt-7a*. *Shh* appeared only after *Wnt-7a* was restricted to a ring pattern (Fig. 2G,H). Hence the expression of *Shh* may mark the axis of the primary row, but *Wnt-7a* expression may be required for the lateral propagation of the feather buds.

Retroviral-mediated ectopic Wnt-7a expression led to plateau-like skin appendages that still matured to form barb ridges

To explore Wnt-7a function in feather development, it was ectopically expressed from a *RCAS-Wnt-7a* retrovirus (Yang and Niswander, 1995). When *RCAS-Wnt-7a* was injected into day 2-4 embryos in ovo, an overall suppression of feather bud formation was observed (not shown). Ectopic expression at different developmental stages may have different functions, as was reported for RCAS-mediated ectopic expression of sonic hedgehog (Morgan et al., 1998). To further examine the effect of Wnt-7a on feather morphogenesis, we developed an in vitro feather reconstitution model using tissues derived from the beginning stages (around H&H stage 30) of feather development. The epithelium were separated from the underlying mesenchyme. The mesenchyme was digested with trypsin/collagenase to a single cell suspension, which was then transduced with *RCAS-Wnt-7a* and recombined with intact epithelium to form a reconstituted explant. In each procedure, the signaling molecules were lost and cells were re-set back to an equivalent state (T.-X. Jiang, H.-S. Jung, R. B. Widelitz and C.-M. Chuong, unpublished). Signaling molecules then reappeared in order. The procedures offer a better opportunity to perturb specific events directly related to feather morphogenesis and also have the advantage of improved viral transduction.

In control reconstituted explants, feather buds reformed and showed normal molecular expression, as exemplified by *Wnt-7a* and *Shh* (Fig. 3A,A′). The efficiency of viral transduction was demonstrated in cultures infected with a *RCAS-alkaline*

phosphatase virus (Fig. 3B,B′). We also examined the expression of the transduced *RCAS-Wnt-7a* gene in explants by RT-PCR using primers specific to exogenous *Wnt-7a* (Fig. 3C). The virus was present in both the epithelium and the mesenchyme, as can be seen by in situ hybridization using *RCAS polymerase* as a probe (Fig. 3D, depicted is a reconstituted explant made from intact epithelium and reassociated mesenchymal cells). The retrovirus was detected by 2 days after transduction (data not shown) and grew more strongly positive by 3 days post transduction. Although *Wnt-7a* was expressed in both the epithelium and mesenchyme, the frizzled receptor was only found in the mesenchyme of H&H stage 34 embryonic skin (data not shown). Hence it appears that Wnt-7a in the epithelium normally signals to the mesenchyme.

The morphology of control reconstituted explants showed that feather buds can reform with normal shapes and were oriented in accordance with the epithelium (Fig. 3E-F′). This was true for explants not transduced as well as those transduced with *RCAS* virus containing no inserted expression sequence. After 2 days in culture, the control and *Wnt-7a* transduced explants were in the small bud stage, although the explants expressing exogenous Wnt-7a were broader with a loss of interbud space (Fig. 3E,E′). After 4 days, the control cultures developed elongated slender feather buds with A-P asymmetry slanting toward the posterior end (Fig. 3F). In contrast, transduction with *RCAS-Wnt-7a* produced broader buds with reduced interbud domains (Fig. 3F′). Occasionally the buds fused together, particularly at the edge. The *Wnt-7a* transformed skin appendages were flatter than the control buds and assumed a plateau-like morphology, nearly forming right angles at the corners (Fig. 4A-D′). Most of them lacked A-P asymmetry (Figs 3, 4). By 8 days in culture, the control reconstituted epithelial explants developed follicles while the *Wnt-7a* transformed skin appendages did not develop follicles (Fig. 4D,D′). Although the *Wnt-7a* transformed buds did not elongate distally, they did have the ability to form barb ridges (Fig. 4C,D′).

Wnt-7a transformed feather buds showed a diffuse distribution of posterior bud markers (*Notch-1* and *Delta-1*) and the middle bud marker (*Shh*) but suppressed expression of the anterior bud marker (tenascin-C)

To analyze the downstream events of ectopic *Wnt-7a* expression, we examined its effects on other molecules involved in feather morphogenesis. We observed the expression of posterior bud markers (*Notch-1* and *Delta-1*) using whole-mount in situ hybridization at 2 days in culture and section in situ hybridization at 2 and 4 days in culture. *Delta-1* was normally restricted to the posterior feather bud mesenchyme. This pattern was re-expressed in the reconstituted buds infected with *RCAS*. In the *Wnt-7a* transduced cultures, *Delta-1* expression became more widely distributed by day 2. There was a greatly expanded region of high *Delta-1* expressing mesenchyme adjacent to the epithelium (Fig. 5). The posteriorly localized *Serrate-1* expression also became diffuse (not shown). In normal buds cultured for 2 days, *Notch-1* was expressed as a central stripe perpendicular to the A-P axis. In the *Wnt-7a* transduced explants, the *Notch-1* expression pattern became diffuse within the buds and was greatly enhanced throughout the mesenchyme. The central stripe did not form or was very weakly visible in some buds (Fig. 5). By day 2, the *Notch-1* expression pattern was expanded in the *Wnt-7a* transformed explants. At day 4, the *Notch-1* expression was clearly elevated in the mesenchyme of *Wnt-7a* transformed explants (Fig. 5). Alterations of *Notch-1* and *Delta-1* at day 2 prior to changes in feather morphology

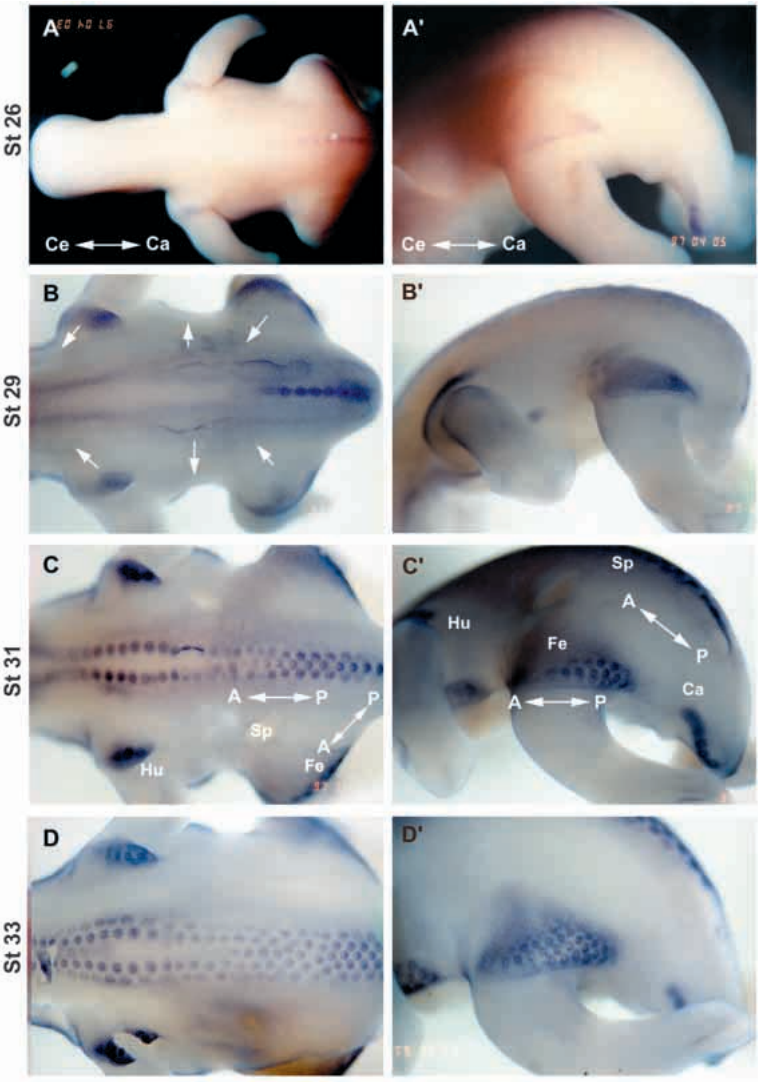

Fig. 1. Expression of *Wnt-7a* transcripts shown by whole-mount in situ hybridization. (A-D) Dorsal views. (A′-D′) Lateral views. Chicken embryos at stages 26 (A,A′), 29 (B,B′), 31 (C,C′) and 33 (D,D′) were used. Staining in the spinal tract (Sp), humeral (Hu), femoral tracts (Fe) and caudal tracts (Ca) can be seen. Different tracts are at different developmental stages. In each tract, *Wnt-7a* staining started as a relatively homogenous stripe (with higher intensity toward the region of the primary row) that progressively segregated into individual feather buds. Cephalic (Ce) and Caudal (Ca) ends of the embryo are indicated. The arrows in B indicate the direction of sequential appearance of new rows of feather propagation. The A-P axes of the spinal tract feathers are parallel to the Ce-Ca axis, but the A-P axes of humeral and femoral tract feather buds are not, as indicated.

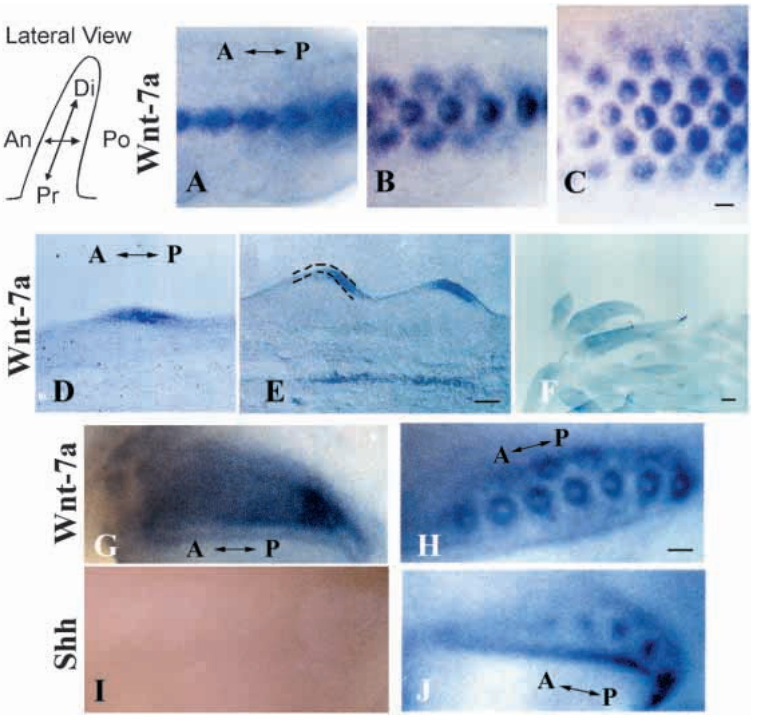

Fig. 2. Progression of *Wnt-7a* expression and comparison with *Shh*. A schematic diagram of feather polarity shows the anterior (An)-posterior (Po) and proximal (Pr)-distal (Di) axes. (A-F) In situ hybridization with *Wnt-7a*. (A-C) Dorsal view of the spinal tract (stages 29, 31 and 33, respectively). (D) Stage 29 and (E) stage 31, longitudinal sections. (F) Whole mount (stage 37). In the dorsal skin, *Wnt-7a* was first expressed as a continuous stripe (A), which then concentrated in the feather buds (B). The pattern shifted to become a ring bordering the bud domain and then became further restricted to the posterior bud (C). In the longitudinal sections, *Wnt-7a* can be seen in the posterior-proximal epithelium of the short feather buds (E). *Wnt-7a* expression was transient. In the feather filament stage, *Wnt-7a* expression became much weaker (F). (G-J) In situ hybridization comparison of *Wnt-7a* (G,H) and *Shh* (I,J) expression in the femoral tract. *Wnt-7a* was expressed throughout the putative femoral tract field at stage 29 (G). The expression pattern became gradually restricted to the feather bud domain by stage 31 (H). *Shh* was absent (I), then appeared as a stripe over the primary row and punctated pattern adjacent to it, similar to that of the spinal tract (J) (Jung et al., 1998). Thereafter, in the lateral rows, de novo *Shh* mRNA synthesis appeared in the center of the feather primordia after *Wnt-7a* expression became restricted to a ring pattern. Bars, 200 μm (A-C); 70 μm (D,E); 100 μm (F); 250 μm (G-J).

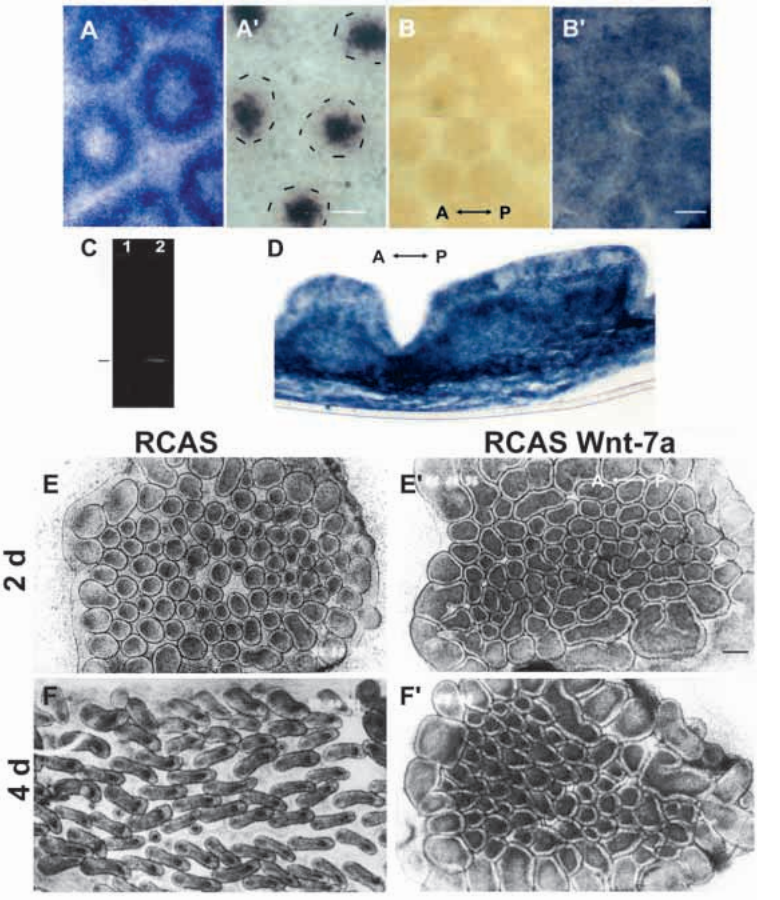

Fig. 3. *RCAS-Wnt-7a* viral infection led to plateau-like skin appendages that did not show anterior-posterior orientation. We used a novel procedure to reconstitute feather buds using a single cell suspension of mesenchymal cells recombined with intact epithelium. This procedure allows specific perturbation of feather development and better viral infectivity. After 1 day in culture, the reconstituted skin explants re-express *Wnt-7a* (A) and *Shh* (A′), which is similar to the in vivo ring and central dot pattern (Fig. 2H,J). The border of the buds is indicated by a black dotted line in A′. Short buds form after 2 days in culture and elongated buds form after 4 days in culture, as shown in (E-F′). (B) Viral infectivity is verified by alkaline phosphatase activity in *RCAS*-control (B) and *RCAS-alkaline phosphatase* (B′) transduced cultures. (C) Infected skins were further analyzed by RT-PCR for the expression of exogenous *RCAS-Wnt-7a*. Control *RCAS* transduced explants (lanes 1) and *RCAS-Wnt-7a* transduced explants (lanes 2) were amplified with primers corresponding to the Cla12NCO shuttle vector and *mWnt-7a*, which would amplify only exogenous *Wnt-7a*. The exogenous band is only visible in the *RCAS-Wnt-7a* transduced explants. The band is at the expected size of 967 bp. The presence of the retrovirus in explants reconstituted from intact epithelium recombined with reassociated mesenchyme was detected by in situ hybridization using the *RCAS specific polymerase* as a probe (D). The virus is present in both the epithelium and mesenchyme. (E-F′) Dorsal view of *RCAS* control (E,F) and *RCAS-Wnt-7a* transduced cultures (E′,F′) cultures made from pelleted mesenchymal cells recombined with intact epithelium (E-F′). (E,E′) cultured for 2 days, (F-F′) cultured for 4 days. Note the *Wnt-7a* transduced buds are larger in size and more symmetric in shape than the controls. At day 4, control feather buds elongated and the feather base was reduced in size with interbud spacing clearly visible. In *Wnt-7a* transduced cultures, few buds showed A-P orientation. Buds remained large and interbud spacing was reduced. The orientation of the A-P axis is indicated. Bars, 100 μm (A,A′); 200 μm (B,B′); 200 μm (D-F′).

suggest that these molecules are involved in shaping the feather bud and not merely responding to subsequent changes induced by morphological change.

We next examined the expression of the middle bud domain markers, *Shh* and *Notch-1* (*Notch-1* is in the posterior and along the midline). *Shh* was examined at 4 days by tissue section in situ hybridization. Sonic hedgehog was normally expressed in the middle feather placode epithelium and shifted to the distal-posterior bud epithelium when feather buds started to elongate (Ting-Berreth and Chuong, 1996b). In Wnt-7a overexpressing buds, the distal epithelial region expressing *Shh* was greatly expanded (Fig. 5).

Next we examined the expression of the anterior bud marker, tenascin-C by immunocyochemistry. Tenascin-C was normally expressed in the anterior feather mesenchyme (Jiang and Chuong, 1992). A lateral section showed that control cultures grown for 4 days had the same anterior immuno-localization pattern (Fig. 5). In explants overexpressing Wnt-7a, tenascin-C expression was inhibited within the bud (Fig. 5). Thus ectopic Wnt-7a expression altered the characteristics of the skin appendages and expanded regions with middle and posterior domain characteristics.

Overexpression of Wnt-7a was accompanied by the lack of localized distribution of proliferating cells

What is the effect of altering A-P polarity in the chicken feather bud on P-D elongation? While control skin explants developed elongated skin appendages, the *RCAS-Wnt-7a* transformed skin did not grow beyond the short bud stage. If localized cell proliferation is required to shape the skin appendage morphology, the localized distribution of proliferating cells observed in these stages could have been disrupted.

We examined the distribution of proliferating cells using anti-PCNA antibodies to stain sectioned reconstituted explant cultures and skin developed in vivo. In control reconstituted explant cultures PCNA staining was present in the anterior and posterior periderm, but was absent from the epithelium and mesenchyme at 1.5 days in culture (Fig. 6A). This time point corresponds to the time when proliferation is suppressed in vivo. A section from control skin developed in vivo from a slightly later developmental stage is also shown (Fig. 6B). Here PCNA staining was present in the posterior mesenchyme at a time prior to morphological asymmetry. Reconstituted skin explants cultured for 3 days still had PCNA staining in the periderm as well as some weak staining of the epithelium and mesenchyme (Fig. 6C). Later, at 4 days in culture, control feathers maintained the peridermal staining and a newly restricted distribution of positive cells in the posterior bud epithelium and posterior-proximal mesenchyme became strong (Fig. 6D). In contrast the *RCAS-Wnt-7a* transduced explants had more PCNA-positive cells present in both the epithelium and mesenchyme as well as positive staining in the periderm (Fig. 6E). The entire epithelium contained proliferating cells after transduction, while mesenchymal staining was highest in the middle bud domain. At 3 days the PCNA staining continued in the epithelium and became more diffuse in the mesenchyme (Fig. 6F). By 4 days, the explants still did not exhibit polarity in the distribution of PCNA as did the controls (Fig. 6G). This lack of localized cell proliferation probably led to the right-angled plateau-like morphology.

DISCUSSION

Molecular heterogeneity of feather buds

Epithelial-mesenchymal interactions start a chain of molecular events leading to a hierarchy of developmental steps progressively determining the fates of the epithelial and mesenchymal cells. As a result, feather bud domains are alternately arranged with interbud domains. Some molecules, such as protein kinase C, are expressed in the interbud domain. Other molecules are expressed within the bud domain, such as *Shh*, Msx-1 and Msx-2 in the epithelium, and FGF-4, NCAM, tenascin and pCREB in the mesenchyme (Jiang and Chuong,

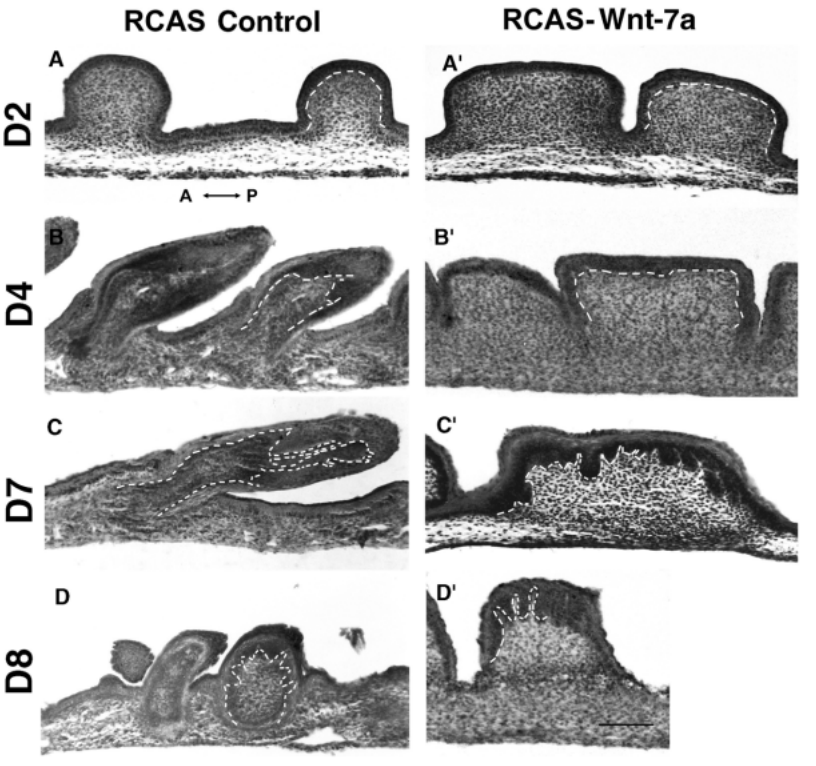

Fig. 4. Histology of *Wnt-7a* transformed skin appendages. Hematoxylin and Eosin stained sections with *RCAS* (A-D) and *RCAS-Wnt-7a* virus transduction (A′-D′). (A,A′) 2 day, (B,B′) 4 day, (C,C′) 7 day and (D,D′) 8 day reconstitution cultures. Control buds first appeared as round buds at day 2 (D2), then elongated and slanted toward the posterior end at day 4 (D4). This was followed by the formation of barb ridges (D7) and follicles (D8). *RCAS-Wnt-7a* transduced buds were initially wider (D2), forming plateaus with nearly right angles at each bend (D4). They proceeded to form barb ridges (D7), but failed to form follicles (D8). Since the base of the bud was not reduced in size, as in controls from D2 to D7, the interbud spacing was reduced. The orientation of the A-P axes are the same in all panels and is indicated in A. The dashed lines highlight the feather follicle. Bar, 100 μm.

Table 1. Summary of molecular expression in different regions of feather buds

	Anterior domain	Middle domain	Posterior domain
Epithelium	Msx-1[c], Msx-2[c] Tenascin[e]	Shh[a]	Wnt-7a[b] FGFR2[d] Cell proliferation[f,g]
Mesenchyme	Tenascin[e]	Notch-1[g]	Delta-1, Serrate-1, Notch-1[g,j,k]
	NCAM[h] Hox C6, D4[i] FGFR1[d] pCREB[l]		Fibronectin[m] Cell proliferation[f,g]

[a]Ting-Berreth and Chuong, 1996; [b]Chuong et al., 1996; [c]Noveen et al., 1995a; [d]Noji et al., 1993; [e]Jiang and Chuong, 1992; [f]Desbiens et al., 1992; [g]Chen et al., 1997; [h]Chuong and Edelman, 1985; [i]Chuong et al., 1990; [j]Crowe et al., 1998; [k]Viallet et al., 1998; [l]Noveen et al., 1995b; [m]Mauger et al., 1982.

1992; Noji et al., 1993; Nohno et al., 1995; Noveen et al., 1995a; Ting-Berreth and Chuong, 1996b). At this stage, the mesenchyma within the bud domain are radially symmetrical and can be molded by epithelial signals to become A-P asymmetrical (Novel, 1973; Chuong et al., 1996).

While signals emanating from the mesenchyme to direct the formation of feather primordia do not have polarity, the epithelium soon develops polarity. Shortly thereafter, at the short bud stage, molecular heterogeneities establish anterior, middle and posterior domains within both epithelial and mesenchymal components (Mauger et al., 1982; Chuong and Edelman, 1985; Kitamura, 1987; Jiang and Chuong, 1992; Noji et al., 1993; Noveen et al., 1995b; Chen et al., 1997; Crowe et al., 1998). These molecular heterogeneities precede the formation of morphological heterogeneities. A summary of these expression modes is shown in Table 1 and Fig. 7A. Some molecules are expressed in radial fashion, such as *Shh*, which is expressed in the distal placode epithelium (Fig. 2I,J). Some molecules are expressed with A-P asymmetry such as *Delta-1*, which is in the posterior bud mesenchyme. Some molecules change from radial symmetry to A-P asymmetry, such as *Wnt-7a*.

What is the functional significance of this molecular heterogeneity? Do they prepare the round flat feather primordia for the next morphogenetic event? Are changes in cell proliferation and gene expression occurring prior to the morphological changes? In Figs 5 and 6, day 1.5 and 2 explants showed clearly that there were already differences in expression of *Delta-1*, *Notch-1* and PCNA distribution before the buds became morphologically asymmetrical. These results support the idea that morphological asymmetry is the result of molecular asymmetry.

The molecules expressed in the P and A feather buds are surprisingly similar to those expressed in the *Drosophila* D and V imaginal discs, respectively. Therefore we hypothesize that the P-A axis in feather is reminiscent of the D-V axis of the *Drosophila* wing. It is then logical to reason that the interactions between these regions are essential to the formation of P-D axis of skin appendage, similar to that of the wing margin and limb bud apical ectodermal ridge (AER) (Saunders, 1948; Couso et al., 1994; Fallon et al., 1994; Kim et al., 1995). One way to address this hypothesis is to overexpress or suppress the expression of a tested molecule and to analyze the perturbed feather buds and molecular consequences. In this study we overexpressed Wnt-7a.

Overexpression of Wnt-7a transformed feather buds into appendages with more middle and posterior bud characteristics and with reduced interbud space

Here we misexpressed Wnt-7a by transducing skin explant cultures with *RCAS-Wnt-7a*. While control explants formed elongated feather buds pointing toward the posterior end, the *Wnt-7a* transduced skin explants produced wide and plateau-like skin appendages. They did not develop A-P asymmetry, nor did they elongate to form the P-D axis even after 8 days when the control explants formed feather follicles (Fig. 4). We then studied the characteristics of these plateau-like appendages. One possible mechanism limiting the growth of skin appendages may be through the arrest of cell proliferation. This is not the case, however, because PCNA staining showed that the Wnt-7a buds had indeed increased cell proliferation. Moreover, the proliferation was uniformly distributed in these appendages and this may explain the loss of both A-P and P-D axes. Earlier we proposed that the differential localization of cell proliferation in the posterior buds may contribute to the formation of A-P asymmetry, because more cells are laid into the posterior bud (Desbiens et al., 1992; Chen et al., 1997). For an appendage to form an elongated P-D axis, it needs a growth zone that can generate new cells continuously, whether new cells are added distally such as found in the progress zone of the limb bud, or proximally such as in the collar of the feather follicle (equivalent to hair matrix epithelia). The diffuse distribution of proliferating cells leads to a limited mode of growth, similar to that of the scale (Tanaka and Kato, 1983).

What are the characteristics of these perturbed appendages? *Delta-1* and *Shh* in the long feather bud stage have been shown to be expressed preferentially in the posterior bud. Here we observed that they too became expressed diffusely in the transformed appendages. In addition to a proximal-posterior expression, *Notch-1* forms a midline stripe in normal feather buds marking a zone that functions through prolonged proliferation potential, probably mediated by the activation of the Notch pathway (Austin et al., 1995; Chen et al., 1997; Dorsky et al., 1997; Henrique et al., 1997). In the *Wnt-7a* transformed buds, *Notch-1* also became diffusely expressed. The middle bud marker, *Shh*, also became more widely distributed, suggesting that the middle bud domain was also expanded in the *Wnt-7a* transformed feather buds. We earlier showed that *Shh* is required for feather formation (Chuong and Edelman, 1985; Ting-Berreth and Chuong, 1996b). The results suggest that Wnt-7a caused the expansion of posterior and middle bud markers into the anterior feather buds. The buds also lost a clear-cut A-P polarity, although in some regions multiple aborted axes can be seen. Together with the expression sequence (Fig. 7A), the results also suggest that Wnt-7a can increase the expression of Shh, Notch-1 and Delta-1. Therefore, the order of the molecular cascade may be Wnt-7a Notch-1, Delta-1, Serrate-1. Wnt7a is probably responsible for the posterior expression of these molecules. In addition, Wnt-7a may suppress tenascin expression in the posterior bud.

Another observed phenotype caused by Wnt-7a overexpression is the reduction of interbud space. This may reflect the perturbation of the earlier phase. *Wnt-7a* was all over the presumptive feather field and later was restricted with enhanced expression in the feather primordial region (Fig. 7A). Ectopic Wnt-7a expression prevented the normal

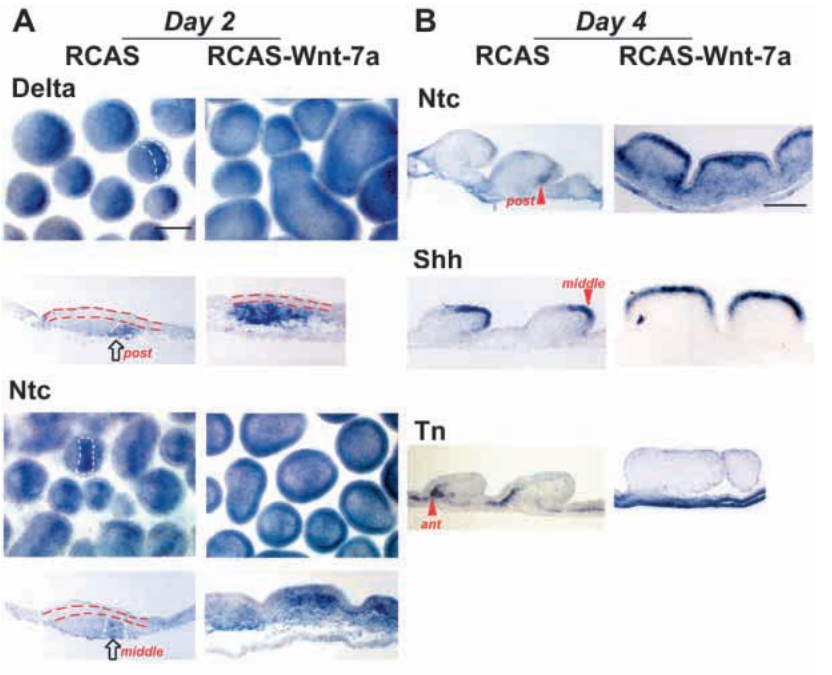

Fig. 5. Overexpression of Wnt-7a led to diffuse expression of *Notch* and *Delta*, expanded expression of *Shh* and suppressed expression of tenascin. The expression of *Delta-1*, *Notch-1* and *Shh* were examined by whole-mount and section in situ hybridization in control RCAS and RCAS-Wnt-7a transformed skin explants. Tenascin-C was detected by immunostaining. (A) Expression after 2 and (B) 4 days in culture. At 2 days after transduction, before the morphololgical changes have occurred, changes in the expression of *Delta-1* (*Delta*) and *Notch-1* (*Ntc*) can already be seen. *Delta-1* is expressed in the posterior (post) feather domain of control buds and its expression is expanded in the *RCAS-Wnt-7a* transformed explants. This can be seen from both whole-mounts and sections. *Notch-1* was expressed in the middle domain and extended into the posterior domain in control explants. Its expression was greatly increased and became widely distributed in the whole bud of *Wnt-7a* transformed explants, as can be seen from both whole-mount and section in situ hybridization. The epithelium is flanked by the broken red line. Regions expressing the genes of interest are indicated by a broken white line. After 4 days in culture, *Notch* was expressed in the posterior region of control explants but expression was elevated in the *RCAS-Wnt-7a* transformed explants. Sonic hedgehog (*Shh*) was expressed in the middle region of control feather buds but the region of its expression was expanded in *RCAS-Wnt-7a* transduced cultures. Tenascin-C was localized to the anterior (ant) mesenchyme of control feather buds. In *Wnt-7a* transduced buds, tenascin was diffusely expressed or suppressed. Bars, 200 μm.

downregulation of *Wnt-7a* in the inter-bud space, which may have resulted in the expansion of feather primordia domains. Thus, depending on the expression patterns of Wnt 7a and its receptors in skin and other tissues, as well as the specific time of perturbation, a spectrum of phenotypes may be observed. In this report our focus is on its roles in A-P formation of the primordia.

Interactions between the anterior and posterior bud domains may be important for the proximal-distal outgrowth of feather buds

In *Wnt-7a* transformed feather buds, as well as in cAMP and anti-tenascin perturbed buds (Jiang and Chuong, 1992; Noveen et al., 1995a), why are the proximal-distal outgrowth and anterior-posterior asymmetries always lost at the same time?

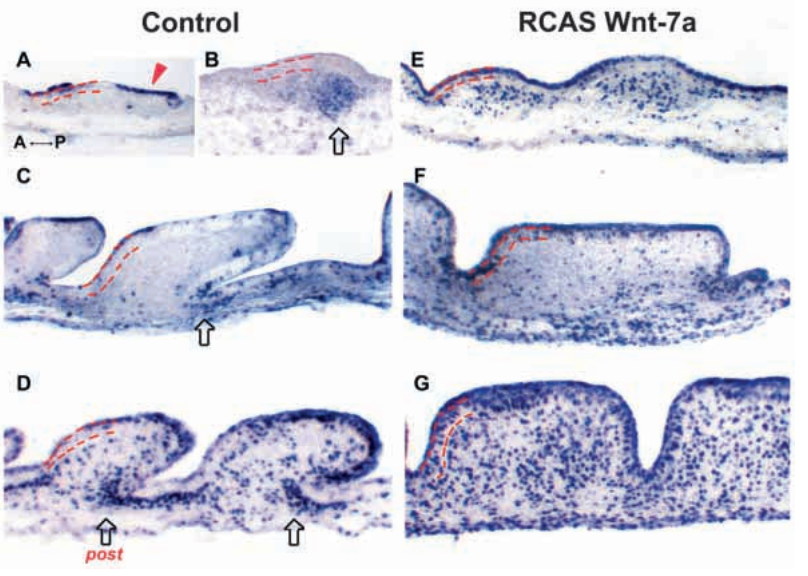

Fig. 6. *RCAS-Wnt-7a* transduced skin explants show more diffuse cell proliferation. Cell proliferation was examined by immunostaining for the proliferating cell nuclear antigen (PCNA) at 1.5 (A,E), 3 (C,F) and 4 (D,G) days after formation of the reconstituted explant cultures and in sections of skin grown in ovo (B). (A) In control cultures staining was found in both the anterior and posterior feather bud periderm but not in either the epithelium or mesenchyme at 1.5 days in culture. (B) Proliferation was present in the posterior feather bud mesenchyme before morphological asymmetry was established in skin from a slightly later developmental stage grown in ovo. (C) Staining expanded to the epithelium and mesenchyme by 3 days in culture. (D) Proliferating cells were in the distal and posterior epithelium and the posterior mesenchyme at 4 days. The pattern was very different for the *RCAS Wnt-7a* transduced buds. (E) At 1.5 days in culture, prior to morphological alterations in the AP axis, PCNA was present in the periderm of *Wnt-7a* transduced feather buds. It was also present diffusely through the epithelium and in the middle mesenchyme domain at this stage. (F,G) At 3 and 5 days, proliferation was still observed in the periderm and was also evenly distributed throughout the feather primordia and did not form differential AP staining as was seen in the controls. A portion of the epithelium is indicated by broken red lines. The PCNA staining in A (arrowhead) mostly represents the periderm. The increased proliferating regions in the posterior buds of control explants are indicated by arrows. The orientation of the A-P axes are indicated in A.

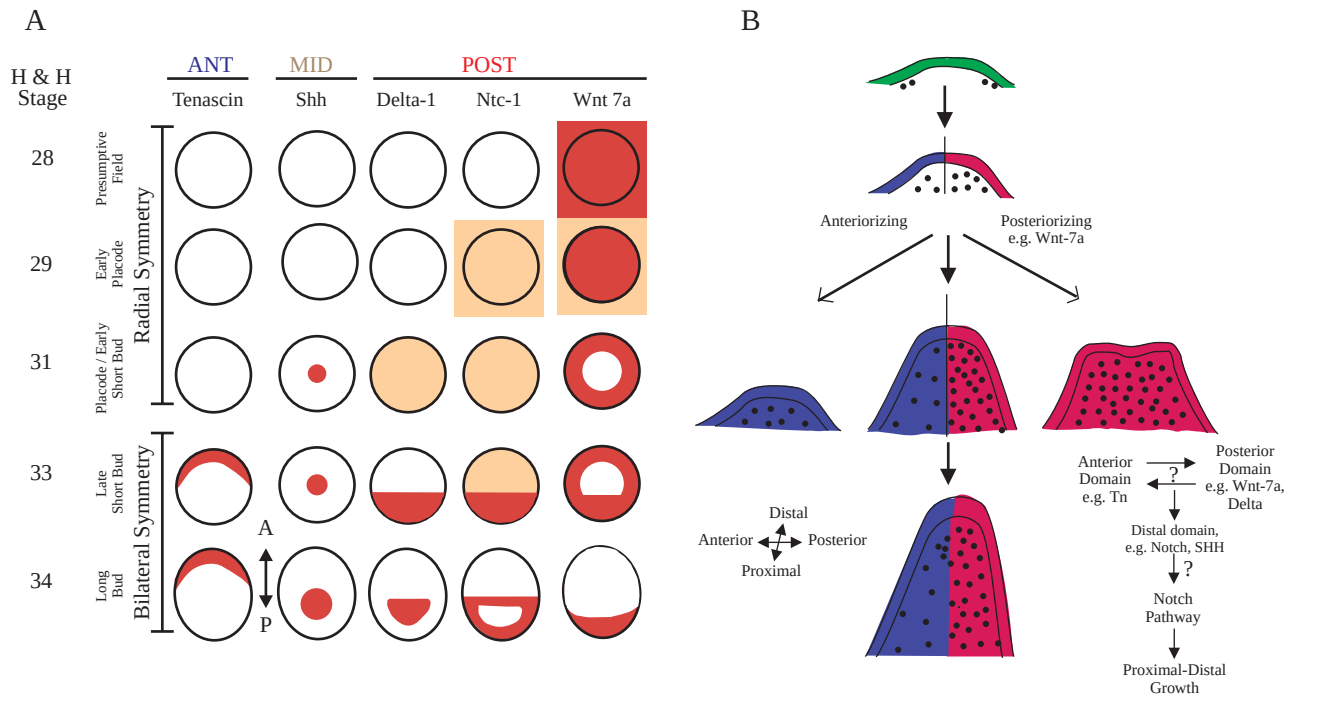

Fig. 7. A working model for the elongation of skin appendages. (A) Schematic summary of molecular expression patterns viewed from the top of feather primordia. *Wnt-7a* was initially expressed throughout the whole tract field. It gradually became restricted to feather primordia regions, enhanced in the primordia and decreased outside the primordia. Within the primordia the expression of *Wnt-7a* gradually became a symmetrical ring pattern. Then, a posterior localization pattern gradually emerged. *Delta-1* and *Notch-1* had weak general expression patterns to begin with (Crowe et al., 1998; Viallet et al., 1998), which are followed by strong posterior bud expression patterns. Additionally, *Notch-1* was expressed from a mid-stripe to circumscribe the posterior bud (Chen et al., 1997; Crowe et al., 1998; Viallet et al., 1998). SHH was expressed de novo in the center of the primordia, which later became posterio-distally localized (Ting-Berreth and Chuong, 1996b). Tenascin-C was expressed slightly later in the anterior feather bud. With reference to H&H embryo staging and feather staging (Widelitz et al., 1997), feather buds formed in different stages over the embryo body surface. Furthermore, changes in feather growth occurred faster than H&H staging during this period. Therefore the bud morphology staging is a more accurate system for staging feather development (Chuong and Widelitz, 1998). The H&H stages indicated are the approximate stages for feather buds of the spinal tract at the lumbar level. The bilateral and radial symmetry on the left side refers to the primordia morphology. The molecular expression sequence we are developing here will eventually help to better define feather developmental stages. (B) A working model for the elongation of feather buds. When feather primordia are first formed, they are radially symmetrical (green). The primordia then develop molecular heterogeneity, first along the A-P axis (as shown in A). Proliferation is higher in the posterior feather bud (red) but lower in the anterior bud (blue), which may contribute to the formation of the asymmetric shape (Chen et al., 1997; Chuong and Widelitz, 1998). We propose that the juxtaposition of anterior and posterior buds may be important for the initiation of new growth in the central and posterior domains, hence producing the P-D axis. We propose that overexpression of posteriorizing signals such as Wnt-7a posteriorizes the whole feather bud and blocks the anterior-posterior interactions necessary for elongation, similar to what has been found for the limbless mutant (Ros et al., 1996). From this work and those reported in the literature (Laufer et al., 1997; Rodriguez-Esteban et al., 1997), we suggest a tentative molecular cascade for feather morphogenesis, as shown at the lower right. Question marks indicate that more evidence is required to establish the hypothetical relationship.

Are these two processes coupled? In *Drosophila*, wg and Dpp act as concentration dependent morphogens. The juxtaposition of wg and Dpp along the dorso-ventral axis of the limb establishes the proximal-distal axes (Lecuit and Cohen, 1997). Misexpression of wg leads to the formation of ectopic appendages (Rulifson et al., 1996; Lecuit and Cohen, 1997). Through interactions between different regions, new axes are produced.

Cell proliferation is essential for the growth of feather buds. The centers of proliferation in feather morphogenesis shift dynamically during feather development and this may shape feather morphology by adding cells at certain locations. Cell proliferation is first all over the putative skin, then becomes absent in the dermal condensation regions during the time feather primordia form (Wessels, 1965). Proliferation reappears in the short feather bud stage, enriched in the posterior half of the bud (Desbiens et al., 1992; Chen et al., 1997). Therefore, the once homogeneous feather primordia progress to become two or more heterogeneous domains. These domains are marked with characteristic molecular expression patterns (Fig. 7A). We propose a feather growth model in which the juxtaposition of anterior and posterior buds produces a zone with higher growth potential toward the posterior region (Fig. 7B). Thus the morphological A-P asymmetry formation is linked to feather elongation along the P-D axis.

We can also re-evaluate previous results with this model. Treatment of the skin with cAMP led to small and round buds (Noveen et al., 1995a), with simultaneously suppressed P-D and A-P axes. However, the morphology is distinct from Wnt-

7a induced plateau bud morphology. The cAMP-treated explants showed increased expression of NCAM and pCREB, markers of the anterior buds (Noveen et al., 1995a), but suppressed expression of *Wnt-7a*, *Delta 1* and *Serrate-1*, markers of the posterior buds (Chen et al., 1997). There are also uniformly very low levels of BrdU labeling and a lack of *Notch-1* and *Shh* expression, characteristics associated with the growth zone (Noveen et al., 1995b, 1996). It seems that cAMP has expanded the regions expressing several characteristics of the anterior bud, and there is suppression of characteristics of both the middle bud growth zone and posterior buds. It remains to be determined which molecule may be the major anteriorizing signal. However, the results here are consistent with the notion that balanced interactions between different feather bud regions are essential for the continuous P-D outgrowth of skin appendages.

Future studies will involve exploration of the molecular signals and interactions in this model. It is possible that the prototype of signaling networks used to form epithelial appendages (wings, legs) in *Drosophila* later evolved to form various vertebrate epithelial appendages. It is unlikely that all the molecular mechanisms involved in insect appendage morphogenesis (Shubin et al., 1997) can apply to the morphogenesis of vertebrate skin appendages. However, the basic principles of appendage morphogenesis may be shared. In the imaginal disc, the interaction between D and V disc signaling appears to be mediated by wg and dpp, and results in the outgrowth of the leg (Lecuit and Cohen, 1997). In skin appendages, an analogous situation may exist between the anterior and posterior domains of feather buds. The results here are examples showing how the morphology of skin appendages can be altered by the imbalance of localized signaling activities.

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