

Involvement of *Hex* in the initiation of feather morphogenesis

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ABSTRACT In a previous study, we showed that the proline-rich divergent homeobox gene *Hex/Prh* is expressed in dorsal skin of the chick embryo before and during feather bud development and that the pattern of *Hex* mRNA expression in the epidermis is similar to that of *Wnt7a* mRNA. In order to study the function of *Hex* and the relationship between *Hex* and *Wnt7a* in feather bud development, sense and/or antisense sequences of *Hex* or *Wnt7a* were ectopically and transiently expressed in the dorsal skin with the epidermal side toward the cathode by electroporation at the placode stage and then the skin was cultured. Increased expression of *Wnt7a* and β -catenin mRNA was observed in the same region where *Hex*-EGFP fusion protein was expressed 2 days after culture, which was followed by extra bud formation a few days later as a result of the stimulation of cell proliferation. Concomitantly, expression of *Notch1* mRNA, which is expressed in normal bud development, increased in *Hex*-overexpressing skin. However, ectopic *Wnt7a* expression induced neither *Hex* expression nor extra bud formation in normal skin. Antisense *Wnt7a* specifically inhibited bud initiation in *Hex*-overexpressing skin but did not in normal skin. Taken together, these results suggest that *Hex* is upstream of *Wnt7a* and β -catenin and regulates the Wnt signaling pathway in feather bud initiation and that some other Wnt signals in addition to *Wnt7a* may be required for bud initiation.

KEY WORDS: homeobox gene, *Hex*, feather bud development, *Wnt7a*, β -catenin, cell proliferation

Introduction

Epithelial appendages including feathers, scales, hair, claws, teeth, etc are induced and shaped through epithelial-mesenchymal interactions (Smola *et al.*, 1993; Chuong *et al.*, 1996; Kishimoto *et al.*, 2000). An inductive signal from the dermis initiates formation of epidermal placodes that, in turn, induce dermal condensation in the underlying dermis (reviewed in Sengel, 1976). Several molecules that mediate inductive signaling during hair and feather tract formation have been identified, including Wnts (Widelitz *et al.*, 1999; Noramly *et al.*, 1999; Huelsken *et al.*, 2001; Andl *et al.*, 2002), bone morphogenetic protein (BMP) in early skin development (Scaal *et al.*, 2002), BMP inhibitor at placode stages (Patel *et al.*, 1999), fibroblast growth factors (FGFs) (Widelitz *et al.*, 1996; Song *et al.*, 1996), Hedgehog (Ting-Berreth and Chuong, 1996) and Notch/Delta families (Crowe *et al.*, 1998; Viallet *et al.*, 1998). Notch/Delta signals refine the patterning of the feather placode (Crowe *et al.*, 1998). *In situ* hybridization studies indicated that development of dorsal feather inducing dermis is dependent on *Wnt11* from the dorsomedial lip, which is induced by *Wnt1* from

the dorsal neural tube (Olivera-Martinez *et al.*, 2001 & 2002). Afterward, β -catenin protein is found transiently and uniformly in the nuclei of the dense dermis underlying the feather tract 1 day before appearance of placode (Noramly *et al.*, 1999). *Wnt7a* is first expressed as a relatively homogeneous stripe and progressively segregated into individual feather placode in the epithelium (Widelitz *et al.*, 1999). Hence, the dermal signal, which might occur uniformly within skin, triggers the activation of promoters and repressors of feather bud fate presumably under the control of *Wnt7a* and Notch/Delta signals from epithelium of early placode, resulting in the establishment of a regular array of buds. Homeobox genes are a large family of transcription factors that play a fundamental role in cell differentiation during development (Gehring *et al.*, 1994). Abnormal hair follicles were observed in transgenic mice overexpressing homeobox gene *Msx-2* (Jiang *et al.*, 1999). Hair defects were observed in *Hoxc13* mutant mice (Godwin &

Abbreviations used in this paper: BMP, bone morphogenetic protein; BrdU, bromodeoxyuridine; EGFP, enhanced green fluorescent protein; FGF, fibroblast growth factor.

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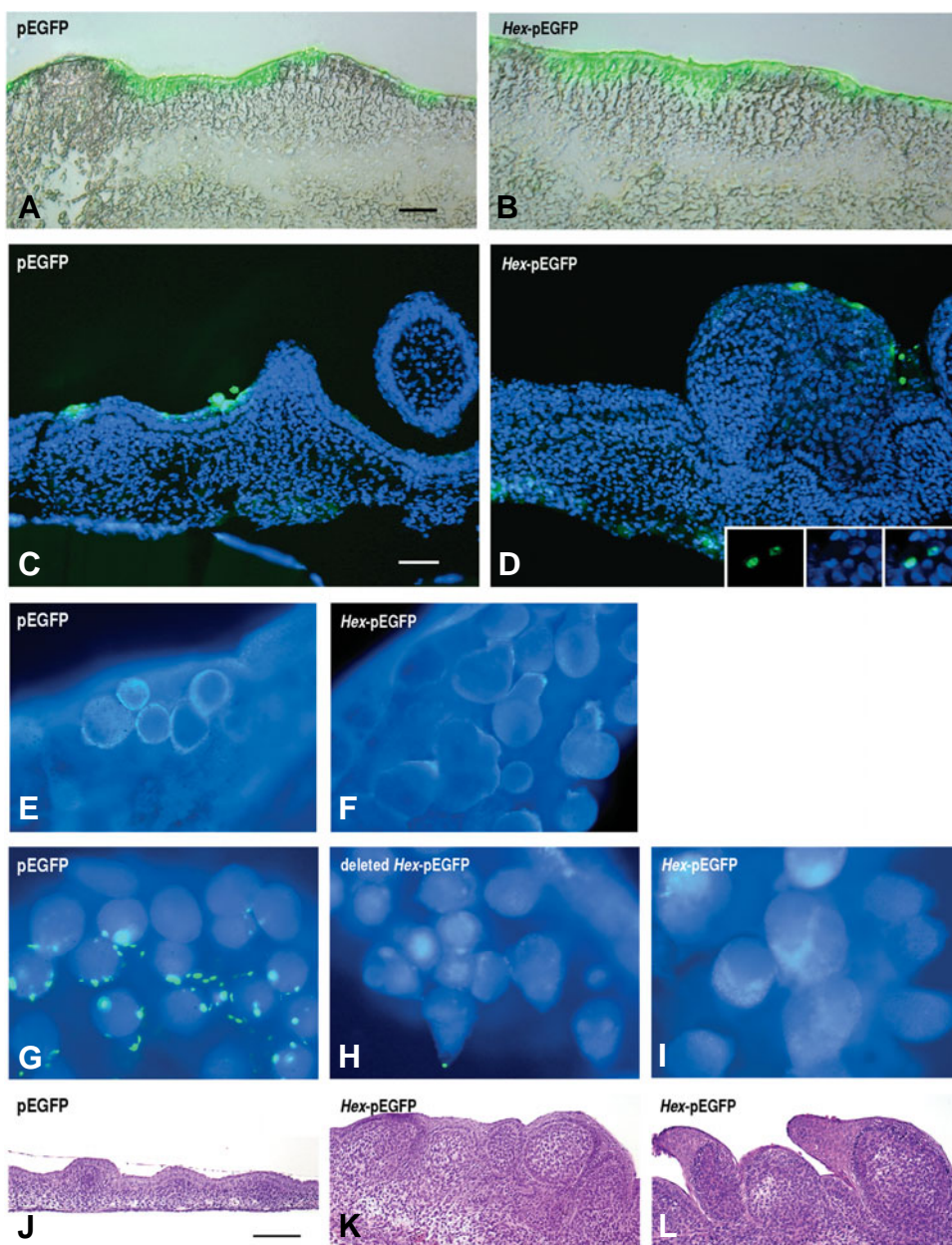


Fig. 1. Perturbation of feather bud formation by ectopic *Hex* expression. The epidermis of the back skin of 8 (A-D, G-L)- or 7 (E, F)-day-old-chick embryo was transfected with pEGFP (A, C, E, G, J), Hex-pEGFP (B, D, F, I, K, L) or deleted Hex-pEGFP (deficient of N-terminal region, H) by electroporation and then cultured for 1 (A, B) or 5 days (C-L). Fluorescent micrographs of skin sections of a single image of luminescence of EGFP (green, A, B) or double exposure images of luminescence of EGFP and DAPI (blue)-stained nuclei (C, D). Insets in (D) from left to right are luminescence of EGFP, DAPI and double exposure image. Fluorescent micrographs of intact skin of double exposure images of EGFP and DAPI (E-I). Hematoxylin and eosin-stained sections (J-L). Bar, 100 μ m.

Capecchi 1998) and Jave-Suarez *et al.* (2002) showed direct involvement of HOXC13 in the regulation of human hair keratin gene expression. The homeobox genes *Msx1* (Noveen *et al.* 1995), *Gbx1* (Obinata *et al.*, 2001) and *HB9* (Kosaka *et al.*, 2000a & 2000b) are expressed in skin and its appendages, such as hair, feather or scale and appear to be candidates for the regulation of the development of these tissues. We showed previously that

divergent homeobox gene *Hex/Prh* is expressed in chick embryonic tarsometatarsal skin and regulates epidermal cell proliferation (Obinata *et al.*, 2002). *Hex* is expressed during early stages of chick embryogenesis, including pharyngeal endoderm, endocardium, liver, thyroid gland primordia and blood islands (Yatskiyevych *et al.* 1999). *Hex* is required for forebrain, thyroid and liver formation and blood differentiation (Keng *et al.* 2000; Martinez-Barbera *et al.* 2000; Martinez-Barbera & Beddington 2001). In liver morphogenesis, *Hex* expression in avian anterior lateral endoderm is regulated by autocrine BMP signaling (Zhang *et al.*, 2002).

In a previous study (Obinata and Akimoto, 2005), we showed *Hex* expressed in dorsal skin of chick embryo before and during feather bud development and that the pattern of *Hex* expression in the epidermis is similar to that of *Wnt7a*. In order to know the relationship between *Hex* and *Wnt7a* in the feather bud morphogenesis, sense and/or antisense gene of *Hex* or *Wnt7a* were ectopically and transiently expressed in the epidermis of the dorsal skin at the placode stage by electroporation and then the skin was cultured. We indicated that *Hex* is upstream of *Wnt7a* and β -*catenin* and regulates *Wnt* signaling pathway in the feather bud initiation and suggested that some other *Wnts* in addition to *Wnt7a* might be involved in the bud initiation.

Results

Perturbation of feather bud formation by ectopic *Hex* expression

To learn the function of *Hex* in the skin, the back skin of a 7- or 8-day-old chick embryo that had been transfected with *Hex*-pEGFP or pEGFP with the epidermal side toward the cathode by electroporation was cultured for 1 - 5 days. Strong luminescence of EGFP was observed discontinuously 1 day

after the culture in the epidermis and the dermis that closely contacts the epidermis (Fig. 1 A, B). Discontinuity of the luminescence was presumably caused by a different current conductivity in the skin structure. The luminescence became weak 4 days later (Fig. 1 C, D), indicating the fusion protein and the plasmid were unstable and/or were diluted by cell proliferation during the culture. The shape of the feather bud expressing *Hex*-EGFP fusion

protein was abnormal (Fig. 1D). Luminescence, which was seen exclusively in many nuclei of skin during culture for 2 days, was seldom seen in the nuclei after culture for 5 days and hence we showed the nuclei in the different region of the skin (Fig. 1D insets). Patterning of the bud after culture for 5 days was perturbed and buds were fused with each other in a snowman-like shape in the skin transfected with *Hex*-pEGFP (Fig. 1F,I) or *dHex*-pEGFP, whose translated product is the truncated protein that contains the DNA binding domain of Hex but lacks most of the amino-terminal domain (Fig. 1H), while patterning of the buds in the skin transfected with pEGFP was normal and was round in shape (Fig. 1E,G,J). As so many buds were formed, no interbud was observed in some regions of the skin (Fig. 1K,L). Little morphological difference was observed, if any, in the skin, when we used either late 7-day-old (Fig. 1E,F) or early 8-day-old skin (Fig. 1G,I). As it is easier to manipulate, early 8-day-old skin was used in many experiments.

Stimulation of epidermal cell proliferation in the interbud region of *Hex*-transfected skin

To examine whether the bud formation in skin transfected with *Hex* was accompanied by cell proliferation, DNA synthesis in the skin was analyzed. When ³H-thymidine incorporation into DNA was measured, DNA synthesis in the skin was stimulated 1.3 fold in the *Hex*-transfected skin ($5,816 \pm 632$ dpm/sheet) compared with control skin ($4,355 \pm 217$ dpm/sheet) after 2 days of culture. While incorporation of BrdU into the nuclear DNA was seen little in the interbud than in the bud in control skin (Fig. 2C,D), it was observed both in bud and interbud region of the skin in the *Hex*-transfected skin (Fig. 2A,B), indicating that the feather bud formation in the interbud epidermis was accompanied by enhanced cell proliferation induced by ectopic *Hex* expression.

Expression of Notch1 and β -catenin in *Hex*-transfected skin

In *Hex*-transfected skin, β -catenin, a placode marker (Widelitz *et al.*, 2000), was seen throughout the epidermis with stronger expression at the lateral of the bud (Fig. 3A) and *Notch-1*, a posterior bud marker (Crowe *et al.*, 1998), was seen in the epidermis of the bud region (Fig. 3C). In pEGFP-transfected skin, however, β -catenin expression was seen at the placode region (Fig. 3B), but there was little, if any, *Notch-1* expression (Fig. 3D). We next examined whether expression of β -catenin will be increased in *Hex*-transfected skin or not in the same section. In *Hex*-transfected skin, β -catenin expression was seen in the lateral of the bud in the epithelium (arrows in Fig. 4B,D), where *Hex*-EGFP fusion protein was expressed (Fig. 4A,C), while the expression (Fig. 4F,H) was little, if any, in pEGFP-transfected skin (Fig. 4E,G). Hence, ectopic expression of *Hex* to the skin enhanced the property characteristics of posterior feather buds.

Induction of *Wnt7a* in the *Hex*-transfected skin

As *Hex* and *Wnt7a* are expressed in the feather primordium regions during development (Obinata

and Akimoto, 2005) and expression of β -catenin increased in the lateral of the bud in *Hex*-transfected skin (Figs. 3A, 4B,D), we next examined whether *Hex* regulates *Wnt7a* expression or not. *Wnt7a* expression increased in the epidermis of the bud in *Hex*-pEGFP (Fig. 3E,I) or *Hex*-pcDLSR α -transfected skin (Fig. 3M) compared with that in the control skin (Fig. 3G,J,N). Figures 3E,F showed that, when we examined the same section, *Wnt7a* expression increased in the epidermal cells expressing *Hex*-EGFP fusion protein or in the neighborhood of the cell (arrow or arrowhead in E and F) but no expression was seen in pEGFP-transfected skin (Fig. 3G,H). In the late 7-day-old chick embryo, there are the different phases of development resulting in the variation of bud development as was seen in Fig. 3E,I. No increase in *Hex* expression was seen in *Wnt7a*-pcDNA-transfected skin compared with control skin (Fig. 3K,L). These results suggested that *Hex* is upstream gene of *Wnt7a* and β -catenin. In the next step, we studied whether or not *Hex*-induced *Wnt7a* is required for the bud initiation.

Inhibition of *Hex*-induced extra bud formation by co-transfection of antisense *Wnt7a* to the skin

Antisense *Wnt7a*-pcDNA was co-transfected with *Hex* to the dorsal skin and then the explants were cultured for 5 days. *Hex*-induced feather initiation (Fig. 5A,B) was inhibited by the co-transfection of antisense *Wnt7a*-pcDNA (Fig. 5C,D) and hence patterning seemed normal (Fig. 5C) compared with those of *Hex*-transfected skin (Fig. 5A). However, buds with abnormal shape (arrowheads in Fig. 5D) were observed. Electron microscopic study indicated that the dermis (D) (Fig. 5N) of the abnormal bud (Fig. 5M) was separated by the epithelium (E) (Fig. 5N) sur-

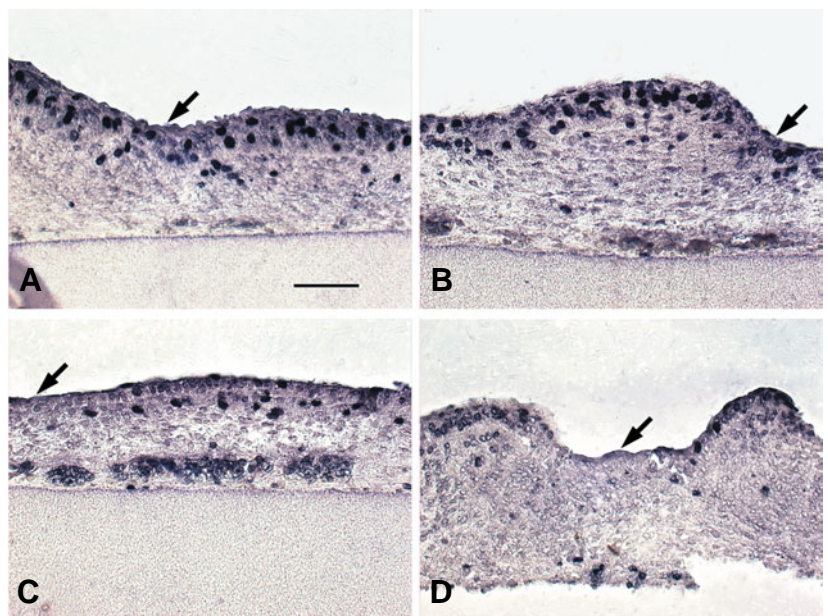


Fig. 2. Stimulation of epidermal cell proliferation in the interbud region of *Hex*-transfected skin. Dorsal skin of early 8-day-old chick embryo that had been transfected with *Hex*-pEGFP (A,B) or pEGFP (C,D) was cultured for 2 days and was labeled with BrdU for 2 h. Skin was analyzed immunohistochemically using anti-bromodeoxyuridine (BrdU) antibody. While incorporation of BrdU into the nuclear DNA was seen little in the interbud than in the bud in control skin (C,D), it was observed both in bud and interbud region of the skin in the *Hex*-transfected skin (A,B). Arrows; interbud region. Bar, 100 μ m.

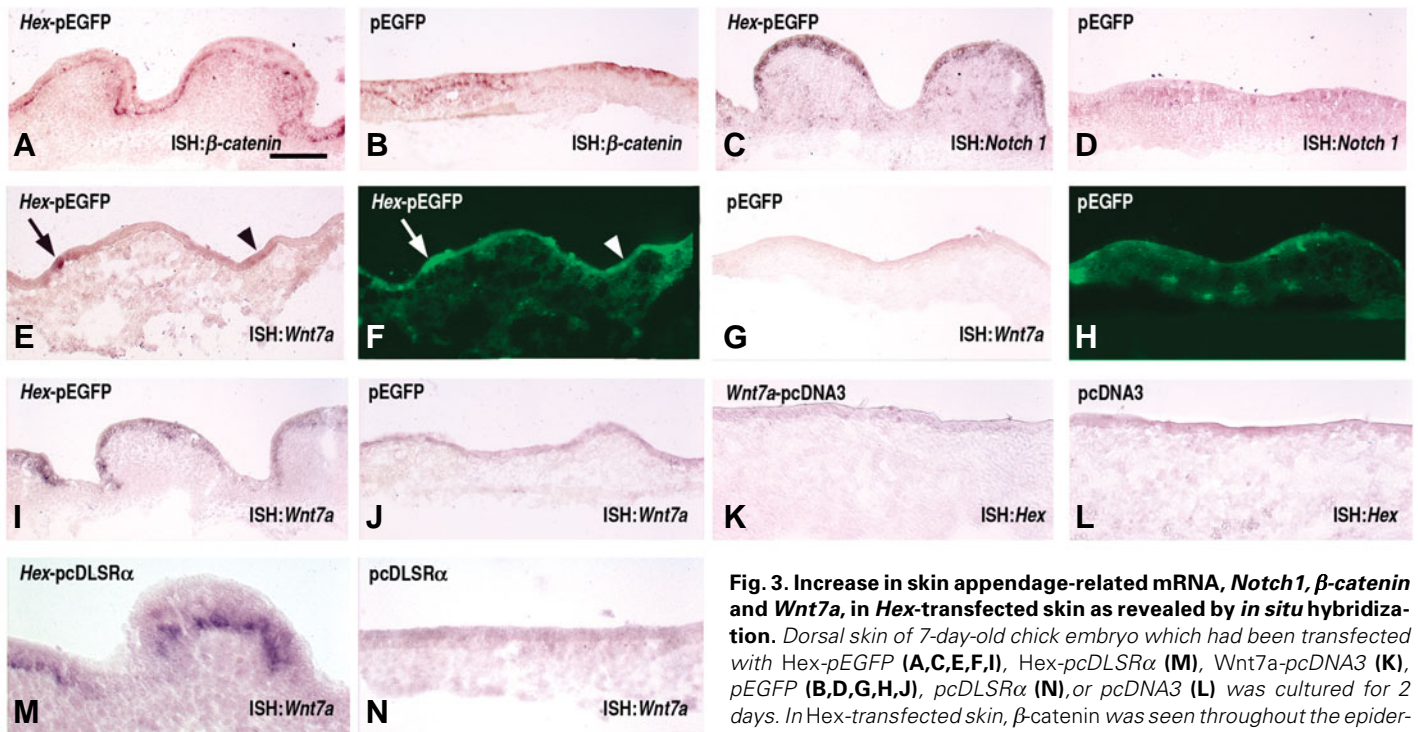


Fig. 3. Increase in skin appendage-related mRNA, *Notch1*, β -catenin and *Wnt7a*, in *Hex*-transfected skin as revealed by *in situ* hybridization. Dorsal skin of 7-day-old chick embryo which had been transfected with *Hex*-pEGFP (A,C,E,F,I), *Hex*-pcDLSR α (M), *Wnt7a*-pcDNA3 (K), pEGFP (B,D,G,H,J), pcDLSR α (N), or pcDNA3 (L) was cultured for 2 days. In *Hex*-transfected skin, β -catenin was seen throughout the epidermis with stronger expression at the lateral of the bud (A) and *Notch-1* was seen in the epidermis of the bud region (C). In pEGFP-transfected

skin, β -catenin expression was seen at the placode region (B) and there was little, if any, *Notch-1* expression (D). *Wnt7a* expression increased in the epidermis of the bud in *Hex*-pEGFP (E,I) or *Hex*-pcDLSR α (M)-transfected skin compared with that in the control skin (G,J,N). Gene expression of *Hex* is not seen in *Wnt7a*-pcDNA3-transfected skin (K) or pcDNA3-transfected skin (L). While *Wnt7a* expression increased in the epidermal cells expressing *Hex*-EGFP fusion protein (arrow or arrowhead in E,F) in *Hex*-transfected epidermis, *Wnt7a* expression did not increase in pEGFP-transfected epidermis (G,H). Bar, 100 μ m.

rounded by basement membrane on both sides (arrowheads in insets of Fig. 5N). Next, we transfected antisense *Wnt7a*-pcDNA or antisense *Hex* to the dorsal skin and then the explants were cultured for 5 days. Neither antisense *Wnt7a* (Fig. 5 E,F) nor antisense *Hex* (Fig. 5 K,L) disturbed the patterning of the feather when compared with control skin (Fig. 5 G,H), presumably because the initiation of bud formation induced by *Hex* and *Wnt7a* in normal skin development had already completed before 8 days. Bud initiation was not seen in *Wnt7a*-transfected skin (Fig. 5 I,J), suggesting that other Wnts in addition to *Wnt7a* might be required for the feather initiation.

Discussion

Epithelial-mesenchymal interactions are important in the morphogenesis of many organs (Sengel, 1976; Smola *et al.*, 1993; Chuong *et al.*, 1996; Kishimoto *et al.*, 2000).

The Wnt/Wg signaling pathway plays an essential role in the early inductive events in hair and feather follicles (Widelitz *et al.*, 1999; Huelsken *et al.*, 2001; Andl *et al.*, 2002) and even in its equivalent (denticle) of fly (Dai *et al.*, 1998; Payre *et al.*, 1999; Li *et al.*, 2002). Wnt signals (reviewed in Wodarz & Nusse, 1998) are activated by binding to the frizzled family of transmembrane receptors and are transferred across the cell membrane. A conserved canonical Wnt signaling pathway causes stabilization of cytoplasmic β -catenin, its translocation to the nucleus and the

formation of active transcription complexes between β -catenin and members of the Lef/Tcf family of DNA binding factors. Several Wnt family members (Wnt3, 3a, 4, 5a, 7a, 10a and 10b) are expressed in distinct patterns and stages in the developing skin of mammals and birds (Millar *et al.*, 1999; Kishimoto *et al.*, 2000; Bonifas *et al.*, 2001; Rodriguez-Niedenfuhr *et al.*, 2003; Reddy *et al.*, 2001; Andl *et al.*, 2002). It has been demonstrated that *Wnt3a* and *7a* expression in epithelium, respectively, maintain anagen gene expression *in vitro* and their signaling to dermal papilla retain the inductive activity of hair in organ culture (Kishimoto *et al.*, 2000). The facts indicated above suggest that activation of the paracrine Wnt signaling pathway may be required for generation of chick feather buds and most pelage hair follicles.

Hex is expressed as early as at 5 days in chick embryonic dorsolateral skin and then diffusely distributed in skin before placode formation (Obinata and Akimoto, 2005). *Hex* expression is restricted to the placode and the dermis under the placode and later in the epidermis and the dermis of the posterior region of the short bud (Obinata and Akimoto, 2005). As the pattern of *Hex* expression in the epidermis is similar spatially and temporally to that of *Wnt7a* expression (Widelitz *et al.*, 1999) and *Hex* protein exists both in cytoplasm and nucleus (Obinata and Akimoto, 2005), it was suggested that *Hex* is important as a transcription factor in the feather bud initiation.

We demonstrated here that ectopic expression of *Hex* in the skin induced expressions of *Wnt7a* (Fig. 3) and β -catenin (Fig. 4),

placode markers, in the same region of Hex-expressing cell during 2 days of culture followed by extra bud formation several days later (Fig. 1). The extra bud formation was accompanied by enhanced cell proliferation in the interbud region of the dorsal skin (Fig. 2) with increased expression of posterior bud marker, *Notch1* (Fig. 3), suggesting an importance of cross talk between Notch1 and *Wnt7a*, both of which are induced by Hex, during feather bud initiation. Over expression of *Wnt7a* by retrovirus transfection to chick embryonic dermis and epidermis induced ectopic bud formation (Widelitz *et al.*, 1999). As the amount of *Wnt7a* expression in the dermis is very small in normal bud development (Widelitz *et al.*, 1999), we tried to express the gene mainly in the epidermis by electroporation. Ectopic *Wnt7a* expression in the skin induced neither extra bud formation (Fig. 5) nor *Hex* expression (Fig. 3). Antisense *Wnt7a* specifically inhibited bud initiation in the *Hex*-overexpressed skin (Fig. 5). However, antisense *Hex* or antisense *Wnt7a* could not inhibit the bud formation in normal development, presumably because the bud formation induced by *Hex* and *Wnt7a* in normal skin development had already completed before 8 days and hence transfection of antisense *Hex* or antisense *Wnt7a* to the skin at early 8 days did not affect bud formation. In fact, expressions of *Hex* and *Wnt7a* in the epidermis of dorsal skin were observed as early as 6-day-old chick embryo (Obinata and Akimoto, 2005; Widelitz *et al.*, 1999). As the dorsal skin at 6 days is so soft and small, we could not remove the skin from the embryonic body in order to study the effect of antisense *Hex* or antisense *Wnt7a* on the feather bud development. Taken together, it was suggested that *Hex* plays an important role in the upstream of *Wnt7a* signaling pathway in the feather bud initiation and some other *Wnts* in addition to *Wnt7a*, which are also induced by *Hex*, might be required for the bud initiation. Indeed, C-H Chang *et al.*, (2004) showed that distinct *Wnt* members have positive and negative roles in forming the dermis, tracts, interbud spacing and the growth and shaping of individual buds.

Promoter sequence of human, mouse or rat *Wnt7a* genome has three to six Hex-binding sites (ATTA) in -9000 ~ -1200 bp upstream of the gene. Hence, it is conceivable that chick *wnt7a* promoter is conserved between mammals and birds. Interestingly, both *Hex* protein and the truncated protein, which contains the DNA binding domain of *Hex* but lacks most of the amino-terminal domain (dHex), similarly affected the morphogenesis of skin appendages in chick embryonic dorsal (Fig. 1) and tarsometatarsal skin (Obinata *et al.*, 2002), although the truncated protein acts as a dominant negative in

HepG2 cells (Denson *et al.*, 2000), suggesting that the amino-terminal domain of *Hex* is not required for the regulation of gene expression in the case of bud formation. Further genetic studies must be done to resolve the function of the *Hex* in the feather (hair) follicle development.

Materials and Methods

Preparation of a digoxigenin (DIG)-labeled RNA probe

The *Hex* RNA probe was prepared as described previously (Obinata *et al.*, 2002). For synthesis of a β -catenin specific probe, a DNA fragment

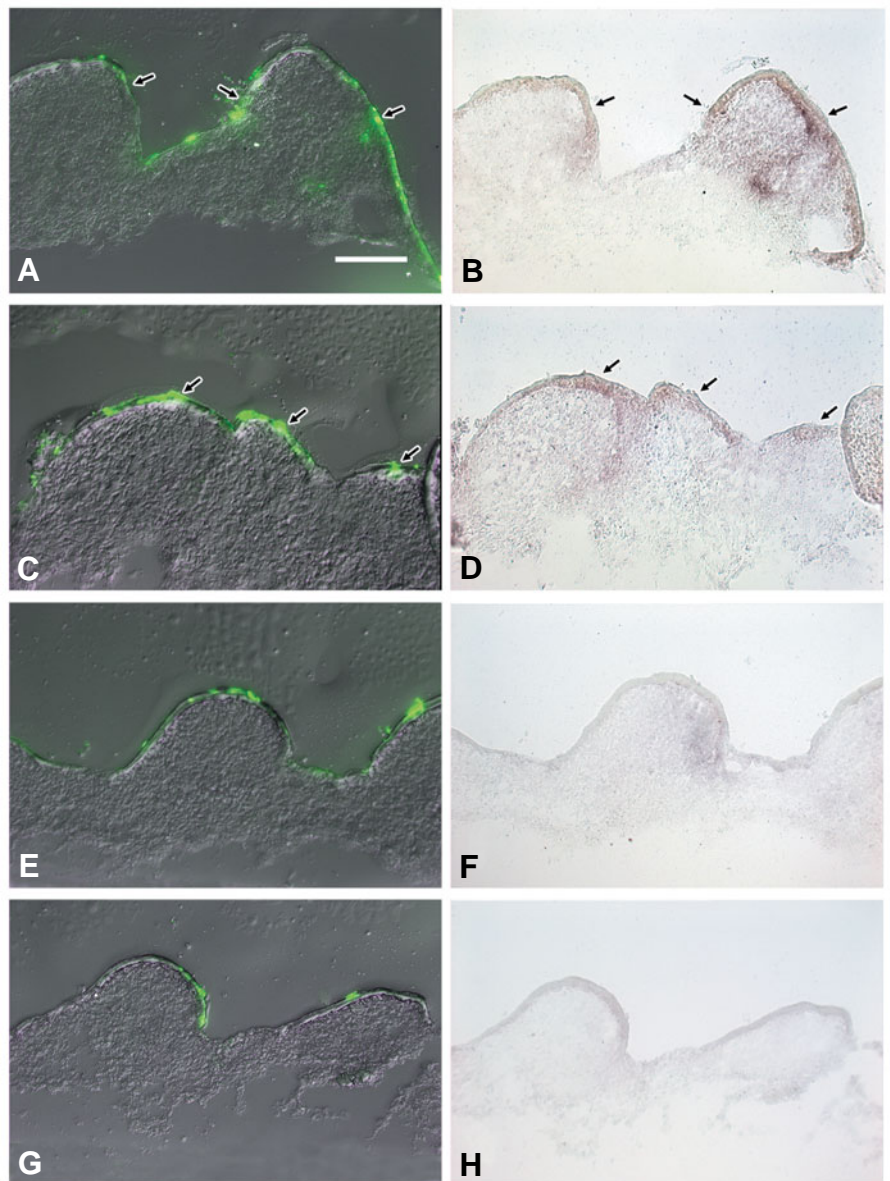


Fig. 4. Increase in β -catenin mRNA in *Hex*-transfected skin as revealed by *in situ* hybridization. Dorsal skin of early 8-day-old chick embryo, which had been transfected with *Hex*-pEGFP (A-D) or pEGFP (E-H), was cultured for 2 days. In *Hex*-transfected skin, β -catenin expression was seen at the lateral of the short bud region (arrows in B,D), where *Hex*-EGFP fusion protein was expressed (arrows in A,C), although β -catenin expression (F,H) was little, if any, in pEGFP-transfected skin (E,G). Bar, 100 μ m.

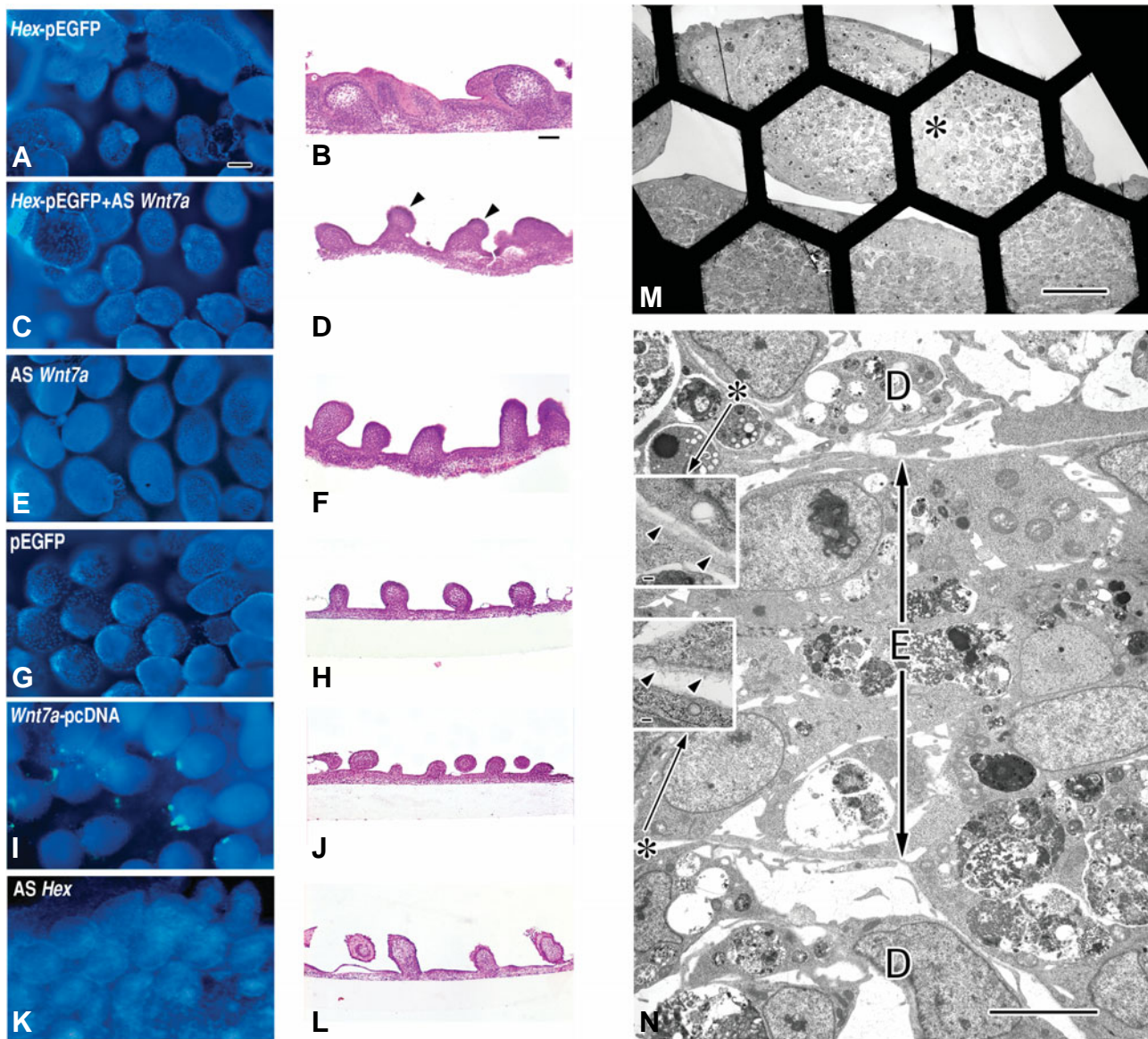


Fig. 5. Hex-induced *Wnt7a* is required for bud initiation. Dorsal skin of 8-day-old chick embryo, which had been transfected with plasmid, was cultured for 5 days. Feather initiation induced by Hex (A,B) was inhibited by the co-transfection of antisense *Wnt7a* (C,D), but abnormal buds (arrowheads in D,M), whose dermis (D) was separated by the epidermis (E) surrounded by basement membrane (arrowheads of insets of N) on both sides, were observed (M). Neither antisense *Wnt7a* (E,F), *Wnt7a*-pcDNA3 (I,J) nor antisense Hex (K-L) disturbed the patterning of the feather in normal skin. pEGFP-transfected skin (G,H). Fluorescent micrographs of intact skin of double exposure images of luminescence of EGFP (green) and DAPI (blue)-stained nuclei (A, C, E, G, I, K). Hematoxylin and eosin-stained sections (B, D, F, H, J, L). Electron micrographs of skin (M, N). Asterisks indicate the regions that are enlarged in the insets. Bars, (A, B) 100 μ m; (M) 50 μ m; (N) 10 μ m and (insets) 1 μ m.

consisting of 431 bp was amplified from skin cDNA using the following primers from 5' to 3' aagggttctctcagtcctt and gctgttccacatcggtt, corresponding to nucleotides 310-740 of the published sequence. The fragment was then cloned into pT7Blue T-vector (Takara, Kusatsu, Shiga, Japan) and the resulting clone was sequenced in its entirety. For synthesis of a *Wnt7a* and a *Notch1* specific probe, a *Wnt7a* cDNA fragment containing the entire coding region and a *Notch1* cDNA fragment containing cDNA of the outside cell membrane domain, which were kindly provided by Dr Y. Wakamatsu (Wakamatsu *et al.*, 2000) and Dr T. Nohno (Kawakami *et al.*, 2000), respectively, were amplified and prepared using a standard protocol.

In situ hybridization

In situ hybridization with the DIG-labeled probe was performed as described previously (Kosaka *et al.*, 2000a).

Microscopy

Skin explants were processed for light and electron microscopic observations as described previously (Obinata *et al.*, 1991).

Transgene construction

A full-length *Hex* cDNA containing the entire *Hex* coding region was generously provided by Dr G. Goodwin (Haddow Laboratories, Institute of

Cancer Research, Sutton, UK). A full length *Hex* and *Wnt7a* cDNA containing the entire *Wnt7a* coding region were constructed with pcDLSR α and pcDNA3. A full length antisense *Hex* and antisense *Wnt7a* were constructed with pcDLSR α and pcDNA3.1/Hygro. *Hex*-pEGFP and deleted *Hex*-pEGFP plasmids were amplified and constructed as described previously (Obinata *et al.* 2002).

Skin culture

Explants of 7- or early 8-day-old chick embryonic dorsal skin attached to a Millipore filter with the dermis side toward the filter were grown in DMEM. Medium was placed in the outside well and the inner chamber. A thin layer of the medium was left in the inner chamber to keep the explants 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.

Electroporation

The back skin of 7- or 8-day-old chick embryo attached to a Millipore filter (0.45 μ m pore size) was placed in a small 1% agarose chamber (10H x 6D x 1W mm³) filled with PBS containing 3-3.3 μ g/ml plasmid with the epidermal side toward the cathode by electroporation. An electro Square Porator T820 (BTX) generated square pulses (30V, pulse length 100 ms, 7 times).

Immunocytochemical assay for the detection of bromodeoxyuridine incorporation into cellular DNA

Skin that had been transfected with *Hex*-pEGFP or pEGFP by electroporation was cultured for 2 days in DMEM. The cultured skin from which fluorescence had been eliminated was cut into a 2mm² area and incubated in the medium containing 10 μ M bromodeoxyuridine (BrdU) for 2 h, fixed with Bouin solution, embedded in paraffin and cut into 5 μ m sections. After being dewaxed and rehydrated, the sections were incubated with anti-BrdU mouse monoclonal antibody (Boehringer Mannheim) and then with anti mouse-Ig alkaline phosphatase followed (according to the manufacturer's instructions) by the substrate reaction

Measurement of DNA synthesis

Nine explants of skin were electroporated with *Hex*-pEGFP or pEGFP followed by culture for 2 days. The area of explant with strong luminescence of EGFP was cut and was pulse-labeled by immersion in 2 μ Ci/ml of [methyl-1',2'-³H] thymidine (86 Ci/mmol; Amersham) for 2 hr at 37°C. After the skin was washed with phosphate-buffered saline, macromolecules in the epidermal homogenate were precipitated with 5% TCA; the precipitate was collected on a Whatman GF/filter and its radioactivity was measured as dpm/sheet. As only very small amount of skin was obtained, we could not determine DNA.

Acknowledgments

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