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Developmental Expression Pattern

Expression of *Hex* during feather bud development

AKIKO OBINATA*,[1] and YOSHIHIRO AKIMOTO[2]

[1]*Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa, Japan and* [2]*Department of Anatomy, Kyorin University School of Medicine, Mitaka, Tokyo, Japan*

ABSTRACT We studied proline-rich divergent homeobox gene *Hex/Prh* expression in the dorsal skin of chick embryo during feather bud development. *Hex* mRNA expression was first observed in the dorsolateral ectoderm and mesenchyme at 5 days, then in the epithelium and the dermis of the dorsal skin before placode (primordium of feather bud) formation and then was restricted to the placode and the dermis under the placode. Afterward, *Hex* expression was seen in the epidermis and the dermis of the posterior region of short bud. In accordance with *Hex* mRNA expression in the placode, Hex protein was observed in the epidermis as well as in the dermis of the placode. Immunoelectron microscopic study indicated that the protein located both in the nuclei and cytoplasm of the epidermis and the dermis at the short bud stage. The Wnt signaling pathway plays an essential role in the early inductive events in hair (Wnt3a and 7a) and feather (Wnt7a) follicles. The pattern of *Hex* expression in the epidermis was similar to that of *Wnt7a*, while little, if any, expression of *Wnt7a* was detected in the dermis under the placode or the dermis of the short bud compared with that of *Hex*, suggesting that Hex plays an important role in the initiation of feather morphogenesis.

KEY WORDS: *Hex, homeobox gene, feather placode, Wnt, feather bud development*

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).

Homeobox genes are a large family of transcription factors which plays 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 and Capecchi, 1998) and Jave-Suarez *et al.,* (2002) showed direct involvement of HOXC13 in the regulation of human hair keratin gene expression. The divergent homeobox genes *Msx1* (Noveen *et al.*, 1995), *Gbx1* (Obinata *et al.*, 2001) and *HB9* (Kosaka *et al.,* 2000a,b) 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. Another divergent homeobox gene *Hex* is expressed during early stages of chick embryogenesis, including pharyngeal endoderm, endocardium, liver, thyroid gland primordia and blood islands (Yatskievych *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). We showed previously that *Hex* is expressed in chick embryonic tarsometatarsal skin and regulates epidermal cell proliferation (Obinata *et al.*, 2002). In this study, to examine whether the *Hex* gene is involved in the feather morphogenesis or not, we performed *in situ* hybridization and immunostaining analyses spatially and temporally in dorsal skin of chick embryo.

Expression pattern of Hex *during feather bud development*

To see the localization of *Hex* during feather bud development, we performed an *in situ* hybridization analysis in developing chick. *Hex* expression was seen in the dorsolateral mesenchyme

***Address correspondence to:** Dr. Akiko Obinata. Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-0195, Japan. Fax: +81-426-85-3744. email: akiobi@pharm.teikyo-u.ac.jp

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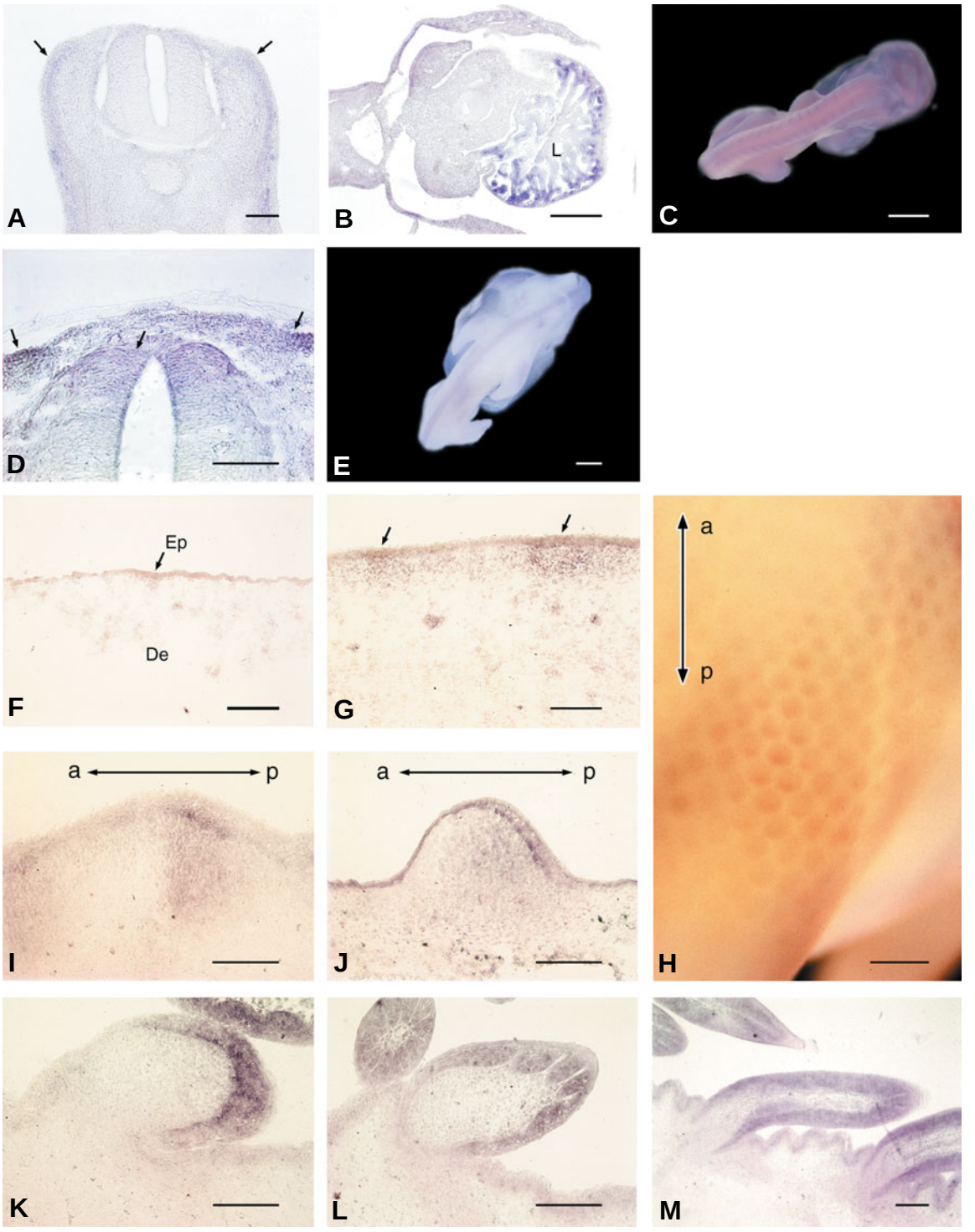


Fig. 1. Expression pattern of *Hex* mRNA during feather development as revealed by *in situ* hybridization. *Transverse sections (A,B,D) through the dorsal ectoderm and trunk region of chick embryos and whole embryo (C,E,H) after whole mount in situ hybridization (WISH)(A-E, H).* Hex *expression is seen in the dorsolateral region (arrows) of the mesenchyme, if any,* **(A,C)** *and is strong in liver (L)* **(B)** *at 4 days. The expression is seen from the dorsolateral to dorsomedial region (arrows) of the ectoderm and mesenchyme* **(D,E)** *at 5 days. Cryostat sections* **(F,G,I-M)**. Hex *expression is seen through the epidermis (Ep) while the expression was very little in the dermis (De) of the dorsal skin before placode formation* **(F)** *at 6 days and is restricted to the epidermis and dermis of the placode region (arrows)* **(G)** *at 7 days. Later, at short bud stage, the stronger expression is seen in both the epidermis and the dermis at the posterior buds* **(I,J)**. *At long bud stage, the signal is stronger in the epidermis than in the dermal cells in the posterior bud* **(K-M)**. *In WISH* **(H)**, *as well as in cryostat sections,* Hex *gene expression is detectable in feather buds with stronger signal in posterior regions, but not in interbud regions in 8-day-old chick embryo. a, anterior; p, posterior. Bars, 100 μm in A,D,F,G,I-M; 500μm in B; 1 mm in C,E; 2 mm in H.*

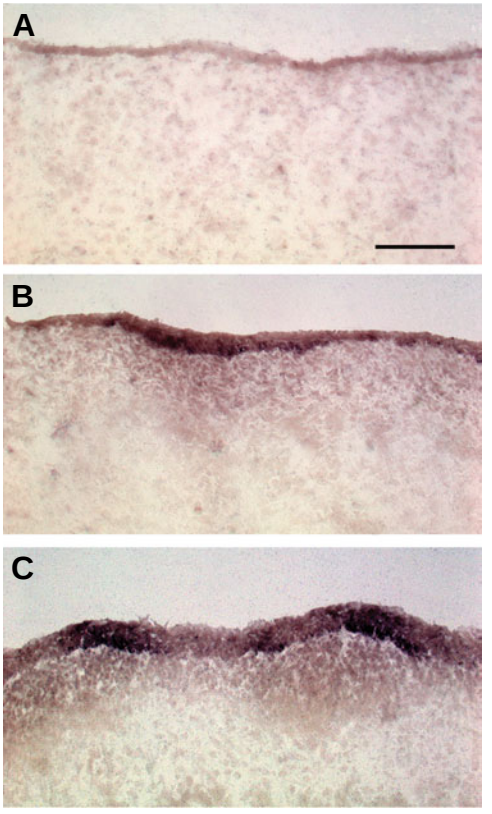
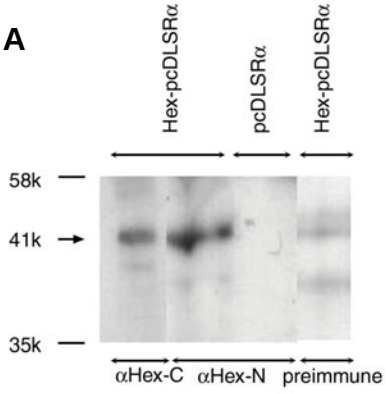


Fig. 2. Expression pattern of *Wnt7a* mRNA during feather development as revealed by *in situ* hybridization. *Sections of dorsal skin from 6- to 8-day-old chick embryos (A-C).* Wnt7a *expression is seen through the epidermis before placode formation* **(A)** *at 6 days and the stronger signal is seen in the epidermis of the placode region* **(B)** *at 7 days. At late placode stage, the stronger signal is seen in the epidermis of the posterior buds* **(C)**. *Bar, 50 μm.*

(arrows) (Fig. 1 A,C), but was strong in liver (B) at 4 days. The expression was seen from the dorsolateral to dorsomedial region (arrows) of the ectoderm and mesenchyme at 5 days (Fig. 1 D,E). *Hex* expression was seen through the epidermis of the dorsal skin before placode formation at 6 days while the expression was very little in the dermis (Fig. 1F) and is restricted to the placode epithelium and the dermis underneath the placode at 7 days (Fig. 1G). Later, at short bud stage, stronger *Hex* expression was seen in both the epidermis and the dermis of the posterior bud (Fig. 1 H,I,J). At long bud stage, the signal was stronger in the epidermis than in the dermis at the distal region of the bud (Fig. 1 K,L) and later an intense signal in the dermis at the bottom region of the bud adjacent to the epidermis was also seen (Fig. 1 M). Surprisingly, the *Wnt7a* expression pattern in the epithelium (Fig. 2 A,B,C and Widelitz *et al.,* 1999) was almost similar to that of *Hex* (Fig. 1 F,G,I), while less expression of *Wnt7a* in the dermis relative to that of the epidermis was observed under the placode (compare Fig. 1G with Fig. 2C).

Immunohistochemical localization of Hex protein during feather bud development

To know whether *Hex* mRNA and Hex protein are expressed correlatively and Hex locates in nucleus as a transcription factor during feather bud development, we used two different kinds of antiserum. One is antiserum raised against glutathione S-transferase fusion protein containing the 76 COOH-terminal amino acids of mouse Hex (Ghosh *et al*., 2000), which was



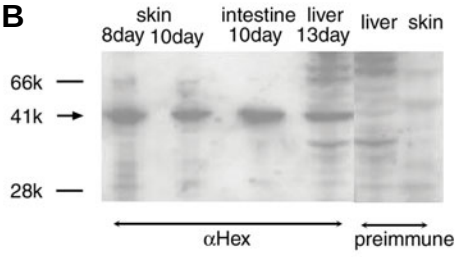
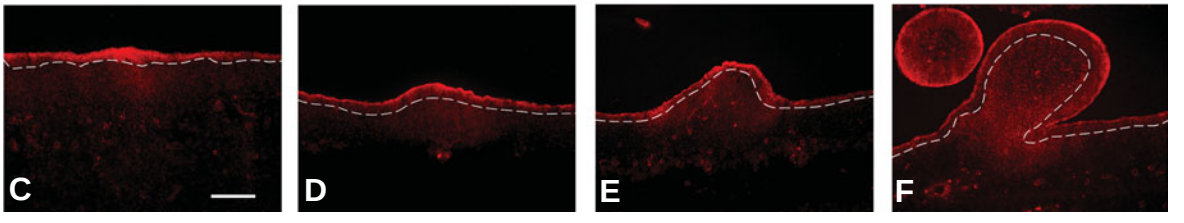


Fig. 3. Western blot and immunohistochemical staining of Hex protein during feather development. *Whole-cell lysates (50 μg)obtained from HeLa cells transfected with Hex-pcDLSRα* **(A)** *and chick embryonic tissues* **(B)**. *See text for details. Numbers used at the left side indicate molecular weights. Hex protein is detected by anti-N-terminal chick Hex antiserum followed by Cy3-conjugated secondary antibody (C-F). Hex was observed through the epidermis and the dermis with a stronger immunoreactivity at the placode* **(C)** *and the short bud* **(D,E)**, *while less immunoreactivity was observed in the dermis* **(C,D,E)**. *At the long bud stage, the immunoreactivity was seen throughout the epidermis and the periderm at the bud with a stronger immunoreactivity in the peridermal cells and the dermis at the root regions of the bud* **(F)**. *Bar, 50 μm.*



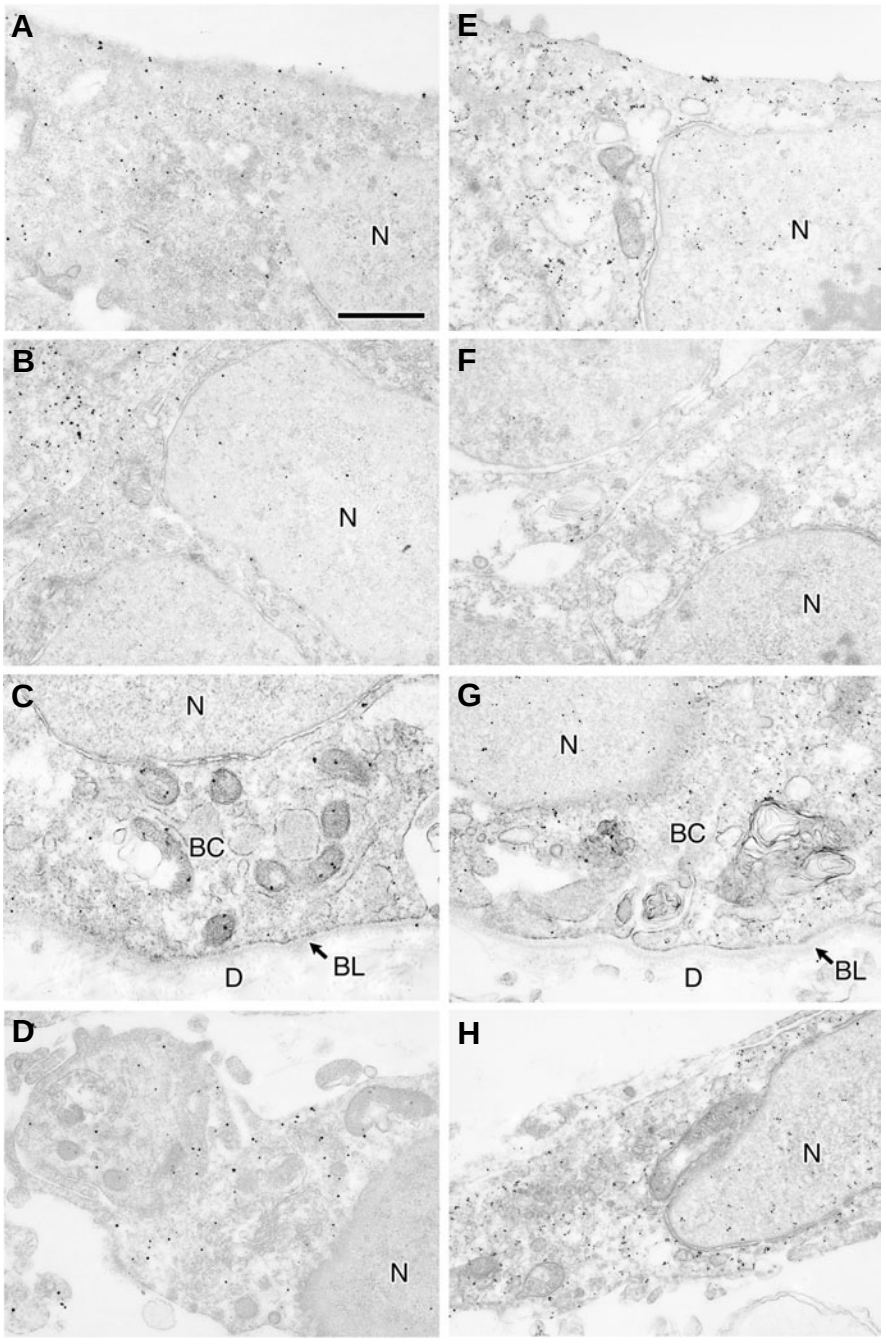


Fig. 4. Immunoelectron microscopic study of feather bud at 10 days. *Anti-N-terminal chick Hex antiserum* **(A-D)** *and antibody against glutathione S-transferase fusion protein containing the 76 COOH-terminal amino acids of mouse Hex* **(E-H)** *were used. Colloidal gold labeling represents the localization of Hex in the epidermal cells of superficial layer* **(A,E)**, *intermediate layer* **(B,F)**, *basal layer* **(C,G)** *and in the dermal fibroblasts* **(D,H)**. *Both antibodies indicated the same localization pattern in nucleus and cytoplasm of the epidermis and the dermis in the bud at 10 days with stronger immunoreactivity in the cytoplasm* **(A-H)**. *BC, basal cell of epidermis; BL, basal lamina; D, dermis; N, nucleus. Bar, 1 μm.*

kindly provided by Dr C.Bogue. The other is anti-N-terminal chick Hex antiserum. By Western blots, they developed a specific strong immune response against a 41k dalton band of Hex recombinant protein (Fig. 3A), the homogenate of chick embryonic dorsal skin, intestine, liver (Fig. 3B), or 5-day-old whole embryo (data not shown). At placode stage and later, Hex was observed throughout the epidermis with a stronger expression at the placode and bud region and in the dermis under these regions (Fig. 3 C-E). At the long bud stage, the immunoreactivity was seen at the bud with a stronger immunoreactivity in the peridermal cells and the dermal cells at the root regions of the bud (Fig. 3F). It is interesting to note that, while tissue localization of Hex protein was correlated with that of its mRNA, the extent of their expression level was different. The periderm is sloughed at later stages in the development. To study more precisely about the localization of the Hex in the cell, immunoelectron microscopic study was performed with 2 different kinds of Hex antibody. Colloidal gold labeling represents the localization of Hex in the epidermal cells of superficial layer (Fig. 4 A,E), intermediate layer (Fig. 4 B,F), basal layer (Fig. 4 C,G) and in the dermal fibroblasts (Fig. 4 D,H) of the long bud at 10 days. Both antibodies indicated the same localization pattern in nucleus and cytoplasm of the epidermis and the dermis with stronger immunoreactivity in the cytoplasm (Fig. 4 A-H). Few colloidal gold labeling was observed in the skin without first antibody (Fig. 5).

In this study, we showed that *Hex* was expressed as early as at 5 days in chick embryonic dorsolateral skin and then diffusely distributed in skin before placode formation and that its expression was 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. The Hex protein localized both in nucleus and cytoplasm of the epidermis and the dermis at the long bud stage with stronger expression in the cytoplasm, suggesting Hex might be actively sequestered in the cytoplasm, either by preventing nuclear import or by promoting a balance of export over import signals, or have a potential for intercellular trafficking by conventional secretion as in the case of Engrailed homeoprotein (Maizel *et al.*2002) or CVC paired-like homeobox proteins (Knauer *et al.,* 2005). The meaning of Hex existence in the cytoplasm must be studied in future. Surprisingly, the pattern of *Hex* expression in the epidermis was similar spatially and temporally to that of *Wnt7a* expression. The Wnt/Wg signaling pathway reviewed in Wodarz & Nusse, (1998) 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) and in many aspects of development such as the tooth (Dassule & McMahon,1998), limb bud (Cygan *et al.,* 1997), lung (Shu *et al.,* 2002), mammary gland (Humphreys *et al.,* 1997),

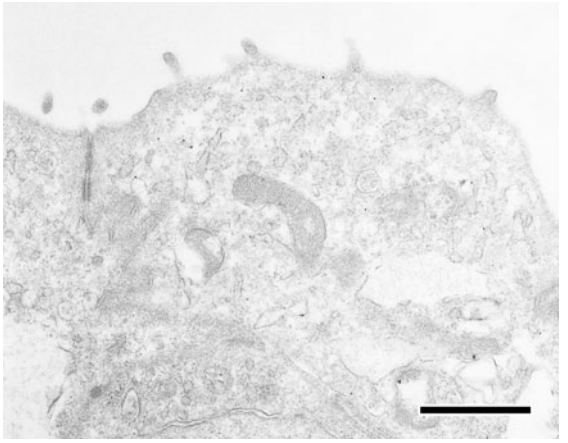


Fig. 5. Cytochemical control. *Immunoelectron microscopic study of the superficial layer of feather bud at 10 days. To check the specificity, anti-chick Hex antiserum was replaced with normal rabbit serum. Scarce colloidal gold labeling was observed. Bar, 1 μm.*

kidney(Torres & Nelson 2000) and liver (Suksaweang *et al.,* 2004). Recent studies have now addressed the issue of whether *Hex* is involved in the Wnt7a signaling pathway or in the initiation of feather bud formation (Obinata and Akimoto, 2005).

Experimental Procedures

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 *Wnt 7a* RNA probe, a *Wnt7a* cDNA fragment containing the entire coding region, which was kindly provided by Dr T.Nohno (Kawakami *et al*., 2000), was 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).

Transgene construction

A full-length *Hex* cDNA containing the entire *Hex* coding region, which was generously provided by Dr G.Goodwin (Haddow Labolatories, Institute of Cancer Research, Sutton,UK), was constructed with pcDLSRα.

Lipofection

HeLa cells were transfected with *Hex* -pcDLSRα using TransIT-LT1 Transfection Reagent (Invitrogen, California, USA) according to the manufacturer's instructions.

Western blotting

A rabbit antiserum was generated against a KLH-conjugated chick Hex NH2 peptide (MQYQAPGAAPAAALC). Western blotting was performed using standard protocols.

Immunostaining for light and electron microscopy

Frozen skin sections were processed for immunohistochemical staining as described previously (Akimoto *et al*.,1992; Kosaka *et al.,* 2000b).

Microscopy

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

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