Skeletal Analysis and Characterization of Gene Expression Related to Pattern Formation in Developing Limbs of Japanese Silkie Fowl

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The Japanese Silkie (Ukokkei) is a breed of domestic fowl with several unique characteristics. Skeletal analysis revealed that an extra cartilaginous digit was apparent transiently in the wing bud of Japanese Silkie embryos between stages 32 and 36. In contrast, an extra digit of the leg resembled digit 2 in terms of its skeletal elements. Whole-mount *in situ* hybridization analysis revealed that *Shh, Bmp-2,* and *Hoxd-13* genes are expressed in the presumptive region of the extra digit in the leg buds at stage 26. Expression of *Shh* and *Hoxd-13* was also apparent in the anterior region, including the presumptive area of the extra digit, in the developing wing bud of Japanese Silkie embryos. The Japanese Silkie fowl thus warrants further investigation as a potential model animal for the study of limb development.

Key words : Japanese Silkie, limb bud, polydactyly, skeletal development

Introduction

Silkie fowls are among the oldest of the rare breeds of domestic fowl. The first unambiguous record of the Silkie fowl is found in a Chinese book, *Wu lei xiang gan zhi,* written by Su Dongpo (1037–1101, according to http : //www.greatchinese.net/famous/schooler/sushi.htm ; http : //su-shi.biography.ms/ ; http : //www.silkqin.com/09hist/other/sushi.htm) in the 11th century. The Silkie fowl was introduced into Japan from China or India early in the 17th century, and the modern breeds of the Silkie fowl are thought to have been established in China and Japan (Roberts, 1997). These breeds are now bred worldwide and varieties are categorized on the basis of the area of their production. The Silkie fowl in Japan is thus known as the Japanese Silkie (Ukokkei in Japanese).

The Japanese Silkie fowl possesses numerous unique characteristics (Tsudzuki, 2003). The feathers of this fowl do not have a flat web. They also have abnormal barbules and no barbicels, resulting in a silky appearance. The skin is blackish, as is the surface of the bones and viscera, and the earlobes are

blue. This breed has five digits per leg. Wada *et al.* (2004) recently compared the mitochondrial DNA of the Japanese Silkie with that of the White Leghorn and found that the similarity between the two breeds was 99.77% (39 nucleotide differences out of a total of 16,784 base pairs).

In tetrapod vertebrates, limb patterning along the proximal-distal (P-D), anterior-posterior (A-P) and dorsal-ventral (D-V) axes is controlled by coordination of signaling centers. Patterning along the P-D axis is controlled by signaling from the apical ectodermal ridge (Crossley *et al.,* 1996 ; Loomis *et al.,* 1998 ; Min *et al.,* 1998 ; Lewandoski *et al.,* 2000 ; Moon and Capecchi, 2000). Signaling along the A-P axis originates from the zone of polarizing activity (ZPA) at the posterior margin of limb bud mesoderm. Grafting of the ZPA to the anterior margin of limb bud of a recipient animal results in the formation of additional digits with a mirror-image sequence along the A-P axis (Tickle *et al.,* 1975 ; Tickle, 1981). The dorsal nonridge ectoderm generates the D-V axis formation (Kengaku *et al.,* 1998).

Several genes including sonic hedgehog (*Shh*), bone morphogenetic protein 2 (*Bmp-2*) and homeobox D13 (*Hoxd-13*) are expressed in the posterior mesenchyme of the presumptive limb buds and are thought to contribute to pattern formation along the A-P axis (Francis *et al.,* 1994). Indeed, a cascade of *Shh, Bmp-2,* and *Hoxd-13* expression was found to occur in the developing chick limb (Francis *et al.,* 1994). Ectopic expression of *Shh* activates expression of 5' members of Hoxd complex and induces digit duplications, suggesting that the product of *Shh* is one of the first signals in the polarizing pathway (Riddle *et al.,* 1993). In contrast, activation of *Bmp-2* and *Hoxd-13* expression appears to occur in response to the polarizing signal (Izpisúa-Belmonte *et al.,* 1991 ; Francis *et al.,* 1994).

Currently, there are two hypotheses on the digit identity of the wing of birds. Traditional naming is that the digits are 2, 3 and 4 based on embryological evidence (Galis *et al.,* 2005) and the alternative one is that the digits are 1, 2 and 3 based on evolutionary evidence (Vargas and Fallon, 2005 a, b). In the present study we adopt the naming of wing digits as 2, 3 and 4.

Abnormal pattern formation in the limbs gives rise to various bird mutants. Preaxial polydactyly, which is characterized by the presence of extra digits on the side of digit 1, is one of the most common such abnormalities and is often coupled with hyperphalangy and malformation of the tibia. It is also associated with duplication of digits in a mirror-image sequence along the A-P axis in chickens (Landauer, 1956). The *talpid*[2] and *talpid*[3] mutant chickens manifest many digits in limbs that lack the characteristic A-P pattern (Ede and Kelly, 1964 ; Hinchliffe and Ede, 1968 ; Dvorak and Fallon, 1991 ; Galis *et al.,* 2005). Hereditary multiple malformation (HMM) is an embryonic-lethal condition of Japanese quail characterized by syndactylous polydactyly in both wing and leg buds with four to eight digits (Tsudzuki *et al.,* 1998). The molecular mechanisms responsible for these forms of preaxial polydactyly are poorly understood, however.

We have now examined the skeletal patterns of the developing wing and leg buds of the Japanese Silkie fowl. Furthermore, we characterized the distribution of *Shh, Bmp-2,* and *Hoxd-13* mRNAs during limb formation in embryos of this breed.

Materials and Methods

Animals

The study was performed in accordance with the policies on animal care developed by the Animal Care and Management Committee of the Faculty of Agriculture at Shinshu University. Fertilized White Leghorn eggs were obtained from Aichi Livestock and Poultry Breeding Center (Anjo, Japan). The black-feathered Japanese Silkie breed was introduced from Tokai Biken (Gifu, Japan) to our laboratory and fertilized eggs were obtained in-house. Eggs were incubated at 37.5℃ and 70% relative humidity, with tilting to a 90° angle at 30-min intervals. Embryos were staged as described by Hamburger and Hamilton (1951).

Skeletal Preparations

Skeletons of the Japanese Silkie and White Leghorn embryos were stained with Alcian blue 8GX (Sigma-Aldrich, St. Louis, MO, USA) and alizarin red S (Wako, Osaka, Japan) for cartilage and ossified bone, respectively. Staining was performed as described by Nakane and Tsudzuki (1999), with some modifications. Embryos at stages 26 and 45 were fixed overnight in Bouin's solution, rinsed with distilled water, and maintained with gentle agitation in 70% ethanol containing 10 mM ammonia. The ammoniated alcohol was changed every 3 h until

most picric acid had been removed. The embryos were then dehydrated by exposure to 95% ethanol for 2 days before stained for 1 day at 37℃ in a freshly prepared solution comprising Alcian blue 8 GX (30 mg), 95% ethanol (80 mL) and acetic acid (20 mL). After dehydration with 95% ethanol for 3 days, the viscera were removed and the embryos were macerated for 1 day in 0.002% alizarin red S in 1% KOH. They were finally exposed for 7 days to each of a series of glycerol solutions of increasing concentration (25, 50, 75 and 100%). Embryos between stages 26 and 35 were not treated with alizarin red S.

RNA Probes for Chicken Shh, Bmp-2 and Hoxd-13

Total RNA was extracted from the limb buds of White Leghorn embryos at stage 26 by the acid guanidium-phenol-chloroform method (Chomczynski and Sacchi, 1987) and was subjected to reverse transcription. The resulting cDNA was subjected to the polymerase chain reaction with forward and reverse primers shown in Table 1 ; the primers were designed on the basis of chicken *Shh, Bmp-2* and *Hoxd-13* cDNA sequences (GenBank accession nos. NM204821, AY237249 and NM205434, respectively). The amplification products were ligated into pCR2.1 (Invitrogen, Carlsbad, CA, USA). Antisense RNA probes labeled with digoxigenin-11-uridine-5′-triphosphate were prepared by transcription of the chicken *Shh, Bmp-2* and *Hoxd-13* cDNAs from the T7 promoter in the linearized vector with the use of a DIG RNA Labeling Kit (Roche Diagnostics, Mannheim, Germany).

Whole-Mount In Situ Hybridization

Whole-mount *in situ* hybridization was performed essentially as described by Ohuchi and Noji (2000) and Arisawa *et al.* (2005). In brief, embryos were isolated from eggs and fixed overnight with 4% para-formaldehyde in phosphate-buffered saline containing 0.1% Tween-20 (PBS-T). The embryos were then washed in PBS-T and dehydrated with a series of ethanol solutions of increasing concentration in PBS-T. After rehydration in a series of ethanol solutions of decreasing concentration in PBS-T and washing with PBS-T, the embryos were treated with proteinase K (10 μg/mL) for 5 to 15 min and then with glycine (2 mg/mL) for 5 min before washing twice with PBS-T. They were then fixed again with 4% paraformaldehyde and 0.2% glutaraldehyde. After incubation for 90 min with a solution comprising 50% formamide, 5× saline sodium citrate (SSC, pH 4.5), yeast tRNA (50 μg/mL) and heparin (50 μg/mL), the embryos were subjected to hybridization overnight at 70℃ in the same solution containing the digoxigenin-labeled RNA probe (100 ng/mL). Embryos were washed consecutively with solutions containing 50% formamide, 5× SSC, and 1% sodium dodecylsulfate (SDS) ; 50% formamide, 2× SSC and 1% SDS ; and 150 mM NaCl, 100 mM Tris HCl (pH 7.5) and 0.1% Tween-20 (TBS-T). For the detection of hybridization signals, the embryos were incubated with alkaline phosphatase-conjugated Fab fragments of sheep polyclonal antibodies to digoxigenin (1 : 2000 dilution ; Roche Diagnostics) in TBS-T containing 1.5% Blocking Reagent (Roche Diagnostics). Specimens were washed consecutively with TBS-T containing 5 mM levamisole ; with a solution containing 100 mM NaCl, 100 mM Tris HCl (pH 9.5), 50 mM $MgCl_2$ and 1% Tween-20 ; and with 5% polyvinylalcohol in the latter solution. They were then stained with the polyvinylalcohol solution containing nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (1 : 50 dilution ; Roche Diagnostics), followed by destaining with ethanol.

Table 1. **Primers used for the polymerase chain reaction in preparation of RNA probes**

Gene	GenBank accession no.	Primer sequence (5′ to 3′)	Product size (bp)
Shh	NM204821	CAGTGACTTCCTCACCTTCCTC TCCCACACTCTGTCTCTGTCC	600
Bmp-2	AY237249	AACAGCACGACGTTTCTTCTTT CATGGCAGTAAAAGCCACTGTA	599
Hoxd-13	NM205434	GCTTAAGGAACTCGAGAACGAA TTTTCCGACCAATACACCAAAT	599

The polymerase chain reaction was performed for 35 cycles of denaturation at 96℃ for 60 s, annealing at 55℃ for 60 s, and extension at 72℃ for 60 s, with a preliminary denaturation at 96℃ for 5 min and a final extension at 72℃ for 10 min.

Results

Skeletal Analysis

Skeletal pattern of the wing buds of the Japanese Silkie and White Leghorn embryos are shown in Figure 1. Three cartilaginous digits were observed in the wings of both types of embryo at stage 31 (Fig. 1 A, B). Ossification of wing digits occurred at stage 35 (data not shown). An extra digit that bifurcated from the root of digit 2 was apparent in the wing of Japanese Silkie from stages 32 and 36 (Fig. 1 C, E, G) but was no longer detectable at stage 37 (Fig. 1 I) and thereafter. Figure 2 shows skeletal patterns of the leg buds of Japanese Silkie and White Leghorn embryos. Four cartilaginous digits were apparent in both types of embryos at stage 29 (Fig. 2 A, B). Ossification in digits occurred at stage 38 (Fig. 2 G, H). An extra cartilaginous digit that bifurcated from the root of digit 1 was detected in Japanese Silkie embryos between stages 30 and 37 (Fig. 2 C, E). There were no differences in digits 2, 3 and 4 between the two types of embryos. Two cartilaginous joints were apparent in the original digit 1 of Japanese Silkie embryos, whereas the extra digit possessed three joints similar to those of digit 2 (Fig. 2 I). The extra digit persisted throughout life.

Gene Expression Patterns

Figure 3 shows whole-mount *in situ* hybridization analysis of the localization of *Shh* mRNA in the limb buds of Japanese Silkie and White Leghorn embryos. At stage 24, both types of embryo manifested marked expression of *Shh* in the posterior regions of the wing and leg buds (Fig. 3 A-D). Expression of *Shh* was also apparent in the anterior region of the leg bud in the Japanese Silkie embryo (Fig. 3 C). As the limb buds elongated at stage 26, *Shh* expression in the wing buds of both types of embryo became localized to the posterior margin (Fig. 3 E, F). In contrast, *Shh* expression in the leg buds became localized to the distal tip (Fig. 3 G, H). In addition, expression of *Shh* was apparent in the anterior region of the leg bud in the Japanese Silkie embryo at stage 26, with no such expression being evident in the White Leghorn embryo.

The expression patterns of *Bmp-2* and *Hoxd-13* in the limb buds of Japanese Silkie and White Leghorn embryos at stage 26 are shown in Figure 4. Expression of *Bmp-2* was apparent at the distal tips of the limb buds of both types of embryo (Fig. 4A-D). In addition, *Bmp-2* expression was detected in the anterior region of the leg bud, including the presumptive region of the extra digit, in the Japanese Silkie embryo (Fig. 4 C). Expression of *Hoxd-13* was also apparent at the distal tips of the limb buds of both types of embryo (Fig. 4 E-H), with expression in both wing and leg buds of the Japanese Silkie embryo also being manifest in the anterior region, including the presumptive area of the extra digit (Fig. 4 E, G).

Discussion

Polydactyly in the Silkie fowl has been shown to be caused by the dominant gene, *Po* (Somes, 1990). We have now detected a cartilaginous extra digit anterior to digit 2 of the wing bud in Japanese Silkie embryos between stages 32 and 36 ; this digit was no longer apparent at stage 37 and thereafter. Barfurth (1911) showed that, in polydactylous chicken embryos, a small rudimentary extra digit is often manifest at the anterior edge of the wing, near the bastard wing, at 8 to 10 days of incubation (approximately corresponding to stages 33 to 36) but not thereafter. In normal chick embryos, the rudimentary extra digit (mesenchymal condensations) is observed transiently posterior to digit 4 both in the wing and leg buds (Burke and Feduccia, 1997 ; Larsson and Wagner, 2002 ; Galis *et al.,* 2005). The transient condensation is also observed in the area anterior to digit 2 in the chick embryonic wing bud at stage 29 but it remains in arrested state throughout life (Larsson and Wagner, 2002). The chondrified extra digit shown in the present study is unlikely the transient condensation shown by Larsson and Wagner (2002) but it is the characteristic feature of the Japanese Silkie embryos.

An extra toe is found in breeds of fowl other than the Silkie, including Dorking, Houdan and Sultan (Somes, 1990 ; Roberts, 1997). The extra toe develops on top of the normal digit 1, arising from the metatarsal, but there is considerable variation in the expression of polydactyly (Somes, 1990). Kaufmann-Wolf (1908) described polydactyly as being apparent at 5 days of incubation (approximately corresponding to stage 26). As described above the transient formation of the anlage of digit 5 posterior to digit 4 was observed in the leg bud of normal chick embryos (Burke and Feduccia, 1997). Burke and Feduccia (1997) did not mention the developmental
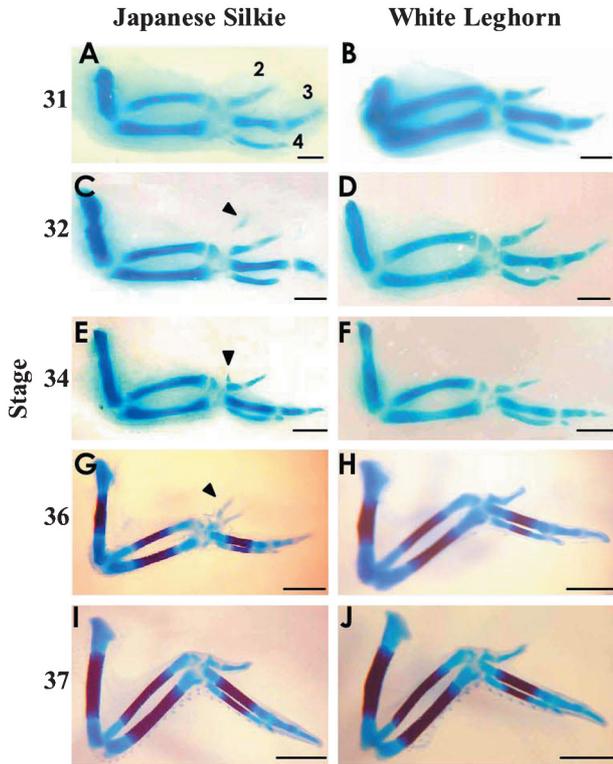
Fig. 1
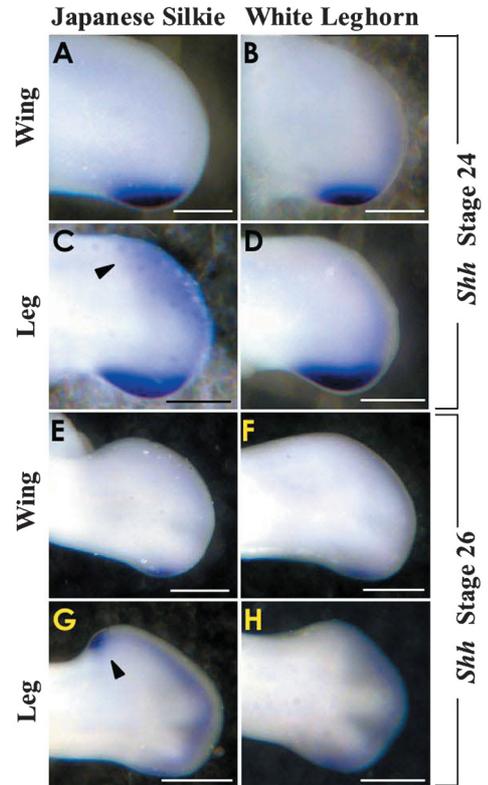
Fig. 2

Fig. 3
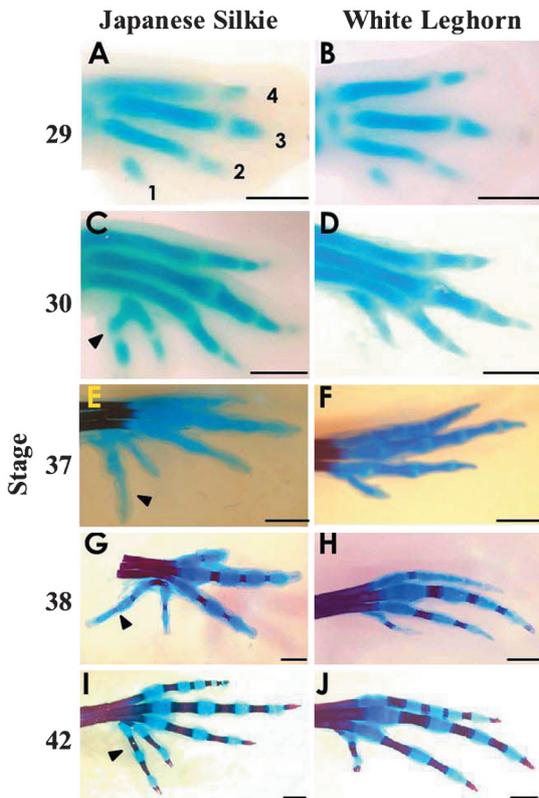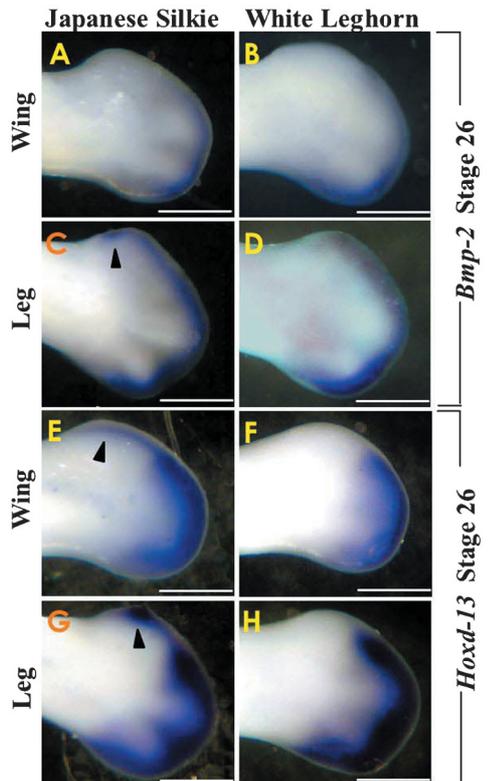
Fig. 4

stages when the anlage of digit 5 appears, but it is estimated to be between stages 27 and 33 according to the figures. In the present study, however, such a cartilaginous rudiment was not observed in the area posterior to digit 4 of the leg bud in the White Leghorn and Japanese Silkie embryos at stage 26 and thereafter. In the Japanese Silkie embryos the extra digit that bifurcated from the root of digit 1 was observed at stage 30 and persisted throughout life.

The *talpid²* and *talpid³* mutant chickens possess polydactylous limbs with partially fused digits that form according to a pattern lacking obvious A-P polarity (Ede and Kelly, 1964 ; Hinchliffe and Ede, 1968 ; Dvorak and Fallon, 1991 ; Galis *et al.*, 2005). In contrast, Japanese Silkie embryos possess an independent extra digit that resembles digit 2 both in size and in that it contains three phalanges.

Transplantation of Shh-secreting cells located in the posterior mesenchyme of the ZPA to ectopic anterior locations induced complete mirror-image duplication of skeletal elements in chicken and mouse embryos (Saunders and Gasseling, 1968). Furthermore, transplantation of ectopic Shh-expressing cells into anterior tissues of the chick wing bud induced

ectopic *Bmp-2* and *Hoxd-13* expression (Yang *et al.*, 1997 ; Drossopoulou *et al.*, 2000). We have now shown that *Shh* is expressed prominently in the anterior region of the leg bud of Japanese Silkie embryos but not in the corresponding region of White Leghorn embryos. This region corresponds to the presumptive site of development of the extra digit in Japanese Silkie embryos. It is thus possible that the extra digit in the leg bud of Japanese Silkie develops as a result of the Shh signal in the anterior region and represents a mirror-image duplication of digit 2.

Bmp-2 is one of the coding genes of bone morphogenetic proteins and *Hoxd-13* is essential for the phalange formation (Francis *et al.*, 1994 ; Fromental-Ramain *et al.*, 1996). Both genes function downstream of *Shh*, which direct positional specification during pattern formation in the limbs (Dollé *et al.*, 1989 ; Izpisúa-Belmonte *et al.*, 1991 ; Nohno *et al.*, 1991 ; Niswander *et al.*, 1993 ; Francis *et al.*, 1994). The domain of *Bmp-2* expression overlaps substantially with that of *Hoxd-13* (Francis *et al.*, 1994). BMP-2 is a downstream component of the polarizing region signaling pathway rather than being the polarizing region signal itself since the application of recombinant BMP-2 protein to anterior margin of

Fig. 1. **Wing skeletons of Japanese Silkie (A, C, E, G, I) and White Leghorn (B, D, F, H, J) embryos between stages 31 and 37.** Dorsal view. Three cartilaginous digits were apparent in both Japanese Silkie and White Leghorn embryos at stage 31. The wing of the Japanese Silkie manifests an extra digit (arrowheads) bifurcated from the root of digit 2 between stages 32 and 36. Digit numbers are labeled in (A). Bars, 1 mm.

Fig. 2. **Leg skeletons of Japanese Silkie (A, C, E, G, I) and White Leghorn (B, D, F, H, J) embryos between stages 29 and 42.** Dorsal view. Four cartilaginous digits were apparent in both Japanese Silkie and White Leghorn embryos at stage 29. The leg of the Japanese Silkie manifests an extra digit (arrowheads) bifurcated from the root of digit 1 at stage 30 and thereafter. Digit numbers are labeled in (A). Bars, 2 mm.

Fig. 3. **Whole-mount *in situ* hybridization analysis of the localization of *Shh* mRNA in the limb buds of Japanese Silkie (A, C, E, G) and White Leghorn (B, D, F, H) embryos.** Dorsal view of wing (A, B, E, F) and leg (C, D, G, H) buds at stages 24 (A-D) and 26 (E-H) are shown. At stage 24, *Shh* expression was apparent in the posterior regions of the limb buds of both types of embryo ; the leg bud of the Japanese Silkie embryo also manifested *Shh* expression in the anterior region [arrowhead in (C)]. At stage 26, *Shh* expression was detected at the posterior margins of the wing buds and at the distal tips of the leg buds of both types of embryo but was also manifest at the anterior margin of the leg bud of the Japanese Silkie embryo [arrowhead in (G)]. Bars, 200 μm.

Fig. 4. **Whole-mount *in situ* hybridization analysis of the localization of *Bmp-2* (A-D) and *Hoxd-13* (E-H) mRNAs in the limb buds of Japanese Silkie (A, C, E, G) and White Leghorn (B, D, F, H) embryos at stage 26.** Dorsal view. Expression of *Bmp-2* was apparent at the distal tips of the limb buds of both types of embryo as well as at the anterior margin of the leg bud, including the presumptive region of the extra digit, in the Japanese Silkie embryo [arrowhead in (C)]. Expression of *Hoxd-13* was apparent in the presumptive regions of the normal digits in the wing and leg buds of both types of embryo as well as in the presumptive region of the extra digit in both wing and leg buds of the Japanese Silkie embryo [arrowheads in (E) and (G)]. Bars, 200 μm.

chick wing buds did not induce digit duplications (Francis *et al.,* 1994). Francis *et al.* (1994) also suggested that cells might activate *Hoxd-13* shortly after they have activated *Bmp-2* and raises the possibility that BMP-2 is involved in the activation of *Hoxd-13.*

Japanese Silkie embryos showed weak expression of *Hoxd-13* without that of *Shh* and *Bmp-2* in the presumptive region of the extra digit in the wing bud, whereas no corresponding region of expression was apparent in the wing bud of White Leghorn embryos. It should be examined whether the expression of *Shh* and *Bmp-2* is below the detectable level or these genes are expressed in the earlier stages of development. Formation of the extra digit was initiated at the anterior tip of the wing bud at stage 32 in Japanese Silkie embryos but it was disappeared at stage 37 with unknown reasons. Our literature survey could not find molecular evidence concerning the transient expression of the extra digit in the chicken. Recent studies have shown that the expression of the *Hoxd-12* and *Hoxd-13* is related to the development of digit 1 (Ros *et al.,* 1992 ; Chiang *et al.,* 2001 ; Litingtung *et al.,* 2002). In the chick embryos, *Hoxd-12* is not expressed in the most anterior digit (digit 2 and 1 in the wing and leg buds, respectively ; as mentioned above there are two hypotheses on the digit identity of the wing of bird) but it is expressed in all other digits at day 5 of incubation (approximately corresponding to stage 26) and later stages (Ros *et al.,* 1992 ; Knezevic *et al.,* 1997 ; Chiang *et al.,* 2001 ; Chen *et al.,* 2004). *Hoxd-13,* in turn, is expressed in all other digits including the most anterior digit (Ros *et al.,* 1992 ; Knezevic *et al.,* 1997 ; Chiang *et al.,* 2001 ; Chen *et al.,* 2004). Vargas and Fallon (2005 a, b) showed both expression of *Hoxd-12* and *Hoxd-13* in the extra digit of the leg bud of the Silkie fowl. The leg buds of the Japanese Silkie embryos in the present study manifested expression of *Bmp-2* and *Hoxd-13* in the presumptive regions of the extra digit as well as in the original digits in the leg bud at stage 26. The expression of both *Bmp-2* and *Hoxd-13* may lead to the formation of the extra ossified bones in the leg of Japanese Silkie embryos. However, it should be examined whether *Hoxd-12* is expressed in the presumptive regions of the extra digit.

In conclusion, we have demonstrated formation of a rudimentary extra cartilaginous digit in the wing bud, mirror-image development of an extra digit in the leg, and expression of *Shh, Bmp-2,* or *Hoxd-13* in the presumptive regions of the extra digits in the limb buds of Japanese Silkie embryos. The Japanese Silkie fowl warrants further investigation as a potential model animal for the study of limb development.

Acknowledgments

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