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[REVIEW]

Evolutionary and Developmental Aspects of Avian-specific Traits in Limb Skeletal Pattern

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The two sets of paired appendages, called limbs, are locomotory organs in tetrapods that are used for various functions (e.g., walking, running, crawling, digging, climbing, diving, swimming, and flying). Unlike such organs as the eye, which contain specialized tissues such as the lens and photoreceptor, the limb does not have any specialized cells or tissues, but consists of common tissues, such as bone, cartilage, muscle, blood vessels, and dermis. However, limb morphology is highly specialized and varies to provide species-specific modes of locomotion. As do the vertebrae and skull, the limb skeleton varies in morphology among species. The diversity of limb skeletal morphology provides examples of material for studies on morphogenesis. Avian forelimbs have evolved into wings for flight. The skeletal pattern in the avian limb has many traits that are unique among extant species of vertebrates; some of such traits are avian-specific, others are shared with more basal members of Theropoda, to which Aves belongs. Since such avian traits generally form during ontogenic development, determining when and how they appear in the developing embryonic limbs or limb buds provides important insights into the mechanisms underlying the generation of vertebrate morphological diversity. Here, we present an overview of several features of the skeletal pattern in the avian limb and discuss the developmental mechanisms responsible for their unique and lineage-specific traits.

Key words: limb, skeleton, pattern, development, evolution

1. UNIQUE TRAITS OF THE SKELETAL PATTERN IN THE AVIAN LIMB

1-1. Skeletal structure of the vertebrate limb

The presence of four limbs (two sets of paired limbs) is a synapomorphy shared by amphibians, mammals, and reptiles including avians, uniting these groups of animals as a clade, Tetrapoda. The skeletal elements of the tetrapod limb can be divided into three segments along the proximo-distal axis (Fig. 1A). The most proximal segment (stylopod) contains a single long bone (the humerus and femur of the forelimb and hindlimb, respectively) (Fig. 1). The middle segment (zeugopod) contains two long bones placed side by side (the radius and ulna of the forelimb and the tibia and

fibula of the hindlimb). The number of bones in these two segments is well conserved among tetrapods. The distal segment (autopod, hand or manus and foot or pes of the forelimb and hindlimb, respectively) can be generally further subdivided into three parts: basipodium, metapodium, and autopodium (acropodium). The most proximal basipodium (wrist/ankle) consists of carpals/tarsals that are multiple short, cuboidal bones. The metapodium (palm/sole) consists of the metacarpals/metatarsals that are long bones connecting the digits and the basipodium. The number of metapodial elements usually corresponds to the number of digits. The most distal segment, autopodium, includes digits (thumb and fingers/toes) that consist of a series of phalanges. The number of phalanges in each digit varies among taxa and is represented by a phalangeal formula that indicates the number of phalanges per digit, beginning with the anteriormost digit (for example, the phalangeal formula of the human forelimb is 2-3-3-3-3.). In general, unguals are present at the ends of the digits and are often covered dorsally by skin derivatives, such as nails, claws and hoofs.

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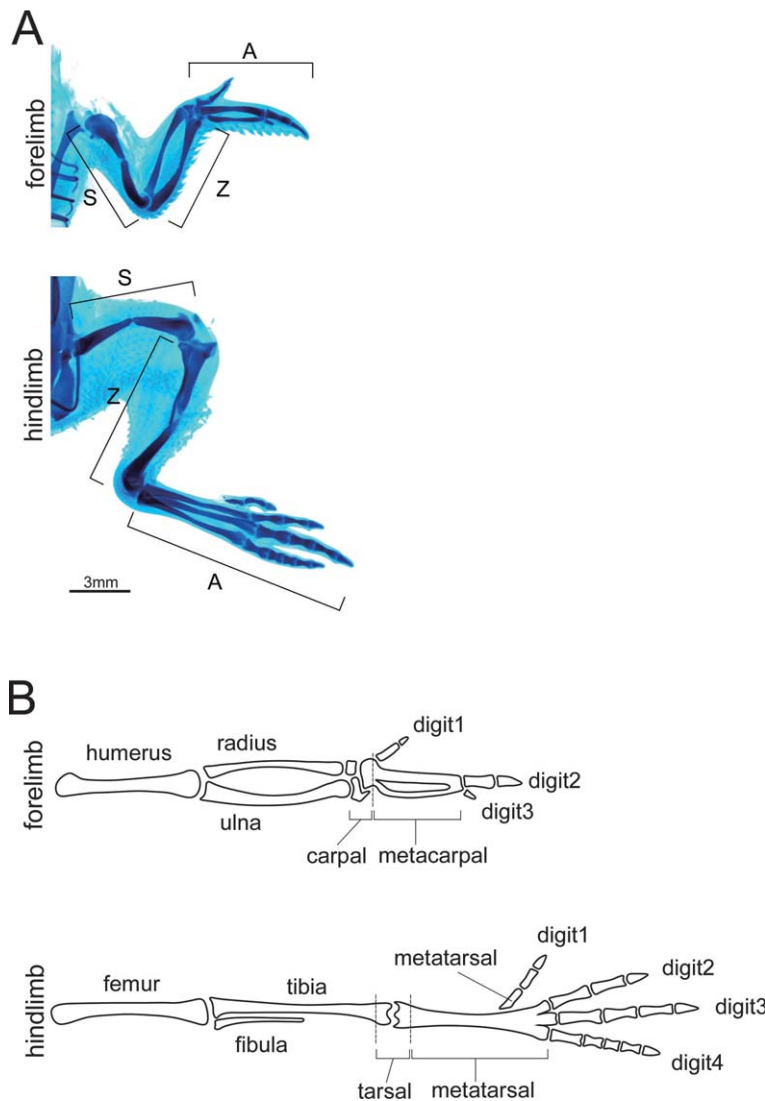


Fig. 1. (A) Skeletal patterns of the fore- and hind-limb in the chick embryo, as shown in one individual stained with Alcian blue for cartilage at E10.5. In this stage, most of the limb skeletal elements are cartilaginous tissue, which are then replaced by bony tissue as development proceeds. Both the fore- and hind-limb have three distinct segments along the proximo-distal axis; stylopod, zeugopod, and autopod. S, stylopod; Z, zeugopod; A, autopod. Scale bar = 3 mm. **(B)** A schematic representation and terminology of the skeleton of the avian fore- and hind-limb. See the text for details.

The typical number of digits in the tetrapod limb is five. Whereas reduction of digit number has occurred independently and repeatedly in many tetrapod lineages, no extant species has more than five digits (we do not include prehallux or prepollex as digits in this review). The terminology for orientation in the autopodium differs between embryology and comparative morphology. In the embryological nomenclature, “anterior” and “posterior” are used for directions toward the first digit (thumb/big toe, or digit 1) and fifth digit (little finger/pinky toe, or digit 5), respectively, as digit 1 is formed in the most anterior (rostral) part of the developing embryonic limb along the rostro-caudal body axis. In the comparative morphological nomenclature, however, “internal (inner)” or “medial” and “external (outer)” or “lateral” are used for directions of digits 1 and 5, respectively, as in most

quadruped animals (using four limbs for locomotion) digit 1 is oriented towards the midline of the body.

All of the skeletal elements of the limbs are derived from the somatic mesoderm of the developing embryo. During ontogenetic development, the somatic mesoderm initially forms as a single sheet of lateral plate mesoderm that is then divided into two layers separated by the coelom. The dorsal layer is called the somatic mesoderm, and the ventral layer is called the splanchnic mesoderm. While the splanchnic mesoderm mainly generates the mesenchyme of the digestive tube, the somatic mesoderm gives rise to the mesenchyme of the body wall. At each level of the cervico-thoracic (or cervico-dorsal) and lumbo-pelvic (or dorso-sacral) boundaries, both sides of the lateral body wall swell out to form a pair of bud-like outgrowths composed of mesenchyme derived from the somatic mesoderm and the overlying ectodermal epithelium. These outgrowths are the embryonic limb primordia called limb buds.

As the mesenchymal cells of the limb bud proliferate, the limb bud steadily elongates laterally from the body. During this process, the skeletal elements of the limbs are formed through endochondral ossification; cartilage models are first formed by mesenchymal condensation, and later replaced by bone (see other reviews for details; Hall, 2005; Yano and Tamura, 2012). Since cartilage formation in the limb bud begins from the proximal end and proceeds toward the distal tip, as the limb bud grows, the skeletal elements of the limb form gradually and sequentially in the following order: stylopod, zeugopod, autopod. The tendons (Chevallier et al., 1977; Christ et al., 1977) and the dermis of the limb (Pearse et al., 2007) are also derived from the somatic mesoderm; other components, such as muscle, blood vessels (vascular endothelium), Schwann cells, and nerve axons, migrate into the limb bud from the trunk tissues (somites, neural tube, and neural crest).

The unique traits of the skeletal pattern in avian forelimbs and hindlimbs, in terms of their structure and embryonic origin, are discussed in

the following sections, with reference to the basic pattern in tetrapods.

1-2. Skeletal pattern of the avian forelimb

The standard number of digits in the avian forelimb is three (digit 1, digit 2, and digit 3) although there are exceptions, such as *Apteryx*, which often have only two metacarpals (Parker, 1891). The most common phalangeal formula is 1-2-1 or 2-2-1 (e.g., Beddard, 1898; Baumel and Witmer, 1993), but there are also variations, as observed in *Apteryx* (0-2/3-0; Parker, 1891) and *Dromaius* (0-3-1; Maxwell and Larsson, 2007, *contra* Parker, 1888a). The phalangeal formula in the chicken is 2-2-1. Three metacarpals are fused with one another as well as with distal carpals, forming the carpometacarpal (e.g., Morse, 1872; Parker, 1888b; Hogg,

1980) (Fig. 1B). Digit 1 is the shortest, and the first metacarpal is rudimentary and fused with the proximal end of the second metacarpal. Digit 2 is the most well-developed. Some birds possess unique, derived features in the manus. Penguins, for example, have large, well-developed forelimbs (Fig. 2A) for underwater “flying,” although they have lost digit 1 and therefore have only two digits while retaining three metacarpals (Fig. 2B; Couse, 1872; Watson, 1883; Shufeldt, 1901). As shown in a forelimb skeleton of Humboldt penguin (*Spheniscus humboldti*), there is a vestigial trace of the first metacarpal fused with the second metacarpal (Fig. 2B, C). It can be speculated that the first metacarpal forms transiently as an independent element in the developing forelimbs, and subsequently fuses to the second metacarpal during late embryonic or post-hatching stages.

The number and morphological pattern of the carpals vary among avian species (e.g., Parker, 1888b). The two small, cuboidal carpals adjacent to the distal ends of the radius and ulna are called the radiale and ulnare, respectively (Baumel and Witmer, 1993). Homologies of these elements with bones of the same names in other tetrapods, however, are yet to be established (e.g., Holmgren, 1955; Hinchliff, 1984). Most recently, for example, Kundrat (2009) regarded the avian “radiale” and “ulnare” as the fused radiale and intermedium in other tetrapods and a neomorph (pseudoulnare), respectively. In the chick forelimb, the other carpal bones are fused together and with the metacarpals, to form the carpometacarpal. While the number of bones in the stylopod and zeugopod is almost invariable, their morphological characteristics, such as length and thickness, vary widely (compare the skeleton of the chick forelimb in Fig. 1A with that of the penguin forelimb in Fig. 2B).

1-3. Skeletal pattern of the avian hindlimb

Most extant species of birds retain four digits, digits 1 through 4, in the hindlimb. The phalangeal formula is generally 2-3-4-5 (Beddard, 1898) with a few exceptions. There are several types of toe arrangement, and the most common arrangement is an anisodactyl foot; digit 1 points in a medial and plantar direction (the digit being turned backward), while the other three digits are spread out in a forward direction. Another is a zygodactyl foot, in which digit 1 and digit 4 are turned backward and digits 2 and 3 point forward (e.g., Finn, 1894; Raikow, 1985).

Whereas the first metatarsal is rudimentary, the other three metatarsals are long and well-developed. These three metatarsals (second through fourth metatarsals) align in parallel and fuse to each other in adults, forming one, single long bone (e.g., Gegenbaur, 1864; Parker, 1888b; Hinchliffe, 1977; Namba et al., 2010). Some groups of birds such as penguins have relatively short and broad metatarsals (Fig. 2A).

A bird has at least three tarsals (Gegenbaur, 1864; Morse, 1872; McGowan, 1985; Müller, 1989): the two proximal components (proximal tarsals) are called the astragalus (tibiale) and calcaneus (fibulare), and the other is the distal tarsal. In adults, no tarsal is present as an independent element, because the proximal tarsals are fused together with the tibia to make the tibiotarsal, and the distal tarsal is fused with the metatarsals to form the tarsometatarsal (Gegenbaur, 1864; Morse, 1872; Hinchliffe, 1977; Müller, 1989). In the

chick, for example, the proximal tarsals fuse with the tibia in a few months after hatching (Hogg, 1982). Therefore, the hinge of the ankle joint in birds runs inside the tarsus (between the proximal and distal tarsals), not between the zeugopod and the autopod (Fig. 3C; Gegenbaur, 1864; Morse, 1872). This type of ankle joint is called a mesotarsal or intertarsal joint (e.g., Wiedersheim, 1883; Goodrich, 1916; Shaeffer, 1941). The primitive type of the mesotarsal joint in which the calcaneus is similar in size with the astragalus and bears a laterally extending tuber is a plesiomorphy for Archosauromorpha (Chatterjee, 1982; Cruickshank and Benton, 1985). However, another, derived type the mesotarsal joint in which the astragalus bears an ascending process articulating with the tibia and the calcaneus reduced in size lacks the tuber characterizes the clade Ornithodira that includes Aves, other dinosaurs, and Pterosauria (Gauthier, 1986). In adult birds, both proximal tarsals are fused with the tibia, with the fibula not reaching the ankle joint. In other words, only one zeugopodial component (tibia) participates in the ankle joints of birds. The fibula is often fused with the tibia, or is tightly united with the later through ligamentous connection (syndesmosis tibiofibularis; Müller and Streicher, 1989).

2. DEVELOPMENTAL MECHANISMS UNDERLYING THE AVIAN DIGIT PATTERN

The first part of this section is an overview of the general developmental mechanisms that establish the limb skeletal morphology and pattern in tetrapods. Although there exists considerable information on these mechanisms, how the avian-specific traits of limb skeletal morphology and pattern are formed remains unclear. The latter part of this section focuses on the developmental mechanisms underlying the production of the avian-specific traits of digit number and identity, as these mechanisms have been partially elucidated.

2-1. General developmental mechanisms for limbs

Most of the skeletal characteristics in the avian limb described in the previous section are produced during the morphogenetic process of embryonic development. However, some morphogenetic changes, including skeletal fusion and deformation, take place in later developmental or post-hatching stages. In this section, we outline the early process of skeletal morphogenesis, and discuss the mechanisms by which the avian-specific morphology of digits is generated.

There are two major signaling centers for pattern formation in the limb bud. One is the apical ectodermal ridge (AER), which is a thickening of the epithelium running along the distal limb bud tip (Fig. 3A). Classical experiments in which the chick AER was removed at various developmental stages resulted in stage-dependent distal truncations, demonstrating that the AER is essential for proximo-distal patterning and growth (Saunders, 1948; Summerbell, 1974; Rowe and Fallon, 1982). Among a number of genes expressed in the AER, some members of the *fgf* gene family (including *fgf2*, *fgf4*, and *fgf8*) can compensate for the removal of the AER, indicating a crucial role of *fgfs* in limb bud outgrowth (Niswander et al., 1993; Fallon et al., 1994; Vogel et al., 1996). Although it remains unclear what regulates the skeletal pattern along the proximo-distal axis, *Fgfs*

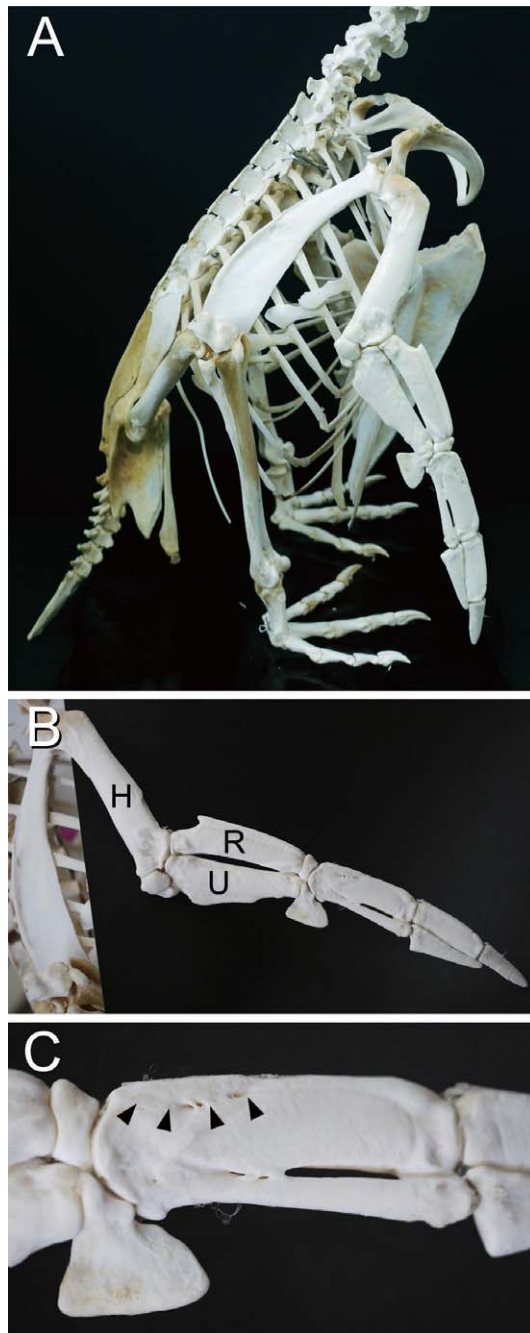


Fig. 2. Skeletal pattern of the penguin forelimb. The skeleton of an adult Humboldt penguin (*Spheniscus humboldti*) preserved at Tokyo Sea Life Park (Kasai Rinkai Suizokuen). **(A)** The post-cervical part of the skeleton of the Humboldt penguin. Right lateral view. Note the peculiar morphology of the forelimb including flat and wide bones. **(B)** Enlargement of the forelimb skeleton. The anterior digit is digit 2, which has two phalanges and a long metacarpal, and the posterior digit, digit 3, has only one phalanx. H, humerus; R, radius; U, ulna. **(C)** Higher magnification of the autopod region. Note that only two digits are visible. However, there is a trace of bone fusion at the anterior-basal metacarpal II (arrowheads), suggesting the presence of the vestige of metacarpal I.

in the AER are known also to be responsible for axis formation (Mariani et al., 2008).

Two major models have been proposed for axis forma-

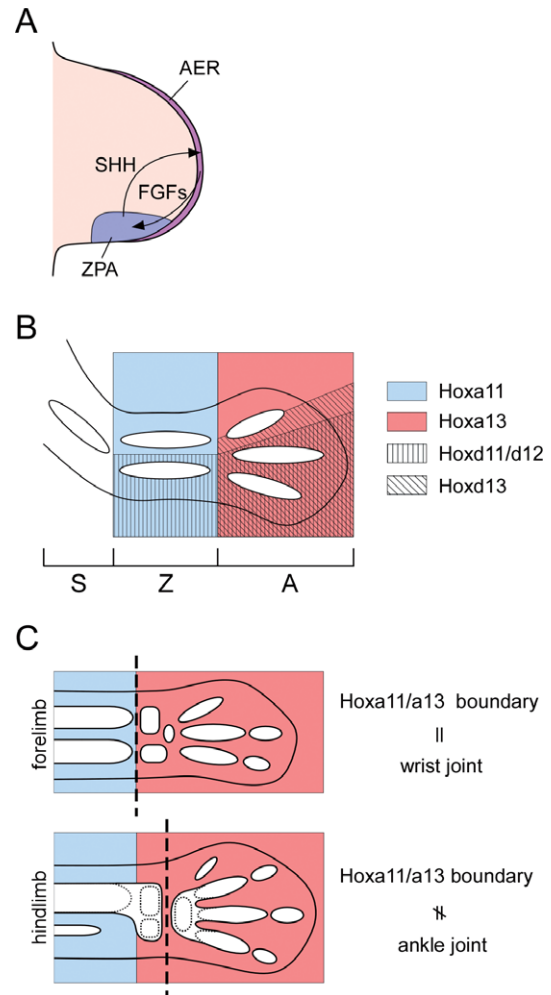


Fig. 3. Feedback loop of ZPA-AER and Hox code in the developing chick forelimb bud. **(A)** Schematic representation of the feedback loop of ZPA-AER (Niswander et al., 1994; Zeller et al., 2009). The AER runs along the distal limb bud tip (pink). The ZPA is located at the posterior margin of the limb bud (purple). FGF proteins such as FGF2, FGF4, and FGF8 are secreted from the AER and maintain expression of the *shh* gene in the ZPA. In turn, the SHH protein from the ZPA maintains the expression of *fgf* genes in the AER. Thus, a positive feedback loop is established between the ZPA and the AER. **(B)** Schematic representation of the expression of *Hox* genes in the developing chick forelimb bud (Yokouchi et al., 1991; Nelson et al., 1996). The expression patterns of five major *Hox* genes, *Hoxa11*, *Hoxa13*, *Hoxd11*, *Hoxd12*, and *Hoxd13* are illustrated. *Hoxa11* is expressed in the prospective zeugopod region, and *Hoxa13* is expressed in the prospective autopod region. Both *Hoxd11* and *Hoxd12* are expressed in the prospective posterior zeugopod region and the prospective autopod region, except for the digit 1 region. *Hoxd13* is expressed in the prospective autopod region with the exception of the region extending from the anterior half of digit 1 to the anterior margin of the limb bud. In the chick hindlimb bud and mouse limb buds, *Hoxd13* is expressed in the entire digit 1 region. S, stylopod; Z, zeugopod; A, autopod. **(C)** Schematic representations of the positional relationship between the expression boundary of *Hoxa11/a13* and the wrist/ankle joint in the chick fore- and hind-limb buds at a later stage than **(B)**. In the forelimb bud, the wrist joint is formed on the expression boundary of *Hoxa11/a13*. In the hindlimb bud, on the other hand, the position of the ankle joint does not correspond to the expression boundary, but is formed between the proximal and distal tarsals (inside the autopod). Broken lines indicate the positions at which the wrist/ankle joint is formed. Dotted lines indicate the skeletal elements that are eventually fused each other.

tion under the control of the AER: the progress zone model and the early specification model (Tabin and Wolpert, 2007; Towers and Tickle, 2009). The former postulates that compartments corresponding to the stylopod, zeugopod, and autopod are progressively produced from proximal to distal (Summerbell et al., 1973; Summerbell and Lewis, 1975). In the latter model, three distinct parts, corresponding to the stylopod, zeugopod, and autopod are already specified in the very early limb bud, and the three parts grow and expand as limb development proceeds (Dudley et al., 2002; Sun et al., 2002). In fact, there is evidence for a combination of these two models, in which the proximal region is specified in the early stage and the distal region is progressively added to the tip of the limb bud (Sato et al., 2007; Cooper et al., 2011; Rosello-Diez et al., 2011). The AER also appears to be involved in controlling the number of digits and phalanges. The AER expresses *fgf2* and *fgf4*, and products of these genes can induce additional digits when over-expressed or applied ectopically (Riley et al., 1993; Wada and Nohno, 2001). Furthermore, Fgf signaling from the AER defines the number of phalanges (Sanz-Ezquerro and Tickle, 2003), and in dolphins, which have many phalanges in their forelimbs (flippers), the AER persists for a longer period during limb morphogenesis than in other animals (Richardson and Oelschlager, 2002).

The other signaling center is the zone of polarizing activity (ZPA), from which SHH protein is diffused (Fig. 3A; Riddle et al., 1993). Both *Shh*-knockout mice and ZPA-removed chicks show a lack of posterior limb skeletal elements (Pagan et al., 1996; Litingtung et al., 2002). Furthermore, implantation of the ZPA into the anterior margin of the chick limb bud induces a complete mirror-image digit duplication, suggesting that the ZPA plays a crucial role in limb skeletal morphogenesis (Saunders and Gasseling, 1968; Tickle et al., 1975). There is a positive feedback loop between the ZPA and the AER (Fig. 3A), which is required for their maintenance for limb bud outgrowth (Laufer et al., 1994; Niswander et al., 1994). *Hox* genes (see below) act downstream of this feedback loop and determine the characteristics of each skeletal element. SHH, a morphogen diffused from the ZPA, mediates the feedback loop to express *Hox* genes, which help define the antero-posterior axis and digit identity in the limb bud (see also the next section for details). When mirror-image digit duplication is organized by experimentally transplanted ZPA or SHH protein application, the expression of posterior *Hox* genes is induced in the anterior limb bud (Nohno et al., 1991; Nelson et al., 1996; Yang et al., 1997). Studies using *Shh*-deficient mice and chicks showed that Shh signaling functions upstream of *Hox* gene expression along the antero-posterior axis in the zeugopod and autopod (Chiang et al., 2001; Ros et al., 2003). In contrast, implantation of the ZPA has little effect on the humerus (Wolpert and Hornbruch, 1987), and *Shh*-KO mice retain a stylopod skeleton (Litingtung et al., 2002), indicating that the stylopod is formed independently of Shh signaling.

The role of the *Hox* gene family, which encodes homeobox transcription factors, is one of the best understood in terms of defining the skeletal morphology of vertebrates. The morphology of axial structures, including vertebrae, depends on the combination of *Hox* genes expressed. For example, the expression of a certain combination of *Hox*

genes leads to the generation of thoracic vertebrae, while the expression of a different combination leads to the generation of sacral ones (Burke et al., 1995; Wellik, 2007; Mello et al., 2010). Similarly, it is suggested that each limb skeletal element is specified by a distinct combination of *Hox* genes in early limb development (Yokouchi et al., 1991). Figure 3B shows the relationships between some *Hox* expression patterns and skeletal elements in the limb bud. In the zeugopod, for example, *Hoxa11* is expressed throughout the zeugopod, and *Hoxd11* and *Hoxd12* are expressed in the posterior zeugopod, giving rise to morphological differences between its anterior and posterior skeletal elements (Yokouchi et al., 1991; Haack and Gruss, 1993; Small and Potter, 1993; Nelson et al., 1996; Suzuki and Kuroiwa, 2002). Such expression patterns of *Hox* genes, called the “Hox code,” are highly conserved among tetrapods. *Hoxa13* expression domain corresponds to the autopod region (Haack and Gruss, 1993). In addition, *Hoxd11*, *Hoxd12*, and *Hoxd13* are expressed in the autopod (Yokouchi et al., 1991; Nelson et al., 1996; Suzuki and Kuroiwa, 2002). There is, however, no distinct Hox code for each digit. Instead, each digit is identified by a developmental mechanism involving Shh signaling (see the next section.). The exception is the region that gives rise to digit 1, which is defined as a *Hoxd13*-positive and *Hoxd11/d12*-negative domain that can thus be distinguished from the regions that will give rise to the other digits (Chiang et al., 2001; Vargas and Fallon, 2005; Vargas et al., 2008). However, the Hox code and resulting compartment for each skeletal element is not sufficient to account for the variation of skeletal morphology within an animal or its species-specific traits. For example, the two posterior zeugopod elements in the forelimb and hindlimb, the ulna and fibula, which are morphologically quite different in adult birds, as described above, are defined by the same Hox code (*Hoxa11/Hoxd11/Hoxd12*-positive). Moreover, these two bones vary in shape among species. It is still unclear how the skeletal elements represented by the same Hox code acquire distinct morphologies.

2-2. Developmental aspects of avian digit identity

In general, it is the *Shh* expression and its diffusive product (SHH protein) that specify the morphological property (identity) of each digit, and studies have elucidated the Shh signaling pathway in detail (McGlinn and Tabin, 2006; Tickle, 2006; Tobin and Beales, 2007; Zeller et al., 2009). In this context, it is important that Shh signaling provides digit identity but does not function in digit formation itself. Even if *Shh* is absent, digits can still form so long as the *Gli3* gene is not functional, although the resultant limbs are polydactyl and have no digit identity. That is, they are morphologically indistinguishable from each other (Litingtung et al., 2002; te Welscher et al., 2002). It is likely that there are separate mechanisms for producing digits, determining their number, and specifying their identities. Combinations of these mechanisms must have enabled tetrapods to evolve their considerable variation in digit morphology.

In mouse, genetic research has revealed how the identity of each of the five digits in the limb is specified (Lewis et al., 2001; Ahn and Joyner, 2004; Harfe et al., 2004; Zeller et al., 2009). Digit 1 develops independently of Shh signal-

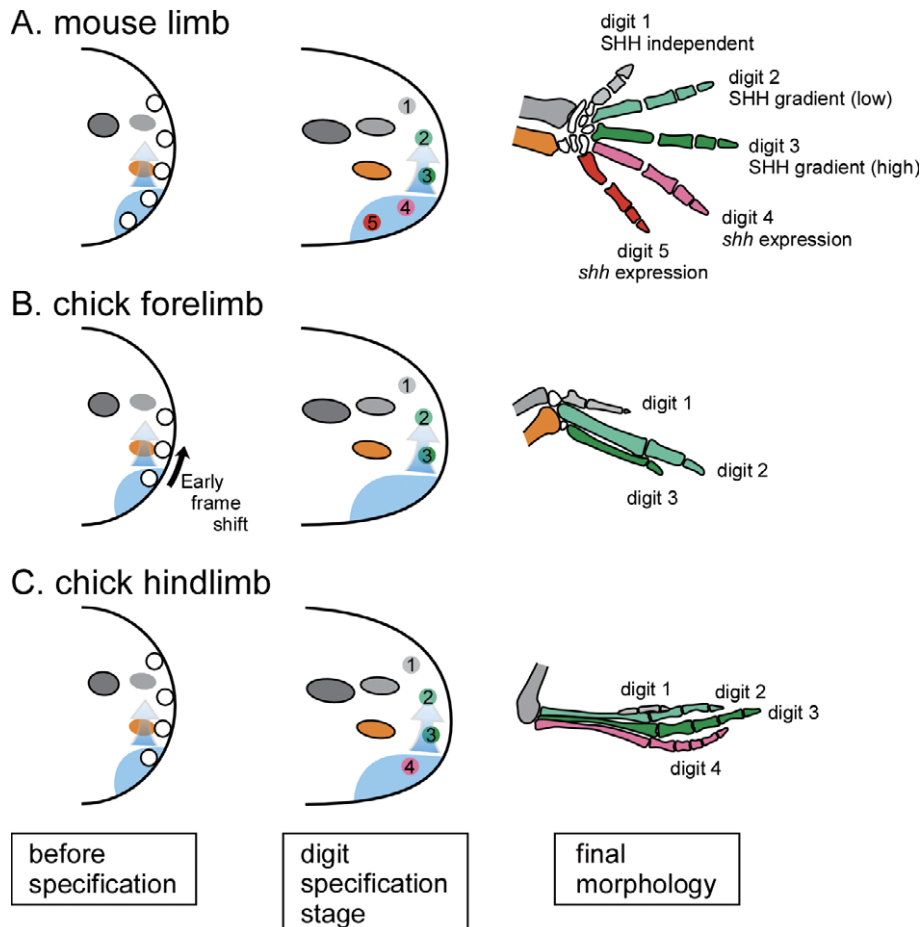


Fig. 4. Formation of digit identity in the (A) mouse limb, (B) chick forelimb, and (C) chick hindlimb. (Left panels) The early limb bud before digit specification. At this stage, the zeugopod region is being specified. White circles indicate foci for the presumptive digit primordium. Note that the early frame shift occurs only in the chick forelimb bud. Blue-gray arrows indicate the concentration gradient of SHH protein. (Middle panels) The limb bud at the digit specification stage. Circles represent digit primordia (the number indicates the digit identity) being specified at this stage. By this stage in the chick forelimb bud, the second, third, and fourth foci have shifted to the positions for digit 1, digit 2, and digit 3. (Right panels) Resultant skeletal pattern of the digits. In the mouse (A), each digit is mediated by a specific Shh signaling mechanism.

ing (Fig. 4A). Digits 2 and 3 are specified outside the ZPA (*Shh* mRNA-expressing region) by a low concentration (for digit 2) and higher concentration (for digit 3) of diffusing SHH. Digits 4 and 5 are specified inside the ZPA and are derived from cells that have experienced *Shh* gene expression in the ZPA. It is thought that cells expressing *Shh* for a longer period give rise to digit 5 (Harfe et al., 2004). Otherwise, the region forming digit 5 loses its competence against SHH at later stage, suggesting that this competence might be involved in digit 5 identity (Ahn and Joyner, 2004).

Based on comparison with such mechanisms of digit specification in the mouse, the identities of the three digits in the avian forelimb (manual digits) can be inferred. A previous study (Amano and Tamura, 2005) using chick embryos suggested that the anteriormost digit in the chick forelimb is specified independently of the ZPA (reviewed by Tickle, 2006). This study demonstrated that when the ectopic and/or additional digits are induced by the ZPA implantation, the posterior two digits are formed from the newly specified mesenchymal cells, but the anteriormost digit is

always formed through the expansion of the region from which the anteriormost digit is originally formed. That is, the newly formed anteriormost digit appears to be due to the proliferative function of the ZPA without specification by the ZPA. Furthermore, it was revealed that the gene expression profile of the anteriormost digit in the chick forelimb is similar to that of digit 1, but not to that of digit 2, in the chick hindlimb and the mouse limb (Vargas and Fallon, 2005; Uejima et al., 2010). Transcriptome analysis on chick limb buds strongly supports this idea (Wang et al., 2011). These classifications identify this digit as digit 1. It is reported that the second and third digits in the chick forelimb can be induced by low and high concentrations of SHH or by small and large numbers of ZPA cells, respectively (Tickle, 1981; Yang et al., 1997). Moreover, the primordium of the third, posteriormost digit is located outside the ZPA at the time of digit specification (Tamura et al., 2011; Towers et al., 2011), suggesting that this digit corresponds to digit 3 in the mouse. Taken together, it can be concluded that the three digits in the chick forelimb correspond to digits 1, 2, and 3 (Fig. 4B). We have such detailed, developmental information concerning digit identity in only one species of birds, the chicken (*Gallus gallus*), but it may be parsimonious to infer that all species of Avialae including *Archaeopteryx* that have three manual digits pos-

sess the same digital identities (digit 1, digit 2, and digit 3) if they share the same number and similar morphology of digits derived from the common ancestor. As discussed below, these arguments on avian digit identity provided an important case study in which homology of a structure is assessed based on the developmental mechanism.

2-3. Frame shift event based on developmental and evolutionary evidence

Contrary to the discussion presented above and morphological/anatomical identification of three digits of avian manual digits as digits 1, 2, and 3, some developmental data, mainly based on topographic relationships of chondrogenic condensations, suggested that the avian manual digits represent digits 2, 3, and 4 (e.g., Hinchliffe, 1984; Burke and Feduccia, 1997; Feduccia and Nowicki, 2002). In this argument, the digit anlage topologically aligned with the extension of the posterior zeugopod and appearing first in ontogeny is generally, which is the first visual digit, is generally identified as digit 4 without evaluation of digit morphol-

ogy. As a solution to these apparently conflicting homology assessments of avian manual digit identities, the occurrence of the frame shift of the digital identity has been predicted by Wagner and Gauthier (1999). These authors proposed a model of the frame shift, in which primordial cartilage condensations formed at the positions of digits 2, 3, and 4 were forced into adult morphology of digits 1, 2, and 3. However, there has been little experimental support for this conceptual model.

As described in section 2-1, the skeleton of the limb is sequentially laid down in order from proximal to distal, as the limb bud outgrows distally. Therefore, the specification of zeugopod identity precedes that of digit identity. In other words, cells that escape from the undifferentiated state (in the progress zone) at an earlier stage form the zeugopod, and the remaining cells in the progress zone escape at a later stage and are specified as digits. During the zeugopod specification stage in the avian forelimb, a group of cells contributing to the posterior zeugopod, from daughter cells of which the posteriormost digit will be formed at the later stage, are located inside the ZPA expressing *Shh* (Fig. 4B; Tamura et al., 2011). By the digit specification stage, however, these cells separate from the ZPA and are then specified as a digit (digit 3), outside the ZPA. This is a rather unique event because progenitor cells of the posteriormost digit (digit 4) in the avian hindlimb remain inside the ZPA until its digital identity is specified. This event in the wing bud, termed the early frame shift, forces each unit of the digit primordia into the position of a more anterior unit. The molecular and cellular mechanisms of the early frame shift remain unknown, but it is likely due to the early disappearance of *Shh* expression in the anterior ZPA where the posteriormost digit forms (Tamura et al., 2011). The developmental changes are already manifest in the signaling stage prior to digit identity specification. Wagner and Gauthier's (1999) model has turned out to be generally correct in explaining avian digit evolution (see also section 3-2).

3. EVOLUTION OF THE NUMBER AND IDENTITY OF MANUAL DIGITS IN THEROPODA

Results of studies showing that the bird wing has digits 1, 2, and 3 described above have ramifications in

the discussion on the phylogenetic origin of birds. Numerous lines of paleontological evidence have demonstrated that ancestral birds originated within the clade of originally carnivorous dinosaurs, Theropoda (Fig. 5A) (e.g., Ostrom, 1976; Gauthier, 1986). In other words, Aves is a subclade of Theropoda. This phylogenetic relationship leads to a hypothesis that the three digits remaining in the avian forelimb are

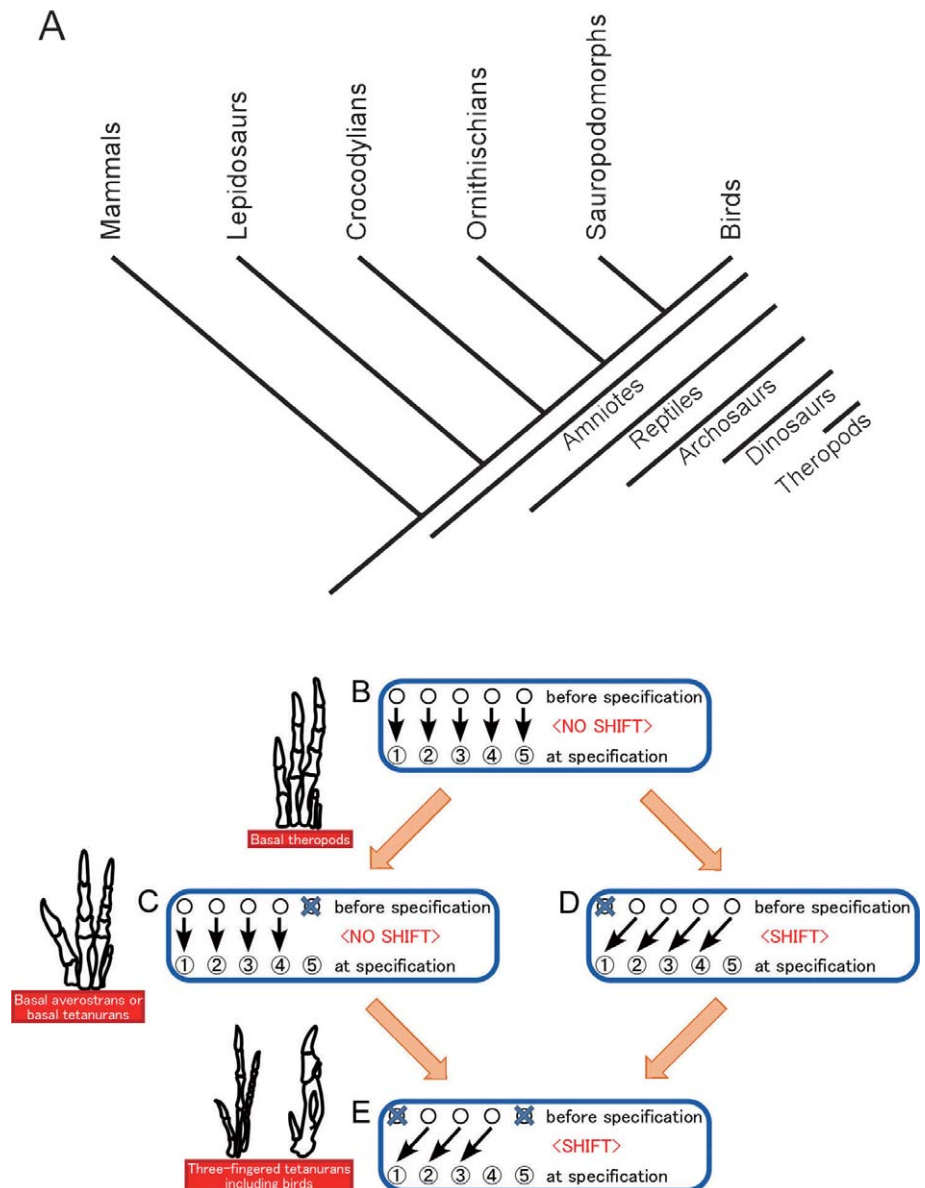


Fig. 5. Phylogenetic tree of amniotes and alternative hypotheses on the pathway of digit loss and early frame shift in the manus during theropod evolution. **(A)** A phylogenetic tree which represents evolutionary relationships among amniotes. See the text for details. **(B–E)** The developmental process of digit formation in an individual is indicated in each rectangle, in which an open circle on the top represents a focus of the presumptive digit before digit specification, and a circle below with a number represents digit primordium with digit identity specified at the digit specification stage. **(B)** Digit formation in basal theropods with five digits in the manus (e.g., *Herrerasaurus*). **(C, D)** Alternative possible processes of digit formation in theropods with four digits in the manus (basal averostrans or basal tetanurans; e.g., *Syntarsus*). In **(C)**, the posteriormost focus is eliminated. In **(D)**, the anteriormost focus is eliminated, and the early frame shift occurs before the digit specification stage. **(E)** Regardless of the pathway, three-fingered tetanurans including Avialae have lost both the anterior and posterior foci and experience an early frame shift (e.g., *Deinonychus* and chicken). All illustrations were adapted and modified from Larsson et al., 2010.

homologous with those of more basal, three-fingered theropods, thus representing digits 1, 2, and 3 (e.g., Ostrom, 1976; Gauthier, 1986). However, some embryologists disagree with this hypothesis, instead proposing that the avian manual digits represent digits 2, 3, and 4 on embryonic grounds as described above. The evidence was used by some to argue against the theropod origin of birds (Hinchliffe, 1984; Burke and Feduccia, 1997; Feduccia and Nowicki, 2002). This apparent conflict in digit homologies suggested by paleontological and embryonic data was solved by demonstration of the frame shift in avian embryos and resulting identification of avian manual digits as digit 1, 2, and 3 (Tamura et al., 2011). It has resolved an issue that “has frequently been cited as the biggest obstacle to the theropod origin of birds” (Prum, 2002).

Recently, however, Xu et al. (2009) proposed that three fingers remaining in the manus of theropods represent digits 2, 3, and 4 based on a paleontological finding. In the following section, this new hypothesis of Xu et al. (2009) is discussed, followed by a discussion on the possible timing of the frame shift on theropod phylogeny.

3-1. Patterns of the reduction in manual digit number in theropod evolution illuminated by paleontological data

The plesiomorphic number of manual digits in Theropoda is five, as observed in basal Triassic forms such as *Herrerasaurus* and *Eodromaeus* (Sereno, 1994; Martinez et al., 2011). In the course of theropod evolution, this number decreased, with most tetanuran (“stiff-tailed”) theropods including birds retaining only three. Except for a few unorthodox views (e.g., Thulborn and Hamley, 1982; Galis et al., 2003), most studies based on paleontological data have indicated that the reduction of manual digits in Theropoda proceeded from posterior to anterior direction, with the three fingers remaining in the manus of tetanurans being digits 1, 2, and 3 (e.g., Ostrom, 1976; Gauthier, 1986).

Recently, however, it was proposed that the three fingers remaining in the manus of tetanuran theropods represent digits 2, 3, and 4 based on the manual morphology of a more basal theropod, ceratosaurian *Limusaurus* (Xu et al., 2009). *Limusaurus* has four metacarpals, with metacarpal I being extremely reduced and lacking phalanges. Xu et al. (2009) proposed that this condition represents an evolutionarily transitional form showing the reduction of the first digit (digit 1) that was eventually lost in the ancestral tetanuran and thus that the three manual digits remaining in more derived tetanurans represent digits 2, 3, and 4. Xu et al. (2009) provided the following lines of evidence regarding the morphology, especially that of the metacarpals, to support their proposition:

Similarities between metacarpal II of basal (pentadactyl or tetradactyl) theropods and the anteriormost metacarpal of tetanurans: The dorsolateral corner of the proximal end of metacarpal II of basal theropods and that of the anteriormost metacarpal of tetanurans both form a flange. Also, the anteriormost metacarpal of basal tetanurans is the most robust in the manus, as is metacarpal II in the more basal theropods.

Similarities between metacarpal III of basal theropods and

the middle metacarpal of tetanurans: The middle metacarpal is longer than the other metacarpals in tetanurans, as is metacarpal III in basal theropods. In addition, a dorsolateral process is present on the proximal end of metacarpal III of basal theropods as well as on that of the middle metacarpal of basal tetanurans.

Similarities between metacarpal IV of basal theropods and the posteriormost metacarpal of tetanurans: The proximal end of metacarpal IV of basal theropods and that of the posteriormost metacarpal of tetanurans are similarly appressed to the ventrolateral aspect of the next anterior metacarpal (metacarpal III of basal theropods and middle metacarpal of tetanurans). Also, the posteriormost metacarpal in tetanurans is short, slender and proximally sub-triangular in outline, as is metacarpal IV in non-tetanuran theropods.

However, some of these features do not robustly support Xu et al. (2009)’s claim, for the following reasons:

Flange on the dorsolateral corner of the proximal end on the anteriormost metacarpal in tetanurans: In fact, not only the anteriormost metacarpal but also the middle metacarpal of some tetanurans bears a flange on the dorsolateral corner of its proximal end (e.g., *Allosaurus*; Madsen, 1976). Therefore, instead of the flange on the anteriormost metacarpal, the one on the middle metacarpal of tetanurans could also be homologized with the flange on metacarpal II in basal theropods.

Robustness of metacarpals: In the basal tetanuran *Torvosaurus*, for example, the middle metacarpal is more robust than the anteriormost metacarpal (Galton and Jensen, 1979). Thus, robustness of metacarpals varies within Tetanurae, and the anteriormost metacarpal is not always the most robust metacarpal in Tetanura, contrary to the claim by Xu et al. (2009).

Lengths of metacarpals: Metacarpal II is longer than metacarpal III in some individuals of *Coelophysis bauri* (Colbert, 1989). Therefore, metacarpal III is not always the longest metacarpal in theropods, contrary to the claim by Xu et al. (2009).

These characteristics, therefore, are not robust criteria for metacarpal homologies. In addition, there are other features of metacarpals instead supporting the hypothesis that the tetanuran manual digits are 1, 2, and 3. Firstly, the proximal end of the anteriormost metacarpal in tetanurans is triangular in profile as is that of metacarpal I in more basal theropods such as *Herrerasaurus*, *Dilophosaurus*, and *Limusaurus* (Sereno, 1994; Xu et al., 2009). Also, the proximal end of the middle metacarpal in basal tetanurans is quadrangular in shape, as is metacarpal II in more basal forms such as *Dilophosaurus* and *Limusaurus* (Xu et al., 2009). Secondly, the anteriormost metacarpal is the shortest among the three metacarpals in tetanurans. In basal theropods, metacarpal I is the shortest among the first three metacarpals; in fact, a marked reduction in length of metacarpal I is a saurischian synapomorphy (Gauthier, 1986).

Taken together, the characteristics of accordingly, the

characteristics of metacarpals are inconclusive at best concerning digital identity, and thus the claim by Xu et al. (2009) that “most metacarpal features support a 2, 3, 4 identification” of the tetanuran manual digits is clearly not supported. With a well-documented evolutionary trend of digit reduction proceeding from posterior to anterior directions in Dinosauria and a highly conservative phalangeal formula (2-3-4 for the three anterior digits) persisting from basal members of Tetrapodomorpha in the Devonian (e.g., *Tulerpeton*) through basal members of Avialae (Wagner and Gauthier, 1999), the reduction of digit 1 observed in *Limusaurus* is most parsimoniously inferred as a derived, specialized feature of Ceratosauria, rather than representing an evolutionarily transitional form that dictates the loss of digit 1 in Tetanurae (Vargas et al., 2009; Bever et al., 2011).

3-2. When and how did the frame shift in the developing hand occur in theropod evolution?

As discussed above, manual digit loss proceeded from posterior to anterior direction during theropod evolution, resulting in three anterior digits (digits 1, 2, and 3) remaining in the manus of the tetanuran theropods. It is not known whether the three-digit tetanuran theropods, including the extinct species of Avialae, experienced an early frame shift during limb development, but it is reasonable to assume that they did, given the strictly conserved numbers and morphology of manual digits in this group (Wagner and Gauthier, 1999). Based on this speculation, we can further predict when the digit loss and developmental frame shift occurred in evolution and what mechanisms were responsible for these transformations.

Two alternative hypothetical pathways for the manual digit loss during the evolution of theropod dinosaurs are proposed here (Fig. 5). In basal theropods that have five digits in the manus, five foci of progenitors of digit primordia (indicated by open circles) exist before specification and each focus produces a digit (Fig. 5B). The basic process of digit development is assumed to be the same as that in the mouse (Fig. 4A). In more derived theropods that have only four digits (digits 1, 2, 3, and 4), there are two possible alternative pathways of digit loss. The first possible pathway (Fig. 5C) is that forelimb buds of such theropods have lost the posteriormost focus for the digit primordium in the early limb bud with no frame shift occurring. The second, alternative pathway (Fig. 5D) is that these theropods have lost the anteriormost focus for the digit primordium in the early limb bud, with simultaneous early frame shift producing digits 1, 2, 3, and 4 in adults. Finally, irrespective of the alternative pathways shown in Fig. 5C, D, three-digit tetanurans, including Avialae, have lost the anterior and posterior slots for digit primordia and experience an early frame shift, giving rise to digits 1, 2, and 3 (Fig. 5E). The chick hindlimb, which has digits 1, 2, 3 and 4, undergoes the developmental process similar to the first pathway, loss of the poste-

rior focus and no frame shift (Fig. 5C). If we assume that developmental processes are somehow linked and occur simultaneously between the fore- and hindlimb, the first pathway may have been more likely to be taken to produce the four-digit manus in theropods. On theropod phylogeny, however, whereas the number of manual digits was reduced to three in basal tetanurans (Gauthier, 1986), the number of metatarsals remained five much further crown-ward, with the metatarsal V lost somewhere near the base of Ornithoraces (e.g., *Patagopteryx*; Chiappe, 2002). Therefore, it is unlikely

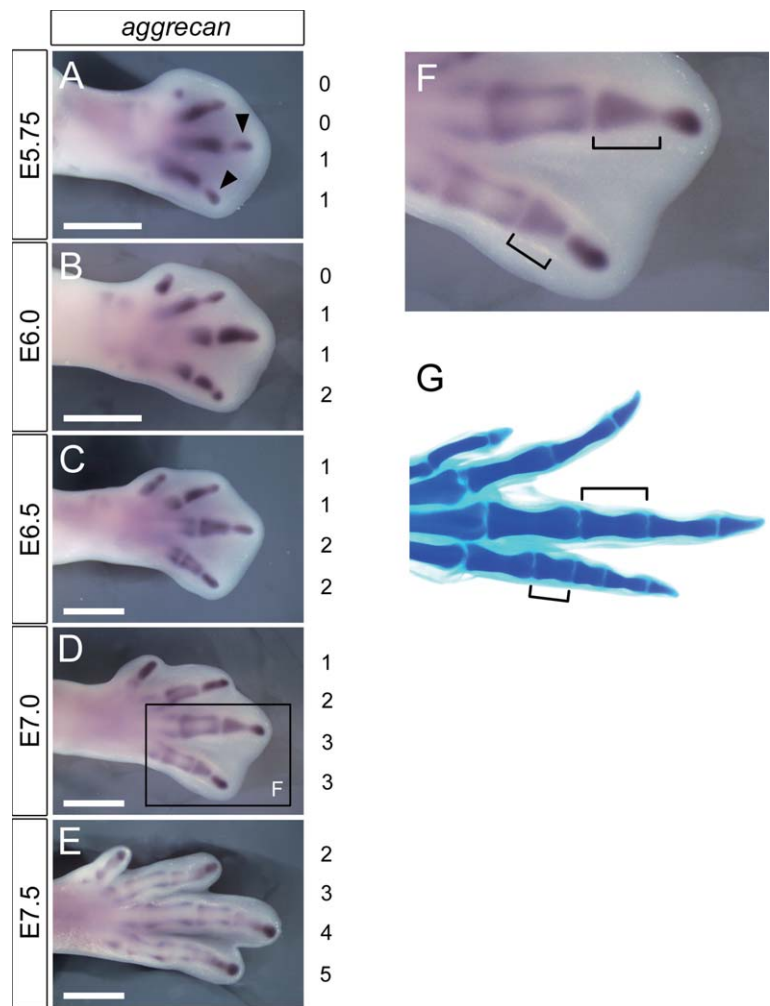


Fig. 6. Digit formation in the developing chick hindlimb bud. (A–E) Whole-mount in situ hybridization for an early cartilage marker, *aggrecan*, showing the skeletal morphology of each phalanx in the chick hindlimb bud. The numerals on the right side of panels indicate the numbers of phalanges in each digit at each developmental stage. Scale bars = 1 mm. Phalanges are formed sequentially in a proximal to distal manner. At E5.75 (A), the first phalanges of digits 3 and 4 are visible (arrowheads), but those of digits 1 and 2 are not. Despite the different numbers of phalanges between digits, the most distal phalanges of all digits are formed at about the same time as shown in (E). (F) Close-up view of the inset in (D). Brackets show the second phalanges of digits 3 and 4 immediately after segmentation. Note that these phalanges differ in length with the second phalanx of digit 3 being longer than that of digit 4. (G) The digital region of a chick hindlimb at E10.0 stained with Alcian blue for cartilage. Brackets show the second phalanges of digits 3 and 4. The second phalanx of digit 3 is longer than that of digit 4, as seen in the specimen at E7.0 (F). All photographs are oriented with anterior to the top and proximal to the left.

that a theropod possessing four metacarpals and four metatarsals existed, suggesting that the process of digit loss in the forelimb was uncoupled with that in the hindlimb. Accordingly, neither hypothetical pathways of the manual digit reduction can be discounted at present.

4. SKELETAL DEVELOPMENT OF THE AVIAN HINDLIMB

Each digit in the avian hindlimb has a different number of phalanges. It is known that different amounts of interdigital BMP signaling activity induced by Shh signaling play a crucial role in determining the number of phalanges, which represent the digit identities (Dahn and Fallon, 2000; Suzuki

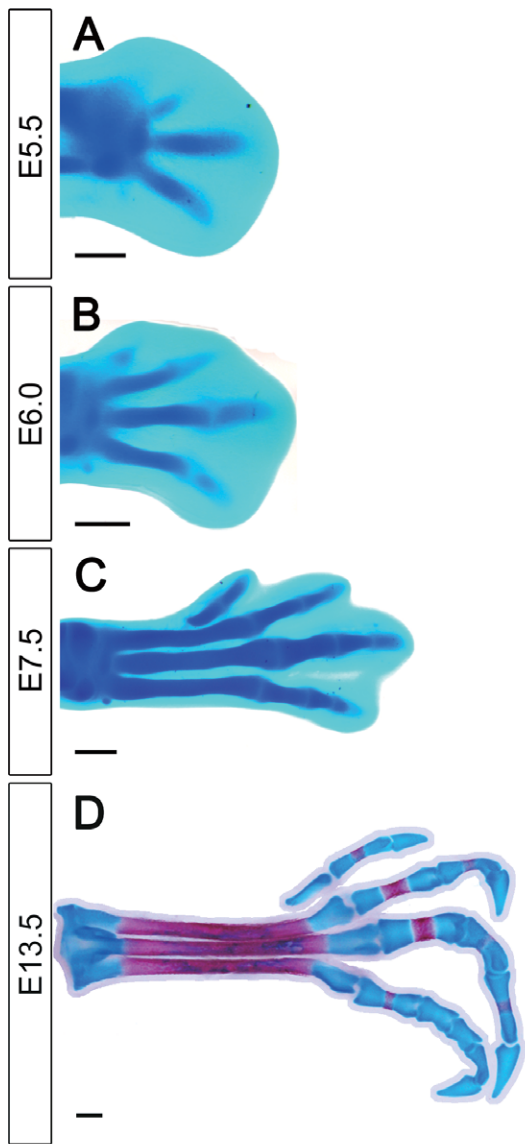


Fig. 7. Parallel rearrangement of metatarsals in the developing chick hindlimb bud. The autopod of chick hindlimbs was stained with Alcian blue for cartilage (dark blue) and Alizarin red for calcified bone (red). Three metatarsals extend radially at first (A), and they grow rapidly and are realigned in parallel (B, C). At a later stage (D), the three metatarsals are bundled together. All photographs are oriented with anterior to the top and proximal to the left. Scale bars = 500 μm.

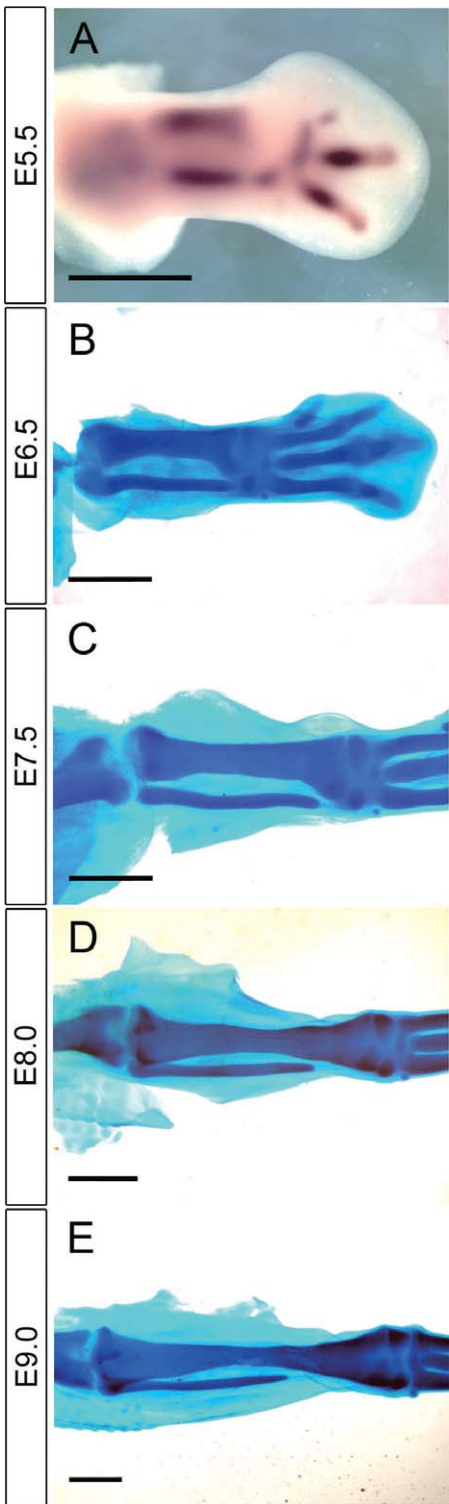


Fig. 8. Change in length of the fibula relative to the tibia in the developing chick hindlimb bud. (A) Whole mount in situ hybridization for *aggrecan* at E5.5. Two foci representing the tibia and fibula at the middle of the limb bud can be seen. (B–E) Cartilage was stained with Alcian blue in dark blue, showing autopod and zeugopod at E6.5 (B) and zeugopod at E7.5–E9.0 (C–E). In the early cartilage stages, the lengths of the tibia and the fibula are almost the same (A, B). After E6.5, however, the tibia gradually becomes longer than the fibula (C–E). All photographs are oriented with anterior to the top and proximal to the left. Scale bars = 1 mm.

et al., 2008). Species-specific numbers of phalanges may also be determined by the amount of BMP signal, but the system for regulating the amount of BMP, which must be mediated by the tissues surrounding the phalanges, remains unknown. The length of each phalanx differs among digits and even within one digit (Fig. 1A; Fig. 6G). In the process of digit development, the phalanges are produced by segmentation/division of a cartilaginous mesenchymal condensation, but the phalanges do not form simultaneously. Rather, cartilage segments for the phalanges are sequentially added from proximal to distal (Sanz-Ezquerro and Tickle, 2003; Fig. 6). The time of onset of the condensation formation for the proximal phalanx differs among digits (Fig. 6A), but formation of the distal-most phalanx is completed in all digits at about the same time (Fig. 6E), despite there being different numbers of phalanges among the digits. Interestingly, the length of each phalanx is already different when these elements appear as a cartilaginous condensation, suggesting that the initial morphology of the cartilage condensation partly reflects the final morphology of each phalanx (Fig. 6F, G). Some genes, such as *fgf8*, *bmps*, and *shh*, are known to be involved in determining the number and length of phalanges (Sanz-Ezquerro and Tickle, 2003). There are two major models for regulating the length of phalanges. In one model, the phalangeal length is controlled by the diffusion length of GDF5 protein (Hartmann and Tabin, 2001; Guo et al., 2004). In the other model, a molecular clock similar to the one controlling somitogenesis regulates the phalangeal length in limb mesenchyme (Pascoal et al., 2007).

In the adult avian hindlimb, three metatarsals (second through fourth metatarsals) are aligned in parallel and fused, but these metatarsals are not initially formed in parallel. When these metatarsals first appear as cartilaginous condensations during hindlimb development, they initially extend radially (Fig. 7A) and then rapidly grow and align in parallel (Fig. 7B–D) before fusing together (see also Müller, 1991; Namba et al., 2010). Although the detailed mechanism for this rearrangement of metatarsals remains unclear, it is possible that the initial radial form represents a plesiomorphy, which was secondarily modified into the parallel form in Aves (Hinchliffe, 1977; Müller, 1991). Regarding tarsals in the avian hindlimb, we have mentioned above that the ankle joint extends between the proximal and distal tarsals, not between the autopod and zeugopod. It is widely accepted that some Hox gene functions are required for both patterning and growth/differentiation of the autopod and zeugopod, and the boundary between the autopod and zeugopod corresponds to the boundary between the *Hoxa11* expression domain and *Hoxa13* expression domain. That is, in general in tetrapods, *Hoxa11* expressed in the prospective zeugopod region and *Hoxa13* expressed in the prospective autopod region have spatially exclusive domains for gene expression in the limb bud when the zeugopod/autopod elements are formed (Yokouchi et al., 1991; Haack and Gruss, 1993; Small and Potter, 1993; Nelson et al., 1996). In the chick hindlimb, cartilaginous anlage of tibiale and fibulare express *Hoxa13* but not *Hoxa11* (Suzuki and Kuroiwa, 2002), indicating that the expression boundary of *Hoxa11/a13* in the avian hindlimb corresponds to the boundary of zeugopod/autopod

elements, but not to the position of the joint hinge. The production of the hinge within the *Hoxa13*-positive domain is specific to the chick hindlimb, and is not observed in the chick forelimb or mouse limbs (Fig. 3C).

Two skeletal elements in the zeugopod of the avian hindlimb are extremely different in length and width, with the fibula being much shorter and thinner than the tibia. During their development, the two cartilage condensations of these elements appear to have similar dimensions (Fig. 8A). The truncated growth of the fibula is a result of the loss of its distal epiphysis (Archer et al., 1983). This epiphysis initially forms in the distal fibula, but is detached from the diaphysis later (Fig. 8B, C). It subsequently becomes fused with the tibia, which allows the tibia to expand along the proximo-distal axis and to form the distal part of the tibiotarsal. The rest of the fibula is left behind proximally (Fig. 8D, E). Some cellular events, including competition for mesenchymal cells, growth of chondrocytes, and chondrogenesis/osteogenesis, seem to play a role in the variation in the dimensions of the two skeletal elements (Archer et al., 1983; Müller, 1989; Goff and Tabin, 1997). However, the molecular regulatory mechanism upstream of such developmental processes remains unknown, although Hox genes may be involved in these processes (Goff and Tabin, 1997). Through a clearer understanding of such molecular mechanisms, we may be able to approach the mechanisms producing morphological differences in a certain limb element among species as well as distinct shapes of serially-homologous elements between the forelimb and hindlimb in one individual in the future.

5. EPILOGUE

Since the fundamental morphologies of vertebrates are constructed through the process of their development, research on morphogenesis during development provides keys to the understanding of their phylogeny and evolution. Developmental biology indeed continues to provide important information on homology, convergence, innovation, and degeneration of structures during animal evolution. In particular, many scientists have been investigating comparative developmental aspects of limb morphology in diverse tetrapod species, as limb skeletal morphology serves as an excellent model of diversification of morphogenesis.

The chick embryo has been most widely used as a model organism for research on features of morphogenesis common to amniotes because of its accessibility, availability of technologies including chimera analysis for detecting cellular origin and electroporation for introducing genes into the embryo, and the similarity of its developmental processes to those of the human embryo. It is no exaggeration to say that it is chick embryology that established the cellular and molecular bases of morphogenesis in amniotes.

The chicken possesses a combination of morphological features, such as (1) amniote plesiomorphies, (2) derived features shared with other dinosaurs, (3) avian synapomorphies, and (4) features specific to the chicken. Each trait reflects embryonic processes and molecular signaling during development. A common signaling mechanism will provide a feature shared by species, although a mechanism different in different species is generally thought to function on a feature specific to the species. Hence, if a trait is an avian synapomorphy, such as a long tibia and a short fibula, infor-

mation on the developmental process producing such a trait obtained from the chick embryo will enable us to infer that the same process is working in other birds without observing the development of the latter. Similarly, we can logically infer the developmental process producing a characteristic observed in fossil dinosaurs if such a characteristic is shared with the chicken even though we cannot directly observe their development. For example, we can infer the developmental and evolutionary processes of digit loss producing three-fingered manus during dinosaur evolution (Figs. 4 and 5). In this sense, we can utilize chicken as a “representative” of dinosaurs (Fig. 5A), at least for the traits common between other dinosaurs and birds. Research on dinosaurs is no longer restricted to the field of paleontology; integration with comparative anatomy at the cellular level, developmental biology, and genome science of extant reptiles and birds should open new avenues for understanding the morphology and evolution of dinosaurs.

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