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Growth Plate Formation and Development in Alligator and Mouse Metapodials: Evolutionary and Functional Implications

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ABSTRACT Mammalian metapodials (metacarpals and metatarsals), unlike most long bones, form a single growth plate, and undergo longitudinal growth at only one end. The growth dynamics of non-mammalian tetrapod metapodials have not been systematically examined in order to determine if unidirectional growth is unique to mammals. Here we compare murine metapodial ossification in growth stages that parallel those of embryonic, juvenile and subadult American alligators (*Alligator mississippiensis*). Safranin O staining was used for qualitative histology, and chondrocyte differentiation and proliferation were assessed via immunohistochemistry for type X collagen and proliferative cell nuclear antigen (PCNA). We establish that growth plates form at both ends of alligator metapodials and are maintained in the subadult. PCNA results show that alligators and mice share common patterns of chondrocyte proliferation during growth plate formation. In addition, while alligators and mice differ initially in the degree of organization and pace of chondrocyte differentiation, these parameters are largely similar in established growth plates. However, the replacement of cartilage by bone is highly irregular throughout growth in the alligator, in contrast to the more uniform process in the mouse. These results indicate that while alligators and mammals share common mechanisms of chondrocyte regulation, they differ substantially in their processes of ossification. Phylogenetic analysis indicates that the direct ossification of one epiphysis and reliance on a single growth plate is a derived character (synapomorphy) in therian mammals and likely indicates an adaptation for erect quadrupedal gait. *J. Exp. Zool. (Mol. Dev. Evol.) 308B:283–296, 2007.*

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The tetrapod hand and foot have undergone significant evolutionary modification in archosaurs (i.e., birds, dinosaurs and pterosaurs), extinct aquatic diapsids (i.e., mosasaurs, pleisiosaurs and ichthyosaurs) and mammals (Carroll, '97). These taxa exhibit a variety of differences in both the number and size of the individual skeletal elements, and one key mechanism by which they have achieved such morphological divergence is the manner in which their metapodials and phalanges ossify and grow longitudinally (Hamrick, 2001; Caldwell, 2002).

Unlike most major mammalian long bones, those of the hands and feet have only one growth plate, located proximally in the first metapodial

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(MP1) and all phalanges, but distally in MP2–5 (Thomson, 1869). At the opposite end of each of these elements the primary center of ossification directly invades and replaces the adjoining cartilaginous epiphysis, thus severely restricting any further contribution to longitudinal growth (Ogden et al., '94; Reno et al., 2006). We previously proposed that this unidirectional mammalian growth pattern qualifies the mouse metatarsal as an ideal model with which to examine mechanisms underlying growth plate formation. For example, we showed that cells in the epiphysis adjacent to the columnar zone (corresponding to the reserve chondrocytes) display a proliferative peak during growth plate formation. In contrast, no similar peak was observed during direct ossification, suggesting that this proliferative peak may represent cellular behaviors specific to growth plate formation (Reno et al., 2006).

In non-mammalian tetrapods, epiphyses often develop at both ends of all five metapodials and phalanges (Moodie, '08). These can either take the form of epiphyses that remain cartilaginous throughout life as in crocodilians, chelonians, anurans and avians, or later ossify as epiphyses with secondary centers of ossification as in lepidosaurs (Haines, '38, '42, '69). The maintenance of identifiable epiphyses at both ends of metapodials and phalanges throughout ontogeny may indicate that these bones also possess growth plates at each end and, therefore, exhibit bidirectional growth. Thomson (1869) conducted the most extensive survey of metapodial and phalangeal ossification. However, his analysis was largely restricted to mammals, and no study has yet compared metapodial and phalangeal ossification in mammals and other tetrapod lineages to determine whether unidirectional or bidirectional growth occurs in these taxa.

Comparative analysis of these patterns of metapodial growth is important for two reasons. First, differences between unidirectional and bidirectional growth of a metapodial necessitate the loss (or gain) of a growth plate. The developmental basis of such a transition furthers our understanding of mechanisms underlying evolutionary change in the vertebrate skeleton and of growth plate biology in general. Second, if mammalian metapodial growth is unique relative to other tetrapod taxa, it probably constitutes a fundamental mammalian adaptation.

To augment the histological descriptions of metapodial ossification that have been provided for humans (Ogden et al., '94) and mice (Reno et al., 2006), we examine in detail the ossification in the metacarpals and metatarsals of an age series of *Alligator mississippiensis*. This taxon makes a particularly useful comparison because it represents a distant tetrapod lineage (archosaurs) relative to mammals, while still maintaining a less derived autopod skeleton compared to other members of its clade (i.e., birds). We explore chondrocyte differentiation and proliferation during metapodial ossification and growth using both histology and immunohistochemistry. We here test the hypothesis that alligator metapodials form two growth plates and thereby grow bidirectionally. In addition, to further clarify the role early chondrocyte behavior plays in growth plate formation, we tested a second hypothesis that chondrocyte proliferative behavior similar to that previously identified in mouse metatarsals also occurs during growth plate formation in the alligator. Finally, we consider unidirectional/bidirectional metapodial growth in a larger phylogenetic context to determine the timing, polarity and functional consequences of this character.

MATERIALS AND METHODS

Specimens

Mid-embryonic [Ferguson ('86) stages 23–25], juvenile and subadult specimens of *A. mississippiensis* were obtained from the Rockefeller Wildlife Refuge (Cameron Parish, LA). Specimen ages and/or sizes are provided in Table 1. The embryonic and juvenile specimens were immediately fixed in 10% buffered formalin when sacrificed. The right hand and foot were dissected and decalcified in EDTA for 5–10 days. In contrast, the subadult specimen was frozen for an indeterminate period, and then thawed overnight prior to removal of a single fore and hind limb. After dissection of excess soft tissue, the limb skeletons were fixed in 10% buffered formalin. Metacarpals and metatarsals were collected individually and decalcified in formic acid for 4–6 weeks. All specimens were dehydrated in graded ETOH and xylene baths, embedded in paraffin, sectioned at 6 μm and mounted on Superfrost slides (VWR Scientific; West Chester, PA). The mouse specimens (C57/B6 strain) were collected and processed as previously described (Reno et al., 2006).

Histology

To assess the progress of ossification and cellular morphology, sections from each age group were

TABLE 1. Alligator specimens used in this study (all lengths in mm)

Age/overall size		Hand size	Foot size
		Embryos $N = 9$	
Days post laying	Ferguson stage[a]	Hand Length[b]	Foot Length[b]
44	23	7.6	12.5
44	23	7.8	13.1
44	23	7.9	13.2
47	24	8.8	14.1
47	24	8.8	14.7
47	24	8.5	14.4
49	25	10.0	15.8
50	25	10.4	16.0
50	25	10.6	15.6
		Juveniles $N = 2$	
Total length		Hand length[b]	Foot length[b]
375		20.9	35.3
385		20.4	34.4
		Subadult $N = 1$	
Total length[c]		Metacarpal length	Metatarsal length
~1200		MC1: 26 MC4: 32	MT2: 61 MT3: 63

[a]From Ferguson ('86).
[b]Measured from proximal extent of palmer/plantar pad to tip of digit 3 not including the claw.
[c]Estimated length; specimen described as 4 ft in total length.

deparaffinized and rehydrated with a reversed series of xylene and ETOH baths. Safranin O/Fast Green staining allows clear contrast between cells, cartilage matrix and bone. Weigert's Iron Hematoxylin (Sigma, St. Louis, MO) was applied and blued in low concentrations of HCl and ammonium hydroxide to define cell cytoplasm and nuclei, and to assess cell preservation and morphology. The sections were then stained in filtered 0.1% Safranin O (Sigma), which specifically targets anionic groups on mucopolysaccharides and glycosaminoglycans. This was followed by a 1% acetic acid wash. Subsequent staining with 0.1% Fast Green (Sigma) demarcated ossified and articular cartilage matrix, and was again followed by an acetic acid wash. The sections were briefly restained in Safranin O and rinsed in acetic acid. They were then dehydrated in graded ETOH and xylene washes and mounted using DPX. Stained sections were imaged using an Olympus BH-2 light microscope attached to an Optronics DEI-750 CE digital image capture system.

Immunohistochemistry

Chondrocyte differentiation and proliferation in the embryonic and juvenile specimens were assessed using immunostaining for type X collagen and proliferative cell nuclear antigen (PCNA), respectively. Due to the methods of collection and preservation, the subadult specimen was not suitable for immunohistological analysis.

For type X collagen immunostaining, the cartilage matrix was digested in 1% bovine testis hyaluronidase (Sigma) for 30 min at 37°C for antigen unmasking, and the endogenous peroxidase activity was quenched using 1% H_2O_2 solution. Sections were preincubated with goat serum for 60 min, and then incubated overnight at 4°C with a mouse polyclonal collagen type X primary antibody (1:200, a generous gift from Dr. A. Robin Poole, Shriner's Hospital, Montreal, Que.). Localization of the primary antibody was accomplished using the ABC Staining System (Santa Cruz Biotechnology) following the manufacturer's protocol. After counterstaining with methyl green (Sigma), the sections were dehydrated and mounted using DPX paramount. All experimental staining procedures were accompanied by control slides omitting the primary antibody to assess the level of non-specific staining. A rabbit polyclonal antibody was used to detect PCNA (1:100, Santa Cruz Biotechnology, Santa Cruz, CA). The above protocol was followed with the exception that antigen unmasking was instead facilitated by digestion with 0.1 units/ml chondroitinase for 10 min at 37°C and thionin counterstain was used to identify nuclei unstained for PCNA.

RESULTS

Histology of alligator metapodial ossification

To test the hypothesis that crocodilian metapodials undergo bidirectional growth distinct from mammals, we stained an age series of mice and *A. mississippiensis* with Safranin O/Fast Green to compare the progress of cartilage differentiation and ossification. The mouse sample included postnatal day 1 to day 14 individuals, while the alligator specimens included mid-embryonic, juvenile and subadult individuals (Table 1). While it is not possible to precisely compare ossification stages between these taxa, nevertheless both samples encompass the entire range from initial primary ossification through growth plate formation and maturation of epiphyses.

Growth plate formation in the neonatal mouse and mid-embryonic alligator

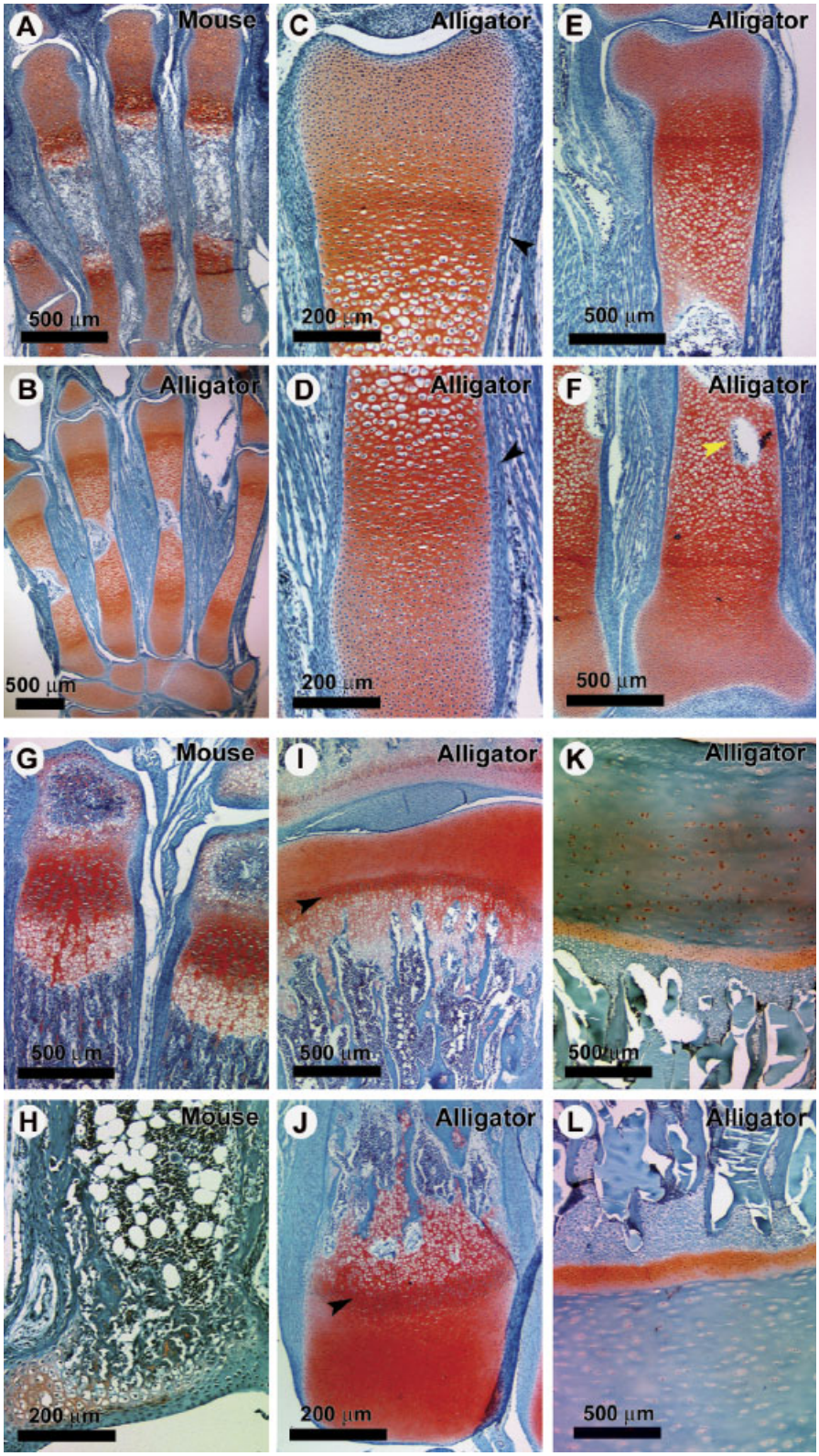
In mouse neonatal metatarsals, the primary center of ossification was flanked at either end by well-organized and clearly defined cartilaginous physes (Fig. 1A). Undifferentiated round chondrocytes resided within both epiphyses. The chondrocytes of both physes displayed clear columnar organization throughout both zones. In addition, distinct linear boundaries were present between the columnar and hypertrophic zones and between the hypertrophic and ossified matrix. Comparison of proximal and distal physes indicated that the heights of the columnar and hypertrophic zones were similar at both ends of the metatarsals at this stage (Fig. 1A, for further details see Reno et al., 2006).

In the alligator, ossification had already initiated in many of the cartilaginous metapodials by day 44 post-laying (Ferguson stage 23) (Fig. 1B). Small primary centers of ossification are present in all four metatarsals [alligators have only a rudimentary digit 5 in the hindlimb (Ferguson, '86; Rieppel, '93)] and in metacarpals 1–3, while metacarpals 4 and 5 were completely cartilaginous at this stage. In contrast to the mouse, the ossification centers invaded the cartilaginous models in a very irregular manner. Recognizable regions of epiphyseal, columnar and hypertrophic chondrocytes lay at each end of all metapodials (Fig. 1C–F); however, these zones were not as clearly defined as in the neonatal mouse. The alligator epiphyseal chondrocytes were small, round and appeared randomly distributed. The columnar zone consisted of flattened chondrocytes with poor columnar organization (Fig. 1C–F, to match convention we will continue to refer to this region of flattened cells as the ''columnar zone''). The expanded hypertrophic zone encompassed most of the total length of each metapodial and was as poorly organized as the adjacent columnar zone. Surrounding the hypertrophic cartilage were elongated bone collars extending to the approximate juncture of the hypertrophic and columnar zones. This expanded region of hypertrophic cartilage has been described as a ''cartilage cone'' in reptiles and birds (Moodie, '08; Horner et al., 2001). Its morphology was clearly distinct from that of the mouse in which the primary center of ossification kept pace with the differentiating cartilage and perichondrial ossification, thus maintaining a much narrower hypertrophic zone and a shorter bone collar. However, as in a similar-staged mouse, the relative sizes and degrees of differentiation of the alligator epiphyseal, columnar and hypertrophic zones were similar between the proximal and distal ends of each metapodial.

Established growth plates in juvenile and subadult mice and alligators

Later in ontogeny, the ossification patterns of the proximal and distal mouse metapodials diverged. As seen in Figure 1G, the day 14 mouse

Fig. 1. Age series of mouse and alligator Safranin O/Fast Green stained metapodials. Distal is towards the top in each image. (**A**) Limited primary centers of ossification are present in neonatal mouse MT2–4 (left to right) that are flanked by cartilaginous hypertrophic and columnar zones at both the proximal and distal ends. Note the generally similar size and level of organization of the proximal and distal chondrocyte zones, and the uniform replacement of the hypertrophic cartilage by the primary center of ossification. (**B**) Embryonic (day 44) alligator hand showing MC1–4 (left to right). The primary centers of ossification (MC1–3) are irregularly shaped as they replace the expansive hypertrophic zones (cartilage cones). Even in MC4, which has yet to form its primary center, the hypertrophic zone encompasses a majority of the bone. A relatively narrow columnar zone lies beyond the hypertrophic zone at each end. (**C**, **D**) Higher magnification image of the distal and proximal ends respectively of MC3 in B illustrating the poor columnar organization and the gradual transition between chondrocyte zones. The black arrowheads indicate the extent of the bone collar. (**E**, **F**) The distal and proximal ends of day 47 MT1 alligator demonstrate the irregularity of the ossification of the hypertrophic cartilage. Note the bleb of the ossification front in the proximal end isolated in this section (yellow arrowhead). The hypertrophic and columnar zones are very similar between each end and continue the poor columnar organization and gradual differentiation. (**G**) At day 14 the distal mouse metatarsal possesses a very large growth plate with well-organized columnar and hypertrophic zones. The ossification front of the primary center uniformly replaces the hypertrophic zone, while the secondary center of ossification has largely replaced the cartilaginous epiphysis. (**H**) In contrast, the proximal end of the mouse metatarsal preserves no growth plate and the primary center has invaded and ossified the epiphysis. (**I**) The juvenile alligator distal MT2 displays an evident growth plate with organized chondrocyte columns. The boundaries between chondrocyte zones are clearly defined producing a uniform-sized columnar zone (black arrow) across the bone. In contrast, the irregular invasion of the ossification front produces a highly variable hypertrophic zone. Above the columnar zone the epiphyseal chondrocytes remain undifferentiated. (**J**) The proximal end of the same bone shows identical histomorphology to the distal end in contrast to what is seen in the mouse (black arrow indicates the columnar zone). (**K**) In the subadult distal MT3 the clear organization of the growth plate is maintained while the hypertrophic zone continues to be replaced unevenly by the primary center of ossification. (**L**) The same morphology is evident at the proximal end indicating that both ends maintain growth plates.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metatarsal had formed an active growth plate at one end that preserved the organized columnar and hypertrophic zones surmounted by an epiphysis ossified via a secondary center. In contrast, the cartilage of the opposite epiphysis (Fig. 1H) had been replaced by the invading primary center of ossification, leaving only a narrow articular region. This pattern differed markedly from juvenile alligators in which cartilaginous epiphyses and growth plates remained at each end (Fig. 1I and J). The large cartilaginous epiphysis was composed of small round chondrocytes sparsely dispersed in cartilage matrix. Underlying the epiphysis (towards the center of the diaphysis) well-defined columnar and hypertrophic zones replaced the cartilage cone. Unlike embryonic specimens, the columnar zone consisted of well-organized chondrocyte columns that were maintained into the hypertrophic zone. The pace of ossification from the primary center had been greater than the progression of chondrocyte differentiation such that both the height of the hypertrophic zone and the length of the bone collar were reduced dramatically (Fig. 1J). However, the ossification front continued to replace the hypertrophic cartilage in a highly irregular fashion, thus making the length and number of chondrocytes composing each column highly variable.

Even after considerable growth, the subadult alligator metapodial maintained a well-defined growth plate at each end (Fig. 1K and L). The absolute sizes of the columnar and hypertrophic zones were similar to those in the juvenile specimens; however, the size of the cartilaginous epiphysis increased substantially (Fig. 1I and J). As previously described for crocodilians (Moodie, '08; Haines, '38, '42, '69), epiphyseal cartilage beyond the columnar zone showed no signs of replacement by a secondary center of ossification.

Type X collagen expression

To test the hypothesis that alligator metapodials are characterized by the same changes in cell behavior and gene expression that occur during mammalian chondrocyte differentiation, we examined the expression of type X collagen via immunohistochemistry. In the mouse, strong type X collagen expression is restricted to the hypertrophic cartilage of both the growth plate and epiphyses, forming a sharp expression boundary with the adjacent cartilage (see Reno et al., 2006 for further details). In embryonic alligator specimens, type X collagen was variably expressed across the matrix of the hypertrophic zone (Fig. 2A). Staining was faint at the columnar/hypertrophic transition, but increased in intensity toward the primary center of ossification, suggesting a gradual increase in the degree to which type X collagen was incorporated into the matrix of the cartilage cone.

In the juvenile alligator specimens type X collagen was expressed by late columnar and hypertrophic chondrocytes and was more abruptly incorporated into the matrix at the columnar/hypertrophic transition (Fig. 2B). In addition, no differences in the degree or pattern of type X collagen expression were detected between distal and proximal ends of the metatarsals at either age.

Chondrocyte proliferation

To further test the hypothesis that alligator metapodials undergo bidirectional growth and to determine if their growth plates show proliferative profiles similar to those previously observed in the mouse (Reno et al., 2006), we used PCNA expression to identify proliferating chondrocytes. Nuclear localization of PCNA identifies chondrocytes in the S-phase of the cell cycle (Yu et al., '92). In embryonic alligator metapodials the highest proportion of PCNA expressing cells was found in the round chondrocytes lying adjacent to the columnar zone (Fig. 3A and B). Lower proportions of PCNA expression were found in epiphyseal chondrocytes near the articular surface and in columnar chondrocytes. Similar expression patterns were observed at both the proximal and distal ends.

In juvenile alligator metapodials, PCNA was most strongly expressed in columnar chondrocytes of both the proximal and distal ends (Fig. 3C and D). In contrast, very few cells within the cartilaginous epiphysis showed PCNA expression. These results confirm that both proximal and distal growth plates were active and contributed to longitudinal growth. In addition, the patterns of chondrocyte proliferation match those that occur during growth plate formation in the mouse metatarsal, but were distinct from the pattern observed during direct ossification where proliferation was highly variable across the entire epiphysis and failed to display an overt proliferative peak (see below, Reno et al., 2006).

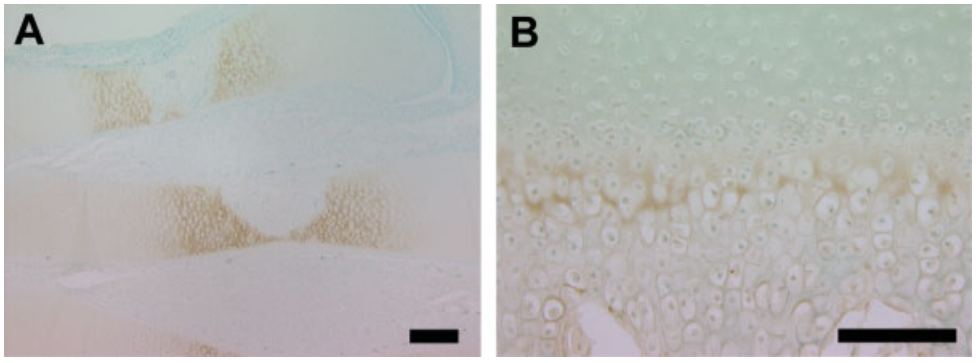


Fig. 2. Immunohistochemistry for type X collagen protein expression in alligator metapodials. (**A**) The hypertrophic matrix of the cartilage cone shows strong expression of type X collagen protein in embryonic metacarpals. A gradient of expression can be seen with weaker staining in earlier hypertrophic matrix adjacent to the columnar zone. (**B**) In the juvenile metatarsal growth plate, type X collagen is expressed abruptly at the transition between the columnar and the hypertrophic zones. Scale bars=200 μm.

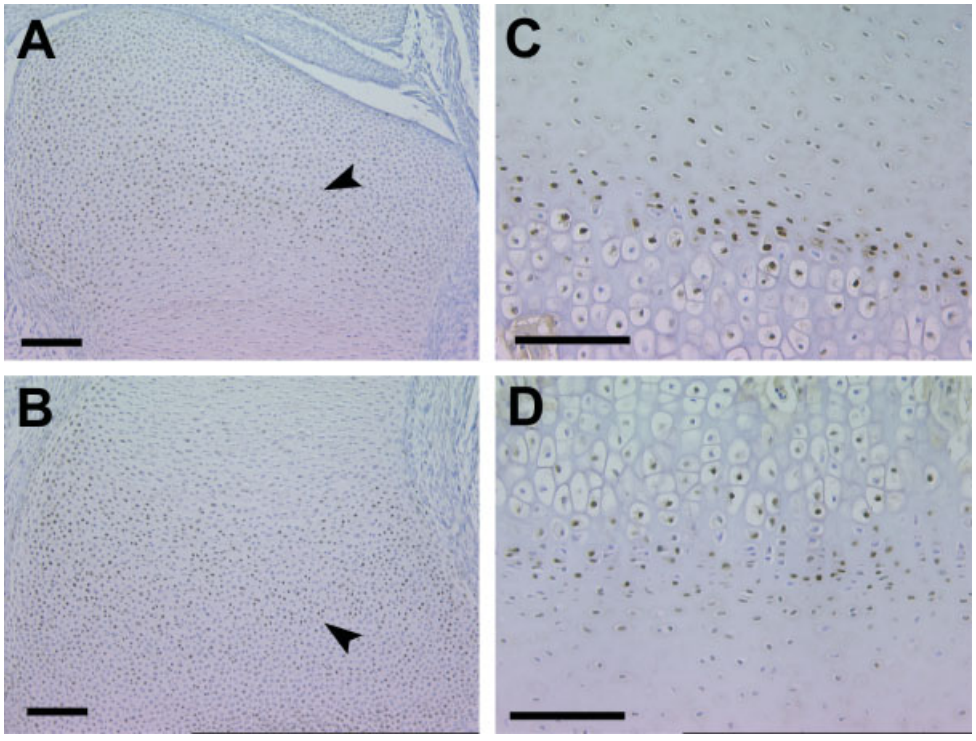


Fig. 3. Immunohistochemistry for PCNA expression in alligator metapodials. In the distal (**A**) and proximal (**B**) embryonic metatarsal, a peak of PCNA expression is located at the boundary between epiphyseal and columnar chondrocytes (black arrowheads). Later in the juvenile, columnar chondrocytes show the strongest proportion of PCNA in both the distal (**C**) and proximal (**D**) growth plate. Scale bars=100 μm.

Taxonomic variation in metapodial growth pattern

To properly understand the evolutionary significance of unidirectional and bidirectional metapodial growth, it is necessary to determine the ancestral tetrapod condition and the timing of any transitions that have occurred within this clade. Unidirectional metapodial growth has long been recognized as being nearly universal in eutherian mammals (Fig. 4) (Thomson, 1869). While a few exceptions are known, these occur in taxa with highly derived skeletal morphology (e.g., Cetacea) in which metacarpal and phalangeal growth is greatly restricted (Ogden et al., '81; Dawson, 2003; Galatius et al., 2006). Other possible exceptions have also been noted [i.e.,

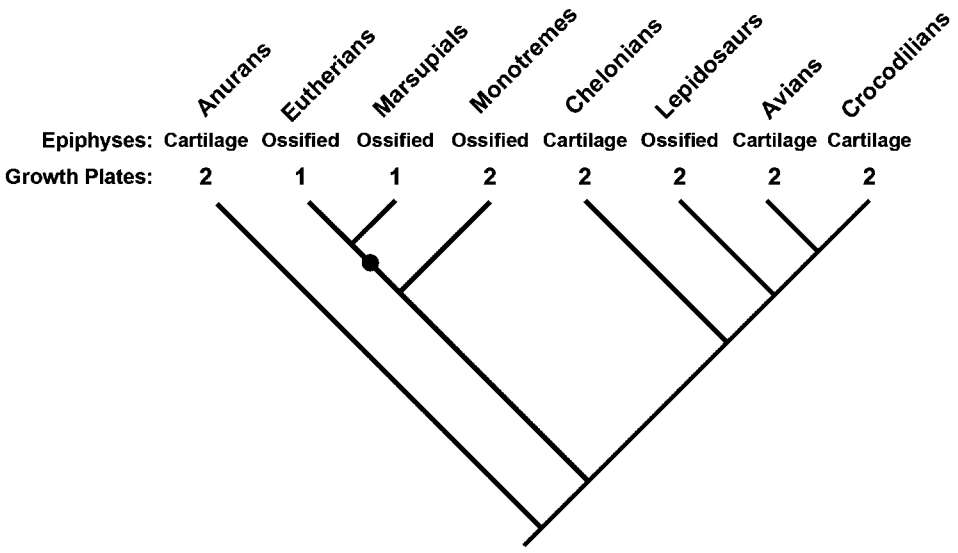


Fig. 4. Cladogram mapping epiphyseal ossification and bidirectional metapodial growth on a simple tetrapod phylogeny. Only eutherian and marsupial mammals display direct ossification of one epiphysis and unidirectional growth. This character evolved in a therian common ancestor (circle) and thus constitutes a shared derived character (synapomorphy) for this clade. In contrast, both mammals and lepidosaurs possess ossified epiphyses. The evolutionary history of this character is more difficult to resolve. Turtles and tortoises (chelonians) clearly maintain cartilaginous epiphyses. However, we are here inferring, based on phylogeny, that they also have two growth plates, because to our knowledge metapodial ossification has yet to be directly observed in these taxa. For sources of other taxa see text.

koalas, kangaroos (Ogden et al., '94) and elephants (Thomson, 1869)], but osteological observations must carefully distinguish ''true'' epiphyses from pseudoepiphyses, an aberrant form of direct ossification that mimics formation of a secondary center of ossification, but does not indicate the presence of a true growth plate (Haines, '74; Ogden et al., '94). Thomson (1869) described the platypus (*Ornithorhynchus*, a monotreme) as possessing ossified epiphyses at each end of all five metapodials (confirmed by personal observation for this study) and thus likely to exhibit bidirectional growth (Fig. 4). However, marsupials [*Dasyurus* (Thomson, 1869), *Didelphis* and *Monodelphis* (personal observation)] share the eutherian pattern of epiphyseal ossification (Fig. 4) (Szalay, '96).

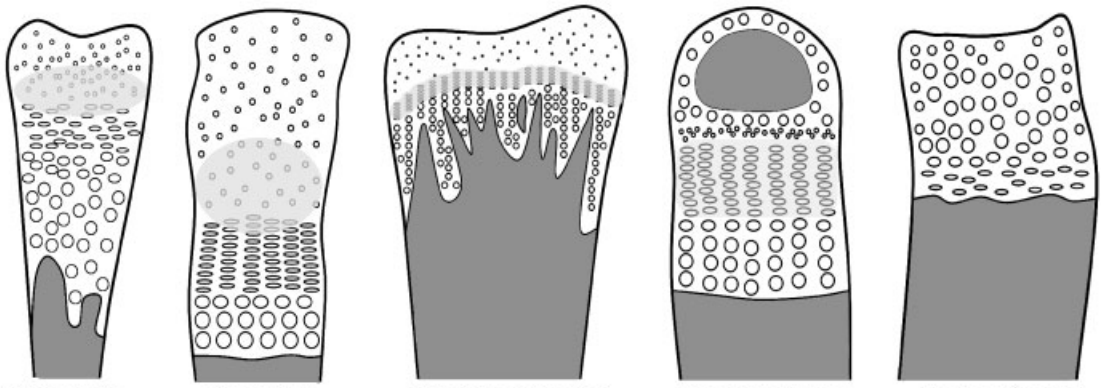
Skeletal development in the metapodials of other non-mammalian taxa suggests they, like alligators, exhibit bidirectional metapodial growth (Fig. 4). Rozenblut and Ogielska (2005) have demonstrated longitudinal growth at the distal end of water frog phalanges (Amphibia: Anura: Ranidae), and formation of secondary centers of ossification in the epiphyses (indicating the presence of growth plates) has been observed at both ends of lepidosaur metapodials [geckos (Rieppel, '92) and monitor lizards (varanids) (Haines, '69; de Buffrénil et al., 2005)]. Moodie ('08), who conducted the largest survey of epiphyseal ossification in reptiles, observed that ossified epiphyses are commonplace at both the proximal and distal ends of lizard metapodials, but he also found that bony epiphyses occur only at the proximal ends of lepidosaurian phalanges (Fig. 4) (see also Fig. 13 in Haines, '69). While the presence of a separate center of ossification would seem to justify the assumption of an underlying growth plate, the lack of epiphyseal ossification obviously does not validate the opposite conclusion (viz., alligators have growth plates but also lack bony epiphyses). Thus, histological analysis will be required to determine whether lizard phalanges undergo bidirectional or unidirectional growth. The cellular kinetics of the avian tarsometatarsus indicate bidirectional growth (Fig. 4) (Kember et al., '90; Cubo et al., 2000), but the highly derived nature of the bird wing and leg skeleton (i.e., fusion of carpal and tarsal elements to the bones of the zeugopod and autopod) complicates their direct comparison to other taxa. Taken together these data indicate that bidirectional growth is the ancestral condition for tetrapods, and that unidirectional metapodial growth is a shared derived character (synapomorphy) for therian mammals (Fig. 4).

DISCUSSION

Using both comparative histology and cellular proliferation, we demonstrate that alligators establish and maintain active growth plates at both ends of their metapodials. Thus, overall pattern of metapodial growth is fundamentally different in alligators than it is in therian mammals, in which only the proximal physis in MP1 and distal physes in MP2–5 develop into active growth plates that account for nearly all longitudinal growth (Lee, '68; Haines, '74; Patake and Mysorekar, '77; Ogden et al., '94; Reno et al., 2006). Such evolutionary diversity in metapodial growth and development, therefore, can serve as an ideal natural model to test hypotheses concerning factors and cellular behaviors specific to growth plate formation. In addition, knowledge of the evolutionary history of metapodial growth pattern in tetrapods enables a more informed exploration of the functional significance of the loss of growth plates in therian mammals.

ALLIGATOR AND MAMMALIAN GROWTH PLATES SHOW COMMON PATTERNS OF CARTILAGE PROLIFERATION AND DIFFERENTIATION, BUT HAVE DISTINCT MODES OF OSSIFICATION

Mice and alligators share a number of elements of growth plate formation and behavior, including common patterns of chondrocyte proliferation prior to growth plate formation (Fig. 5). We previously identified a region of peak PCNA expression in the round epiphyseal chondrocytes adjacent to the columnar zone in the distal (i.e., growth plate) end of mouse MT3 (Reno et al., 2006), confirming a similar pattern described for BrdU incorporation during embryonic long bone formation (Smits et al., 2004). However, no peak of PCNA expression was observed in the opposite end undergoing direct ossification. Instead, chondrocytes proliferated more evenly across the entire proximal epiphysis (Reno et al., 2006). We concluded that the restricted peak of proliferation above the columnar zone was a feature specific to



	Embryonic Alligator	Neonatal Mouse	Juvenile Alligator Growth Plate	Juvenile Mouse Growth Plate	Mouse Direct Ossification
Proliferative Peak	Reserve	Reserve	Columnar Zone	Columnar Zone	Absent
Columnar Organization	Poor	High	High	High	Poor
Chondrocyte Differentiation	Gradual	Abrupt	Abrupt	Abrupt	Gradual
Ossification Front	Irregular	Uniform	Irregular	Uniform	Progressively Irregular

Fig. 5. Comparison of alligator and mouse early growth plate formation, active growth plate function and direct ossification (mouse only). The solid gray areas indicate the extent and form of primary and secondary centers of ossification. The shaded translucent regions indicate the site of maximal chondrocyte proliferation. Small circles represent undifferentiated chondrocytes, oval represents columnar chondrocytes and large circles indicate hypertrophic chondrocytes. Articular chondrocytes are not shown. Illustrations not drawn to scale.

growth plate formation, and agreed with Smits et al. (2004) that this region may represent the progenitor population of the future reserve zone of the growth plate. As in the mouse, the embryonic alligator metapodials display this same localized pattern of PCNA expression during growth plate formation; however, they do so at both ends, each of which forms a growth plate. Thus, alligators form an independent test confirming that this band of PCNA expression is specific to growth plate formation and may play a central role in patterning and establishing subsequent proliferative behavior.

Once growth plates have formed, the earlier differences between mice and alligators in chondrocyte organization and differentiation disappear (Fig. 5). The alligator displays clear chondrocyte columns, and the differentiation process from reserve to hypertrophy occurs in a manner generally similar to its counterpart in birds and mammals (Haines, '69). The transition between columnar and hypertrophic chondrocytes becomes better defined than in embryonic metapodials. This change is reflected in the immunostaining of type X collagen, which becomes more distinct than in the hypertrophic matrix of the juvenile (Figs. 2 and 5). Also in common with the mouse, the region of peak proliferation shifted from reserve to columnar chondrocytes in the established growth plate (Figs. 3 and 5) (Reno et al., 2006).

Differences in ossification, however, continue throughout growth. The replacement of the hypertrophic cartilage by the primary center's ossification front is much more irregular in the alligator than in the mouse (Fig. 5) (Haines, '42). This results in great variation in the height and number of chondrocytes that compose the individual columns of the hypertrophic zone, which is in turn distinct from the more uniform height of the columnar zone. Barreto et al. ('93) observed similar differences in ossification between mammals, lizards and birds. Both the dog and monitor lizard maintain a linear transition between hypertrophic cartilage and bone, while the chick (and dinosaurs) shows the irregular chondro-osseous junction that we observed in alligators. Barreto et al. ('93) concluded that this irregular osseous replacement is associated with the rapid long bone growth in birds and dinosaurs. However, alligators and birds on one hand, and mammals and lizards on the other, share these distinct ossification patterns, and thus they are not likely specific to growth rate. Since alligators and birds (archosaurs) maintain cartilaginous epiphyses throughout life while those of mammals and lizards ossify, these observations suggest that the fundamental differences between these taxa in skeletal development lie in their overall patterns of ossification rather than in the regulation of chondrocyte differentiation and proliferation (which are more likely to determine growth rate).

Indian Hedgehog (*Ihh*) controls the rate of chondrocyte terminal differentiation by regulating Parathyroid Hormone related Peptide (*PTHrP*) expression (Vortkamp et al., '96). However, *Ihh* also performs a number of *PTHrP*-independent functions including the promotion of columnar chondrocyte proliferation (Karp et al., 2000), the differentiation and proliferation of epiphyseal (reserve) chondrocytes (Kobayashi et al., 2002, 2005) and ossification of calcified cartilage (St-Jacques et al., '99). Thus, it plays a key role in coordinating various chondrocyte and osteoblast functions during growth plate formation and activity. Although these integrated functions have been predominantly investigated in the mouse model, the similarities in chondrocyte proliferation and differentiation in alligators and mice suggest that *Ihh* may also play the same integrative role in reptiles. On the other hand, the irregular pattern of osseous replacement in the alligator suggests that the roles of *Ihh* in modulating ossification may differ significantly from those in the mouse. In particular, the progression of perichondral and endochondral ossification shows poor coordination in the embryonic alligator (and birds, Barreto et al., '93). Further analysis of those factors known to regulate chondrocyte proliferation and differentiation (i.e., Ihh and PTHrP) and ossification [i.e., matrix metallproteinase-13 (MMP-13) and vascular endothelial growth factor (Colnot and Helms, 2001; Zelzer et al., 2001, 2002; Maes et al., 2004; Stickens et al., 2004)] will be required to resolve such issues.

Unidirectional growth is derived in therians and likely an adaptation to erect gait

Haines ('38) argued that the crocodilian and chelonian growth plate, with its narrowed columnar and hypertrophic regions residing directly beneath its cartilaginous epiphyses, best represents the primitive tetrapod condition. Previously, we concurred with Ogden et al. ('94) that direct ossification in the mammalian metapodial appears to share characteristics of this primitive growth

pattern, and suggested that comparison of direct ossification to growth plate formation might shed light on the emergence of derived endochondral ossification in tetrapods (Reno et al., 2006). However, the present analysis shows that growth plate formation and behavior in mice and alligators share more similarities than either does with mammalian direct ossification (Fig. 5). Both the proliferative dynamics (as indicated by PCNA expression) during growth plate formation, and the tight regulation of chondrocyte differentiation within the growth plate, are shared by mammals and alligators and differ from mammalian direct ossification. This further demonstrates that direct ossification in metapodials and phalanges is a novel condition (apomorphy) and is not a primitive prototype of the mammalian growth plate (Fig. 4).

Bidirectional metapodial growth in alligators and most other tetrapods has significant implications for interpreting the evolution of therian mammals. The latter's digit-specific unidirectional growth patterns (distal in metapodials 2–5 but proximal in all other bones of the digits) have long been recognized as being virtually universal (Thomson, 1869), and yet no adequate functional explanation has been posed to account for this pattern (Szalay, '96).

Carter and colleagues have proposed models that rely on mechanical loading responses of chondrocytes in order to account for the development of an epiphysis and physis (Wong and Carter, '90). They have suggested that a mechanical stress response by chondrocytes residing within the distinct geometries of perichondrial (bone collar) and endochondral ossification in embryonic mammals and alligators may underlie their different patterns of epiphyseal ossification (Carter et al., '98). However, in the juvenile stage the location of the alligator growth plate and level of cartilage replacement relative to the bone collar more closely approximate the mammalian condition, and it is only after growth plate formation that secondary centers form in mammals. Thus, such a model would have to further account for why alligator and other archosaur epiphyses fail to ossify later in life. In addition, as we have previously discussed, models relying on chondrocyte mechanical stress responses do not explain the ''inverted'' pattern of growth plate formation in the first metapodial of mammals, which is geometrically similar to the posterior metapodials, but exhibits only a proximal, rather than a distal, growth plate (Reno et al., 2006). While these models rely on anabolic interactions between stress and genetic responses during bone formation (Henderson and Carter, 2002), both unidirectional and bidirectional metapodial growth patterns are conserved in taxa that exhibit a variety of diverse locomotor styles and limb morphologies. This suggests that positional information plays a far more fundamental role than does mechanical stress response in defining growth plate location and secondary center of ossification formation in all tetrapods.

The co-evolution of a single growth plate occurring only distally in the posterior metapodials but proximally in the first ray in both limbs would seem to suggest an overarching adaptive basis in therian mammals, which is not shared by other crown taxa. While a significant degree of erect stance had evolved in the hindlimb of cynodont antecedents to mammals, complete erect gait (with motion of both limbs largely restricted the parasagittal plane) was only fully achieved by therian mammals with the elimination of sprawling posture of the forelimb and the superposition of the talus (astragalus) upon the calcaneus in the ankle (Jenkins, '71; Jenkins and Parrington, '76; Kemp, '82). Thus, the loss of a growth plate in each metapodial in the common therian ancestor coincides with the evolution of fully erect quadrupedal stance.

The tarsometatarsal and carpometacarpal joints of rays 2–5 in therians involve substantial ligamentous complexity (Jenkins, '71). While initial stages of this pattern in the form of the transverse tarsal arch is seen in cynodonts (Jenkins, '71), it has been further elaborated in therian mammals as indicated by their flattened articular surfaces and notches for the interosseous ligaments lacking in other taxa (compare the mouse and alligator proximal metapodial articulations depicted in Fig. 1A and B). These articular conformations and elaborated ligamentous supports effectively link the four posterior metapodials and carpus/tarsus into a single strut for fulcrumation at the metapodiophalangeal joints. Joint structures are complex and derive from early modifications of mesenchymal positional information (Storm and Kingsley, '99; Hartmann and Tabin, 2001; Sanz-Ezquerro and Tickle, 2003), and thus, the emergence of such novel syndesmotic complexity would require reorganization of its determinant positional information. In addition, Lacroix ('51) observed that the ligamentous insertions on epiphyses (i.e., the proximal end of the medial collateral ligament, MCL) invade deep into

the underlying cartilage and bone, while those that insert on the diaphysis adjacent to growth plates (i.e., distal MCL) tend to invade only the periosteum, thus enabling more rapid remodeling during growth. Elaborated Sharpey's fiber (or other) (Benjamin et al., 2006) insertional complexes proximal and distal to a growth plate are likely to have confounded simple linear growth during rapid diaphyseal elongation in early mammals. Elimination of proximal growth plates may have facilitated this reorganization, while any reduction in growth velocity could have been accommodated by compensatory acceleration at the retained distal physis. Interestingly, a functionally parallel adaptation occurred within the avian and dinosaur lineage. With the evolution of erect stance and metatarsal fulcrumation, the ankle joint in dinosaurs and birds became greatly simplified with the ultimate fusion of all tarsal elements to the tibia and metatarsus (Romer, '56). The requirement for maintaining eversion/inversion of the ankle in arboreal or scansorial therian ancestors (Ji et al., 2002) likely precluded similar parallel reductions in mammals.

Of course, this raises the issue of why growth plate location is reversed in the first ray. First, loss of a growth plate could be due to selection for a mechanism of ''growth balance,'' i.e., all long bones of both autopods (including the phalanges) have only a single growth plate—a condition that would seem to permit relatively facile maintenance of overall proportions between equivalent proximodistal segments throughout the autopod during both individual growth [i.e., hormonal signals such as growth hormone and insulin like growth factor-1 (Lupu et al., 2001)] and evolutionary modifications of growth patterning [i.e., selector genes of the HoxA and HoxD complexes (Gilbert et al., '96; Lovejoy et al., 2003)]. A relatively uniform growth gradient can be relied upon to facilitate maintenance of overall element proportions, even in the face of substantial anatomical modifications of the autopod as a unit structure [we observed a similar growth phenomenon between the radius and ulna within the anthropoid forearm (Reno et al., 2000)]. In support of this hypothesis Zuidam et al. (2006) observed that in people with triphalangeal thumbs where the MC1 possesses growth plates at both the proximal and distal ends, the MC1 undergoes disproportionate longitudinal growth relative to the other metacarpals.

Second, it is possible that the growth plate and separate epiphysis are retained proximally and not distally in the first ray because it may have been a site of greater kinematic mobility (but not necessarily opposability) in primitive therian mammals (Jenkins and Parrington, '76), and therefore has been retained for the same reasons they are maintained at both ends of most long bones—the requirement of exacting rigid body surface conformity to maintain tangential joint velocities in highly mobile and stable synovial joints (Burstein and Wright, '94). Such conformity is established during growth by cartilage modeling (Hamrick, '99; Lovejoy et al., '99), which may be facilitated by the presence of secondary epiphyses, and which isolate the primary region of cartilage modeling from the principal focus of long bone elongation.

In conclusion, these experiments have shown that alligator metapodial growth is fundamentally different from the mammalian condition, because it relies on bidirectional growth in proximal and distal growth plates. Moreover, cartilage replacement by bone also differs in the two taxa examined here, yet their patterns of chondrocyte proliferation (including a reserve zone proliferative peak) and differentiation during growth plate formation are generally similar. Comparison of the growth patterns between these divergent lineages strongly indicates that the loss of a growth plate and direct ossification in the metapodial are derived characters that evolved early in therian mammals and are linked to the evolution of erect quadrupedal gait.

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