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The pisiform growth plate is lost in humans and supports a role for *Hox* in growth plate formation

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Abstract

The human pisiform is a small, nodular, although functionally significant, bone of the wrist. In most other mammals, including apes and *Australopithecus afarensis*, pisiforms are elongate. An underappreciated fact is that the typical mammalian pisiform forms from two ossification centers. We hypothesize that: (i) the presence of a secondary ossification center in mammalian pisiforms indicates the existence of a growth plate; and (ii) human pisiform reduction results from growth plate loss. To address these hypotheses, we surveyed African ape pisiform ossification and confirmed the presence of a late-forming secondary ossification center in chimpanzees and gorillas. Identification of the initial ossification center occurs substantially earlier in apes relative to humans, raising questions concerning the homology of the human pisiform and the two mammalian ossification centers. Second, we conducted histological and immunohistochemical analyses of pisiform ossification in mice. We confirm the presence of two ossification centers separated by organized columnar and hypertrophic chondrocyte zones. Flattened chondrocytes were highly mitotic, indicating the presence of a growth plate. *Hox* genes have been proposed to play a fundamental role in growth plate patterning. The existence of a pisiform growth plate presents an interesting test case for the association between *Hox* expression and growth plate formation, and could explain the severe effects on the pisiform observed in *Hoxa11* and *Hoxd11* knockout mice. Consistent with this hypothesis, we show that *Hoxd11* is expressed adjacent to the pisiform in late-stage embryonic mouse limbs supporting a role for *Hox* genes in growth plate specification. This raises questions concerning the mechanisms regulating *Hox* expression in the developing carpus.

Key words: African ape; epiphysis; homology; *Hoxd11*; human evolution; ossification; wrist.

Introduction

The human wrist consists of eight short bones, so named for their lack of longitudinal growth due to the absence of a growth plate. Much of the growth in these bones occurs by subchondral and subperiosteal deposition (Dainton & Macho, 1999). In humans, the pisiform is a short pea-shaped spheroid that articulates solely with the triquetral (Fig. 1a). It provides a modest palmar projection and serves as the distal attachment site for the tendon of the powerful flexor carpi ulnaris (FCU) muscle (its tendon continues distally to insert into the hamate and base of metacarpal 5 via the pisohamate and pisometacarpal ligaments). These features have led to the common misconception that the pisiform is essentially a sesamoid and may not have a homolog in primitive carpals (Keibel & Mall, 1910; Haines & Hughes, 1944; Harris, 1944; Standring, 2005). However, the pisiform's small size belies its functional significance as the only carpal with an insertion for an extrinsic flexor of the hand (FCU), as well as serving as the attachment site for the abductor digiti minimi (ADM) muscle and the flexor and extensor retinacula. Additionally, the pisiform defines the medial boundaries of the carpal tunnel and ulnar canal. Compared with humans, the pisiform of most other mammals, including primates, is substantially enlarged and elongated (Fig. 1). A long, rod-shaped pisiform has been attributed to *Australopithecus afarensis* (A.L. 333-91; Fig. 1b; Bush et al. 1982). Thus, a shortened pisiform is a derived trait in *Homo* and represents one of the most dramatic anatomical differences between the human and chimpanzee wrist. Currently, the functional consequences of pisiform reduction are poorly understood.

Discerning the evolutionary and mechanical relevance of pisiform reduction relies on an understanding of the genetic and developmental processes that result in

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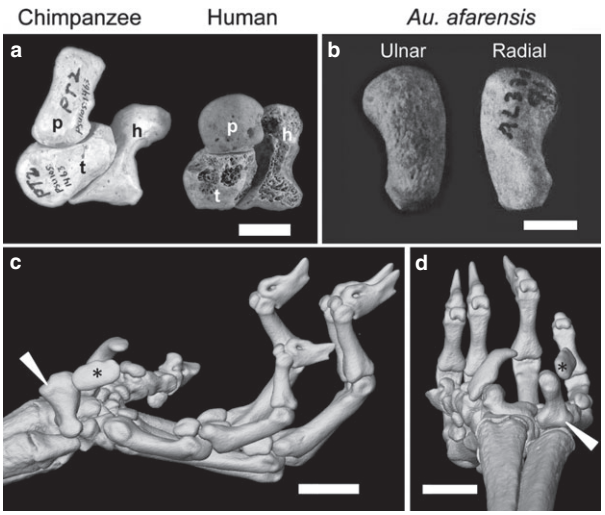

Fig. 1 Comparative anatomy of the pisiform. (a) Articulated pisiform (p), triquetral (t) and hamate (h) in a chimpanzee and human. Note the substantially elongated pisiform in the ape. Scale bar: 1 cm. (b) An elongated pisiform (A.L. 333-91) is attributed to *Au. afarensis* establishing that reduction of the human pisiform occurred after 3.2 my. Scale bar: 5 mm. Modified from Bush et al. (1982). (c) Ulnar and (d) proximal views of an 8-week-old mouse wrist generated from micro-CT. Note the enlarged pisiform (white arrowheads) and its articulation with the ulnar styloid proximally and the triquetral distally. The asterisks in (c) and (d) indicate a large palmar sesamoid that does not articulate with the pisiform (see also Fig. 3c,d). Scale bars: 1 mm.

the derived anatomy. It has long been observed, though remains generally underappreciated, that many mammalian pisiforms form from two centers of ossification (Retterer, 1898; Sieglbauer, 1931; Harris, 1944; Jouffroy, 1991). It is possible that this separate center implies the existence of a growth plate typical of long bones. However, separate centers also occur in the hamate and capitate and within many long bone epiphyses without forming a growth plate (Frazier, 1920). Alternatively, the two centers of the pisiform could represent late-fusing cartilage condensations similar to the os centrale and scaphoid of African apes (Kivell & Begun, 2007). While a brief radiographic description has been provided for the macaque (Eckstein, 1944), we are not aware of a systematic analysis of mammalian pisiform ossification. We addressed this by surveying pisiform ossification in juvenile chimpanzees (*Pan troglodytes*) and gorillas (*Gorilla gorilla*) to verify the timing of ossification of the two centers. To test the hypothesis that the pisiform forms a growth plate, we also conducted a histological analysis of pisiform development in the mouse. If there is indeed a growth plate in the mammalian pisiform, then its reduction would constitute one of the more profound developmental modifications of the human hand and wrist since our last common ancestor with chimpanzees.

The potential existence of a growth plate has particular significance for hypotheses concerning the patterning and formation of the wrist. *Hox* gene expression levels regulate both the pattern of a mesenchymal condensation and its subsequent growth (Morgan & Tabin, 1994; Davis et al. 1995; Boulet & Capecchi, 2002, 2004; Woltering & Duboule, 2010). In particular, *Hox* genes have been implicated in the specification and regulation of growth plates (Boulet & Capecchi, 2004). Posterior *Hoxa* and *Hoxd* genes are expressed in two distinct phases in the developing tetrapod limb; the early phase corresponds to expression in the stylopod and zeugopod, and the later phase the autopod (Zakany & Duboule, 2007). The wrist, or mesopodium, forms at the junction of the zeugopod and autopod. This region corresponds to a gap in *Hoxd* expression (Nelson et al. 1996; Reno et al. 2008). Woltering & Duboule (2010) propose that this 'no *Hoxd* zone' is responsible for the lack of growth plates in the carpals. Therefore, we hypothesize that if the secondary center of the pisiform simply represents a separate, late-fusing carpal element, then this region will be devoid of late-stage *Hoxd* expression similar to the rest of the mesopodium. Alternatively, if the pisiform does form an active growth plate we hypothesize that targeted later-stage *Hoxd* expression should be detectable adjacent to the developing pisiform.

Materials and methods

African ape comparative analysis

To confirm the presence of a secondary center in apes and to determine the relative timing of ossification compared with that in humans, we surveyed pisiform ossification in juvenile chimpanzees (*Pan troglodytes*, $n = 18$) and gorillas (*Gorilla gorilla*, $n = 27$) housed at the Cleveland Museum of Natural History, Ohio, USA. Specimens were either assessed visually when cleaned and disarticulated, or by X-ray when ligamentous. Pisiforms were staged on the following ordinal scale: no primary ossification center; primary ossification center only; unfused secondary center; partial fusion of two centers; and complete fusion of the pisiform. To assess relative age, specimens were scaled based on dental eruption and basilar suture closure: deciduous dentition only; first molar (M1) erupting; M2 erupting; M3 erupting; and patent basilar suture/canine erupting (McCollum, 2008). Despite variation in pisiform orientation across taxa, we will refer to the end that articulates with the triquetral as dorsal and the opposite end as palmar throughout this manuscript.

Mouse whole-mount and histological analysis

FVB/NJ mice were fed solid food and water *ad libitum*, and exposed to a 12 h day/night cycle. Animals were killed using CO_2 following protocols approved by the Penn State IACUC. Gross morphology was assessed in skeletons cleared and stained for alcian blue/alizarin red following standard protocols. Histological analysis was conducted on C57BL/6 forepaws collected for a previous study (Reno et al. 2006, 2007). Sections were stained with Safranin O/Fast Green to provide clear contrast between cells, cartilage matrix and bone as previously described (Reno et al. 2006). We assessed cellular proliferation via immunohistochemistry for proliferative cell nuclear antigen (PCNA) using a rabbit polyclonal antibody (sc-7907, Santa Cruz Biotechnology). Nuclear staining for this protein identifies cells

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in the S-phase of the cell cycle (Yu et al. 1992). For younger specimens (< postnatal day P10), procedures were as previously described (Reno et al. 2006). However, at later time points (> P10), enzymatic trypsin unmasking replaced chemical unmasking with sodium citrate, resulting in better tissue and cellular integrity. Negative antibody controls are provided for both protocols.

Hoxa11 and *Hoxd11* mutants and *in situ* hybridization

Two *Hoxa11*+/del;*Hoxd11*−/− and two *Hoxa11*+/del;*Hoxd11*+/− adult (8 weeks old) mice were provided as a kind gift from Anne Boulet and Mario Capecchi (HHMI, University of Utah, USA). The *Hoxd11* mutant line has been previously described (Davis & Capecchi, 1994; Boulet & Capecchi, 2002, 2004). The *Hoxa11-del* allele was derived from a novel conditional allele (K. Wong and M. Capecchi, unpublished). The null genotype (del) was attained in the limbs by breeding to Hoxb1-IRES-Cre (Arenkiel et al. 2003). Skeletons were cleared and stained as described above. For simplicity, we refer to the wild-type *Hoxa11* and *Hoxd11* alleles as 'A' and 'D', and refer to the mutant allele as 'a' and 'd'.

In situ hybridization was conducted on mouse embryos dissected from the uterine horn of pregnant FVB/NJ females at embryonic day (E) 13.5 and 15.5 and fixed in 4% paraformaldehyde. Embryos were dehydrated in graded methanol and stored at −20 °C. Skin was removed from E15.5 limbs by manual dissection in ice-cold methanol prior to *in situ* analysis. Expression patterns were confirmed in three repeated *in situ* analyses containing at least two experimental specimens and one sense control. Whole-mount *in situ* hybridization for a *Hoxd11* riboprobe (a gift from Denis Duboule, University of Geneva, Switzerland) was performed as previously described (Nieto et al. 1996). Proteinase-K treatment prior to hybridization consisted of 10 μg mL^{-1} for 30 min (E13.5) or 1 h (E15.5).

Results

Ossification of ape pisiforms

The chimpanzee and gorilla pisiforms are distinctly elongate and easily identifiable, even in juvenile specimens (Fig. 2). Evident subchondral surfaces can be visually discerned at each end of many juvenile ape pisiforms. Dorsally this corresponds to the triquetral articular surface, while palmarly it represents the surface underlying an unfused epiphysis. We confirmed this by identifying and refitting the free epiphysis in a sample of cleaned and well-curated specimens (Fig. 2a). The close fit of the secondary and primary center, and the presence of a subchondral surface where they join strongly indicates that the smaller, later-appearing element represents an epiphysis overlying a growth plate.

Radiographic analysis confirmed an early appearance of the primary ossification center in the great apes (Fig. 2b–i). The initial ossification of the dorsal primary center occurs prior to M1 eruption in both chimpanzees and gorillas (Fig. 2b,f). The secondary center makes its first appearance typically during M2 eruption, and complete fusion is seen during M3 eruption. However, there appears to be greater variability within gorillas as one female was observed with complete fusion prior to M2 eruption and two males were only partially fused after completion of M3 eruption. Such variability may reflect greater bimaturation and sexual dimorphism in the gorilla (Shea, 1985; Leigh & Shea, 1995).

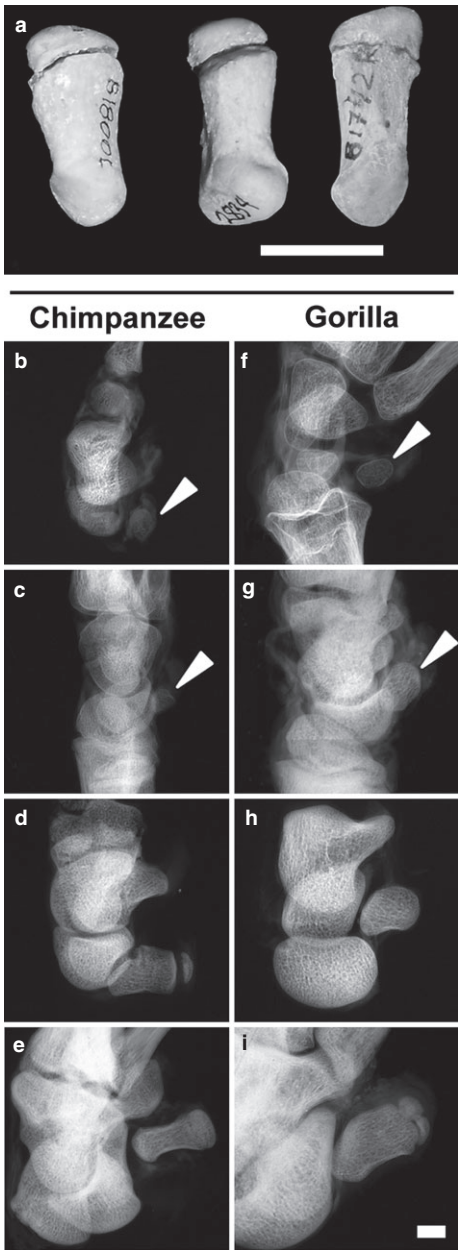

Fig. 2 Ape pisiform ossification. (a) Three juvenile chimpanzee pisiforms illustrating the separate epiphyses (palmar is to the top). For the left and center specimens the epiphysis is completely unfused but can be confirmed by the close fit to the pisiform diaphysis. On the right, the epiphysis is partially fused. For reference the antimere of this specimen is shown in (e). Scale bar: 1 cm. (b–i) Radiographs showing the progressive ossification of the pisiform in chimpanzees and gorillas. The arrowheads indicate the primary center of the pisiform in the very young specimens. Palmar is to the right. Scale bar: 5 mm. Dental eruptions stages are (b, f) deciduous only, (c, g) M1 erupting, (d, e and h) M2 erupting, (i) M3 erupting.

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Pisiform ossification in the mouse

We traced pisiform ossification in an age series of cleared and stained (alcian blue/alizarin red) mice. This procedure allows for easy distinction of cartilage and bone. Even in very young animals (P4), the cartilaginous pisiform is beginning to undergo calcification at the primary center of ossification (Fig. 3a). A separate secondary center begins to form at the palmar margin of the cartilaginous epiphysis by P7 (Fig. 3b). The secondary center expands and comes in close conformity with the primary center by P13 (Fig. 3c). By P19 the pisiform attains its general adult shape with an intervening strip of cartilage between the primary and secondary centers (Fig. 3d). The two centers eventually fuse between the fourth (P28) and fifth (P35) week (not shown).

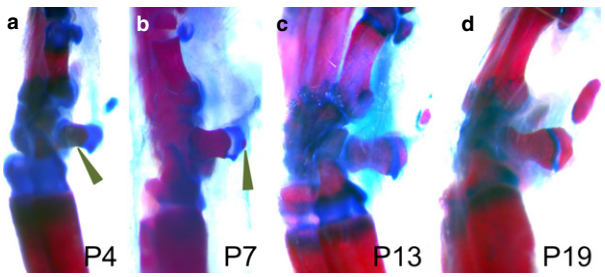

Fig. 3 Age series of the ossification of the mouse pisiform. (a) Alcian blue/alizarin red-stained mouse forelimb showing the initial ossification of the pisiform at P4 (green arrowhead). (b) At P7 the primary center is largely ossified while an incipient secondary center can be seen as a separate ossification forming within the blue cartilaginous epiphysis (arrowhead). (c) By P13 and continuing to P19 (d) the secondary epiphysis has expanded and is separated by the primary center by a band of thin cartilage. Note the progressive ossification of a palmar sesamoid to the upper right of the pisiform in each image. All images are at the same magnification.

The delayed formation of the secondary center and preserved zone of cartilage is consistent with epiphyses overlying a growth plate. To verify this, we conducted a histological analysis of mouse pisiform ossification. At birth (P0), the pisiform is a slightly elongated cartilage model completely composed of undifferentiated chondrocytes (Fig. 4a). Both the dorsal and palmar ends contain a narrow green stained zone about three chondrocytes deep that foreshadows the future articular zone (Reno et al. 2006; Villavicencio-Lorini et al. 2010). In contrast, surfaces along the margins of the pisiform shaft consist of a thinner fibrous perichondrium (Villavicencio-Lorini et al. 2010). At P4, the central chondrocytes begin to undergo hypertrophy and form the primary center of ossification (Fig. 4b), which begins to be replaced by invading bone by P7 (Fig. 4c). At the palmar end, an arc of remaining cartilage contains flattened columnar and hypertrophic chondrocytes that eventually organize (P9) into narrow columnar and hypertrophic zones (Fig. 4d). Similar to other growth plates, a perichondrial ring (zone of Ranvier) can be seen surrounding the bone collar, which extends to the boundary between the columnar and hypertrophic zones (Fig. 4d; Reno et al. 2006). The distinctive nature of the palmar end of the pisiform is apparent when compared with the ossification of the tubercle of

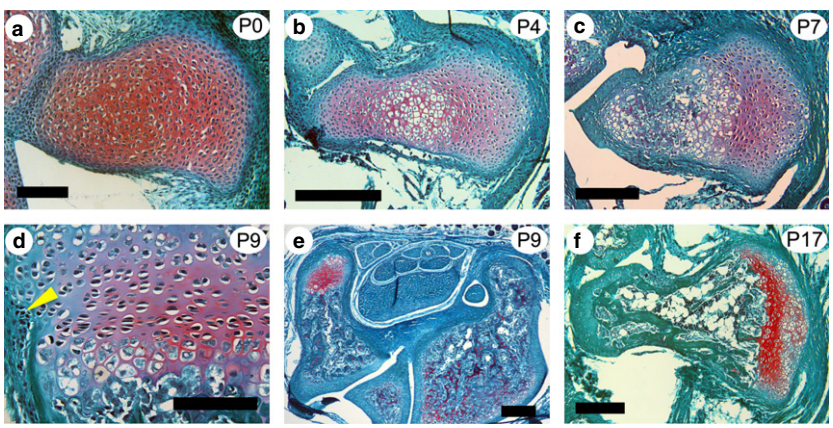

Fig. 4 Safranin O series of pisiforms illustrating the formation of a growth plate. (a) At birth (P0) the pisiform largely consists of undifferentiated hyaline cartilage. Note the future articular surfaces adjacent to the triquetral (right) and the transitional region near the insertion of the FCU (left). Each of these is distinct from the fibrous periosteal layers that surround the future pisiform shaft. (b) At P4 the cartilage has undergone differentiation to flattened columnar and hypertrophic chondrocytes. It is the calcified hypertrophic matrix that is staining red in Fig. 3(a). (c) By P7 the primary center of ossification begins to be replaced by bone. A broad region of flattened columnar and hypertrophic chondrocytes is preserved at the palmar end (right). (d) At P9 the preserved strip of cartilage displays all of the hallmarks of a growth plate: organized columnar and hypertrophic zones and a perichondrial ring (yellow arrowhead) adjacent to the bone collar. (e) A transverse section through the carpal tunnel demonstrates the unique ossification of the pisiform (left). Note the preserved region of red stained cartilage at the palmar end. In contrast the scapholunate (right) has ossified as a single primary center extending into the projecting tubercle. (f) At P17 the growth plate appears to be losing its activity, as there is no longer an identifiable hypertrophic zone underlying the columnar chondrocytes. Palmar is to the right in (a–c) and (f), and to the top in (d) and (e). Scale bar: 100 μm (a and d); 200 μm (b, c, e and f).

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the scapholunate (radiale) (Fig. 4e). While a generally similar structure morphologically and functionally, the scapholunate shows no remaining cartilaginous zones indicative of a growth plate. The pisiform growth plate appears to decrease in activity by P17 with the loss of the hypertrophic chondrocytes (Fig. 4f). While certainly narrower than those of other long bones, the pisiform growth plate shows all of the key hallmarks typical of longitudinal bone growth.

To further verify growth plate activity, we assayed PCNA expression via immunohistochemistry. At early stages, nuclear expression of PCNA is found throughout the population of undifferentiated chondrocytes (Fig. 5a). However, within the growth plate of older specimens, nuclear expression of PCNA was restricted to the thin band of columnar chondrocytes (Fig. 5c). This same pattern of proliferation was previously observed in mouse metatarsals (Reno et al. 2006), and confirms the presence of an active growth plate within the developing mammalian pisiform.

Hoxd11 expression around the developing mouse pisiform

Hox genes are known to be necessary for normal pisiform development in mice. Full deletion of *Hoxa11* or *Hoxd11* results in a highly penetrant phenotype with shortened pisiforms that often fuse to the triquetral (ulnare) or less commonly to the scapholunate and triquetral (Small & Potter, 1993; Davis & Capecchi, 1994; Favier et al. 1995). Double heterozygous animals also show similar pisiform/triquetral phenotypes to the individual *Hoxa11* and *Hoxd11* homozygous knockouts (Davis & Capecchi, 1996). Double homozygous deletion of *Hoxa11* and *Hoxd11* results in the absence of both the pisiform and triquetral (Davis et al. 1995), while in triple homozygous *Hoxa11*/*Hoxc11*/*Hoxd11* mutants, the scapholunate and triquetral (presumably along with the pisiform) involute into the radius and ulna, respectively (Koyama et al. 2010). There is also complementary function of other *Hox* paralogs as *Hoxd11* and *Hoxa10* double mutants show further reduction of the pisiform than does inactivation of *Hoxd11* alone (Favier et al. 1996). In each of these cases, the distal carpals are generally unaffected.

Given the knowledge of the existence of the pisiform growth plate, we further inspected the form of this bone in double heterozygous AaDd and triple allele Aadd mutant mice. Our observations largely confirm the previous results. AaDd mutants have a substantially reduced pisiform. While previous studies have reported occasional pisiform–triquetral fusion, we did not see evidence of this in our four specimens (Fig. 6a; Davis & Capecchi, 1996). This difference could be a result of our small sample or be due to differences between the *Hoxa11* mutant lines. Triple allele mutant (Aadd) lack separate elements for the pisiform and triquetral, and instead form a much reduced and misshapen bone in their place. This suggests that the pisiforms and triquetral are fused as previously described for AAdd mice (Fig. 6a; Davis & Capecchi, 1994). Further analysis is necessary to determine a dosage effect on pisiform growth between

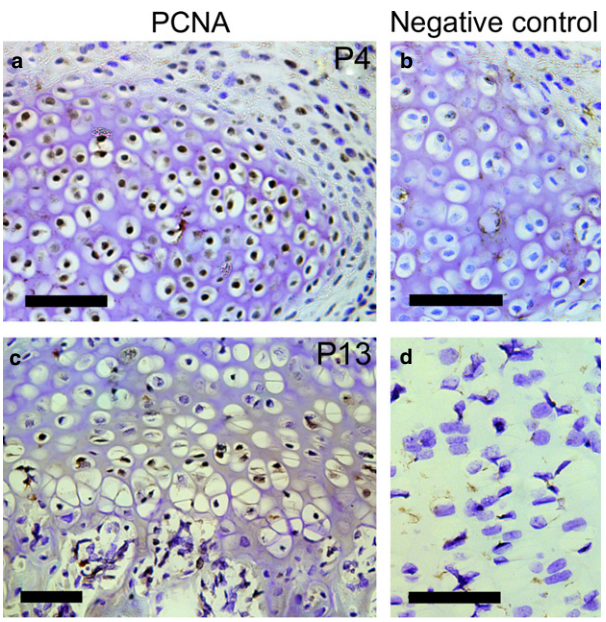

Fig. 5 Proliferation within the developing pisiform. (a) At P4 a majority of round undifferentiated chondrocytes at the palmar end of the pisiform show nuclear staining of proliferative cell nuclear antigen (PCNA) indicating proliferation. (b) There is a lack of background staining in chondrocytes in the absence of the PCNA antibody. (c) Within the established growth plate, PCNA staining is concentrated to the narrow band of columnar chondrocytes indicating tight control of cellular proliferation. (d) Columnar chondrocytes show no staining in the absence of the 1 ° antibody. Scale bar: 50 μm.

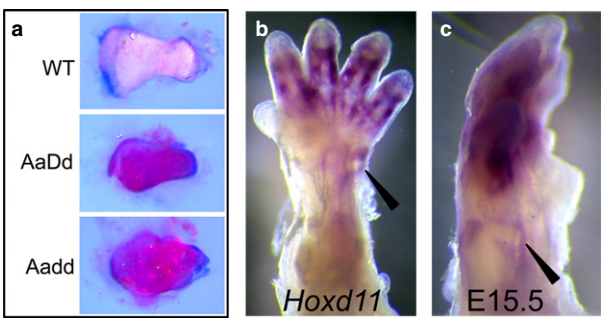

Fig. 6 *Hox* gene expression and the developing pisiform. (a) *Hox11* deletion results in reduced and malformed pisiforms in the adult (8 week) mouse. Palmar is to the left. Top: wild-type (WT) FVB/NJ pisiform for reference. Middle: double heterozygous *Hoxa11*+/del; *Hoxd11*+/− mouse shows reduced size and outgrowth compared with wild-type pisiforms, but otherwise appears generally normal. Bottom: three allele mutant *Hoxa11*+/del;*Hoxd11*−/− mouse shows severe malformation with likely fusion of the pisiform to the triquetral. (b) *In situ* hybridization of *Hoxd11* at E15.5 illustrates the typical autopod expression in the posterior digits but not in digit 1. A region of proximal expression is observed in the ulnar side of the wrist focused around the pisiform (arrowhead). (c) Ulnar view of a different specimen showing *Hoxd11* expression surrounding the pisiform (arrowhead) and ulnar styloid.

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homozygous and three allele mutants. These experiments in mice confirm that *Hox11* has a profound effect on pisiform development and outgrowth.

Given the identification of a growth plate in the mammalian pisiform, the effect of *Hox11* genes on pisiform development is particularly intriguing. As discussed above, previous work has demonstrated that *Hox* regulates both initial patterning and eventual growth of the skeleton though expression in the surrounding mesenchyme and perichondrium (Morgan & Tabin, 1994; Villavicencio-Lorini et al. 2010; Swinehart et al. 2013). At both E11.5 and E12.5, there is a clear reduction of *Hoxd11* expression corresponding to the developing carpal region (Koyama et al. 2010). We previously observed and confirmed with new experiments (not shown) a similar gap of *Hoxd11* expression at E13.5, although the breadth of the gap may be smaller on the ulnar side of the carpus (Reno et al. 2008). We hypothesize that the more subtle pisiform phenotypes on two allele *Hoxa11*, *Hoxd11*, or heterozygous mutant mice may be due to the loss or altered development of the growth plate. Given the previously identified role for *Hoxd11* in growth plate regulation and skeletal elongation (Morgan et al. 1992; Davis et al. 1995; Boulet & Capecchi, 2002, 2004), and the proposed significance of the 'no *Hoxd* land' for short bone morphology (Woltering & Duboule, 2010; Andrey et al. 2013), we surveyed the expression of this gene in the E15.5 mouse wrist when the initial condensation of the pisiform can be readily identified. The forelimbs were skinned prior to *in situ* analysis to facilitate penetration of the probe and visualization of skeletal elements. *Hoxd11* shows typical expression at this stage in digits 2–5 and a lack of expression in digit 1 (Fig. 6b,c). *Hoxd11* expression appeared strongest in the tissues adjacent to cartilaginous epiphyses and presumptive growth plates of the phalanges as in previous studies (Reno et al. 2008). As predicted, *Hoxd11* also showed notable expression in the ulnar side of the wrist, while little *Hoxd11* was detected on the radial side. Expression of *Hoxd11* surrounded the pisiform, consistent with a role in patterning pisiform elongation via growth plate specification and regulation.

Discussion

The mammalian pisiform growth plate

Here we demonstrate that the mammalian pisiform contains a growth plate separating the two centers of ossification. Although small, the growth plate displays well-organized columnar and hypertrophic zones, and is surrounded by a perichondrial ring and developing bone collar (Fig. 4). The pisiform is thus very similar to metapodials (metacarpals and metatarsals) and phalanges in forming a growth plate at only one end, while the cartilage at the opposite end is replaced directly by the primary center of ossification (Reno et al. 2006).

The existence of a secondary center has been noted in a number of mammals including the rabbit, rat, dog, Old World monkeys, chimpanzee and gorilla; however, its development has not undergone systematic assessment (Retterer, 1898; Major, 1899; Strong, 1925; Sieglbauer, 1931; Ayer, 1940; Eckstein, 1944; Harris, 1944; Smith, 1960; Jouffroy, 1991). Eckstein (1944) provided a brief radiographic description of pisiform ontogeny in the Rhesus macaque. He observed that the primary center is present at birth, and the secondary center first appears between 16.5 and 20.5 months. Between 22.5 and 36 months, the two centers remain separate; however, they fuse prior to year 6 (Eckstein, 1944). This timing is generally comparable to that of the apes based on correlated dental eruption (Cheverude, 1981). The difference in life history between mice and primates makes direct comparison across mammalian orders difficult. We observed that the primary center of the pisiform ossifies relatively late compared with other carpals in apes; however, it is one of the first to ossify in the mouse. Regardless, in both rodents and primates, the secondary center forms substantially later than the primary center.

The first signs of ossification in humans are observed as early as year 7 in girls and as late as year 12 in boys (Francis, 1940; Gilsanz & Ratib, 2005). Interestingly, the initiation of ossification in the human corresponds approximately to the period of secondary center ossification in apes and the macaque. This discrepancy in timing suggests that either: (i) growth plate loss is accompanied by a heterochronic shift in the timing of pisiform formation; or (ii) that it is the primary center of ossification that fails to form and the human bony pisiform is actually homologous to the epiphysis of other mammals. In addition to the similarity in timing, several sources of evidence support the latter hypothesis. First, the general shape and radiological appearance of the human pisiform is more reminiscent of both the ape and mouse epiphysis. Second, there can be substantial irregularity in the formation of the human pisiform. It has occasionally been described in radiographs as appearing 'crumbly' or forming from multiple centers (Debierre, 1886; Vasilas et al. 1960; Freyschmidt et al. 2003). We observed at least two cases of gorilla secondary centers forming from two points of ossification, suggesting that this may be a common variant of pisiform epiphyses (Fig. 2i). Third, the mammalian pisiform is actually an apophysis for the insertion of the FCU and ADM (Sieglbauer, 1931; Jouffroy, 1991). Given the functional significance of these muscles, the preservation of the insertion site may have required the palmar apophysis to be constrained. If the human pisiform is homologous to the remaining mammalian apophysis, it is not surprising that it has regularly been confused with a sesamoid bone. Further study and a larger sample are required to better quantify the timing of pisiform development in apes and other primates, and to clarify the issue of human homology.

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This is not the first instance of growth plate loss in mammalian evolution. The presence of a single growth plate in metapodials and phalanges is actually a derived trait in therian mammals (Reno et al. 2007, 2013). While the loss of the pisiform growth plate appears to be a human-specific trait, this raises the interesting question of when did the pisiform growth plate originally evolve during tetrapod evolution? Comparisons to other amniotes, such as alligators, could be informative and further verify that the mechanisms specifying the presence or absence of a growth plate are highly evolvable.

Role of *Hox* in growth plate specification and the developing wrist

Ossification within a cartilage model starts with the differentiation, hypertrophy and apoptosis of central chondrocytes, which are eventually replaced by invading osteoprogenitor cells (Maes et al. 2010). These cells ossify the previously existing cartilage scaffold to form trabecular bone. In typical long bones, the wave of chondrocyte differentiation is directed towards each end (Long & Ornitz, 2013). The columnar and hypertrophic chondrocyte zones expand to form an active growth plate. Around the periphery of developing long bones, flattened perichondrial cells are organized in parallel layers surrounding the cartilage model (Villavicencio-Lorini et al. 2010). At the boundary of the columnar and hypertrophic chondrocytes, the perichondrium lays down a surrounding cortical bone collar (Reno et al. 2006; Bandyopadhyay et al. 2008).

In short bones and epiphyses, the initial process of chondrocyte differentiation is similar. However, expanded cartilaginous growth plates or active perichondrial rings do not form (Reno et al. 2006; Villavicencio-Lorini et al. 2010). Instead, the periphery of these regions largely consists of a narrow three to four cell layer of round chondrocytes that anticipate the future articular zone (Reno et al. 2006; Villavicencio-Lorini et al. 2010). In each of these respects (organized chondrocyte zones, active perichondrial ring and deposition of the bone collar), the pisiform is more similar to long bones (Reno et al. 2006).

The mouse mutant synpolydactyly homolog (*spdh*) encodes a polyalanine expansion in *Hoxd13* that has a negative effect on the function of other *Hoxd* genes in the autopod (Villavicencio-Lorini et al. 2010). Villavicencio-Lorini et al. (2010) recently demonstrated that the metacarpals of *spdh* mutant mice have a malformed perichondrium with reduced expression of numerous perichondrial genes. These metacarpals are dramatically shortened and resemble typical carpals surrounded by presumptive articular chondrocytes, indicating an important role for *Hox* genes in perichondrial patterning and the regulation of skeletal growth.

Numerous experiments have shown that *Hox* genes function at later stages of patterning to modulate longitudinal growth of skeletal elements (Morgan & Tabin, 1994; Yokouchi et al. 1995; Davis & Capecchi, 1996; Capecchi, 1997; Goff & Tabin, 1997; Papenbrock et al. 2000; Zhao & Potter, 2001). Specifically, mice with reduced *Hoxa11*/*Hoxd11* expression display decreased proliferation within mesenchymal condensations and dramatic shortening of the radius and ulna such that they also resemble short bones (Davis et al. 1995; Boulet & Capecchi, 2002, 2004). In contrast, duplication of *Hoxd11* results in elongation of the metacarpals (Boulet & Capecchi, 2002). These actions appear to be mediated by regulating the expression of Indian hedgehog (*Ihh*) and parathyroid hormone-like hormone (*Pthlh*) associated genes that form a crucial feedback loop ensuring coordinated chondrocyte proliferation and differentiation within the growth plate (Vortkamp et al. 1996; St-Jacques et al. 1999). Double heterozygous deletion of *Hoxa11* and *Hoxd11* results in perturbed expression of *Ihh*, *Pthlh* and parathyroid hormone receptor (*Pthr*; Boulet & Capecchi, 2004). Components of this feedback loop are regulated by signals from the perichondrium, which was recently shown to be patterned by *Hox* (Minina et al. 2002; Kronenberg, 2007; Villavicencio-Lorini et al. 2010).

Given its similarities to long bones, the pisiform serves as a natural experiment to further confirm the role that *Hox* genes play in growth plate specification and performance. The biphasic regulation of both the *HoxD* and *HoxA* clusters produces a region of decreased expression in the developing wrist (Montavon et al. 2011; Andrey et al. 2013; Woltering et al. 2014). Woltering & Duboule's (2010) hypothesis that this 'no *Hoxd* land' would explain the lack of longitudinal growth in the mesopodium would have been challenged if the pisiform had formed a growth plate in the absence of *Hoxd* gene expression. However, the demonstration here of persistent *Hoxd11* expression on the ulnar side of the wrist adjacent to the pisiform further strengthens the association between growth plate formation and *Hoxd* expression.

The role of *Hox* genes in pisiform elongation raises interesting questions regarding the patterning of the tetrapod wrist. The limb expression of the posterior *Hoxd* genes is controlled by regulatory landscapes located on the opposing telomeric and centromeric domains of the cluster (Fig. 7; Montavon et al. 2011; Andrey et al. 2013). The telomeric enhancer domain regulates *Hoxd9–12* expression during early phases of limb development that correspond to the stylopod and zeugopod, while an expansive centromeric regulatory archipelago of deeply conserved elements controls *Hoxd10–13* expression in the autopod (Montavon et al. 2011; Andrey et al. 2013). The latter enhancer drives *Hox* expression in a pattern of reversed collinearity due to its proximity to *Hoxd13*, and also acts to inhibit *Hoxd* gene expression in the zeugopod (Tschopp & Duboule, 2011). While the operation of this regulatory archipelago appears to be coordinated, the isolated greatly conserved enhancer islands drive

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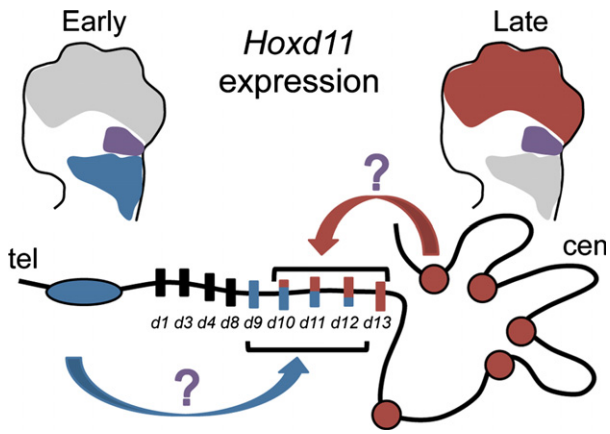

Fig. 7 Schematic of the regulatory landscapes controlling limb expression of the *HoxD* cluster. Separate telomeric and centromeric regulatory landscapes guide early (blue) and late (red) *Hoxd* expression in the tetrapod limb. The centromeric enhancer is better characterized and consists of a regulatory archipelago of a few highly conserved islands (red circles). *Hoxd11* is expressed in the ulnar side of the wrist around the developing pisiform further demonstrating the association between this gene and growth plate formation and performance (purple). Currently it is uncertain whether *Hox* expression in the wrist is determined by the early telomeric, late centromeric, or interaction of both landscapes.

gene expression within specific regions of the developing limb (Gonzalez et al. 2007; Montavon et al. 2011). Similar regulatory structure has also recently been identified for the *HoxA* cluster (Woltering et al. 2014). It has been proposed that this biphasic regulatory structure has facilitated the evolution of the tetrapod wrist by establishing the 'no *Hoxd* land' (Woltering & Duboule, 2010; Andrey et al. 2013). However, our results indicate that the transition between the dominance of the telomeric and centromeric regulatory landscapes does not in itself preclude *Hoxd* expression in the wrist. Instead, *Hox* expression in the wrist is likely to be tightly regulated and may play an important role in carpal evolution.

While the wrist is traditionally considered part of the autopod, the embryology of the proximal carpal row is intimately related to the distal ulna and radius (Keibel & Mall, 1910; Holmgren, 1952; Koyama et al. 2010). In addition, the *Hoxd11* carpal expression domain appears to be separated from the region of *Hoxd11* expression in the digits (Fig. 6). However, it is currently unknown whether expression of *Hoxd* in the ulnar wrist is controlled by the early telomeric landscape, later centromeric archipelago or a combination of both (Fig. 7). Further consideration of the morphology of the pisiform in various *Hox* mutants such as *spdh* could help resolve this issue.

Significance for the evolution of human pisiforms

The phenotypes of mice with reduced expression of *Hoxa11* and *Hoxd11* suggest that modification of these genes or their downstream targets could serve as potential mechanisms for pisiform reduction in humans (Small & Potter, 1993; Davis & Capecchi, 1994; Favier et al. 1995, 1996). We previously proposed a model suggesting that the co-residency of the fingers and forearm within the *Hoxd11* expression territory may underlie their co-evolution in hominoids (Reno et al. 2008). A long pisiform is observed in *Au. afarensis* at 3.2 Ma, and the Bouri skeleton indicates that forearms did not shorten until after 2.5 Ma (Bush et al. 1982; Asfaw et al. 1999; Reno et al. 2005). Modern human-like forearm proportions are observed in *H. erectus* at 1.5 Ma (Walker & Leakey, 1993). This may suggest that both forelimb shortening and pisiform reduction were coincident between 2.5 and 1.5 my. The human pisiform initially develops as a mesenchymal condensation adjacent to the ulna before migrating to the palmar surface of the triquetral (Keibel & Mall, 1910). Thus, it is conceivable that shared developmental processes of the digits and forearm could result in pleiotropic reduction of the pisiform (Reno et al. 2008).

Alternatively, the coincident reduction of forearm, finger and pisiform lengths could reflect independent selection associated with stone tool manufacture and use. Despite the drastic change in pisiform length, its effect on wrist function has not been thoroughly studied. There are at least three reasons to suggest that the pisiform may have important functional consequences for the hominoid wrist. First, the human, and potentially African ape, pisiform has a substantial degree of sliding mobility across its relatively simple articulation with the triquetral, which may exceed that of other primates (Moojen et al. 2001; Jameson et al. 2002). In monkeys and early Miocene hominoids such as *Proconsul*, the pisiform articulates into a socket between the triquetral and ulnar styloid as in the mouse (Fig. 1; Napier & Davis, 1959; Beard et al. 1986; Jouffroy, 1991; contra Lewis, 1972). Similarly, the gibbon pisiform is buttressed proximally by a novel bone, the os Daubentonii, despite the withdrawal of the ulnar styloid. In apes and humans, the articulation of the pisiform has migrated distally to lie on the palmar surface of the triquetral. The orangutan pisiform, however, is stabilized by a direct articulation with the hamate hamulus (Lewis, 1972).

Second, among hominoids, the African ape pisiform is quite long (Fig. 1). While the orangutan pisiform is short relative to those of other apes, it is still substantially longer than that of humans (Lewis, 1972; Sarmiento, 1988). In great apes and humans, the pisiform is oriented generally palmarly, in contrast to its more proximal orientation in hylobatids (Lewis, 1972; Sarmiento, 1988). These changes in length and orientation can have substantial effects on the lever-arm of the FCU and the ADM. The fossil record indicates that the pisiform reduced sometime between *Au. afarensis* and Neandertals (Trinkaus, 1983). This anatomical change may be related to shifts in locomotor habitus. It had been previously suggested that the

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long pisiform in *Au. afarensis* implies maintenance of powerful forearm musculature and continued arboreality (Stern & Susman, 1983). However, other anatomical evidence suggests that dependence on climbing had already been reduced in *Australopithecus* (Lovejoy, 1988, 2005). Perhaps the evolution of this part of the wrist anatomy reflects a different type of repetitive behavior, one relatively unique to the human lineage, tool use. Though still a very broad interval, the start of this window approximately corresponds to the adoption and intensification of stone tool manufacture and use starting approximately 2.6 Ma (de Heinzelin et al. 1999; Semaw et al. 2003). The FCU is one of two muscles recruited in both hands during percussive tool manufacture (Marzke et al. 1998). As such, the reduced lever-arm in humans has been proposed to limit wrist flexion when stabilizing the carpus (Marzke et al. 1992); however, such a conformation may also result in increased axial loads. Alternatively, reduced projection of the pisiform may improve palmar grip and opposition between the thumb and fifth digit (Marzke et al. 1992; Young, 2003; Lovejoy et al. 2009).

The third possible explanation for pisiform reduction results from the pisiform being a component of multiple anatomical complexes with important functional and clinical significance. The flexor retinaculum attaches along its radial border (Manley et al. 2013). The pisiform defines one of the boundaries and potentially the depth of both the carpal tunnel and ulnar canal (Fig. 4e; Marzke, 1971; Pevny et al. 1995). Both passageways can be involved in traumatic and repetitive use injuries. Fracture of the pisiform, though infrequent relative to other carpals, can result in chondromalacia, degenerative osteoarthritis, and chronic pain and weakness if left untreated (Fleege et al. 1991). Tasks that require frequent use of the power squeeze grip such as racquet sports, which are similar to the motions utilized during stone tool production (Williams et al. 2010), can result in pain and disability when associated with pisiform instability (Helal, 1978a,b). While dislocation is also relatively rare due to the sturdiness of the associated pisotriquetral, pisohamate and pisometacarpal ligamentous complex, instability can result in compression of the ulnar nerve with paraesthesia of the hand and weakness of the hypothenar musculature (Pevny et al. 1995; Rayan et al. 2005; Sharma & Massraf, 2005). Therefore, given the substantial mobility of the human and potentially African ape pisiform, the importance of the attached musculature, and its close relationship to clinically relevant structures, it is reasonable to hypothesize that pisiform reduction occurred so as to increase stability of the pisotriquetral articulation. Limiting chronic and repetitive use injury associated with stone tool manufacture and use would likely be of significant selective advantage (Marzke, 2013). Thus, mechanisms that could lead to the specific reduction of the pisiform could have been advantageous in early *Homo*. Elimination of the growth plate and primary center of ossification via reduction of the *Hox11* genes in conjunction with other factors could be a potential mechanism. Further work is necessary to explore this hypothesis.

Concluding remarks

To our knowledge, this is the first histological description of the pisiform growth plate. Verification of its existence has implications for the evolution and development of the hominoid wrist. Growth plate loss provides a mechanism for the dramatic reduction of the human pisiform, one of the more profound changes in the evolution and development of the human wrist and hand. Furthermore, the presence of a growth plate in the carpus has consequences for the interpretation of *Hox* gene function in skeletal development. This provides a plausible explanation for the greater effects that *Hoxa11* and *Hoxd11* mutant alleles have on pisiform size compared with other carpals. Further study of the development of the pisiform in these mice is necessary to determine the effect that modified *Hox* function has on pisiform growth plate formation. We also establish the continued expression of *Hoxd11* on the ulnar side of the developing wrist. This confirms our understanding that *Hox* gene expression is tightly regulated, and suggests that the reduction of *Hox* expression in the mesopodium between the zeugopod and autopod is not a simple consequence of a complex biphasic regulatory motif of the *HoxA* and *HoxD* clusters. Instead, the precise regulation of *Hox* genes is necessary to 'sculpt' wrist morphology (Davis & Capecchi, 1996). Such mechanisms may lie in either of the centromeric or telomeric regulatory landscapes, and are likely to have been fundamental to tetrapod evolution (Woltering & Duboule, 2010; Andrey et al. 2013).

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Author contributions

KMK, COL and PLR conceived of the study; KMK, JEH and PLR conducted the experiments or collected the data; KMK and PLR analyzed the data; KMK and PLR drafted the manuscript; KMK, JEH, COL and PLR revised and approved the article.

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