The Tetrapod Limb: A Hypothesis on Its Origin

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A wrist joint and structures typical of the hand, such as digits, however, are absent in [Eustenopteron] (Andrews and Westoll, '68, p 240).

Great changes must have been undergone during evolution of the ankle joint; the small number of large bones in the fin must somehow have developed into a large number of small bones, and it is very difficult to draw homologies in this region, or even be certain of what is being compared (Andrews and Westoll, '68, p 268).

ABSTRACT The tetrapod limb is one of the major morphological adaptations that facilitated the transition from an aquatic to a terrestrial lifestyle in vertebrate evolution. We review the paleontological evidence for the fin-limb transition and conclude that the innovation associated with evolution of the tetrapod limb is the zeugopodial-mesopodial transition, i.e., the evolution of the developmental mechanism that differentiates the distal parts of the limb (the autopodium, i.e., hand or foot) from the proximal parts. Based on a review of tetrapod limb and fish fin development, we propose a genetic hypothesis for the origin of the autopodium. In tetrapods the genes Hoxa-11 and Hoxa-13 have locally exclusive expression domains along the proximal-distal axis of the limb bud. The junction between the distal limit of Hoxa-11 expression and of the proximal limit of Hoxa-13 expression is involved in establishing the border between the zeugopodial and autopodial anlagen. In zebrafish, the expression domains of these genes are overlapping and there is no evidence for an autopodial equivalent in the fin skeleton. We propose that the evolution of the derived expression patterns of Hoxa-11 and Hoxa-13 may be causally involved in the origin of the tetrapod limb. J. Exp. Zool. (Mol. Dev. Evol.) 291:226–240, 2001. © 2001 Wiley-Liss, Inc.

In this article the evolution of tetrapod limbs is discussed from a morphological and developmental point of view. In accordance with the majority view (Andrews and Westoll, '68; Andrews and Westoll, '70; Ahlberg and Milner, '94; Coates, '94; Sordino and Duboule, '96; Capdevila and Izpisúa-Belmonte 2001) the origin of the tetrapod limb is considered to be coincidental with the origin of the autopodium, i.e., distinct hands and feet in the paired appendages. The present review is aimed at answering two major questions that follow from this view, namely: "What is an autopodium?" and "How did it originate?" The article is also an attempt to exemplify the concept of evolutionary innovation. We therefore want to start with a short summary of the innovation concept as it is used in this article.

Müller and Wagner have argued that the concept of an evolutionary innovation is intimately connected to the notion of homology, or in more neutral terms, to the notion of character identity (Müller and Wagner, '91). Following the lead of

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Shubin and Alberch (Shubin and Alberch, '86), we think that the best way to characterize a character is to identify the morphogenetic rules underlying the development of the characters. This idea is the core of the biological notion of character identity, also known as the biological homology concept (Wagner, '89).

A character is a part of the body that develops according to a coherent (i.e., phylogenetically stable) set of morphogenetic rules which make a distinct range of phenotypic states accessible to this body part, but which are inaccessible (or nearly so) to other parts of the organism.

This formulation is new, but essentially expresses the same ideas as the definition of "bio-

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logical homology" in Wagner ('89). The only differences are that we do not explicitly invoke the notion of developmental constraints but instead directly refer to developmental processes, i.e., morphogenetic rules. By implication, an evolutionary innovation is a part of the body that follows a set of phylogenetically derived morphogenetic rules, which make a distinct set of character states accessible to natural variation. From a developmental point of view this definition implies that an innovation has to be, or at least in many cases will be associated with a certain morphogenetic field (for a definition of a morphogenetic field see Gilbert et al., '96). An innovation can be realized by an ancestral field, which has acquired a new set of morphogenetic rules, or by the origin of a morphogenetic field that has no correspondence in the ancestral lineage and which executes a new set of morphogenetic rules. Based on this way of thinking we will argue that the key developmental difference between a fin and a tetrapod limb is the existence, in limbs, of a morphogenetic field that does not exist in fins, i.e., the autopodial field. We will review evidence showing that the autopodium in fact develops from a morphogenetic field distinct from the proximal parts of the developing limb bud.

In the argument below, we use paleontological and morphological data to examine the plausibility of this hypothesis and to map the hypothesized developmental event onto the vertebrate tree. While direct developmental data from creatures like Acanthostega and Ichthyostega is not available, we note that implicit to an evolutionary developmental hypothesis are predictions about the morphology of the species that possess the developmental innovation. Thus a hypothesis on the origin of a new character, though essentially developmental, is testable with morphological observations in a phylogenetic framework. The basis for this link is that an evolutionary developmental hypothesis has to have implications for the morphological states accessible to the affected lineages. These states have to be distinct from those accessible to the ancestral species. Hence morphology is, in addition to direct comparative developmental data and molecular sequence evolution, a test bed for evolutionary developmental models.

MORPHOLOGICAL PATTERN OF THE FIN-LIMB TRANSITION

The paleontological and anatomical evidence that connects tetrapod limbs with fins has been reviewed repeatedly in recent years as important new fossil evidence has emerged (Milner, '88; Coates, '91; Vorobyeva, '91; Carroll, '92; Coates, '93; Milner, '93; Ahlberg and Milner, '94; Coates, '94; Shubin, '95). The present summary, therefore, is based predominantly on previously published review articles (see preceding list) and a limited number of original contributions (Long, '89; Lebedev and Coates, '95; Coates and Clack, '90; Cloutier and Ahlberg, '96; Coates, '96; Daeschler and Shubin, '98; Paton et al., '99; Berman, 2000). The purpose of the present summary is to clearly define the morphological transformation we seek to explain. It has been proposed that the autopodium is the innovation separating the limb from a fin (Ahlberg and Milner, '94; Coates, '96; Sordino and Duboule, '96; Capdevila and Izpisúa-Belmonte 2001). But what exactly is the autopodium? To answer this question we have organized the comparative anatomical evidence in the phylogenetic framework of vertebrate evolution. While the phylogenetic branching patterns of some lineages remain unresolved, there is an emerging consensus on the relationships among taxa (see discussion later in this article) that are most critical for elucidating the fin-limb transition.

Limbs and fins, a basic taxonomy of terms

The archetypal limb of a tetrapod consists of three major segments: the upper limb or the stylopodium, the lower limb or the zeugopodium, and the hand/foot or the autopodium (Fig. 1A). The stylopodium consists of one long bone, the humerus in the forelimb, attached to the shoulder girdle and the femur in the hind limb, attached to the pelvic girdle. The zeugopodium is primarily composed of two long bones, the radius and ulna, and the tibia and fibula in the fore and hind limb, respectively. The autopodium consists of two segments, a proximal mesopodium and a distal acropodium. The mesopodium is a complex of nodular elements in most tetrapods, and is called the carpus in the hand and tarsus in the foot. The acropodium is a series of small long bones, the metacarpals and metatarsals as well as the digits (Fig. 1A).

The typical paired fin of a teleost (e.g., zebrafish) has no specific skeletal elements in common with the tetrapod limb (Fig. 1B). The proximal endoskeletal elements are an anterior-posterior series of bones called radials. Distal to these radials is a row of small cartilages called distal radials. The most distal skeletal elements are the fin rays,



Fig. 1. Comparison of paired appendage skeletons. (A) typical tetrapod limb with three main segments, the stylopodium, zeugopodium and autopodium. (B) Pectoral fin of a blenny, a perciform fish. The distal elements are fin rays derived from dermal scales and have no counterpart in the tet-

rapod skeleton. The proximal elements are endoskeletal, but are not homologous to any bone in the tetrapod skeleton. (C) Endoskeleton of *Eusthenopteron*, a fossil sarcopterygian relative of tetrapods. Note the branching pattern of skeletal elements, similar to the proximal elements in the tetrapod limb.

lepidotrichia and actinotrichia, which belong to the dermal skeleton. The tetrapod limb contains no skeletal elements derived from fin rays. The connection between the fin and the limb, however, becomes more evident upon examination of the more complex endoskeletal fin structures of sharks, basal ray-finned fishes such as the sturgeon, and the sarcopterygian (lobe-finned) fishes, from which the tetrapods are derived (Janvier, '96) (Fig. 1C).

The tetrapod limb is derived from a posterior part of the fin endoskeleton of elasmobranchs and basal bony fish, the so-called metapterygium, a series of endoskeletal elements that is the first to form in the developing paired fins (Braus, '06; Shubin, '95; Mabee, 2000). It arises in close connection to the girdle and, in turn, gives rise to a series of variable elements, usually at its anterior edge. In addition, there is an independent endoskeletal element called the protopterygium that develops anterior to the metapterygium in many basal fishes (e.g., the bichir and sturgeon). Teleosts have lost the metapterygium whereas the sarcopterygians, on the other hand, have lost the protopterygium. Sarcopterygians thus develop all their endoskeletal structures from the metapterygium and consequently the tetrapod limb skeleton is derived from the metapterygium. The difference between the tetrapod limb and the teleost fin may be explained, then, by a complementary trend in the importance of the metapterygium. These observations thus limit the usefulness of comparisons between zebrafish fin development and limb development to the most general features, such as the presence of a ZPA. But, no specific comparisons are possible between the skeletal elements of these paired appendages.

Phylogenetic position of tetrapods

The hypothesis that tetrapods and sarcopterygian fishes form a clade is widely supported, (Hedges et al., '90; Schultze and Trueb, '91; Cloutier and Ahlberg, '96; Zardoya and Meyer, '97). The question as to which of the two extant sarcopterygian fish lineages, the lungfish (three extant genera) or coelacanth (Latimeria), is closer to the tetrapods remains open (Rosen et al., '81; Panchen and Smithson, '87; Chang, '91; Schultze, '91; Hedges et al., '93; Zardoya et al., '96; Zardoya and Meyer, '97). Since neither of these taxa represents the character state from which the limb is derived (Vorobyeva, '91; Shubin, '95), this uncertainty is not relevant to our discussion. There is strong evidence that the panderichthyids comprise the sister group to the tetrapods and that the osteolepiforms, with the well known Eusthenopteron as a typical representative, are the sister group to the panderichthyid-tetrapod clade (Long, '89; Coates, '91, '94; Vorobyeva and Schultze, '91; Shubin, '95; Ahlberg and Milner, '94; Cloutier and Ahlberg, '96) (Fig. 2).

The panderichthyids are a group of Devonian sarcopterygians which share a number of cranial and postcranial characters with the early tetrapods, with the exception of the structure of the



Fig. 2. Hypothesis about the phylogenetic branching pattern among sarcopterygian relatives of tetrapods. The phylogeny is simplified from Fig. 4 of Cloutier and Ahlberg, '96.

distal parts of their paired appendages (Schultze and Arsenault, '85; Vorobyeva and Schultze, '91). This group can thus be viewed as tetrapods with paired fins. Like the most basal tetrapods, these creatures were shallow water predators (Coates and Clack, '90; Ahlberg and Milner, '94), much like extant crocodiles.

A third group of lobe-finned fish are the rhizodontids, which have an interesting fin structure (see later discussion). However, the phylogenetic position of this group is still debated. It may be the sister taxon to the {[(tetrapod)panderichthyd]osteoleopiform} clade, as proposed by Schultze, '87, Long, '89, and Cloutier and Ahlberg, '96. The other sarcopterygians (such as lung fish, coelacanths, and the porolepiforms), have fin structures that bear little resemblance to the early tetrapods and their immediate relatives.

Phylogeny of tetrapods

There is strong support for monophyly of all extant and fossil tetrapods (Panchen and Smithson, '88; Ahlberg and Milner, '94; Carroll, '95; Coates, '96; Janvier, '96; Laurin, '98b), of anmiotes, and of the lissamphibians, i.e., the frogs, salamanders, and gymnophions (Gauthier et al., '88; Laurin, '98a; Cannatella and Hillis, '93; Hedges and Maxson, '93; Fig. 3). Relationships among the many Carboniferous amphibians and the Lissamphibia, however, remain unresolved (Ahlberg and Clack, '98; Laurin, '98a,b; Carroll, '92, '95; Milner, '93; Berman, 2000; Coates et al., 2000; Laurin et al., 2000).

Evidence from trace fossils of the middle Upper Devonian, about 370 MA, indicates that tetrapod limbs originated in the Devonian (Vorobyeva, '77, cited after Ahlberg and Milner, '94). Most of the anatomical evidence about the structure of primitive tetrapod limbs stems from the fossils of three Devonian tetrapods, Acanthostega, Ichthyostega, and *Tulerpeton*, found in Late Famennian layers about 362 MA (Ahlberg and Milner, '94). According to the cladistic analyses the Devonian tetrapods are offshoots from the tetrapod stem lineage (Ahlberg and Clack, '98; Laurin, '98b). They thus diverged before the most recent common ancestor of the extant tetrapods (Fig. 3). This phylogenetic hypothesis positions the most recent common ancestor of extant tetrapods in the Lower Carboniferous period at about 340 MA (Paton et al., '99; Laurin et al., 2000).

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Fig. 3. Phylogenetic branching patterns among major tetrapod taxa, simplified according to the hypothesis by Ahlberg and Milner ('94). All recent forms are derived from an ancestor more recent than any of the known Devonian tetrapods.

This hypothesis implies that the pentadactyl autopodium arose only once, prior to the most recent common ancestor of living tetrapods (arrow) (Laurin, '98b). The bar indicates the fin-limb transition.

Stages in the acquisition of tetrapod limb characters

From the phylogenetic history outlined previously it is clear that the origin of modern tetrapod limbs was not a single event. Instead it resulted from a series of transformations that continued after the origin of the first unambiguous tetrapod limbs, as shown by the difference between the Devonian and Carboniferous tetrapods. There are at least two major steps to be distinguished. First, the origin of the tetrapod autopodium and second, the transformation of the archaic autopodium of Devonian tetrapods into the pentadactyl autopodium of the extant tetrapods (Coates, '94, '96). Later in this article, we propose a morphological definition of the first step, i.e., the fin limb transition.

The three main outgroup taxa of tetrapods, panderichthyids, osteolepiforms, and rhizodontids, have endoskeletal elements corresponding to the stylo- and zeugopodial elements in a tetrapod limb (Andrews and Westoll, '68; Andrews and Westoll, '70; Coates, '91; Vorobyeva, '91; Ahlberg and Milner, '94; Shubin, '95). In addition, there are elements that share the position and possibly the developmental derivation of the

ulnare and the intermedium. From these observations, most authors have concluded that the stylo- and zeugopodial elements as well as the proximal mesopodial elements have counterparts in the fins of tetrapod ancestors, but there are no indications of wrist or ankle joints (Andrews and Westoll, '68). In addition it is noteworthy that the lineage that leads to the tetrapod ancestor shows a tendency to reduce the complexity of the endoskeleton distal to the zeugopodial segment (Michael Coates, paper given at the meeting of the Society for Integrative and Comparative Biology in January 2001 in Chicago). This trend culminated in the pectoral fin of Panderichthys which has only two distal elements, an elongated element corresponding to the intermedium and large bony plate corresponding to the ulnare (Vorobyeva and Schultze, '91). This pattern suggests that the autopodium did not arise from a transformation of distal fin skeleton but consists largely of new elements with only few homologues in the fin skeleton.

The neomorphic nature of the autopodium is also reflected in the problems of homologizing the digits. Most authors identify them as homologues to the radials of a sarcopterygian fin, e.g., (Coates,

'94), but see (Coates et al., 2000). If digits are "segmented radials that do not support fin rays" (Coates, '94), the question remains as to which radials they correspond. Given that in most recent tetrapods the digits derive from the digital arch in a sequence from posterior to anterior (at least digits DIV, DIII, and DII, but not DV (Burke and Alberch, '85); and perhaps not even DI, see original data on the alligator (Figs. 4 and 7 in Müller and Alberch, '90), it is tempting to assume that the digits correspond to postaxial radials (Ahlberg and Milner, '94). Postaxial radials, however, are not described among the close outgroups of tetrapods. Osteolepiforms tend to only have anterior radials and rhizodontids have terminal radials, much like digits (e.g., *Barameda*: Long, '89; Sauripterus: Daeschler and Shubin, '98). Postaxial radials are present in lungfish, Latimeria, and the shark Xenacanthus (Braus, '06; Shubin, '95) but these lineages are not directly ancestral to tetrapods. Cell fate mapping in bird limb buds does not show a "bending" of the posterior growth axis in the autopodium (Vargesson et al., '97). Hence, it is not obvious that digits and the digital arch can be understood as a bent metapterygial axis as proposed by Shubin and Alberch ('86). We argue that the digital arch may have evolved during the stabilization of the pentadactyl autopodium, rather then during the fin-limb transition itself.

In comparing sarcopterygian fins and the variety of primitive tetrapod limbs, the only consistent differences between these structures are the mesopodial-acropodial pattern of skeletal elements in the autopodium and the absence of finrays. This implies the origin of digits and the mesopodium, carpus and tarsus. The typical mesopodium of extant tetrapods consists of a complex array of three kinds of nodular elements: the proximal tarsals, ulnare/fibulare, intermedium, and radiale/tibiale, the central carpals/tarsals and the distal carpals/ tarsals supporting the metapodial elements. Nothing comparable to this arrangement has been described in Eustenopteron (Andrews and Westoll, '68). Among the recent tetrapods there are a few examples in which tarsal or carpal elements are secondarily elongated (Blanco et al., '98). We will discuss these exceptions below.

Not all mesopodial elements of crown group tetrapods are found in the most basal stem tetrapods. The elaboration of the mesopodium occurred after the origin of the digits (Smithson et al., '93; Coates, '96). The carpus of *Acanthostega* is not known except for the presence of an elongated

fibulare (Coates and Clack, '90), but the tarsus of Acanthostega has been described (Coates, '96). It consists of elements corresponding to the proximal tarsals (tibiale, intermedium, and fibulare) and four distal tarsals supporting digits DII, DIII, DIV, and DV. The preservation of the specimen suggests that some additional elements may have been lost (Coates, '96). The hind limb of Ichthyostega also has the proximal tarsals and two distal tarsals, as well as one central element wedged between the intermedium and the two distal carpals (Coates and Clack, '90). Even among stem amniotes the tarsus is still more primitive than in crown amniotes (Smithson et al., '93). Hence the mesopodium consists of plesiomorphic elements that got integrated and transformed into the mesopodium (the ulnare/fibulare and the intermedium) as well as of new elements, many of them arising after the origin of the autopodium. Clearly, not all mesopodial elements of Devonian forms are nodular (carpal intermedium of Acanthostega) and many elements were added later. But all tetrapod limbs have some nodular elements inserted between the zeugopodium and the digits, which is the structure we call the mesopodium in this article, regardless of the degree of elaboration.

We conclude that a developmental scenario for the origin of the autopodium has to account for the origin of a zeugopodial-mesopodial transition but not necessarily for the completely elaborated mesopodium seen in modern tetrapods. This transition corresponds to a marked difference in skeletogenetic mode, from the development of large elongated elements to smaller and most often nodular elements, that occurs in all tetrapod limbs but not in any sarcopterygian fin.

After the establishment of the meso-acropodial pattern in the Devonian, the tetrapod limb continued to evolve. The Devonian forms have an autopodium that is structurally distinct from all the limbs of extant tetrapods as well as all known limbs of Carboniferous forms (Coates, '91, '94). They are all polydactylous, ranging from eight digits in Acanthostega (Coates and Clack, '90) to six digits in Tulerpeton (Lebedev and Coates, '95). Furthmore, the *Ichthyostega* foot (the hand is not known) is heterodactylous, which means that the digits are heterogeneous in size trends and cross section (Coates and Clack, '90). Finally the number of mesopodial elements is smaller, as discussed above. Similarly, the urodeles have a radically different mode of hand/foot development than all other extant tetrapods (Braus, '06). From this is it clear that the pentadactyl tetrapod limb morphology stabilized after the actual fin- limb transition (Ahlberg and Milner, '94; Coates, '94; Coates, '96; Laurin, '98b; Paton et al., '99; Laurin et al., 2000). The question thus arises, how does the development of extant tetrapod limbs relate to the morphology of stem tetrapods with their polydactylous limbs and primitive mesopodium (Wagner et al., 2000).

Recent tetrapods differ in the mode of digit development. There are at least four modes for deployment of digits (Fig. 4). In the most common mode, the digital arch grows from the ulnare/ fibulare in a posterior to anterior direction and digits sprout from the postaxial side from the digital arch (Fig. 4A). Digit forming condensations without connection to the digital arch have also been observed. These give rise to the most posterior digits in the amniotes, DV, and sometimes also to the most anterior digit, DI (see for instance Burke and Alberch, '85; Müller and Alberch, '90; Burke and Feduccia, '97). A single digit, the prehallux or the prepollex, can develop from the radiale and tibiale, respectively (Figs. 4B and 5). Digits I and II in urodeles are developmentally derived from the intermedium (Schmalhausen, '10; Hinchliffe, '91; Blanco and Alberch, '92; Vorobyeva and Hinchliffe, '96; Hinchliffe and Vorobyeva, '99) (Fig. 4C). Hence, the digital arch is certainly not the only mode for deployment of digits in limb development, and there is variation in the "digitogenic pathways" among recent tetrapods. We thus suggest that in the limb buds of Devonian tetrapods several digitogenic pathways might have been used simultaneously, which may account for the higher digit number compared to extant tetrapods. This hypothesis may also account for the heterodactly situation in *Ichthyostega* (Coates, '91) assuming that digits with different morphologies are derived from different digitogenic pathways. These suggestions are testable with loss of function mutations (see later discussion). The stabilization of the autopodial morphology may have then resulted from suppression of some the digitogenic pathways, like the one from the radiale/tibiale in amniotes, and the expansion of the digital arch. Consequently the extent of the digital arch found in amniotes and frogs may be a derived developmental character of extant eu-tetrapods. We therefore conclude that the digitogenic pathway of most recent tetrapods may not be a guide to the developmental mechanisms for the transformation of fins to limbs. In particular, the digital arch may not be a defining feature of the autopodium.



Fig. 4. Modes of digit development, corresponding to the three proximal mesopodial elements in the tetrapod limb, which are the radiale/tibiale, the intermedium, and the ulnare/fibulare. (A) In almost all recent tetrapods most digits are derived from the digital arch which is emanating from the ulnare/fibulare. (B) One digit also can arise from the radiale/tibiale and is called the pre-pollex or pre-hallux respec-

tively. A pre-digit is a common feature of anuran feet, and is an occasional natural variant in newts (Rienesl and Wagner, unpublished, see Fig. 5) and some lizards. (\mathbf{C}) The third mode of digit development is connected to the intermedium and is seen in many extant urodele species (Blanco and Alberch, '92; Schmalhausen, '10; Vorobyeva and Hinchliffe, '96; Vorobyeva et al., '97).



Fig. 5. Cleared and stained hind limb of *Triturus sp.* Note the pre-hallux on the preaxial side (right). This is a rare variant in newts, but it can lead to a well formed digit with multiple phalanges, as in this case. This digit is most obviously derived from the tibiale, as seen by the close association with the element Y. (Photo by J. Rienesl)

Definition of the autopodium

According to our hypothesis the autopodium can be defined as the distal segment of a vertebrate paired appendage that consists of two types of elements, mesopodial elements, which are mostly nodular, and acropodial elements, which are an anterior-posterior series of small long bones (metacarpals, metatarsals, and phalanges). It is characteristic that the acropodial elements are separated from the zeugopodium by one or more rows of mesopodial elements. This is the only consistent morphological difference between fins and limbs, regardless of whether one interprets the acropodial elements as radials or not.

There are a few recent tetrapod groups in which the proximal mesopodial elements have been transformed into two long bones, resembling zeugopodial elements. These are the anurans and crocodilians, with elongated tarsal (Blanco et al., '98) and carpal (Rieppel, unpublished) elements, respectively. In both cases, the proximal tarsals/ carpals are true long bones with a bony collar and cartilaginous distal and proximal ends. These elements ossify together with the other long bones rather than with the other mesopodial elements. There is evidence that the transformation of the anuran tarsal elements represents a distal shift in the zeugo-autopodial border (Blanco et al., '98). Among the primates, elongated bones develop in the tarsus of galago (Otolemur) and tarsier (Tarsius), but there is no conclusive evidence as to the mode of ossification (Wagner and Chiu, unpublished). These transformations are complementary to the "mesopodialization," i.e., a possible proximal shift of the zeugo-autopodial border, observed in aquatic reptiles (Caldwell, '97). These exceptions are likely due to evolutionary variation in the zeugo-autopodial border, as suggested by Blanco and collaborators (Blanco et al., '98), and are not in contradiction with the definition of the autopodium as a configuration of mesopodial and acropodial segments.

Based on this definition of an autopodium, the critical questions regarding the origin of the autopodium are the following two: (1) What are the genetic and developmental mechanisms which establish the zeugo-mesopodial boundary? (2) Is the origin of these mechanisms also involved in the evolutionary origin of the autopodium? These questions are addressed in the following sections.

DEVELOPMENT OF THE AUTOPODIUM

A morphological innovation can be defined as a character(s) that is derived for a clade (autapomorphic), i.e., not present in the ancestor of a more inclusive clade (Müller and Wagner, '91). It follows that a morphological innovation is correlated with changes to an existing developmental program or creation of a new developmental pathway. Elucidating how the zeugopodial-mesopodial transition, which is the innovation in tetrapod limb evolution, arose requires a brief review of the genetic factors involved in limb development.

Tetrapod limbs originate from groups of cells in the lateral plate mesoderm and develop into mesenchymal buds surrounded by ectoderm (Searls and Janners, '71). As growth continues distally, part of the ectoderm thickens, forming the apical ectodermal ridge (AER). In amniotes the AER is essential for continued cell proliferation during limb bud growth. If the AER is removed, cell proliferation is reduced, leading to truncated limbs (Saunders, '48). However, not all tetrapods have an AER, for instance urodeles (Karczmar and Berg, '51) and the directly developing frog *Eleu*terodactlyus coqui (Richardson et al., '98). Most strikingly, removal of the distal ectodermal cup of the limb buds of urodeles and Eleuterodactlyus does not lead to an arrest of limb bud growth (Lauthier, '85; Richardson et al., '98). Furthermore, it is still a question of debate whether fish (e.g., zebrafish) have an "AER-like" structure (Geraudie, '78; Grandel and Schulte-Merker, '98). Recent genetic evidence, however, suggests that the fin fold has AER activity (Neumann et al., '99). A group of mesenchymal cells located in the posterior margin of the developing limb bud forms the zone of polarizing activity (ZPA), which controls sonic hedgehog (shh)-mediated patterning along the anterior-posterior axis (Saunders and Gasseling, '68; Riddle et al., '93). The mesenchymal cells at the distal end of the developing limb bud form the progress zone (PZ), where cell proliferation is maintained by signaling from the AER (Summerbell et al., '73), which in turn is maintained by the ZPA as development proceeds. This signaling mechanism provides cells of the PZ positional clues as they later develop most of the endoskeletal elements of the limb in a proximal to distal sequence (Summerbell et al., '73).

Several signaling molecules [e.g., shh, engrailed, retinoic acid, wnt, fibroblast growth factors (fgf), and transforming growth factors (tgf)] that are involved in developmental patterning and growth of the limb have been identified (reviewed in Schwabe et al., '98). In this article, we focus on evidence for the developmental autonomy of the autopodium and on the current evidence about the developmental origin of the zeugopodial-autopodial transition.

Evidence for the developmental autonomy of the autopodium

Three distinct phases of expression of the *Abd-B*-like HoxA and HoxD group 9–13 genes in developing chick and mouse limb buds have been described (Nelson et al., '96). In the first phase, group 9 (*Hoxa-9, Hoxd-9*) and 10 (*Hoxa-10, Hoxd-10*) genes are expressed uniformly in the mesoderm. Group 11 through 13 genes are not expressed in phase one. In phase two, the *Hoxd-9* through *Hoxd-13* genes are sequentially activated at the posterior-distal edge of the limb bud. With the exception of *Hoxa-13*, which is expressed only during phase three, the *Hoxa* genes are expressed uniformly in phase two.

During phase three, which corresponds to the stage in development when autopodium skeletal elements are formed, Hoxd-13 through Hoxd-10 are sequentially activated in reverse order, breaking the "temporal" and "spatial" colinearity rule (Nelson et al., '96). Transgenic experiments have shown that whereas expression of *Hoxd* genes in the early phases is regulated by several enhancer elements of each locus (Beckers et al., '96; Hoeven et al., '96) in the third phase, expression of all Hoxd genes is controlled by a single "global" enhancer (Hérault et al., '99). The phase three expression of Hoxa-13 depends on FGF secreted from the AER in the chick limb but FGF-4 soaked beads cannot activate *Hoxa-13* expression in phase two (Vargesson et al., 2001). This indicates that Hoxa-13 is under different control in phase three than in phase two. Interestingly, phase three expression of *Hoxa-13* appears earlier than that of *Hoxd*-13, suggesting that genes on the HoxA cluster may be "upstream" to genes on the HoxD cluster and that paralogous genes of the A and D clusters are under different regulatory controls during autopodial development (Nelson et al., '96). This is supported by analysis of mice mutant for posterior genes of the HoxD or HoxA clusters where loss of function alleles lead to polydactyly (HoxD) or loss of digits (HoxA) (Zákány et al., '97).

Hoxa-11/Hoxd-11 double knockout mice have relatively normal upper limbs and hands, but the long bones of the lower arm are reduced to nodular elements (Davis et al., '95). In contrast, Hoxa-13/Hoxd-13 double knockout mice have relatively normal upper and lower limbs, but their hands/ feet are severely abnormal (Fromental-Ramain et al., '96). Over-expression of Hoxa-13 in the chick wing leads to a loss of the long bone character of the ulna and radius (randomization of the orientation of mitosis of chondrocytes) coupled with the development of several small ectopic cartilages, reminiscent of mesopodial elements (Yokouchi et al., '95).

There is also evidence that chondrification of the mesenchymal condensations in the proximal limb bud or in the autopodial anlage is caused by different molecular mechanisms. Activin A is a member of the TGF β superfamily of growth factors (Stern et al., '95) which is antagonized by follistatin (DeWinter et al., '96). Activin A plays a role in chondrogenesis during digit formation (Merino et al., '99). This activity can be inhibited by follistatin treatment. Interestingly, activin A is not able to induce ectopic chondrogenesis in early stages of limb development. In addition, follistatin inhibits cartilage formation in the autopodium but not in the proximal regions of the limb bud (Merino et al., '99). This indicates that chondrogenesis is induced through a different molecular pathway in the autopodium than in the proximal parts of the limb.

Development of the zeugopodialautopodial transition

The expression domains of *Hoxa-11* and *Hoxa-*13 in mouse (Haack and Gruss, '93) and chick (Yokouchi et al., '91) are mutually exclusive, i.e., Hoxa-11 is restricted to the zeugopodium and Hoxa-13 is expressed only in the autopodium proper. The restriction of *Hoxa-11* to the zeugopodial-autopodial boundary has also been shown for Xenopus (Blanco et al., '98), supporting the hypothesis that this expression dynamic was already present in the most recent common ancestor of extant tetrapods. Distal displacement of the Hoxa-11 expression domain in chick limbs leads to a loss of the zeugopodial-autopodial transition (Mercanter et al., '99). These findings suggest that Hoxa-11 and Hoxa-13 are involved in determining the hand/foot field, i.e., the limit between the developing zeugopodium and the developing autopodium.

In striking contrast to tetrapods, the expression domains of *Hoxa-11* and *Hoxa-13* orthologs in the paired fin development in the teleost zebrafish are overlapping (Sordino et al., '95; Sordino and Duboule, '96). However, the situation is complicated by the recent discovery that zebrafish possess two HoxA clusters (a and b), each containing a group 11 (*Hoxa-11a*, b) and 13 (*Hoxa-13a*, b) gene (Amores et al., '98). In addition *Hoxa-11* is also expressed in the cells that enter the fin fold (Chiu and Pazmandi, unpublished), which is a cell population not found in limb buds. We argue in the following section that evolution of *Hoxa-11* and *Hoxa-13* regulation may have been a key step in the fin-limb transition.

GENETIC HYPOTHESIS FOR THE ORIGIN OF THE AUTOPODIUM

Two specific hypotheses have been put forth to explain the origin of the autopodium by a genetic mechanism. One is related to the maintenance of the progress zone and its associated interactions between the ZPA and AER (Thorogood, '91; Sordino and Duboule, '96) while the other focuses on Hox gene regulation in the autopodial anlage (Gerard et al., '93; Hoeven et al., '96).

The development of the distal parts of the tet-

rapod limb depends on the sustained activity of the progress zone, which in turn depends on the activity of the AER (at least in most tetrapods, see above). The AER in turn is dependent on a sustained interaction with the ZPA (see preceding discussion). Geraudie ('78) and Thorogood ('91) have proposed that the absence of distal endoskeletal structures in the actinopterygian fin is due to the premature cessation of AER-like activity because the ectoderm folds onto itself to become the fin fold. In support of this hypothesis, Sordino and Duboule (Sordino et al., '95) reported that the expression dynamics of sonic hedgehog (shh), a genetic marker of the ZPA, differ in developing fin and limb buds. In zebrafish, shh expression remains in a proximal location, which is consistent with the idea that fish lack a "distal" phase of ZPA-AER-like interaction. This could explain the absence of progress zone mediated growth and formation of distal endoskeletal structures. In tetrapods, in contrast, *shh* expression moves distally as the limb bud grows. From these observations, it has been hypothesized that the origin of the autopodium is due to a distalization of the ZPA.

The second hypothesis is based on the surprising discovery that the inverted co-linearity of HoxD-gene expression in the autopodium (Nelson et al., '96) is caused by a single enhancer element (Gerard et al., '93; Hoeven et al., '96). It is thus easy to imagine that the autopodial expression pattern of *Hox* genes resulted from a few mutations. Indeed, the acquisition of this enhancer element may have been a key step in the origin of the autopodium and may be responsible for the posterior to anterior direction of digital arch development. We evaluate these two hypotheses and propose a third, which is complementary, rather than alternative, to at least one of the proposals reviewed above.

The paleontological evidence reviewed in the second section indicates that the tetrapod limb is derived from the paired fins of sarcopterygian fishes. As reviewed previously, the closest known relative of tetrapods, the panderichthyids, only possess two distal endoskeletal elements, but all the other outgroups have many more, for instance *Eusthenopteron* and *Sauripterus*. From this observation we conclude that the origin of the autopodium is not coincidental with the first appearance of additional endoskeletal elements distal to the putative zeugopodial homologue in *Panderichthys*. Hence, the hypothesis of Thorogood, as well as those of Sordino and Duboule, may account for the stunted development of the actinopterygian fin, as exemplified by zebrafish. This mechanism, however, cannot account for the origin of the autopodium because the tetrapod limb is not derived from the actinopterygian fin. The appendages ancestral to the tetrapod limb possessed endoskeletal elements distal to the zeugopodium. Therefore it is not the lack of distal skeletal elements per se that accounts for the difference between a sarcopterygian fin and a tetrapod limb. Rather, the distal skeletal elements in the sarcopterygian fins do not form an autopodial configuration and are not obviously individualized from the proximal parts, as is the autopodium of tetrapods.

From this reasoning, it is possible that the origin of the global enhancer element in the HoxD cluster may have caused the origin of the digital arch and other osteological features specific to the autopodium. Interestingly, knockout phenotypes of AbdB-like genes from the A and D cluster of the mouse show that the deletion of D cluster genes leads to a polydactylous phenotype with fully formed but shortened digits, while the deletion of A cluster genes leads to digit loss (Zákány et al., '97). Zákány and coworkers have suggested that the autopodial enhancer acts downstream of Hoxa-13, which determines the distal part of the limb bud to become an autopodium and that the HoxD cluster gene function is phylogenetically derived relative to the functional role of Hoxa-13 in autopodium development. We therefore propose that the critical developmental change underlying the morphological innovation (zeugopodialmesopodial transition) is the origin of the genetic mechanism responsible for determining the autopodial field.

The developmental genetic evidence reviewed above indicates that the spatially exclusive expression of *Hoxa-11* and *Hoxa-13* is involved in the determination of the autopodial field. We therefore propose that the evolution of the Hoxa-11/ *Hoxa-13* expression pattern may be causally involved in the origin of the autopodium. This view is consistent with the hypothesis that the third phase of Hox gene expression is involved in the origin of the tetrapod limb (Zákány and Duboule, '99) but different from this idea in several respects. In particular it is clear that function of the 5 HoxD genes is not necessary for digit development (Zákány et al., '97). Further we assume that the *Hoxa-13* function is an apomorphic character of tetrapods rather than plesiomorphic, as suggested by Zákány and Duboule (Zákány et al., '97).

The expression patterns of *Hoxa-11* and *Hoxa-13* in forms basal to the tetrapod lineage are not

known. In the zebrafish pectoral fin bud, these genes have overlapping expression domains (Sordino et al., '95; and see preceding discussion). Interestingly, Hoxa-11 (the b paralog, Chiu, unpublished) and *Hoxa-13* (it is not yet clear which paralog) overlap completely in the distal part of the fin bud, but not proximally (Neumann et al., '99). Hence there is already a proximo-distal difference in the *Hoxa-11/Hoxa-13* expression with *Hoxa-13* being expressed only distally. But there is no local exclusivity of *Hoxa-11* and *Hoxa-13*. There is also some indirect evidence that Hoxa-13 is regulated by *shh* and hence by the ZPA of the fin bud. In the zebrafish mutant sonic you (syu), which is a loss of function mutation of the zebrafish ortholog of shh (Schauerte et al., '98), expression of *Hoxa-13* is lost in the fin bud (Neumann et al., '99). In addition, in syu mutants *Hoxa-11* is only expressed proximally but not in the region where it overlaps with *Hoxa-13* in the wild type. This could mean that in fish there is already a ZPA-dependent expression of *Hoxa-13*, just as in the tetrapod autopodium (Vargesson et al., 2001), but no distal suppression of Hoxa-11. It must be noted, however, that the regulation of Hoxa-13 by shh has not been demonstrated beyond reasonable doubt, since the lack of expression of *Hoxa-13* in *syu* mutants could also be due to the stunted fin bud development typical for these mutant phenotypes. Regardless of whether Hoxa-13 is directly regulated by shh (Neumann et al., '99) or by the FGFs from the AER (Vargesson et al., 2001), the zebrafish expression patterns suggest that the main genetic change necessary to establish a tetrapod like expression pattern may be *cis*-regulatory mutations at the *Hoxa-11* locus, leading to the derived status of distal repression of *Hoxa-11*.

The situation in zebrafish and other teleost fishes, however, is more complex because these taxa possess at least two copies (paralogs) of *Hoxa* genes of which there is only a single counterpart (ortholog) in tetrapods (Amores et al., '98). This fact has not yet penetrated the developmental literature since much of the expression data has been reported before the discovery of the additional gene copies in zebrafish and even some recent developmental articles do not take notice of this fact.

Another caveat in interpreting the zebrafish results is that it is not clear which cell types express *Hoxa-11* in the fin bud. Antibody staining in our lab (Chiu and Pazmandi) clearly shows that *Hoxa-11* is also expressed in the cells that enter the fin fold and presumably contribute to the development of the fin rays. In tetrapods there is no corresponding cell population. This makes the comparison of "expression patterns" between teleosts and tetrapods difficult since they may reflect expression in nonhomologous cell populations.

All these reasons make it imperative to move from zebrafish to nontetrapod species that represent more closely the character state ancestral for tetrapods. The candidates are the extant sarcopterygians, lungfish and coelacanth, as well as basal ray finned fishes like bichir and sturgeons. The closest relatives of tetrapods among extant taxa are the lungfish and a study on gene expression is difficult but technically possible. On the other hand the lungfish paired fin skeleton is quite different from that of osteolepiforms. Among extant forms the metapterygium most similar to that known form osteolepiforms is found in the sturgeon and the paddle fish (*Polyodon*; Mabee, 2000). Hence the closest relative to the tetrapods (the lungfish) does not necessarily represent the character state most similar to the ancestral situation. This implies that developmental data from both extant lungfish as well as several basal ray finned fish are needed to infer the ancestral genetic regulatory network. This recommendation, however, is complicated by the phylogenetic analysis by Cloutier and Ahlberg ('96), which implies that lungfish fins may represent the ancestral fin skeleton for the Tetrapodomorpha (Michael Coates, paper given at the meeting of the Society for Integrative and Comparative Biology in January 2001 in Chicago). Thus the similarity between the paddle fish metapterygion and that of the osteolepiforms might not reflect inheritance of an ancestral character state.

Predictions and implications

From a population genetics point of view the proposed transformation to locally exclusive Hoxa-11/Hoxa-13 expression patterns could only have been subject to natural selection if the transformation had phenotypic consequences. Indeed, an isolated ("abstract") change in positional information of gene products is not evolvable by natural selection. Consequently, the proposed hypothesis requires an ancillary assumption. Here, we propose a simple way in which change(s) in the expression of regulatory genes can lead to phenotypic effects that may affect fitness. In the case of *Hoxa*-11 and Hoxa-13, we assume that these genes interact with different downstream target genes in the fin bud. For simplicity, let us assume that Hoxa-11 and Hoxa-13 have completely nonoverlapping sets of target genes. This functional difference between Hoxa-11 and Hoxa-13 has no consequence when the expression domains overlap in the developing fin bud. If the expression domains of *Hoxa-11* and *Hoxa-13* segregate, however, the different target genes of Hoxa-11 and *Hoxa-13* are differentially expressed, potentially effecting immediate morphological consequences (Fig. 6).

This hypothesis implies a number of predictions that are, to some degree, readily testable. (1) The phylogenetic timing of the genetic and developmental changes coincide with the origin of the autopodium. This implies, for instance, that the most recent common ancestor of extant tetrapods



Fig. 6. Hypothesis about the genetic mechanisms for the origin of the autopodium. We assume that the ancestral state was one in which *Hoxa-11* and *Hoxa-13* had an overlapping expression domain, but already interacted with different downstream target genes. The derived state is the one seen

in amniotes today, in which these genes have a locally exclusive expression domain. The segregation of expression domains would have had immediate morphological consequences because of the different set of genes regulated by *Hoxa-11* and *Hoxa-13*.

had the same expression pattern of Hoxa-11 and Hoxa-13 as that described for mouse and chick. This can be tested by character state reconstructions of expression patterns on the tetrapod phylogeny. Furthermore, Hoxa-11 and Hoxa-13 expression domains are predicted to be overlapping in all outgroups to the tetrapods, i.e., the lungfish, the coelacanth, and primitive ray finned fish like the bichir (Polypterus) or Polyodon. (2) The molecular elements responsible for the tetrapod specific expression patterns of Hoxa-11 and Hoxa-13 are located on the HoxA cluster and are not present in upstream genes. In this context it is interesting to note that we found an association between the presence of a putative repressor domain in the HOXA11 protein and the derived expression pattern (Chiu et al., 2000b). Similar clade specific differences in the noncoding regions of Hoxa-11 and Hoxa-13 are expected (Chiu et al., 2000a). (3) Some of the downstream target genes of Hoxa-11 and Hoxa-13 in tetrapods and fish are the same. (4) The replacement of tetrapod limb enhancers with fish counterparts in a mouse will lead to a loss of autopodial differentiation. (5) The most critical prediction is also the most difficult to test. Changes in the expression pattern of these genes in an outgroup, such as the bichir or lungfish, will lead to partial autopodial transformation of the distal elements in fins. If all these predictions can be confirmed, we would conclude that Hoxa-11 and Hoxa-13 played a causal role in the fin-limb transition.

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