Full regeneration of the tribasal *Polypterus* fin

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Full limb regeneration is a property that seems to be restricted to urodele amphibians. Here we found that *Polypterus*, the most basal living ray-finned fish, regenerates its pectoral lobed fins with a remarkable accuracy. Pectoral *Polypterus* fins are complex, formed by a well-organized endoskeleton to which the exoskeleton rays are connected. Regeneration initiates with the formation of a blastema similar to that observed in regenerating amphibian limbs. Retinoic acid induces dose-dependent phenotypes ranging from inhibition of regeneration to apparent anterior–posterior duplications. As in all developing tetrapod limbs and regenerating amphibian blastema, Sonic hedgehog is expressed in the posterior mesenchyme during fin regeneration. Hedgehog signaling plays a role in the regeneration and patterning processes: an increase or reduction of fin bony elements results when this signaling is activated or disrupted, respectively. The tail fin also regenerates but, in contrast with pectoral fins, regeneration can resume after release from the arrest caused by hedgehog inhibition. A comparative analysis of fin phenotypes obtained after retinoic acid treatment or altering the hedgehog signaling levels during regeneration allowed us to assign a limb tetrapod equivalent segment to *Polypterus* fin skeletal structures, thus providing clues to the origin of the autopod. We propose that appendage regeneration was a common property of vertebrates during the fin to limb transition.

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Paired appendages (i.e., fins and limbs) are a common characteristic of most members of the subphylum Vertebrata, which includes chondrichthyans, actinopterygians, and sarcopterygians. Chondrichthyans encompass very ancient vertebrates such as sharks, and actinopterygians include all ray-finned fishes (e.g., teleosts), whereas the sarcopterygian class contains lobe-finned fishes and tetrapods. Appendages are supported by a cartilaginous endoskeleton that usually ossifies in actinopterygians and sarcopterygians. Appendages of different vertebrate species (extant and extinct) have distinct patterns of endoskeletal elements. Although the identity and homology of bony elements can be assigned with confidence in many cases, uncertainties arise when comparisons are performed between members of different classes or subclasses (1). In fishes, distal to the endoskeleton, there is a dermal extension supported by ossified fin rays (i.e., lepidotrichia) that usually comprises the entire web of the fin. It is known that the dermal skeleton disappeared during the evolution from tetrapod-like fishes to early tetrapods; however, the origin of the autopod, the most distal part of a limb, is still an area of controversy.

Polypterus has several primitive ancestral as well as derived features that historically have complicated its phylogenetic positioning and hence its classification. *Polypterus* shares several morphological and developmental characteristics with extinct early tetrapods and extant amphibians (e.g., paired lungs originating from the ventral foregut, external gills, the structure of the gill arches, stapes bone, spiracular openings, and pituitary gland) (2–4). In the description of its early development, J. S. Budgett mentioned the egg segmentation, movements during gastrulation, and neural fold development as being astoundingly frog-like (5). Recent molecular analysis places *Polypterus* as the most basal actinopterygian fish (6). The pectoral fin of polypteriformes and

other basal actinopterygians (Acipenseriformes, Semionotiformes, Amiiformes), as well as most Chondrichthyes and fossils of the earliest gnathostomes, can form a tribasal structure (7, 8). The endoskeleton of a tribasal fin is composed of three proximal skeletal structures named propterygium (anterior), metapterygium (posterior), and mesopterygium (middle). Different authors have postulated that the tribasal fin represents the basal condition for the gnathostome pectoral fin (1, 8).

The accuracy and reproducibility of amphibian limb regeneration has astonished many scientists since its discovery more than 200 y ago (9). After limb amputation and healing, a structure known as the blastema forms. From this structure limb growth begins and, after approximately 30 d, a complete limb with the exact pattern and size of the original is regenerated. This regeneration implies the formation of all bones along the proximo-distal (PD) axis of the limb: humerus/femur (or stylopod, the most proximal part of the limb), radio and ulna/tibia and fibula (or zeugopod, the middle part of the limb), and carpals, metacarpals, and digits/tarsals, metatarsals, and digits (or autopod, the most distal part of the limb).

The plesiomorphic and derived characters found in Polypteriformes make these fishes attractive subjects for performing evolutionary and developmental comparisons. In the present work, we evaluated the ability of the *Polypterus* fin to regenerate upon a quasi-complete amputation. We observed that *Polypterus* regenerates its fins with remarkable accuracy, only comparable to the regeneration observed in amphibian urodeles. The effects observed on fin patterning caused by increasing retinoic acid (RA) or altering Sonic hedgehog (Shh) signaling during regeneration supports similarities between the distal segments of the tetrapod limb and *Polypterus* fin.

Results

Bone Structure of the *Polypterus* Pectoral Fin. On the basis of the anatomical similarities between the larval fins of *Polypterus* and *Heptanchus* sharks, Budgett concluded that the large bone of the *Polypterus* fin corresponds to the metapterygium and the short bone to the propterygium (5). Proximally, the propterygium and metapterygium bones meet and articulate with a convex head bone that protrudes from the scapulocoracoid, whereas distally they articulate with small skeletal elements (Fig. 1A). The radials form a serial of transverse-aligned rod-like bones (approximately 13.6 radials, $n = 20$) forming a common axis of flexion and extension (Fig. 1A); each radial articulates in a one-to-one manner

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Fig. 1. Anatomy of the *Polypterus* fin. (A) Frontal view of alcian blue- and alizarin red-stained right fin and pectoral girdle, indicating the main fin skeletal components: cleithrum (cl), lepidotrichia (lp), mesopterygium (ms), metapterygium (mt), propterygium (pr), radials (rd), and scapulocoracoid (sc). (Scale bar, 5 mm.) Note the small bone element (*) that articulates to each of the large bone elements metapterygium and propterygium. Segments that may be equivalent to the autopod and zeugopod of tetrapods are also indicated (autopodial- and zeugopodial-like; *Discussion*). (B) Close-up of A to visualize the distal radials (drd) attached to each radial bone element. Posterior is to the bottom in A and to the left in B.

Role of RA in Pectoral Fin Regeneration. RA treatment during regeneration resulted in fin phenotypes characterized by the loss of distal structures. The severity of the effect depended on the stage at which blastema were exposed to RA (Fig. 3 and Table 1). Early treatments (3–6 dpa) with 100 $\mu\text{g/g}$ drastically reduced the

Role of *Shh* in Pectoral Fin Regeneration. Data reported recently demonstrate a correlation between the morphological diversity of limbs and fins and the evolutionary changes in the expression and regulation of the Sonic hedgehog gene (*Shh*) (15, 16). We detected *Shh* expression in the posterior region of regenerating *Polypterus* fins that extends and fades anteriorly below the regenerating fin fold (Fig. 4A and Fig. S3), which is similar to the expression pattern observed in fin buds of different fish embryos (16) or regenerating urodele limbs (17). To determine the role of *Shh* in fin regeneration and patterning, regenerates were exposed to cyclopamine, an inhibitor of *Shh* signaling, or Hh-Ag1.3, a hedgehog agonist. Treatment with cyclopamine (2 $\mu\text{g/mL}$) at early stages (1–6 dpa) impaired regeneration (Fig. 4B). All fins showed a severe reduction in the number of radials and lepidotrichia (20 of 20; Fig. 4C and E); the most-affected fins showed only one skeletal element and no lepidotrichia (Fig. 4C, upper fin). Treatment at late stages (9–12 dpa) with a lower dose (1 $\mu\text{g/mL}$) resulted in narrow fins with reduced number of radials (19 of 19; Fig. 4D and E). Maintaining a fixed concentration of cyclopamine (2 $\mu\text{g/mL}$) from different regeneration stages on fins of *Polypterus ornatipinnis*, we obtained stage-dependent sequential radial reduction phenotypes (Fig. 4H). Regeneration upon

On the other hand, treatments with Hh-Ag1.3 from early stages (2 dpa) of fin regeneration produced dose-dependent phenotypes characterized by broader fins with increased number of radials and lepidotrichia (100 nM, Fig. 4 *E* and *F*; 200 nM, Fig. 4*G*). It is worth mentioning that, despite the consistent increase in the number of radials with this Shh agonist, we never observed phenotypes resembling AP duplications. The Shh agonist did cause an effect on tail fin lepidotrichia regeneration, but their number appeared unchanged (Fig. 5 *A* and *C*).

deles has attracted a lot of attention because of its astonishing precision, only comparable to the development of an organism. Patterning, cellular components, and size are perfectly regulated, such that a fully functional limb develops. Relevant advances regarding the development of the regenerated limb have been reported; however, the basis of why certain species are able to regenerate their limbs is still unknown. Relevant questions to answer are why/how this property was gained and why/how it was lost. The ability to regenerate appendages does not seem to be a property of early members of the vertebrate lineage because the paired fins of sharks are unable to regenerate (18). As shown here, *Polypterus* regenerates its pectoral lobed fins with remarkable accuracy, reminiscent of limb regeneration in urodele amphibians, fulfilling the concept of epimorphic regeneration. Even though we used small fishes for most of the experiments described, *Polypterus* is able to rebuild the complex structure of its lobed fins at sizes approximately 35 cm long (close to the reproductive stage), when fins are larger than an adult *Ambystoma* autopod and the lobulated section is at least 2 cm wide. To our

Members of the hedgehog family have key functions in the development of all animals. In the limb of vertebrates, Shh is the essential component of the zone of polarizing activity. The phenotype of mouse limbs carrying null *Shh* alleles shows distal abnormalities, characterized by a zeugopod with a single bone, possibly the radius, and a reduced number of digits, leaving what seems to be digit one (28, 29). Similarly, cyclopamine induces a posterior to anterior digit loss in a dose-dependent fashion in

Fig. 4. Fin regenerates treated with cyclopamine and Shh agonist. (A) Frontal view of a 10 dpa right fin regenerate hybridized with an Shh probe (same position as Fig. 1A). Arrows point to the cleithrum bone and scapula. (B) Ventral and dorsal view of control (Top and Bottom Left, respectively) and cyclopamine-treated (Middle and Bottom Right, respectively) pectoral fins after 35 d of regeneration. (C) Skeletal staining of early regenerates treated with 2 μ g/mL cyclopamine (Cyc). Right: Magnifications of fins shown at Left. Arrowhead marks the limit of the anterior (to the right) region affected. (D) Skeletal staining of late regenerates treated with 1 μ g/mL cyclopamine. (E) Skeletal staining of an early regenerate treated with DMSO (Control). (F) Skeletal staining of regenerates treated with 100 nM Hh-Ag1.3. (G) Skeletal staining of a regenerate treated with 200 nM Hh-Ag1.3. Arrowhead marks the AP limit at which the posterior region appears more affected. (H) Skeletal staining of left fin regenerates and pectoral girdles of *P. ornatipinnis* after treatment with 2 μ g/mL cyclopamine at 8, 10, 12, and 14 dpa and then left regenerating for 3 wk; control is an untreated fin regenerated for 42 d. Note the sequential reduction of the mesopterygial plate and lepidotrichia, with minor disturbances of propterygium and metapterygium bones. **Materials and Methods** provides details about treatment protocols. a, anterior; cl, cleithrum; d, dorsal; p, posterior; sc, scapulocoracoid; v, ventral.

regenerating axolotl limbs (15, 30), phenocopying the autopod abnormalities observed in the Shh mutant mice. On the other hand, ectopic Shh added to the anterior region of developing chick limbs produces extra digits with a mirror digit identity (31), and ectopic expression of *Shh* (32) or treatment with a Smoothed agonist in axolotl regenerating limbs does not induce AP duplications but causes extra digits, carpals, and tarsals to form. In addition, lack of Gli3, a fundamental target of Shh signaling in the limb, produces polydactyly (28). From these data, it has been concluded that Shh regulates the patterning and number of distal bone elements of the limb, starting from the zeugopod. In *Polypterus*, inhibiting or activating Shh signaling during fin regeneration affected the number of radials, in parallel with the effects observed in the number of digits in terrestrial vertebrates, including the posterior–anterior responsiveness to Shh dose.

Homology Between *Polypterus* Pectoral Fin and Tetrapod Limbs: A Proposal with Evolutive Implications. The detailed comparison of *Polypterus* fin bone elements with radius/radiale, ulna/ulnare, and intermedium, the mesopodial most conserved elements in tetrapod-like fishes and extinct and extant tetrapods, is difficult

Fig. 5. Effects of altering Shh signaling on regeneration of fin tail lepidotrichia. (A) Alizarin red staining of control (DMSO-treated) tail fin. (B) Tail fin treated with 2 μ g/mL cyclopamine. (C) Tail fin treated with 100 μ M of Shh agonist. (D) Regeneration of tail fin before and 3 wk after cyclopamine withdrawal. Fin pictures were taken at 3 wk after amputation, except for the picture in D. Cyclopamine impairs the regenerative process (A and B), but it is resumed after cyclopamine withdrawal (D). The Hh-Ag3.1 agonist did not affect the number, but the morphology of the regenerating tail fin lepidotrichia showed a zigzag pattern (C).

to address without bias. According to the bone arrangement described here, the large and small bone elements (metapterygium and propterygium, respectively) may lie at an equivalent segment as the tetrapod radius/ulna and radiale/ulnare (Fig. 1A); the nodular bone observed between these long bones can be homologized with the intermedium (Fig. 1A). In addition, the articulation between the small skeletal elements (radials) and the propterygium and metapterygium, forming a common axis of flexion and extension, might constitute a “primordial wrist.” In several early tetrapods, wrist bones are almost absent, and it seems that the evolution of ankle and wrist joints lagged behind the formation of digits (1). Currently, the presence of more than five digits (hence, metacarpals) is accepted as a plesiomorphic character of the autopod (33); in *Polypterus*, approximately 12–14 radials are commonly found. In concordance with an equivalence between the distal *Polypterus* fin and the autopod (Fig. 1A), it has been noted that the only elements of the fin whose development resembles that of the autopodial elements are the distal radials, a cartilaginous structure similar in appearance to distal phalanges (34).

On the basis of the similarities described above and the observations resulting from altering RA and Shh signaling during *Polypterus* fin regeneration, radials would seem equivalent to metacarpals of the tetrapod autopod (see below), and the propterygium and metapterygium seem equivalent to the zeugopod bone elements (autopodial- and zeugopodial-like, respectively; Fig. 1A). The homology proposed is in concordance with recent data showing that treatment of skate embryos with RA provokes the expression of *Shh* in the anterior region of fins and consequently the propterygium acquires the identity of the metapterygium (16). This change in identity resembles the transformation of radius to ulna of tetrapod limbs treated with RA, Shh, or through transplantation of the polarizing zone (31, 35, 36).

A corollary of the equivalence of radials to metacarpals is that the autopod is an ancient structure that existed before the emergence of tetrapods. This contrasts with a commonly accepted view in which the autopod is considered a structure appearing de novo in tetrapods. The proposed primitive origin of the autopod is in agreement with the autopodial-like *Hox* expression pattern

recently determined in *Polyodon spathula* fin, another basal actinopterygian fish (37), and in *Scyliorhinus canicula*, a member of the most basal lineage of jawed vertebrates (38). Also, *Hoxd13* expression in the Australian lungfish closely matches the late expression patterns observed in the tetrapod autopod (39). These data together suggest that fin radials and tetrapod digits may be patterned by shared mechanisms that are distinct from those patterning the proximal fin/limb bone elements. This gene expression pattern homology has led to the deep homology concept in which a specific gene regulation mechanism is used to build structures with no evident phylogenetic or morphologic relationship (40). Thus, shared gene expression patterns may not necessarily indicate structural homology or evolutionary origin (41). In the present report we observed that comparative analyses of the RA and Shh responses provide additional clues to understand appendicular skeleton evolution. Thus, in terms of deep homology, we must consider, in addition to shared genetic programs, shared responses of developing/regenerating structures to morphogens. The remarkable ability of *Polypterus* to regenerate their pectoral fins will allow a detailed comparative analysis of gene expression patterns in combination with growth factor responses and, in this manner, to experimentally test whether the tribasal structure of *Polypterus* is the ancestor of the tetrapod zeugopod and autopod.

Materials and Methods

Whole-Mount Skeletal Preparations. Commercially obtained juvenile bichirs, *Polypterus senegalus* and *P. ornatipinnis*, ≈ 6 cm long shortly after gill reduction phase (2), were used for the experiments. Fishes were maintained in glass tanks at 28 °C. Amputations were performed with nail clippers after anesthesia by immersion in cold water containing a 0.1% tricaine solution. Amputations were performed closer to the body. Bichirs were killed with an overdose of tricaine; the fins were dissected, fixed and, dehydrated overnight (ON) in 100% ethanol. Fins were incubated in acetone for 24 h and then stained for 3 h at 37 °C plus ON at room temperature in alcian blue and alizarin red in 70% ethanol with 5% acetic acid. Fins were rinsed in tap water before clearing in 1% KOH and 20% glycerol for 24 h and then in graded glycerol.

Histology. Regenerates were fixed in modified Karnovsky for 5 h at 4 °C and then washed ON in sodium cacodylate buffer at 4 °C. After a postfixation in 1% osmium, samples were dehydrated in graded ethanol series and embedded in Epon. Half-micrometer sections were stained with toluidine blue and visualized by bright-field microscopy.

Whole-Mount in Situ Hybridization. Fins were fixed ON with MEMPPA [0.1 M Mops (pH 7.4), 2 mM EGTA, 1 mM MgSO₄, 4% paraformaldehyde]. The in situ

hybridization protocol used was based on that described by Nieto et al. (42), with two major modifications: proteinase K treatment was extended up to 45 min for large pieces comprising the cleithrum/scapulocoracoid bones, and the last wash in TBST [for 1 L of 10 $\times$ TBST: 8 g NaCl, 0.2 g KCl, 25 mL of 1 M Tris-HCl (pH 7.5), 10 mL Tween 20] before signal development was performed for 48 h. Although in general signal development was performed for less than 24 h, occasionally it was necessary to extend it for 48 h; in these latter cases, control probes did not show any signal (Fig. S3). *Polypterus* Shh probe was obtained by nested RT-PCR using total RNA from regenerating fins and the first-choice RLM-race Kit (Ambion). After cDNA synthesis, we performed the first amplification round using the 3'-RACE outer primer and a specific Shh oligonucleotide 5'-GAGAAGACCTAGGGGCCAGCGCAGA-3' (based on nucleotide sequence identity among different species including mouse, chick, zebrafish, and paddlefish). In the second round of amplification the following oligonucleotides were used: 5'-GGGCCAGCGGAGATACGAG-3' and 5'-TAGACCCAGTCAAATCCAGC-3', which encompass codons for amino acids 23–136 of mouse Shh. The 348-bp fragment obtained was cloned in pTopo2.1 (Invitrogen) and the identity determined by sequencing (GenBank accession no. HM190156).

RA, Cyclopamine, and Shh Agonist Treatment. Bichirs were transferred on small glass containers with 100 mL of water. Cyclopamine (BIOMOL) was administered by adding it directly to water at 2 μ g/mL or 1 μ g/mL final concentration from a 5 mg/mL stock in DMSO. Hh-Ag1.3 (Curis) was used at 100 and 200 nM from a 1-mM stock in DMSO. Treatments with a commercially available Smoothed agonist SAG (Calbiochem) at 200 nM final concentration gave similar effects. Equivalent amounts of DMSO were added to controls. Treatments were performed for 3 wk starting at 1–9 d (early stages) or 12–18 d (late stages) after amputation. Water containing cyclopamine or Hh-Ag1.3 was changed every day during treatment. After treatment, regeneration was allowed to continue for a further 3 wk, before fishes were killed and fins dissected.

The protocol for RA injection was as reported for urodeles (14). All-trans-RA (Sigma) dissolved in DMSO (15 mg/mL) was injected i.p. (100, 75, or 50 μ g/g of body weight), on day 3 after amputation. For time-dependant studies, subsequent injections were done at 6 and 9 d after amputation with 100 μ g/g of body weight.

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