PERSPECTIVE

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Conserved mechanisms regulate outgrowth in zebrafish fins

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Regulation of size is one of the fundamental problems in biology. One general strategy has been to identify molecules required for cell growth and cell proliferation within an organ. This has been particularly revealing, identifying cell-autonomous pathways involved in cell growth, survival and proliferation. In order to identify pathways regulating overall limb growth and morphology, experiments have evaluated gene expression, transplanted and removed tissues, and knocked out genes. This work has provided a vast amount of information identifying molecular mechanisms regulating limb axis formation, outgrowth, and pattern formation. Using the zebrafish fin, genetic, cellular and molecular strategies have also been employed to follow both normal patterns of fin growth and growth in fin mutants. This review will focus on cellular and molecular regulation of the outgrowth and patterning of the zebrafish caudal fin during regeneration, and will emphasize similarities to other systems. Future perspectives describe opportunities using the zebrafish fin to reveal mechanisms underlying the regulation of final size.

Introduction

What's the difference between a *Drosophila* leg or wing, a mouse limb, and a zebrafish fin? Differences in structure, form and size are obvious, and they often provide insights into the diversity of gene expression and function. An equally interesting, but perhaps less intuitive, question is, "What's the same?" Decades of research in limb development reveal striking similarities at the gene level, providing insights regarding the conservation of molecular interactions and cellular events during the development of these key structures. This is especially interesting as appendages in the fly and the mouse are nonhomologous structures, apparently having evolved independently[1]. *Drosophila* limbs (wings and legs) arise from outpockets of epithelium derived from ectoderm, termed imaginal discs. In contrast, skeletal elements in the mouse and chick limbs are derived from the mesoderm. Still, it is evident that these and other animals use an evolutionarily conserved set of genes during limb growth and patterning[1–3]. Members of the fibroblast growth factor (Fgf) family, hedgehogs, and members of the wingless/Wnt family are secreted morphogens that participate in extracellular signaling events and are found across animal species[4]. Signaling through each of these pathways is important during limb development, and cross-talk often occurs between them.

Different model systems for limb development provide both unique and common contributions to the field of limb development. *Drosophila* genetics has revealed numerous genes and pathways regulating axis formation, patterning, and autonomous control of cell proliferation. The chick embryo may be visualized and physically manipulated *in ovo*, permitting the identification of inductive and receptive signaling areas within the limb. The mouse was the first vertebrate model organism where roles of specific genes could be determined by mutant or knockout mice. Moreover, comparative analyses of gene expression between the mouse and chick added a powerful descriptive tool demonstrating the substantial similarities in limb growth between these two vertebrates. A comprehensive understanding of the molecular signaling pathways during the early developmental events of the *Drosophila* leg or wing therefore contributes significantly to our understanding of the underlying mechanisms during early mouse limb development, and vice versa. This provides reason to believe that continuing to probe the molecular events of limb development in multiple species is worthwhile and will substantially increase our comprehension of both conserved and unique aspects of these complex processes.

More recently, studies of limb growth have been initiated using the zebrafish fin, contributing to the base of knowledge in this broad area of limb growth. Genetic mutations in zebrafish are easily generated using a variety of strategies, and zebrafish require less space and incur lower costs than other vertebrates, permitting the routine completion of large-scale genetic screens[5] and facilitating use of the zebrafish as a model system[6]. Indeed, standard chemical and retrovirus-based screens have identified adult-viable fin length mutations[7,8]. Additionally, overcoming the potential problem of lethality when genes involved in limb growth are inactivated, temperature-sensitive screens for fin regeneration have been fruitful[9,10]. Even before the zebrafish was developed as a model organism, morphological and cellular movements were described in regenerating fins of the ray-finned fishes[11,12]. Conversely, the majority of molecular details have been provided only recently, owing largely to the identification of several new mutations affecting the process of fin regeneration and to recently developed methods of manipulating gene function *in vivo* (**Box 1**). This Perspective will review the current literature describing cellular and molecular pathways required for fin growth, highlighting the similarities with signaling pathways regulating growth in other organisms.

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Regeneration recapitulates ontogeny

The zebrafish fin, among other zebrafish tissues such as the heart, spinal cord and retina, has the capacity to regenerate, or to rapidly replace lost tissue. Many researchers opt to evaluate mechanisms underlying fin growth during regeneration, because the timing may be controlled, fin surgeries are facile, and the growth rate is three to five times faster than during ontogenetic growth. In cases where gene expression has been evaluated during both ontogeny and regeneration, expression patterns are similar (albeit at higher levels, reflecting the more rapid rate of growth, during regeneration), suggesting that molecular mechanisms regulating cell proliferation and differentiation are comparable[13–15]. For these reasons, much of what is known about the molecular aspects of fin growth has been revealed during regeneration. Thus, in this review, the mechanisms underlying fin regeneration will be considered to represent ontogeny.

The fin is comprised of an endoskeleton (inside the body wall) and an exoskeleton comprised of many segmented fin rays (the visible fins; **Fig. 1a**). Only the fin rays have the ability to regenerate. The caudal fin is comprised of 16–18 fin rays, and each fin ray grows autonomously by the addition of bony segments at the distal end of the fin (**Fig. 1a**). The fin ray consists of an inner core of mesenchymal cells, blood vessels and nerves, protected by hemirays of bone and surrounded by an epithelium[16] (**Fig. 1b**). When a ray is amputated, epithelial cells migrate to cover the wound, forming the apical epidermal cap (AEC). A specialized mesenchymal structure called the blastema develops directly beneath the AEC and is populated by mesenchymal cells originating in the stump tissue[17] (**Fig. 1c**). Blastema formation is required for epimorphic (that is, dependent on cell proliferation) regeneration, and is likely to distinguish the robust regenerative process in salamanders, newts and zebrafish from the poor-quality regeneration in mammals.

In the immature blastema cell proliferation is slow, with the G2 cell cycle stage having a median length of >6 h. Within 24 h after amputation, the blastemal cells segregate into two morphologically indistinct compartments[18] (**Fig. 1d**). The cells residing in the distalmost 50 μm are referred to as the distal blastema. This population proliferates very slowly, providing the direction for outgrowth and contributing daughter cells to the proximal blastemal, located in the next 200 μm. The latter population proliferates rapidly (median G2 of 30 min), and recently divided cells migrate to new positions and ultimately differentiate to replace the amputated tissue[18]. During this time, the fin grows at a rate of ~1 segment (~200 μm) per day, on average.

The goal of research in the area of fin regeneration, and indeed, in all models of limb growth, is to identify the signaling pathways required to regulate each of these events. Specifically, it is of interest to identify the molecules responsible for establishing the distal and proximal compartments in the blastema (axis formation), regulating cell proliferation in the proximal blastema (outgrowth), and guiding differentiation into the required cell types (patterning or pattern formation). During limb growth in vertebrates, an essential first step is the establishment of 'signaling centers', localized areas in the limb bud that contribute to the mechanisms regulating outgrowth and pattern formation by secreting signaling molecules such as Fgfs, Wnts, transforming growth factor (TGF)-βs or bone morphogenetic proteins (BMPs), and hedgehogs (Hh). In the zebrafish, gene profiling studies have shown that the expression levels of these and other signaling molecules are regulated during the course of regeneration[19]. Combining such analyses with the gene localization studies already in progress will provide insights into the network of signaling events required during outgrowth.

Box 1 Strategies for the manipulation of gene function during fin growth

Mutations. The most traditional method mutagenizes the spermatogonia of male fish with a chemical such as *N*-ethyl-*N*-nitrosourea, resulting in both loss-of-function and gain-of-function mutations. More recently, retroviral insertion, mobilization of transposable elements, or TILLING (targeting induced local lesions in genomes) methods have been developed in order to eliminate gene function more reliably. Traditional and newly developed methods for mutagenizing zebrafish are reviewed in ref. 5.

Transgenics. Germline transformation of linear DNA containing genes of interest has been accomplished. Typically, genes of interest are expressed under either their own promoter or a heat shock promoter (often the hsp70 promoter) for inducible overexpression. The fluorescent protein EGFP is included to identify transgenic fish. For examination of Fgfr1 function, a dominant negative Fgfr1 transgenic fish has been created (hsp70:dnFgfr1-EGFP)[21]. For examination of Wnt–β-cat function, several transgenic fish have been created[23], involving overexpression of the secreted inhibitor of Wnt–β-cat signaling Dikkopf1 (*hsp70:dkk1-EGFP*); overexpression of a dominant negative Tcf3, resulting in inhibition of Wnt–β-cat target genes (*hsp70:dntcf3-EGFP*); and overexpression of the Wnt molecule Wnt8 (*hsp70:wnt8-EGFP*).

Antisense-mediated gene knockdown. Morpholino-modified antisense molecules have been used extensively for transient knockdown of gene function in embryos. Thummel *et al.*[25] recently developed a method that permits uptake of 'morpholinos' during fin regeneration. Therefore, rapid evaluation of the loss of gene function in the adult fin is now possible.

Ectopic gene expression. Injection of plasmid DNA containing genes of interest results in uptake and gene expression in cells of the regenerating fins[28] and is another valuable method for evaluating gene function. Classic studies in limb development used protein-soaked beads to monitor similar effects of ectopic gene function.

Pharmacological application. Some small-molecule drugs are known to inhibit particular signaling pathways and may be applied to fins during growth or regeneration. Drugs are typically applied in fish water. The drug SU5402 blocks Fgfr pathway signaling[20], and cyclopamine inhibits Shh function[28]. Nonautonomous effects of the drugs may be evaluated in independent gene studies once a pharmacological effect has been observed.

Chemical genetics. The zebrafish embryo is used for the study of whole-body screening for drug discovery, with particular emphasis on the zebrafish as a model system for the study of human disease[6]. Several embryos may be tested in a single well of a microtiter dish, permitting high-throughput analysis and small drug volumes. Further, toxicity may be tested in coordination with screening for particular phenotypes. Similar applications may be used for fin studies in the future (that is, either for the identification of small molecules that perturb fin growth or for the identification of enhancers or suppressors of particular signaling pathways).

Establishment of signaling centers in the fin

Fgf signaling in the distal blastema and basal epidermis. The Fgf receptor Fgfr1 mediates various signaling events during fin regeneration (**Fig. 2**) and is required for the early establishment of two signaling centers in the regenerating fin: the distal blastema and the lateral basal epidermal layer[10,14,18]. In particular, the distal blastema serves as a signaling center regulating cell proliferation in the proximal compartment and is demarcated by expression of *msxb*[10,18]. Inhibition of Fgfr1 function also prevents specification of the second signaling center, the lateral basal epidermal layer[20]. This center is required for bone cell differentiation (patterning) and for continued fin outgrowth, and is represented by expression of sonic hedgehog (*shh*). The Fgfr1-positive cells of both signaling centers are nonproliferative, as parallel signaling pathways inhibit the mitogenic Erk serine/threonine kinase signal transduction pathway[21,22].

Inhibition of Wnt–β-catenin (β-cat) signaling (**Box 1**) also prevents the early specification of the distal blastema and the lateral basal epidermal layer, probably by acting through Fgfr1 (ref. 23), (**Fig. 2**). This is evidenced by the failure to express Fgfr1 target genes when Wnt–β-cat signaling is inhibited. Interestingly, the Wnt–β-cat pathway also appears to function downstream of Fgfr1: a mutant in at least one Fgfr1 activating gene loses expression of the Wnt–β-cat target *lef1* (ref. 24), (**Fig. 2**). Reciprocal signaling between the Fgf and Wnt–β-cat pathways is therefore integral to the establishment of the distal blastema and specification of the lateral basal epidermal layer, and therefore to continued outgrowth.

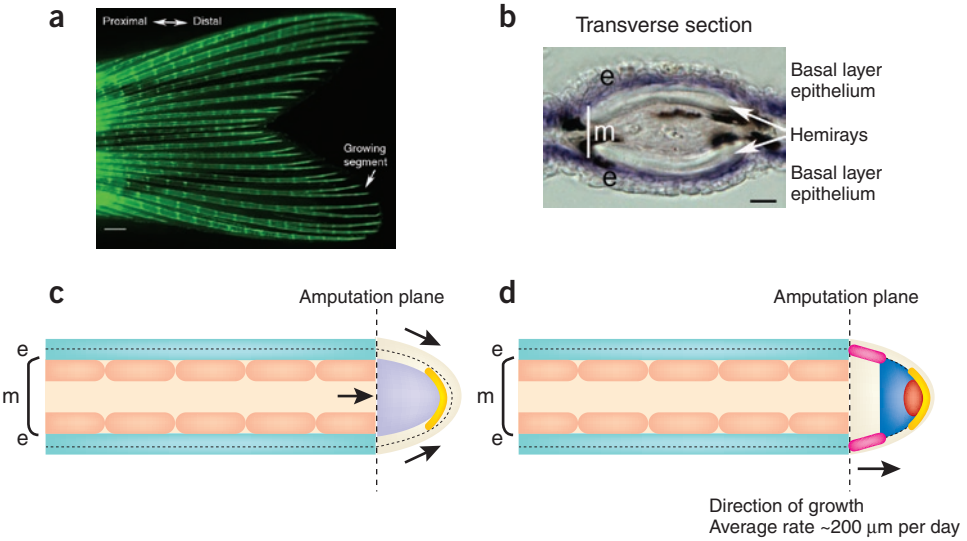

Figure 1 Tissues and structures in the zebrafish fin. (**a**) Zebrafish caudal fin stained with calcein so that bony fin rays are visible. Scale bar, 200 μm. (**b**) Transverse section through a single fin ray. Hemirays (white arrows) are visible as crescents of bone surrounding mesenchymal tissue (fibroblasts, undifferentiated cells, artery, nerve, melanocytes). The bony rays are surrounded by an epithelium, and the basal layer of the epidermis has been labeled (purple stain and arrowheads). Scale bar, 15 μm. (**c**) Cartoon of a longitudinal section of a single amputated fin ray after formation of the immature blastema. The basal layer of the epithelium is indicated by the dotted line. Cells from the epithelium (light blue) cover the wound (side arrows), forming the wound epidermis and apical epidermal cap (AEC). Cells from the stump mesenchyme migrate distally (center arrow), forming the immature blastema (purple). (**d**) Cartoon of a longitudinal section of a single fin ray following blastemal reorganization. The basal layer of the epithelium is indicated by the dotted line, the AEC in yellow. The distal blastema (red) is distal to the proximal blastema (dark blue). Proliferating cells from the proximal blastema will migrate laterally, toward the basal epidermal layer (pink), and differentiate as bone forming cells. e, epidermis; m, mesenchyme.

The distal blastema is required for cell proliferation. Any treatment that perturbs *msxb* expression also prevents cell proliferation in the proximal blastema and prevents further fin outgrowth[20,23–26], suggesting that *msxb* is not simply a physical marker for the distal blastema but also a functional marker. Indeed, targeted gene knockdown of *msxb* prevents cell proliferation and fin outgrowth, indicating that *msxb* function directly or indirectly regulates cell division in the proximal cells of the blastema[25].

In addition to the Wnt–β-cat and Fgfr1 signaling pathways described above, at least one Fgfr1-independent mechanism also appears to regulate *msxb* expression, and therefore outgrowth (**Fig. 2**). Ectopic expression of chordin, which inhibits BMP function, results in reduced expression of *msxb* and in reduced cell proliferation[27]. Multiple BMP pathways are required for fin regeneration (see below and **Fig. 3**). However, only *bmp4* is expressed in the distal blastema[27], indicating that it may be a candidate for the regulation of *msxb* expression. Taken together, signaling by Wnt–β-cat, Fgf and Bmp is required for function of the distal blastema, and therefore for subsequent cell proliferation and fin outgrowth.

Hedgehog signaling regulates patterning and proliferation

The *shh* gene is upregulated in the lateral basal epidermal layer, surrounding the area where new bone will develop (**Fig. 3**). Genes acting downstream of *shh* are also induced both in the basal epidermal layer and in the mesenchymal cells underlying the *shh*-expressing cells. Indeed, ectopic expression of a downstream target of Shh, *bmp2b*, leads to an upregulation of early osteoblast transcription factors and other genes required for osteoblast differentiation[27] (**Fig. 3**). Further, ectopic expression of *shh* or *bmp2b* between fin rays results in the formation of ectopic bone, indicating that either gene is sufficient for bone development[28]. Thus, Shh activity appears to be mediated directly by Bmp2b.

Interestingly, knockdown of Shh function using the pharmacological inhibitor cyclopamine does not perturb bone formation[28]. This is somewhat unexpected, as Hedgehog molecules in other systems are required for normal differentiation and patterning. It suggests instead that redundant pathways for bone formation are in place in the regenerating fin, probably working through Bmps. For example, one possibility is that the expression of one or more *bmp* genes is regulated in both Shh-dependent and Shh-independent manners. Indeed, chordin-mediated inhibition of BMPs prevents expression of genes involved in osteoblast differentiation, development of mature osteoblasts, and mineralization[27] (**Fig. 3**).

Inhibition of Shh function does result in reduced *msxb* expression and reduced cell proliferation[28], suggesting that maintenance of the basal epidermal layer is also required for maintenance of the distal blastema. The molecular pathway regulated by Shh is unknown. One possibility is that reciprocal signaling occurs between Shh in the basal epithelium and Fgfr1 signaling in the distal blastema. Abrogation of Shh activity may thereby result in reduced *msxb* expression and

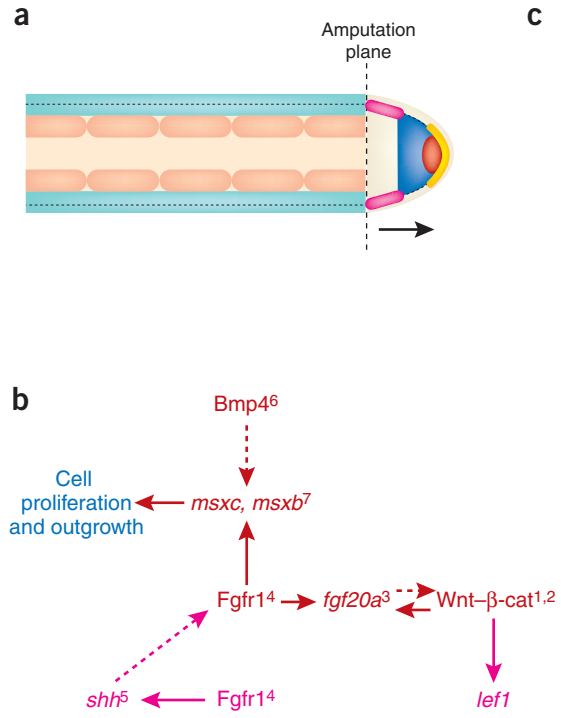

Figure 2 Establishment of signaling centers and initiation of outgrowth. (**a**) Cartoon of longitudinal section described in **Figure 1d**. (**b**) Molecular pathways required for establishment of distal blastema, lateral basal epidermal layer and cell proliferation and outgrowth. Tissue of expression is color-coded to match that in **a**; superscripts refer to list in **c**. Dotted arrows indicate signaling events that seem to occur but are not clearly defined. (**c**) Genetic or chemical modifiers of the molecular mechanism shown in **b** and their effects on gene expression, outgrowth, or both.

coincident failure to maintain cell proliferation during outgrowth[28] (**Fig. 2**). Such a mechanism may facilitate the coordination of outgrowth and patterning.

Similarities to vertebrate and invertebrate limb outgrowth

In mouse or chick limb development, a protrusion of cells called the limb bud forms initially. The bud is comprised of mesodermally derived cells covered by an epidermis (**Fig. 4a**). The apical ectodermal ridge (AER) refers to a morphologically discrete region of epithelium at the distalmost end of the limb bud and is required for continuous proximal-distal outgrowth of the limb and for the establishment of a second relevant signaling center in the posterior mesoderm, the zone of polarizing activity (ZPA). ZPA function is required for patterning along the anterior-posterior axis, and is mediated by Shh activity. Early in limb development, Wnt–β-cat signaling is required upstream of Fgf signaling for the formation of the AER[29], similarly to the early requirement for Wnt–β-cat signaling in the zebrafish fin (**Fig. 4a**,**b**). Continued signaling through Fgfs is required for the establishment of *Shh* expression in the ZPA and therefore for skeletal patterning[22]. Therefore, as in the fin, Fgf activity contributes to the establishment of the *Shh*-based signaling center. Furthermore, Shh signaling is required for the maintenance of Fgf activity in the AER and continuing cell proliferation and limb outgrowth[30]. Indeed, while in the zebrafish direct evidence for reciprocal signaling between the distal blastema and basal epidermal layer is lacking, it is clear that signaling from both areas is required for continued proliferation and outgrowth.

Hh molecules mediate anterior-posterior patterning and outgrowth in the limbs of the mouse, chick, *Drosophila*[3], and also zebrafish. Recall that during zebrafish fin regeneration, *shh* is upstream of *bmp2b*, and either gene may induce ectopic bone formation. Similarly, in mouse and chick the effects of *Shh* in the ZPA are mediated by *Bmp2*, as *Bmp2* can be induced by ectopic expression of *Shh*[2,3]. However, in mouse and chick limb development this interaction may be indirect. For example, ectopic expression of *Shh* in the anterior limb bud (the area opposite that where *Shh* is typically expressed) leads to mirror-symmetric duplications of skeletal elements, while loss of *Shh* leads to limb truncations. In contrast, ectopic Bmp2 itself is not sufficient to induce duplications, suggesting that Shh function is not mediated directly by Bmp2.

In *Drosophila*, Hh is typically expressed on the posterior side of the leg and wing imaginal discs, activating Dpp (a TGF-β molecule in the same family as the BMPs) in the cells along the border of the anterior and posterior regions (**Fig. 4c**). As in the mouse or chick limb, ectopic expression of *hh* in the anterior region also leads to mirror-symmetric duplications, and loss of Hh function causes limb truncations[3].

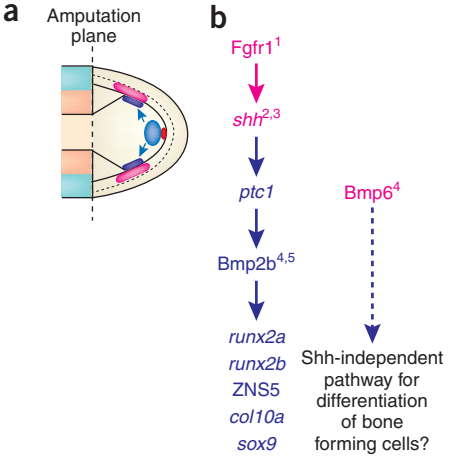

Figure 3 Bone patterning in the regenerating fin. (**a**) Cartoon of longitudinal section of fin ray, with emphasis on blastema and lateral basal layer of the epidermis. Cells from proximal blastema (dark blue) migrate laterally, come in contact with basement membrane adjacent to the lateral basal layer of the epidermis (pink) and differentiate as bone forming cells (purple). (**b**) Molecular pathways required for bone formation and patterning. Dotted arrows indicate signaling events that seem to occur but are not clearly defined. Tissue of expression is color-coded to match that in **a**; superscripts refer to list in **c**. (**c**) Genetic, mutant or chemical modifiers of the molecular mechanism shown in **b** and their effects on gene expression, outgrowth or both.

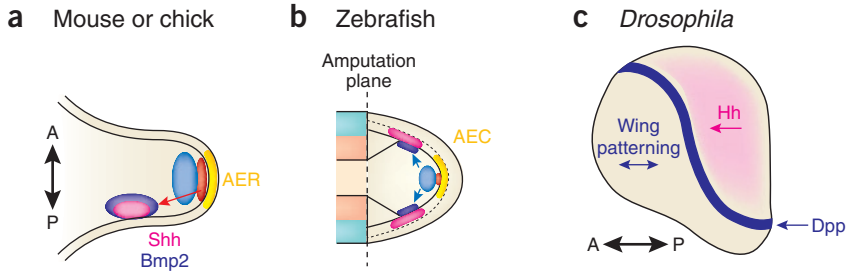

Figure 4 Conserved paradigms during limb growth. (**a**) Vertebrate limb bud showing AER (yellow), underlying mesoderm (red), proliferation zone (blue) and ZPA (pink). Signaling between the mesoderm and AER establishes Fgf signaling from the AER, which is required for cell proliferation and for establishment of the ZPA (red arrow). Shh signaling from the ZPA is required for maintenance of the AER and for skeletal patterning along the anterior-posterior (A-P) axis (mediated in part by Bmp2, purple). (**b**) Zebrafish fin ray during regeneration. See **Figure 3a** for details. (**c**) *Drosophila* wing disc. Hh signaling (pink) is required posteriorly for induction of Dpp in cells along anterior-posterior border. Hh signaling is mediated by Dpp directly.

In contrast to the case in the mouse and chick, ectopic or loss of Dpp in anterior regions is sufficient to phenocopy ectopic or loss of Hh, indicating that Dpp directly mediates Hh signaling[2,3]. Interestingly, as zebrafish *bmp2b* can induce ectopic bone (similarly to ectopic *shh*), patterning by *shh* may be more similar in this regard to that in *Drosophila* than to that in mouse (although in all systems, Hh signaling is mediated at least in part by TGF-β molecules). A further similarity is that in all three systems, continued Hh activity is required for continued cell proliferation and outgrowth.

Future directions for research on fin growth

Termination of growth. In contrast to the large amount of information regarding the molecular details of limb growth itself, the mechanisms underlying the cessation of limb growth remain poorly understood. In mouse and chick, reciprocal signaling between the AER and ZPA is required for continued outgrowth. Therefore, one possibility is that termination of signaling by either Fgfs or Shh would naturally lead to termination of outgrowth[30]. Another possibility is that a more active mechanism inhibiting outgrowth is induced as distal limb elements are completed[31]. More recent studies in the zebrafish fin also shed light on how final size may be achieved.

Ontogenetic growth of the zebrafish body and fins are indeterminate, meaning that zebrafish grow throughout their lifetimes. However, when fins are amputated, fin growth proceeds at a rapid rate only until the precut size is achieved, and then makes a transition back to ontogenetic growth. Interestingly, the amount of time it takes for a fin to regenerate is similar whether most of the fin or only a small amount has been amputated. Thus, stepwise amputations (that is, both proximal and distal amputations on a single caudal fin) achieve the final size at the same time, revealing that the rate of regeneration depends on the proximal-distal level of the amputation[21]. The rate of cell proliferation is correspondingly higher in the proximal amputees, indicating that the remaining stump tissue retains a 'sense' of how much growth is required to achieve its target size.

Given the precedent for the importance of Fgf signaling during fin regeneration, expression levels for Fgfr1 target genes were examined[21]. In proximal, compared with distal regenerating fins, expression of three Fgfr1 target genes is upregulated and these genes are expressed in expanded domains. To test the possibility that levels of Fgfr1 signaling regulate the rate of outgrowth, the *hsp70:dn-fgfr1* transgenic fish was developed (**Box 1**) and precise regulation of Fgfr1 attenuation achieved. As predicted, inhibition of Fgfr1 restricts target gene expression, cell proliferation and fin growth in a dose-dependent manner, providing strong evidence that the Fgfr1 pathway is at least in part responsible for regulating the rate of outgrowth in the fin. Interestingly, inhibition of Fgf signaling has also been shown to prevent heart regeneration in this transgenic line, apparently by blocking the recruitment of progenitor cells into the regenerating tissue[32]. It is tempting to speculate that Fgfr1 has a similar role during fin regeneration, and that the amount of Fgfr1 activity directly regulates the number of proliferating progenitor cells. Continued research in both organ systems will further elucidate the underlying mechanism of Fgfr1 activity.

Further layers of size control in the fin. Fin rays are comprised of bony segments, so it is not surprising that fin structure is regulated by independent mechanisms controlling the length and number of those segments[33]. For example, the fin length mutant *short fin* has short segments, and it is the result of mutations in the gap junction gene *cx43* (ref. 15). Gap junctions permit the exchange of small molecules (≤1,200 Da) among neighboring cells; however, it is not clear how defects in this communication affect cellular activities. Interestingly, mutations in human *CX43* also cause skeletal malformations[34], suggesting that the function of *cx43* is conserved and zebrafish bone growth may therefore serve as a model system to identify the underlying mechanism of *CX43*-based phenotypes. For example, cell proliferation is reduced in *short fin* mutants[15], and strong alleles have more severe defects in gap junctional communication than weak alleles[35]. Further research may demonstrate that local cell-cell communication is more directly related to the proliferative state of progenitor cells. Identification of the types of molecules that can modify gap junctional communication may be facilitated by a chemical genetic screen (**Box 1**) whereby small molecules may be found either to inhibit or enhance the *short fin* phenotype (that is, gap junctional activity).

Final words

Zebrafish fin regeneration represents a convenient and useful model system in which to examine the cellular and molecular mechanisms underlying normal growth control. In combination with studies completed in other species, a comprehensive understanding of the common and species-specific principles of limb growth may be revealed. Furthermore, the zebrafish provides advantages for the examination of growth control mechanisms not yet understood, including the achievement of final size and the elucidation of disease phenotypes.

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COMPETING INTERESTS STATEMENT

The author declares no competing financial interests.

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