A developmental transition in growth control during zebrafish caudal fin development

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Abstract

A long-standing question in developmental biology is how do growing and developing animals achieve form and then maintain it. We have revealed a critical transition in growth control during zebrafish caudal fin development, wherein a switch from allometric to isometric growth occurs. This morphological transition led us to hypothesize additional physiological changes in growth control pathways. To test this, we fasted juvenile and adult zebrafish. Juvenile fins continued allometric growth until development of the mature bi-lobed shape was completed. In contrast, the isometric growth of mature adult fins arrested within days of initiating a fast. We explored the biochemical basis of this difference in physiology between the two phases by assessing the sensitivity to rapamycin, a drug that blocks a nutrient-sensing pathway. We show that the nutrition-independent, allometric growth phase is resistant to rapamycin at 10-fold higher concentrations than are effective at arresting growth in the nutrition-dependent, isometric growth phase. We thus link a morphological transition in growth control between allometric and isometric growth mechanisms to different physiological responses to nutritional state of the animal and finally to different pharmacological responses to a drug (rapamycin) that affects the nutrition-sensing mechanism described from yeast to human.

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Introduction

Elucidating the fundamental biology of growth control remains one of the most significant problems challenging developmental biologists. Size and form are achieved and maintained through the appropriate coordination of isometric (relative growth at a constant proportion or proportionate growth) and allometric (relative growth at varied proportions or disproportionate growth) growth. Different body parts may utilize either or both types of mechanisms in achieving final form. Although the problem of growth and form has interested biologists for centuries (Thompson, 1992), the underlying mechanisms remain largely unsolved.

Environmental factors (e.g. nutrition, light, population density) profoundly impact the physiology of growth control, although the mechanisms by which such information is integrated into an overall hierarchy of growth control pathways is also poorly understood. For example, nutrients are essential building blocks for cell growth and proliferation, yet little is known about how nutritional status is integrated into an overall network of growth regulatory signals. In vertebrates, it has been suggested that a constant pool of circulating nutrients is required to maintain cellular homeostasis (Conlon and Raff, 1999; Raff, 1992). Growth is a metabolically costly process, and animals have evolved checkpoints that serve to curtail growth when nutrient supplies are limited. Interestingly, at times, the need to grow may be paramount, regardless of whether or not a state of nutritional sufficiency exists. Under these circumstances, growth continues, but at a cost. For example, the Dutch famine at the end of the second world war illustrated that fetal growth proceeded largely unabated until extreme maternal caloric restriction and maternal weight loss ensued (Stein and Susser, 1975). As additional examples, salamanders (Morgan, 1905)
and planarians (Morgan, 1901) are both capable of regenerative growth during fasting. The regenerating parts increase in size at the expense of the remainder of the starving animal, again illustrating an interesting problem of nutrient allocation during periods of suboptimal nutritional availability.

Zebrafish offer a unique opportunity to investigate the mechanisms controlling growth and form. First, they are a genetically tractable species that demonstrate continuous (indeterminate) growth (Iovine and Johnson, 2000; Jordan, 1905). In addition, their fins are anatomically and histologically simple structures that are unessential for life in the relatively pampered environs of a laboratory fish; moreover, fins are easily amputated for detailed study without needing to sacrifice the animal. Finally, mutations that affect fin growth have been developed without impacting on viability or fecundity (Goldsmith et al., 2003; Iovine et al., 2005; Iovine and Johnson, 2000, 2002).

Fin rays are composed of multiple segments. Each segment in turn is comprised of two concave hemirays (lepidotrichia) of dermal bone which lie in apposition, surrounding an intra-ray fin mesenchyme (Santamaria et al., 1992). In teleosts, including zebrafish, fin growth occurs via the sequential addition of new segments of bone to the distal end of each nascent fin ray (Haas, 1962; Nabrit, 1929; Santamaria and Becerra, 1991). We previously demonstrated that growth (segment addition) of the adult zebrafish caudal fin is episodic (alternating cycles of growth and rest), synchronous (all fin rays within a given fin grow simultaneously) and isometric (proportionate to growth of the body) (Goldsmith et al., 2003; Iovine and Johnson, 2000). We report here that the zebrafish fin undergoes a fundamental morphologic and physiologic transition in growth control during post-larval development. Fin growth in juvenile zebrafish, in contrast to adults, is continuous, asynchronous and allometric. During fasting, fin growth abates rapidly in adult zebrafish, consistent with what has been observed in other teleosts (Duan and Plisetskaya, 1993). In contrast, juvenile fin growth during fasting persists until the adult bi-lobed fin form has been established. Finally, we show that the continuous growth of the juvenile caudal fin, but not the episodic growth of the adult caudal fin, is resistant to the drug rapamycin, a drug known to block the nutrient-sensing mechanism in all eukaryotic cells. This developmental transition from allometric to isometric growth supports an important notion that critical changes in both morphology and physiology punctuate development, even well after organogenesis is complete (Burggren, 2005).

Materials and methods

Fish husbandry and general methods

Wild type fish stocks used for these studies were from the C32 and AB strains. Fish were reared at a constant temperature of 25°C and maintained on a 14L:10D photoperiod. Fish were fed three times daily (except fish that were fasted for purposes of the studies described below) with both micropellets (Hikari, Aquatic Eco-Systems) or flake food (Tetramin, Aquatic Eco-Systems) and brine shrimp (Biomarine, Aquafauna Biomarine). Fasted fish were maintained on flowing water at all times. Microscopy was performed with a Nikon SMZ1500 stereomicroscope. Photography was performed with a Nikon DXM1200F digital camera. Images were captured using Nikon Act-1 software and processed in Photoshop CS2 (Adobe). Chemicals were from Sigma (Sigma, St. Louis) unless otherwise specified.

Morphometry

To facilitate visualization of individual segments, fish were first stained with the vital fluorophore calcine (Du et al., 2001). Briefly, calcine solutions (0.2%) were made by dissolving 2 g of calcine in 1 l of MilliQ water and adjusting pH to ~7.0 with NaOH. Fish were immersed in calcine solution for 5–10 min and then washed in fresh system water for 10 min to clear away excess dye. For segment number measurements (as well as fin amputations), fish were anesthetized for several minutes in Tricaine. Segment numbers were counted using a Nikon SMZ1500 stereomicroscope.

Detection of proliferating cells using BrdU

Fish were labeled with BrdU in vivo by allowing them to swim for 6 h in fresh water containing BrdU (50 μg/ml). Fins were harvested by anesthetizing fish in Tricaine as described above and amputating approximately 50% of the caudal fin. Detection of BrdU incorporation was done on whole mounts (Goldsmith et al., 2003; Nechiporuk and Keating, 2002). For whole mount BrdU detection, fins were harvested as described above then fixed overnight in Carnoy’s solution (60% EtOH, 30% chloroform, 10% acetic acid) followed by dehydration in 100% MeOH. Fins were subsequently rehydrated into PBS containing 0.3% Triton X-100 (PBTx), washed in PBTx, rinsed twice in 2 N HCl in PBTx and then incubated for 30 min in 2 N HCl/PBTx at room temperature. After two more PBTx washes, fins were blocked for several hours in PBTxB (PBTx containing 0.25% BSA) then incubated overnight at 4°C in anti-BrdU (Roche, 1:50 dilution in PBTxB). Fins were then washed extensively (4–24 h) in multiple changes of PBTxB, blocked for 30 min in PBTxB then incubated overnight at 4°C in secondary antibody (Molecular Probes, goat anti-mouse conjugated to either Alexa-488 or Alexa-546, 1:200 dilution in PBTxB). After 4–24 h of additional washes in PBTxB, fins were stored in the dark in Vectashield (Vector Laboratories) until ready for mounting. Microscopy was performed as described above.

Preparation of riboprobes and ISH

Riboprobe synthesis and ISH were performed as described elsewhere (Goldsmith et al., 2003; Poss et al., 2000). ISH was either performed manually or with the assistance of an automated ISH robot (Abimed In Situ Pro, Intavis AG). All color development was performed manually.

Rapamycin treatment

Rapamycin (LC Laboratories) stock solutions (100 μM in DMSO) were stored at −20°C. For experiments, rapamycin stock solutions were first diluted in DMSO to a 1000× working concentration. Groups of fish were placed in tanks with 500 ml fresh system water. Following this, 500 μl of the appropriate concentration of rapamycin (1:1000 dilution) or 500 μl of DMSO (control) was added to each tank. Fish were fed once daily with brine shrimp and maintained off flowing water for the course of these experiments. Water and drug (or DMSO) were refreshed daily.

Results

Asymmetric growth of the juvenile caudal fin

Fin shape is the result of differences in the length of individual fin rays within a given fin. The bi-lobed shape of the adult zebrafish caudal fin, resulting from shorter fin rays in the cleft and longer fin rays in the dorsal and ventral lobes (Fig. 1F), provides one opportunity to investigate pathways that determine
shape and size. To understand how the bi-lobed fin morphology develops, we monitored segment addition in larval and post-larval (juvenile) zebrafish.

As the caudal fin develops, it changes from a paddle to a bi-lobed shape (Figs. 1A–F). The first segments form simultaneously in all fin rays of the nascent dorsal lobe, ventral lobe and cleft (Fig. 1A). Monitoring segment addition in the lobe and cleft fin rays throughout fin development reveals subsequent asynchronous growth, wherein lobe and cleft fin rays add segments asynchronously until the fin rays in the dorsal and ventral lobes have \( \sim 12-14 \) segments while the fin rays in the cleft have \( \sim 8-10 \) segments (Figs. 1A–F, Figs. 2A, B). This difference of \( \sim 4 \) segments is then maintained through synchronous segment addition, allowing the bi-lobed shape of the mature fin to persist as the adult fish continues to grow throughout its life (Figs. 2A, B) (Goldsmith et al., 2003; Jordan, 1905).

**Two models for asymmetric fin ray growth**

We are interested in how differences in length are established between fin rays from the dorsal and ventral lobes and fin rays from the cleft. Two models could explain this asynchronous growth in juvenile caudal fins. In one model, cleft fin rays could skip or “opt out” of segment addition cycles. That is, while segment addition proceeds in the nascent fin rays of the dorsal/ventral lobes, fin rays in the neighboring cleft remain quiescent. In this manner, fin ray segments are added asymmetrically and a bi-lobed shape develops. This model predicts that we will find

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**Fig. 1. Development of caudal fin shape by asynchronous fin ray segment addition.** (A–F) Zebrafish caudal fin development from first fin ray segment (A) to 14 (F) segments. A caudal fin has 18 primary fin rays (9 dorsal and 9 ventral). The longest (D3/V3) and shortest (D9/V9) rays and their segments (1, 2, etc.) are labeled in panels A–D. (A) Each primary ray has formed its first segment. (B) Each primary ray has formed its first joint, highlighted in D3 (arrow) and D9 (arrowhead). Formation of the second segment of each ray is underway. (C) Asymmetric development is clearly evident. The longest ray (D3) has 4 joints (arrows) and 5 segments, while the shortest ray (D9) has 3 joints (arrowheads) and 4 segments. (D) Asymmetry of the fin increases. In this higher magnification view of the dorsal lobe, D3 has 6 joints (arrows) and 7 segments while D9 has 4 joints (arrowheads) and 5 segments. (E–F) Asymmetry of the fin increases over time with the difference in segment number between longest (D3) and shortest (D9) ray increasing to 3 (E) and finally 4 (F) segments. There is no further increase in asymmetry after this time and the bi-lobed shape is mature.
cleft fin ray segments lacking dividing mesenchymal cells in ~1/3 of the fins (i.e. 4/12 segment cycles) where segment addition-associated cell division is observed in fin rays of the dorsal and ventral lobe. In an alternative model, all fin rays grow continuously, however, the rate of growth is faster in the dorsal and ventral lobes, relative to the cleft. The second model predicts that we will observe ~1/3 fewer dividing mesenchymal cells in the cleft fin rays, when compared to fin rays in the dorsal or ventral lobe.

Fin rays do not “opt out” of segment addition cycles

To distinguish between these two models, we examined bromodeoxyuridine (BrdU) incorporation in 100 juvenile caudal fins. We have previously shown in the adult zebrafish that bursts of proliferating cells in the distal fin ray mesenchyme delimit periods of segment addition in the caudal fin (Goldsmith et al., 2003) and, furthermore, that these proliferating mesenchymal cells can be labeled in vivo with BrdU. We counted BrdU-labeled mesenchymal cells in 3 fin rays from the dorsal or ventral lobe and 3 fin rays from the cleft in all 100 fins (Fig. 3A). All fin rays (300 total) from the dorsal/ventral lobe contained BrdU-labeled cells in the distal fin ray mesenchyme. In the cleft, we found a total of 6 fin rays lacking BrdU-labeled mesenchymal cells. While this provides evidence that fin rays in the cleft may occasionally skip segment addition cycles (“opt out”), this frequency (6/300) is far smaller than the frequency (100/300) we predicted necessary to achieve the bi-lobed shape.

A gradient of proliferating cells in the distal fin ray mesenchyme

Next, we tested the alternative model of asynchronous growth that all fin rays grow continuously but the rate of growth...
in fin rays from the dorsal and ventral lobes exceeds the rate of growth of fin rays from the cleft. In 5 fins from juvenile fish, we counted the total number of BrdU-labeled cells in the distal mesenchyme of 3 fin rays from the cleft and 3 fin rays from the dorsal or ventral lobe (Fig. 3B). We found that fin rays in the dorsal or ventral lobe have \( \sim 25\% \) (55.0 ± 3.3 [lobe] versus 41.2 ± 2.4 [cleft], \( P = 0.0037 \), two-tailed \( t \) test) more dividing cells in the distal fin ray mesenchyme when compared with fin rays from the cleft, supporting the model that a gradient of proliferating cells, increasing bi-directionally from the cleft, to the dorsal and ventral lobes, underpins asynchronous growth in the developing zebrafish caudal fin. A further prediction of this model is that, once the mature bi-lobed fin morphology is established and synchronous growth ensues, differences in the number of dividing cells between the dorsal or ventral lobes and the cleft will disappear. We tested this prediction by counting, in 5 adult fins, BrdU-labeled distal fin ray mesenchymal cells from the dorsal or ventral lobe (3 fin rays) and the cleft (3 fin rays) (Fig. 3B). While the overall number of BrdU-labeled cells is somewhat lower in adult than in juvenile zebrafish, adult caudal fins undergoing synchronous growth demonstrate no difference in the number of BrdU-labeled cells between fin rays from the dorsal or ventral lobe and fin rays from the cleft (±29.2 ± 0.8 [lobe] versus 29.8 ± 1.6 [cleft], \( P = 0.66 \), two-tailed \( t \) test).

A physiological transition in the growth response to nutritional sufficiency accompanies the change from allometric to isometric growth

We wondered whether changes in the growth response to physiologic stimuli, specifically nutritional status, accompany the developmental transition from allometric to isometric growth that we have characterized. First, we tested the growth response of the juvenile and adult caudal fin to fasting. To assess growth status of the fin, we used whole mount in situ hybridization with the fin growth marker fa93e10. We have previously shown that expression of this marker delimits periods of fin ray growth (Goldsmith et al., 2003). When fasted, adult zebrafish exit the fin ray segment growth cycle over 6 days (Fig. 4A). In contrast, in a mixed population of juvenile zebrafish, it took 2 weeks before all of the fins extinguished expression of the growth marker (Fig. 4A). To replicate and further refine these data, zebrafish spanning the morphologic transition (12–14 fin ray segments in the dorsal/ventral lobe and 8–10 fin ray segments in the cleft) from juvenile to adult caudal fin were fasted for up to 4 weeks (Fig. 4B). Segment number was directly assessed in the longest fin rays from either the dorsal or ventral lobe at weekly intervals. In adult zebrafish (≥12 segments in either the dorsal or the ventral lobe at the onset of the experiment), approximately one fin ray segment was added during the 4-week experiment. Furthermore, addition of this segment occurred during the first week of fasting with no segment addition occurring over the subsequent 3 weeks (data not shown), consistent with the data from adult zebrafish described above. In contrast, juvenile zebrafish (<12 segments in the dorsal or ventral lobe at onset of fasting) continued to add new fin ray segments after the first week of fasting. Indeed, the total number of segments added to either the dorsal or the ventral lobe in juvenile zebrafish was tightly and inversely correlated to the number of segments present at the beginning of the fast (\( R^2 = 0.92 \), \( P = 0.002 \), with adult zebrafish (≥12 segments) demonstrating no such correlation (\( R^2 = 0.09 \)). These results show that juvenile zebrafish continue to add fin ray segments during fasting, until a point when the adult bi-lobed fin morphology is achieved.
Rapamycin selectively abrogates isometric growth of the adult caudal fin

Increasing evidence in eukaryotes suggests roles for insulin/insulin-like growth factor (IGF), the target of rapamycin (TOR) kinase, and their various signaling partners as playing pivotal roles in regulating nutrient homeostasis and growth (Jacinto and Hall, 2003; Oldham and Hafen, 2003). We asked whether abrogating TOR signaling using the specific TOR inhibitor rapamycin inhibits fin ray growth in non-fasting juvenile and adult zebrafish. Juvenile (<12 dorsal/ventral lobe fin ray segments) and adult (>14 dorsal/ventral lobe fin ray segments) zebrafish were treated with either vehicle (DMSO) or rapamycin (5 nM and 50 nM) for 6 days (Fig. 5). Zebrafish were fed throughout these experiments. After rapamycin treatment, the zebrafish were labeled in vivo with BrdU. Fins were then harvested, and cell proliferation (growth status) was assessed in the distal fin ray mesenchyme. A fin was scored as BrdU-positive when 3 fin rays from the cleft and 3 fin rays from the dorsal/ventral lobe all contained ≥5 BrdU-labeled mesenchymal cells. In adults, rapamycin significantly decreased the number of BrdU-positive fins relative to vehicle (DMSO) ($P = 0.001$ for one-way ANOVA). The effect was significant at concentrations of 5 nM ($P < 0.01$) and 50 nM ($P < 0.01$) compared with vehicle, although these two concentrations were not significantly different from each other (Bonferroni’s multiple comparisons test). In contrast, no difference was observed between treated (5 nM or 50 nM rapamycin) and untreated (DMSO) juvenile zebrafish ($P = 0.54$ for one-way ANOVA). BrdU labeling in juvenile zebrafish was also unaffected by 100 nM rapamycin (data not shown).

Discussion

A morphological transition from allometric to isometric growth control

We have shown that distinct physiologies regulate fin growth in juvenile and adult zebrafish and, furthermore, we have defined the mechanism generating asynchronous fin ray growth in the juvenile caudal fin. Specifically, asynchronous growth arises from differences in numbers of proliferating cells in the distal fin ray mesenchyme. This differential in proliferating mesenchymal cell numbers is lost in the adult zebrafish caudal fin as synchronous growth ensues. This transition in growth control mechanisms has a profound affect on fin morphology. A bi-lobed fin is generated by continuous, asynchronous, allometric growth (juvenile). This period begins at approximately 2 weeks post-fertilization and continues through the previously described larval to adult transition in pigment pattern that is complete at 3–5 weeks (Johnson et al., 1995). The developmental transition from allometric to isometric growth control and from nutrition-independent to nutrition-dependent growth control occurs later, generally, at about 6–7 weeks of development or soon before the onset of sexual maturity. From this stage forward, fin growth is episodic, synchronous and isometric (adult), and the bi-lobed shape of the caudal fin is maintained throughout the life of the continuously growing zebrafish.

A physiological transition during zebrafish caudal fin growth

We hypothesized that important changes in the physiologic/metabolic regulation of growth might parallel the aforementioned morphological transition during caudal fin development. When zebrafish of different ages were fasted and caudal fin growth was assessed, a striking transition was observed. In adult zebrafish, caudal fin growth was inextricably dependent upon nutrient intake. We presume that metabolic checkpoints peg fin growth to nutritional sufficiency, allowing resources to be conserved for essential homeostatic processes during times of limited nutrient availability. In contrast, juvenile fin growth continued despite an interruption of nutrient intake during fasting, implying that a fundamental, physiologic transition in growth and nutrient homeostasis occurs during development. Clearly, nutrients must be re-allocated from other parts of the body to support the continuously growing fin.

What are the molecular mechanisms allowing the juvenile caudal fin to bypass nutrition checkpoints that rapidly abrogate fin growth during fasting in adult zebrafish? In fed Drosophila larvae, dTOR mutations or rapamycin (a specific inhibitor of TOR) treatment phenocopy starvation (Oldham et al., 2000; Zhang et al., 2000). Furthermore, constitutive PI3 kinase (a signaling partner in the insulin/IGF/TOR pathway) activity will drive growth in starved Drosophila larvae (Britton et al., 2002). Indeed, all eukaryotic cells appear to use TOR to “sense” their nutrient status (Raught et al., 2001; Schmelze and Hall, 2000), although the exact “sensing” mechanism is unknown (Klein and Proud, 2000). Our rapamycin data do not necessarily imply that...
TOR plays a less pivotal role in juvenile fin growth. For example, strong up-regulation of a canonical growth pathway utilizing TOR could indeed account for persistent growth during fasting, although the failure of rapamycin at concentrations up to 100 nM to inhibit the growth of juvenile fins argues against this. A more definitive understanding of the role that TOR might play in regulating juvenile and adult fin growth will need to await the development of tools that allow us to specifically interrogate the activity of the TOR signaling pathway in zebrafish.

We note that, while growth status during either food deprivation (Fig. 4) or rapamycin treatment (Fig. 5) was not measured using identical parameters, we feel that the results are directly comparable. We have assessed BrdU incorporation in the distal fin ray mesenchyme of juvenile and adult fish during fasting (data not shown), and the results corroborate the results presented here using the surrogate growth marker fa93e10 (Fig. 4A) and the direct measurement of segment addition (Fig. 4B). In addition, although the short time course of the rapamycin experiments precludes direct measurement of segment addition, our previous studies (Goldsmith et al., 2003) carefully demonstrated that fa93e10 expression in the fin clearly delimited BrdU labeling of cells in the distal fin ray mesenchyme.

It is interesting, although perhaps not surprising, that the response to nutritional sufficiency vis-à-vis fin growth in juvenile zebrafish is distinct from that of adult fish. It has often been assumed that physiological parameters (e.g. metabolism, heart rate) scale to animal size in a predictable manner (Burggren, 2005). For example, many biological relationships can be described by the equation, \( Y = aX^b \), where \( Y \) is the variable of interest, \( X \) is the body mass and \( b \) is an allometric constant. Typically, \( b \) is \( \approx 0.75 \), thus the variable of interest \( Y \) increases more slowly than mass. This relationship most famously describes metabolism (Schmidt-Nielsen, 1984). Developing animals, including post-larval zebrafish, may flout these physiological assumptions (Burggren, 2005). For example, during larval zebrafish development, heart rate (HR) and oxygen consumption (MO2) increase relative to increasing body mass (i.e. \( b \) is >1). In contrast, during early post-larval development, HR and MO2 decrease relative to increasing mass, but at a much greater rate than would be predicted if \( b = 0.75 \) (Barionuevo and Burggren, 1999). Interestingly, the slope of the line relating HR/MO2 to mass in zebrafish undergoes an abrupt change at \( \sim 50 \) days of age, similar to the time that the switch from allometric to isometric (and nutrition-independent to nutrition-dependent) growth control occurs (Barionuevo and Burggren, 1999). We are currently exploring whether these changes in the relationship of HR/MO2 to mass are causally linked to the switch from allometric to isometric growth.

Why might physiologic checkpoints linking growth to nutritional status be bypassed under some circumstances (juvenile fin growth, fetal growth) but imposed during others (adult fin growth)? Superficially, the relationships relating nutrient intake to growth seem uncomplicated. Animals eat, grow and mature. In reality, “decisions” must constantly be made regarding the allocation of nutrients for either growth or storage and presumably these “decisions” have evolved in every species to maximize reproductive success (Stern, 2003).

In summary, we have identified a discrete morphological and physiological transition that demarcates juvenile from adult fin growth in the zebrafish Danio rerio. This transition highlights the fundamental, yet poorly understood problem of coordinating nutrient allocation and growth. Integration of genetic and metabolic studies using the zebrafish affords us an opportunity to dissect the fundamental pathways coordinating growth, nutrition and energy homeostasis.

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