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REVIEW

Osteogenic programs during zebrafish fin regeneration

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Recent advances in genomic, screening and imaging technologies have provided new opportunities to examine the molecular and cellular landscape underlying human physiology and disease. In the context of skeletal research, technologies for systems genetics, high-throughput screening and high-content imaging can aid an unbiased approach when searching for new biological, pathological or therapeutic pathways. However, these approaches necessitate the use of specialized model systems that rapidly produce a phenotype, are easy to manipulate, and amenable to optical study, all while representing mammalian bone physiologies at the molecular and cellular levels. The emerging use of zebrafish (*Danio rerio*) for modeling human disease highlights its potential to accelerate therapeutic and pathway discovery in the mammalian skeleton. In this review, we consider the potential value of zebrafish fin ray regeneration (a rapid, genetically tractable and optically transparent model of intramembranous ossification) as a translational model for such studies.

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Introduction

Technological advances in large-scale biological research (including rapid whole-genome sequencing, high-throughput chemical discovery and high-content imaging) hold promise to open powerful new avenues for bone biological discovery. The potential to benefit from these technologies is directly related to the availability of model systems suitable for large-scale approaches. Zebrafish (*Danio rerio*) represent a unique combination of genetic flexibility, low cost, optical transparency, small size and ease of compound administration via water. These features make zebrafish an attractive model for biomedical research by facilitating powerful experimental approaches that are highly challenging in other vertebrate model systems. These approaches include genome-scale genetic screens,[1] high-content imaging of cellular dynamics,[2] *in vivo* small molecule discovery[3] and rapid interrogation of human mutant gene function.[4]

The utility of zebrafish, medaka and other laboratory fish as translational models for bone and mineral research depends on understanding the degree to which they exhibit genetic and phenotypic homology with the mammalian skeleton. For example, the lack of hematopoietic tissue-containing bone marrow, diminished participation in calcium homeostasis and a reduced role in resisting gravitational loading in the zebrafish skeleton underscores the fact that its translational value is likely to differ across different contexts (for a thorough review of similarities and differences between the teleost and mammalian skeletons, see Apschner *et al.*[5]). However, it is becoming clear that many molecular and cellular features of mammalian bone are conserved in the zebrafish skeleton, pointing to their largely unexplored potential to provide insights into both native and diseased states in humans. For example, at the cellular level, zebrafish bone is composed of many of the same components as mammalian bone, including osteoclasts, osteoblasts and osteocytes.[5] Physicochemical commonalities include the presence of type I collagen[6] and hydroxyapatite.[7] At the subcellular level, a growing number of genes mediating bone development, homeostasis and regeneration in mammals appear to be highly conserved in zebrafish, both in regard to their amino acid sequence and spatiotemporal expression profiles.[8] Several orthologous genes implicated in mammalian skeletal disease are expressed in zebrafish[9] and there is accumulating evidence that zebrafish mutants can exhibit traits of human bone diseases as a result of alterations in these genes.[10] Finally, compounds that are osteoactive in humans can also elicit changes in zebrafish bone.[11,12] These similarities provide both a foundation and rationale to clarify the degree to which zebrafish may be used to model mammalian bone physiologies during development, maintenance, regeneration and disease.[8,13,14]

To date, the majority of efforts to use zebrafish to examine bone formation have focused on craniofacial development and

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axial skeletogenesis.[10] Experimentally, the use of developing zebrafish larvae affords several important benefits, including their small size (which facilitates high-throughput experiments), optical clarity (which is lost during the larval-to-juvenile transition), and the greater number of tools/methods in zebrafish that have been established for early development (for example, injection of DNA or mRNA into the embryo, chemical screening in 96-well plate format, and so on). However, there are several limitations as well. For example, a gene may be critical to a given developmental process outside of its role in bone formation. In this case, skeletal phenotypes caused by a genetic alteration may be obscured by gross developmental defects. Further, mutations in a gene may not appropriately reflect its function in a mature organism after development, nor will it lend insight into gene function if the mutation is lethal. Thus, an ideal skeletal structure would be non-essential, retain optical clarity post-development, accessible (that is, near the outside of the body), and sufficiently stereotyped such that different stages of bone formation (for example, progenitor proliferation/recruitment, differentiation, matrix maturation and mineralization, and so on) may be independently investigated.

Of the bony structures in zebrafish, the regenerating tail fin is uniquely suited for such studies. Zebrafish fins possess a relatively simple anatomical structure, consisting of segmented bone rays, nerves, blood vessels, pigment cells and fibroblastic/mesenchymal cells residing within the intra- and inter-ray spaces. Each fin ray is composed of two concave-shaped hemirays. The hemirays are composed of multiple segments joined by fibrous ligaments and lined by a monolayer of osteoblasts. Like other teleosts, following fin amputation, zebrafish possess a remarkable capacity to regenerate their fin bone rays through epimorphic regeneration. This process occurs through three sequential phases: (i) inflammation and wound healing, (ii) formation of the blastema (a proliferative mass of progenitor cells) via dedifferentiation and (iii) a redevelopment phase consisting of intramembranous bone outgrowth, patterning and mineralization[15,16] (**Figure 1**). The regenerative capability of the fin is not dependent on developmental stage,[17] nor the age or number of amputations in adult fish.[18] In adults, bone regeneration is rapid, as newly synthesized bone appears within two to three days after amputation, and subsequent nerve, joint, circulatory and mature bone tissue are largely restored by 2 weeks. Although the regenerative capacity of the fin depends greatly on the blastema, recent studies suggest that the redevelopment phase involves major pathways known to be involved in mammalian bone growth,[19,20] patterning[21] and mineralization.[22]

Several excellent reviews have described the value of fin regeneration as a tractable model of regenerative biology,[18,23,24] as well as the broader utility of fish models for bone biomedical research.[5,8,10,24] However, an updated review of the potential applications for fin regeneration as a model of bone growth and mineralization has been lacking. In this review, we survey evidence of genetic and pathway similarities specifically between zebrafish fin regeneration and mammalian osteogenic physiologies. We also discuss emerging imaging technologies that may help advance this model as a rapid-throughput and high-content model for skeletal research. Finally, we consider the unique experimental requirements of genetic and chemical screening in the zebrafish skeleton, and discuss opportunities for innovation that may help advance new approaches to rapid and high-content analyses in this model system.

Osteoblast Differentiation and Activity

Over the past decade, efforts to elucidate the genetic mechanisms underlying epimorphic regeneration have led to an increased understanding of the molecular events governing early osteoblast differentiation during zebrafish fin regeneration. In mammals, one of the earliest events governing the differentiation of mesenchymal cells into bone-forming osteoblasts is the activation of the transcription factors RUNX2 and OSX (also known as SP7). RUNX2 is critical to the formation of ossified bones[25] and is important for the differentiation of mesenchymal cells into preosteoblasts. Similarly, OSX is required for bone formation by regulating differentiation from preosteoblasts into mature osteoblasts, and is used as a marker of osteoblastic cells in both mammals[26] and in zebrafish.[27] Expression of zebrafish orthologs for these transcription factors (*sp7*, *runx2a* and *runx2b*) is upregulated following fin amputation in zebrafish in coordination with bone outgrowth.[19,28–30] Moreover, the sequential activation of *runx2a*/*runx2b* followed by *sp7* during fin regeneration parallels the temporal activation of these genes during mammalian osteoblastic differentiation,

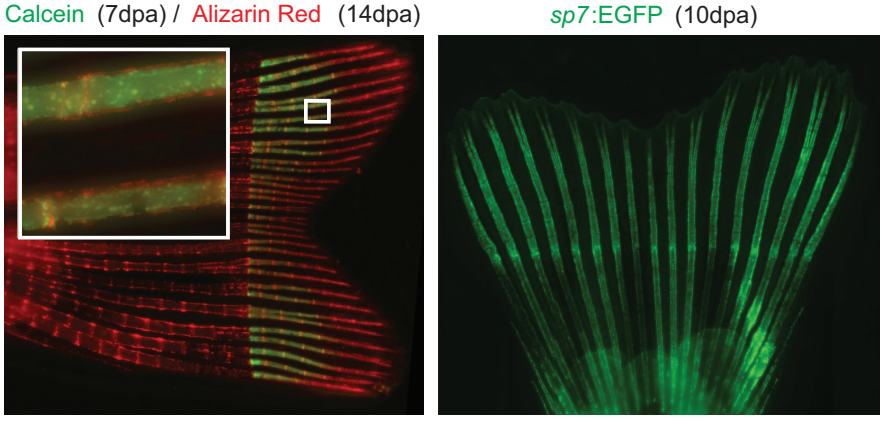

Figure 1 *In vivo* imaging of bone tissue and cell dynamics during zebrafish fin regeneration. (Left) Zebrafish tail fin subjected to double fluorochrome labeling. Regions of regenerated bone labeled by alizarin red, but not calcein, indicate new bone regenerated between 7 and 14 days post amputation (d.p.a.). (Left inset) Magnification of boxed region reveals post-outgrowth bone apposition. (Right) EGFP expression in *sp7*:EGFP zebrafish reveals osteoblasts in both native and newly regenerated bone tissue.

suggesting conserved roles for these genes in modulating the progression of the differentiation program.[31] Orthologs for established markers of later osteoblast differentiation and mineralization such as osteopontin,[32] osteocalcin,[30] collagen 1a1[6] and alkaline phosphatase[33] have been detected in the fins of zebrafish (or related species) as well. The expression of these factors has been less well-characterized relative to earlier markers and further studies are needed to determine if fin regeneration can lend insight into the molecular events underlying late-stage osteogenic processes such as osteoblast maturation, mineral accrual and post-outgrowth apposition.

Blastema Formation and Bone Cell Dedifferentiation

Investigations into key events that permit appendage regeneration (in zebrafish fins, as well as other vertebrate structures such as salamander limbs and mouse digits) have centered on blastema formation. In addition, both the pluripotency, as well as the source of progenitor cells in the blastema have been questioned (with hypotheses ranging from cells in circulation, resident stem cells or dedifferentiated cells). Recent studies point to dedifferentiated osteoblasts as a significant contributor to blastemal progenitors in zebrafish fin regeneration,[28,30] and also to fracture healing in zebrafish fin and skull.[34] These studies suggest that dedifferentiation is neither process specific (regeneration vs fracture), nor location specific (fin vs skull). Importantly, the pool of dedifferentiated osteoblasts in the blastema remain fate-restricted and re-differentiate exclusively into osteoblasts during regenerative outgrowth.[28]

In mammals, there is increasing evidence that bone formation can be mediated by fate-restricted progenitors in multiple contexts. In the mouse digit, which can regenerate P3- but not P2-level amputations (similar to digit tips in young children), it has been demonstrated that proliferative cells are formed by a local pool of fate-restricted cells.[35,36] Further, lineage-tracing studies in mice suggest that both bone maintenance and regeneration is mediated (at least in part) by osteolineage-restricted progenitor cells,[35,37] indicating that some cases of mammalian bone regeneration may not require the involvement of a pluripotent cell population.

The processes in which mammalian bone cells might undergo dedifferentiation are currently unresolved. Dedifferentiation has primarily been thought to be specific to organisms with high regeneration potential. However, murine cells isolated from bone chips have provided evidence of dedifferentiation from osteocytes into fate-restricted progenitors.[38] Interestingly, these cells re-differentiate into mature bone cells upon implantation *in vivo*. Studies have also documented the conversion of mature murine bone cells into osteoblasts as a result of PTH treatment both *in vivo*[39] and in culture.[40] In contrast, in adult mice, lineage-tracing studies suggest that the majority of osteoblasts mediating bone maintenance/remodeling are not derived via dedifferentiation, and a subset of resident, fate-restricted mesenchymal stem cell progenitors are the source of osteoblastic cells during fracture healing.[37] Cells from the pre-amputation tissue have also been implicated as the osteoblastic progenitors in murine digit regeneration, but whether these are derived by dedifferentiation or from a local pool of adult progenitors is unclear.[35,36]

Despite this context dependence, a recent study comparing limb regeneration in two salamander species (*axolotl* and *newt*) reveals that the source of skeletal muscle progenitors differs between the two.[41] The authors find that progenitor cells in *newts* are derived from myofiber dedifferentiation, whereas *axolotl* progenitors are derived from resident satellite cells inferring that although the source of progenitor cells may differ, the ultimate ability of these cells to differentiate into mature tissue does not. In this context, it remains to be seen whether bone cell progenitors not derived from dedifferentiation may also participate in blastema-mediated bone regeneration.

Osteogenic Signaling Pathways

Osteoblast differentiation during fin regeneration may involve a similar panel of canonical markers of osteoblastic differentiation as in mammals and raises the question of the degree to which pathways regulating these factors may also be conserved. For example, BMPs are central to the regulation of bone formation and development, likely by mediating osteoblast differentiation.[42,43] Canonical BMP signaling in bone (see Chen *et al.*[44] for a review) is dependent on SMAD1/5/8 phosphorylation and downstream regulation of transcription factors including RUNX2, OSX and DLX5. In zebrafish, *bmp2b* has specifically been shown to be expressed and regulate skeletogenesis in the regenerating fin[19,29] along with other BMPs including *bmp4*[29,45] and *bmp6*.[29] In mammals, loss of BMP2 and BMP4 also result in skeletal defects, sometimes causing severe osteogenic impairment as seen in a double *Bmp2*/*Bmp4* conditional knockout.[43] Activated Smad1/5/8 has been detected in the regenerating zebrafish fin[19,46] and has been indicated in directing BMP signaling in differentiating osteoblasts.[19] Finally, upregulation of *dlx5* has been seen in regenerating zebrafish fin[46] although its exact role in modulating bone formation in the fin is less clear.

Wnt signaling also has a central role in osteogenesis in mammals,[47] and in the regenerating zebrafish fin.[19,20,48] Expression of orthologs for important mammalian osteogenic molecular players such as LRP5, β-catenin and AXIN2 have been detected in the regenerating fin[19,20,46] in addition to orthologs for downstream targets such as CX43,[46,49] BAMBI[20,46] and TWIST2.[19] Furthermore, *dkk1b* was found to inhibit Wnt signaling and reduce the number of osteoblast precursors in the regenerating fin.[19] In mammals, canonical Wnt signaling is well established to be essential for osteoblast differentiation. However, its role in regulating bone formation in differentiated osteoblasts appears to be much more limited. Mouse mutants with stabilized β-catenin signaling in differentiated osteoblasts have been shown to exhibit osteopenia through alterations in resorption rather than formation.[50] This limited bone anabolic role for canonical Wnt signaling in differentiated osteoblasts may be conserved during zebrafish fin regeneration, as β-catenin signaling is spatially restricted to the blastema[51] and is not observed in newly formed bone tissue.

Interestingly, the Wnt signaling inhibitor Sost, which is found nearly exclusively in osteocytes in mammalian bone, is expressed in the blastema during early fin regeneration.[20] While other bones within the zebrafish skeleton possess osteocytes, the fin bone rays do not.[52] In other species, osteocytic and non-osteocytic bone has been observed in the bony rays of the fin, and it has been suggested that the degree of osteocyte cellularity depends on the bone ray segment thickness.[53] In this context, an important question is whether the expression of *sost* or other osteocytic genes in the zebrafish fin is reflective of divergent function, or conserved function in different cell types.

Evidence for Conserved Gene Function

Much of our current knowledge about the involvement of the pathways and physiologies during osteogenesis in regenerating fins is limited to gene expression data. Although the functional roles for these genes are best examined through knockout/knockdown models, in most cases mutant phenotypes for these genes in the regenerating fin have yet to be examined. However, several zebrafish mutants that mimic human bone disorders have been characterized and can help set a precedent for linking zebrafish gene expression and function data to known mammalian physiologies. Zebrafish mutants with molecular links to skeletal disease have been identified,[10] and can be broadly grouped into a few categories: (i) disrupted craniofacial development, (ii) effects on cartilage or collagen formation and (iii) altered mineralization and bone density.

For example, both the *chihuahua* (*chi*)[54] and *frilly fins* (*frf*)[55] mutants have been shown to resemble human osteogenesis imperfecta owing to mutations in the *col1a1a* and *bmp1a* genes, respectively. In addition, regenerated *chi* fins showed a greater impairment in fin ray structure compared with non-regenerated *chi* fins.[6] In another example, a forward genetic screen revealed mutants for ectodysplasin (*eda*) and ectodysplasin receptor (*edar*) genes.[56] These genes are frequently mutated in the human hereditary disease hypohidrotic ectodermal dysplasia that affects the development of integumentary appendages such as hair and teeth. In fish, mutations in these genes resulted in loss of adult dermal bone structures such as the rays of the fins and the scales, as well as the pharyngeal teeth. Another forward genetic screen revealed two zebrafish mutants, *no bone* (*nob*) and *dragonfish* (*dgf*) that displayed altered mineralization as a result of modifications in phosphate homeostasis.[57] In *nob*, mutations in the *entpd5* gene (which acts to hydrolyze extracellular diphosphates) cause a loss of mineralization, presumably due to a lack of extracellular phosphate. Interestingly, *dgf* mutants show the opposite phenotype with ectopic mineralizations, and have a mutation in the *enpp1*, a gene known to promote pyrophosphate (which inhibits hydroxyapatite formation) in both zebrafish and mammals.[58] Furthermore, the balance between phosphate and pyrophosphate can be restored in these fish by the generation of a double *nob*/*dgf* mutant.[57]

Parallels with Limb Development

Evaluating the translational potential of zebrafish bone regeneration (or other skeletal physiologies) requires not only assessing whether individual genes and pathways mediating this process are conserved, but also whether they are integrated within conserved paradigms of molecular control. There have been significant efforts to understand the degree to which fin regeneration utilizes common paradigms as those regulating limb development (for an excellent review, please see Iovine[21]). One example of this is in the specification of bone patterning via sonic hedgehog signaling. Briefly, during fin regeneration, epithelial cells from an amputated ray migrate to cover the wound and form a structure known as the apical epidermal cap. Next, mesenchymal cells from the stump tissue form the blastema directly under the apical epidermal cap.[59] Several signaling centers have been established during this process, one of the most well-characterized of which is the *shh*-positive center at the basal epidermal layer. In mammals, SHH is known to regulate bone cell differentiation, patterning and growth, thus promoting its expression where new bone will eventually form.[16] In zebrafish, Shh appears to mediate bone formation through Bmp2b as exogenous expression of either results in ectopic bone development.[16] Similarly, during the development of mouse and chick limbs, mesodermally derived cells create a small protrusion, which is also covered by an epidermal region, and form the limb bud. In this case, there is a distinct region at the distal end of the limb bud called the apical ectodermal ridge and another, more posterior, signaling center called the zone of polarizing activity. Shh signaling is predominant in the zone of polarizing activity, which is necessary for anterior–posterior patterning, and its effects are also mediated (although perhaps indirectly) by Bmp2.[60,61]

Neuromuscular-bone Crosstalk

The above studies point to the potential for zebrafish fin regeneration to recapitulate pathway dynamics essential to mammalian osteogenesis, warranting an exploration into the ability of this model to identify mechanisms of crosstalk with these pathways by extraskeletal tissues and organs. As neuromuscular function is a principal natural factor governing bone mass and strength, an interesting question is whether experimental associations between impaired neuromuscular activity and altered osteogenic function would be recapitulated during fin regeneration. Intramuscular injection of botulinum toxin (BTx, an inhibitor of synaptic fusion within cholinergic nerves) has been shown to induce focal and transient muscle paralysis with concomitant skeletal alterations observed in a variety of contexts. Under conditions of bone anabolism, BTx alters bone formation in broad conditions of osteogenesis including bone and joint development,[62] healing,[63] appositional growth,[64] mandibular development[65] and heterotopic ossification.[66] The diversity of contexts in which BTx-induced osteogenic dysfunction is manifested suggests that this physiology may be conserved in broad conditions of osteogenesis, and consequently, may be ultilized to identify underlying mechanisms of nerve- and muscle-bone crosstalk during zebrafish fin regeneration.

Toward this goal, we recently developed a novel model of BTx-induced neuromuscular dysfunction in adult zebrafish, and showed that BTx inhibits multiple aspects of bone formation during tail fin regeneration.[22] Using a combination of motor activity and behavioral assays, we showed that the paralytic effects of BTx in adult zebrafish were site-dependent, transient and focal, mimicking the paralysis observed in both animal and human studies. When subjected to tail fin regeneration, BTx impaired bone outgrowth, patterning and mineral accrual. Interestingly, despite the rapid onset of BTx-induced paralysis within 24 h, no effects on bone outgrowth were observed earlier than 5 days post amputation. This suggests that cholinergic transmission (either in adjacent neuromuscular tissues, or in skeletal nerves themselves) may be required for late-stage regenerative functions associated with bone redevelopment, but not early-stage (or 'preparatory phase') functions associated with wound healing and blastema formation. Stage-dependent effects have been previously observed during zebrafish fin regeneration in response to other pathway antagonists, such as the inhibition of bone outgrowth (but not blastema formation) by the hedgehog pathway inhibitor cyclopamine.[16] Given that osteoblast differentiation and activity

is a conserved feature of mammalian bone formation (whereas blastema formation is not), identifying and discerning stage-dependent functions during fin regeneration may have implications in inferring regulatory mechanisms to broader contexts.

One possible advantage to using zebrafish fin regeneration as a model of osteogenesis is the opportunity to draw parallels between identified skeletal deficits, and phenotypes attributed to known regenerative signaling pathways. For example, in our BTx model we observed several phenotypic similarities as those previously identified in fish administered cyclopamine[16] (for example, lack of effect on early regeneration, impaired outgrowth during late regeneration, and decreased bone ray bifurcations). Subsequently, we found that regenerative deficits in response to BTx were preceded by overexpression of the hedgehog/gli pathway genes *gli1* and *ptch1*. The latter is a known repressor of Shh signaling, suggesting a role for this pathway in mediating the neuromuscular regulation of late-stage osteogenic functions (for example, bone maturation and mineral accrual) downstream.

Quantitative Bone Imaging in the Regenerating Zebrafish Fin

The demonstrated conservation of osteogenic pathways suggests integration of this model with emerging imaging technologies may enable new approaches for bone functional analysis. In this regard, the optical transparency of the fin, coupled with the growing number of skeletal transgenic fluorescent reporter lines (see Hammond and Moro[67] and Spoorendonk *et al.*[13] for excellent reviews), provides a unique opportunity to examine how tissue-level phenomena (for example, osteoid secretion, mineral accrual and remodeling) are linked to cell-level functions (for example, cell signaling, proliferation and migration). For example, microCT (a three-dimensional x-ray-based technique) is widely considered the gold standard for non-invasive quantification of bone morphology and mineralization in mammalian bone. Although we have recently demonstrated the ability to distinguish newly regenerated bone in in fixed fins, the difficulties in immobilizing adult zebrafish for sufficiently long periods to obtain high-resolution scans (which are required to distinguish the lowly mineralized tissue in recently regenerated bone) makes *in vivo* imaging challenging. In addition, the technical challenges in integrating microCT with fluorescence imaging limits its utility for screening- and systems- based explorations in the zebrafish skeleton.

Recently, Mahamid *et al.*[7] demonstrated that zebrafish bone biomineralization occurs through an amorphous calcium phosphate phase, which then crystallizes with time (a process believed to occur, but had yet to be resolved, in vertebrate bone formation). As part of these studies, the authors showed that zebrafish bone rays exhibit birefringence owing to accumulation of mature (crystalline) bone mineral, suggesting the utility of polarized light imaging to quantify bone mineral maturation and accrual during fin regeneration. However, quantitative birefringence analysis using traditional polarized light microscopy is difficult because image intensity is dependent on not only birefringence but on specimen transmittance and orientation, which cannot be easily controlled for between specimens. To overcome this challenge, we recently developed a novel approach to quantify bone mineral accrual during zebrafish fin regeneration using Rotopol microscopy.[22] In this technique, a custom microscope is used to acquire sequential images of the fin under a step-wise rotating polarizer. By applying the appropriate relations,[68] birefringence, transmittance and orientation can be independently computed on a pixel-by-pixel basis, directly enabling quantitative analysis. Using this technique, we found that birefringence was significantly decreased in the regenerated bone rays of BTx-treated fish.[22] More recently, by imaging the same fin using Rotopol microscopy and microCT, we showed that birefringence is highly correlated with TMD (**Figure 2**), except between joints (where there is additional birefringence from the inter-segment ligament[7]).

The development of a rapid ($\sim 2\times$ to $10\times$ faster than microCT), light-based modality for bone mineralization imaging during fin regeneration opens new opportunities for multi-scale and systems-based investigations in this system. Unlike microCT, Rotopol technology is readily integrated with fluorescence microscopy. The integration of Rotopol imaging into fully motorized microscope systems can give rise to high-content, multi-modal (birefringence and fluorescence) imaging systems for simultaneous examination of 'microscopic' (for example, osteoblast numbers) and 'macroscopic' (for example, growth and mineralization) phenotypes (**Figure 3**). Such imaging strategies may provide valuable opportunities to gain a unique, systems-level perspective into the interplay between genomics, cell-level functions and tissue-level bone properties. In addition, as fin regeneration and imaging can be performed in live animals, this assay could be integrated into efficient phenotyping pipelines where developmental, ontogenetic and regenerative osteogenesis may be serially examined in the same animals. Given the availability of mutant fish from systematic efforts to knockout every single protein-coding gene in the zebrafish genome,[1] the ability to easily and quickly generate mutant fish using CRISPR/Cas, and the ability for individual labs to house thousands of adult fish, such pipelines may be valuable in accelerating the functional annotation of genes mediating osteogenesis.

Genetic and Chemical Screening During Fin Regeneration

The amenability of zebrafish to unbiased genetic and chemical screening is one of the defining features of this model system (there are several outstanding reviews in regard to zebrafish screen design and methods[69–71]). However, given the fact that the majority of the zebrafish skeleton ossifies post-embryonically, the use of screens to examine bone regeneration (or other adult traits) is faced by several unique challenges. First, the resource requirements are significantly greater in adult animals. For example, in a F_3 genetic screen for recessive mutations, a large number of random crosses are required in the F_2 generation. Because the F_3 animals must be raised to adulthood this greatly increases the requirements for housing, as well as labor (to raise the fish through a full feeding program). The greater size of adult fish also poses a problem for chemical screens. The most successful zebrafish chemical screens have been performed by housing embryos/larvae into 96-well plates, and adding compounds into the water. Most commonly, these approaches have enabled screens on the order of ~ 1000 compounds, with $\sim 10\,000$ compounds possible through automation.[3] However, as adult fish require more water for housing, this significantly increases the amount of chemical required to achieve an active concentration in the water (and thus reduces cost-effectiveness). Although the capacity to

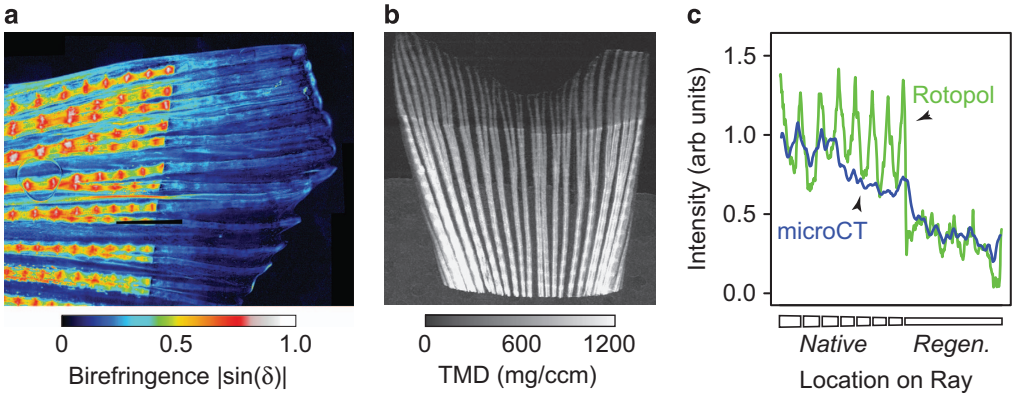

Figure 2 Visible light-based quantification of bone mineral accrual via Rotopol imaging. (**a**) Birefringence image of regenerated fin (8 d.p.a.) obtained using Rotopol microscopy. (**b**) MicroCT image of the same fin in **a**. (**c**) Pixel-by-pixel comparisons of intensity profiles in a single ray reveals a high correlation between Rotopol and microCT measurements (except for joints, where there is additional birefringence owing to the inter-segment ligament; see text for details).

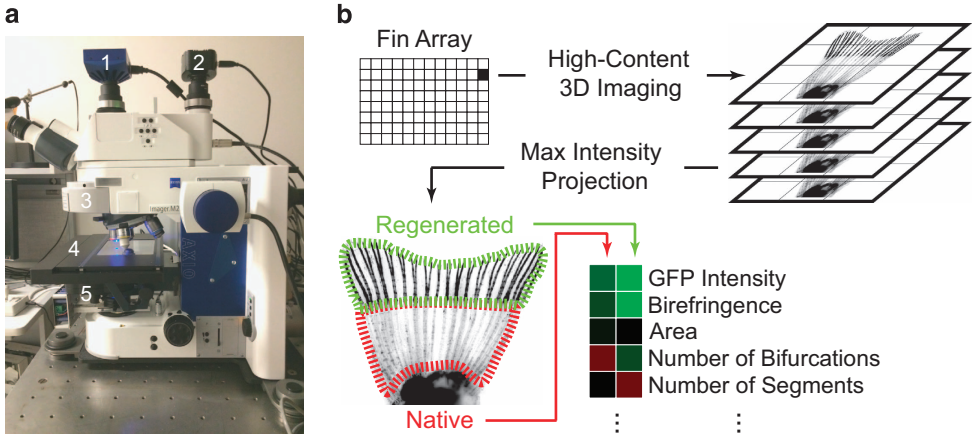

Figure 3 Multi-modal imaging platform for rapid and high-content imaging in the regenerating zebrafish fin. (**a**) Fully motorized microscope with fluorescence and Rotopol imaging capabilities. (1) Camera #1 (fluorescence); (2) Camera #2 (polarized light); (3) Motorized filter wheel; (4) XY stage with piezo motor drives; (5) Motorized rotating polarizer. (**b**) Schematic demonstrating imaging pipeline for high-content screening.

regenerate fin tissue is not age-dependent, it remains unclear how mechanistically similar later stages of fin regeneration (during bone maturation) are in young and adult fish. In larval tissue, the fin regenerates more quickly than in older fish and early molecular events appear to be similar to those in an adult,[72] but fully differentiated cell types are lacking in this model[17] and there is no mineralized bone. Therefore, adult fish are presumed to be required for the detection of fully mineralized, mature bone in a chemical or genetic screen, although it is possible younger fish may be utilized in the future with more insight into these mechanisms.

Despite these limitations, the ability to identify novel pathways mediating fin regeneration in genetic and chemical screens has been established. Johnson and Weston[52] performed a genetic screen in zebrafish subjected to a high temperature following fin amputation. By screening for temperature-sensitive mutations affecting fin regeneration, this enabled the possibility of identifying genes in which mutations would otherwise be lethal during development or ontogenesis. To reduce housing requirements in their screen, early pressure-induced parthenogenesis was used to render homozygous mutations in F_1 animals. More recently, Oppedal and Goldsmith[73] performed a chemical screen in zebrafish to identify novel inhibitors of caudal fin regeneration. These authors screened 520 compounds and identified 2 novel inhibitors: budesonide and the imidazoline receptor antagonist AGN192403. Although the number of screened compounds was less than that typically screened in embryos/larvae (~1000), this was the first study demonstrating the feasibility of chemical screening in adult animals. In combination with successful screens in adult zebrafish for phenotypes in other skeletal structures (such as those described previously), these studies provide the rationale to continue to develop new screening strategies and methods in the adult zebrafish skeleton. Such advances include innovations in housing,[71,74] as well as automation technologies (for example, for injury induction[74]). In addition, the identification of experimental 'windows' in which juvenile animals are small, yet their skeletons are sufficiently mineralized to be detected by quantitative approaches, may aid in reducing housing/chemical-dosing requirements. Finally, although a scalable genetic strategy for inducing tissue-specific mutations at a

genome-wide scale has yet to be established, advances in genome editing may one day make tissue-specific mutational screens possible, and would have significant impact in expanding the range of zebrafish for not only fin regeneration but other adult physiologies.

Conclusion

In conclusion, zebrafish bone maintains much of the complexity of mammalian bone on a structural, cellular, molecular and genetic level, and yet possesses important differences as well. Emerging strategies for cross-species pathway mapping will provide new opportunities to efficiently translate across zebrafish and mammalian bone physiologies, as well as identify regenerative stages in the fin with high translational utility. By examining these pathways in a rapid, genetically tractable and optically transparent system, zebrafish fin regeneration may enable novel paradigms for rapid-throughput and high-content analysis with potential to enhance our understanding of osteogenesis and accelerate bone therapeutic discovery.

Conflict of Interest

The authors declare no conflict of interest.

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