



HHS Public Access

Author manuscript

J Orthop Res. Author manuscript; available in PMC 2021 May 01.

Published in final edited form as:

J Orthop Res. 2020 May ; 38(5): 925–936. doi:10.1002/jor.24539.

Zebrafish: An Emerging Model for Orthopaedic Research

Björn Busse^{1,6}, Jenna L. Galloway^{2,6}, Ryan S. Gray^{3,6}, Matthew P. Harris^{4,6}, Ronald Y. Kwon^{5,6}

¹Department of Osteology and Biomechanics, University Medical Center Hamburg-Eppendorf, 22529, Hamburg, Germany

²Center for Regenerative Medicine, Harvard Stem Cell Institute, Department of Orthopaedic Surgery, Massachusetts General Hospital, Harvard Medical School, 185 Cambridge Street Boston, MA 02114, United States of America

³Department of Pediatrics, Dell Pediatric Research Institute, The University of Texas at Austin, Dell Medical School, Austin, Texas, United States of America

⁴Department of Genetics, Harvard Medical School; Department of Orthopedic Research, Boston Children's Hospital, 300 Longwood Avenue, Boston, MA, 02115, United States of America.

⁵Department of Orthopaedics and Sports Medicine; Department of Mechanical Engineering; Institute for Stem Cell and Regenerative Medicine, University of Washington, Seattle, Washington, United States of America

⁶all authors contributed equally to this work and are listed in alphabetical order

Abstract

Advances in next generation sequencing have transformed our ability to identify genetic variants associated with clinical disorders of the musculoskeletal system. However, the means to functionally validate and analyze the physiological repercussions of genetic variation have lagged behind the rate of genetic discovery. The zebrafish provides an efficient model to leverage genetic analysis in an *in vivo* context. Its utility for orthopaedic research is becoming evident in regard to both candidate gene validation as well as therapeutic discovery in tissues such as bone, tendon, muscle, and cartilage. With the development of new genetic and analytical tools to better assay aspects of skeletal tissue morphology, mineralization, composition, and biomechanics, researchers are emboldened to systematically approach how the skeleton develops and to identify the root causes, and potential treatments, of skeletal disease.

Keywords

zebrafish; bone; tendon; muscle; cartilage

Corresponding author: Ronald Y. Kwon, Address: 850 Republican St, Seattle WA 98109, Phone: 206 897 5906, Fax: 206 685 1357, ronkwon@uw.edu.

Author Contributions: BB, JLG, RSG, MPH, and RYK drafted and revised the manuscript. All authors have read and approved the final submitted manuscript.

1. INTRODUCTION

Small animal models amenable to rapid-throughput biology are needed to accelerate the discovery of new treatments for clinical disorders of the musculoskeletal system. Complex, multi-cellular interactions are difficult to recapitulate in a dish. While such processes can be studied in animal models, ready-made mutant lines often do not exist (e.g., see section 5.3). The zebrafish (*Danio rerio*) is a small, tropical freshwater fish that, by virtue of its unique experimental attributes (e.g., small size, low cost, genetic tractability, and optical transparency), has opened powerful avenues for biomedical research (including studies of development^{1;2}, neuroscience³, regeneration⁴, and disease⁵) that are difficult in other vertebrate models. Such avenues include *in vivo* imaging of cell dynamics, and genetic and chemical screens. Moreover, zebrafish can be used as a pre-screening tool to prioritize more labor and cost-intensive studies that require *de novo* mutant mouse generation. The potential benefits of incorporating zebrafish into a research program must be weighed with limitations, including infrastructure costs (which vary depending on institution), differences in zebrafish and human genetics and physiology, and the fact that many experimental approaches are still in their infancy, and thus remain to be rigorously validated. Indeed, the use of zebrafish to understand clinical disorders of the musculoskeletal system has only begun to be established. Here, we introduce experimental advantages of the zebrafish, discuss its genetic and physiological similarities and differences to humans, and survey recent applications to musculoskeletal development and disease. This review elaborates on a workshop of the same name at the 2019 Orthopaedic Research Society Meeting, conducted by the authors, the purpose of which was to introduce emerging orthopaedic research in zebrafish to facilitate cross-talk, establish foundations, and develop new models of clinical disorders.

2. GENETICS

2.1 Genetic similarity to humans

A key criterion in the selection of an appropriate disease model is its genetic similarity to humans. Approximately 71% of human protein coding genes possess at least one zebrafish ortholog⁶. This is comparable to mouse, as ~80–90% of human protein coding genes possess at least one ortholog in mouse (<http://www.informatics.jax.org/homology.shtml>). Because zebrafish arose from a common ancestor that underwent an additional round of whole-genome duplication relative to mice and humans, zebrafish can have multiple co-orthologs for human genes (e.g., human *RUNX2* has two zebrafish co-orthologs, *runx2a* and *runx2b*). While this can complicate testing of gene function due to issues such as functional redundancy, such difficulties can be alleviated through simultaneous knockdown or knockout of co-orthologs^{1;7}. In other cases, maintenance of two copies of a gene in the zebrafish often is balanced by partitioning function of a gene, or subfunctionalization. As such, the retention of co-orthologs in the zebrafish often permits nuanced analysis of gene function in genes that might be lethal in mice. In mouse, analysis of many genes often requires combinatorial genetic techniques to provide conditional spatial or temporal regulation of gene function, whereas in the zebrafish simple genetic alterations can be studied (e.g. *Fgfr1*⁸). The often partitioned function of paralogues in the zebrafish also

permits loss-of-function analysis to model effect of more nuanced alleles such as regulatory shifts in gene function underlying many common skeletal pathologies. Such pathologies cannot easily be modeled with knock out strategies in the mouse and due to the complex anatomical nature of many skeletal disorders, often cannot be modeled *in vitro* even when allele-specific cell lines are constructed. A large number of skeletal disease models have been identified in zebrafish (recently reviewed in ⁹⁻¹¹), and this number is steadily increasing.

2.2 Techniques

The ease of forward genetics in zebrafish gives this model an advantage for unbiased discovery of mutant phenotypes. N-ethyl-N-nitrosourea (ENU) mutagenesis screens in zebrafish have uncovered a large number of variants relevant to fundamental aspects of skeletogenesis ¹²⁻¹⁴, morphological evolution ¹⁵, and human skeletal diseases ¹⁶⁻²⁰. Early examples involved the identification of a large collection of mutants with defects in formation of the jaw and branchial arches ¹²⁻¹⁴. The same screen also identified mutations specifically affecting the adult form ²¹. The mutants identified in these early large-scale screens served as foundation for experimental analysis and were proof that not only could specific mechanisms of skeletal development be identified, but that mutations could be in genes homologous to human genes associated with skeletal diseases. Such screens often yield specific defined point mutations which provide nuanced changes in gene function that simple loss-of-function mutations or frameshifts cannot. Screens for dominant mutations affecting skeletogenesis in which mutations often lead to dominant negative properties as well as alleles with increased functions (hyper- or neomorphic alleles), are proving to be informative and useful in disease modeling. For example, recent screens have identified dominant mutants closely mirroring collagenopathies and Osteogenesis Imperfecta (OI) (*coll1a1a/b*, *coll1a2*), Adams-Oliver Syndrome (*dll4*), and Hyperhidrotic Ectodermal dysplasia (*edar*) ¹⁶. Finally, the Zebrafish Mutation Project²², which has phenotyped a large number of zebrafish mutant alleles and made them available to the community, demonstrates the feasibility of systematic genome-wide analysis.

In addition to forward genetics, zebrafish are also readily amenable to reverse genetics, i.e., testing for phenotypic consequences following the targeted interference of gene function. The advent of TALEN-²³ and CRISPR-based gene editing has substantially expanded the means by which the scientific community can approach reverse genetics in zebrafish. For gene editing using CRISPR, administration of Cas9:gRNA ribonucleoproteins (RNPs) generates double stranded breaks at defined loci. Errors in the non-homologous end joining (NHEJ) repair mechanism lead to insertions and deletions (indels) at the cut site, often leading to loss of function (e.g., due to non-sense mediated decay triggered by a premature stop codon). Alternatively, multiple RNPs can be used to induce site-spanning deletions that delete promoter regions or entire gene loci, which may help reduce activation compensatory pathways triggered by mRNA degradation ²⁴. Moreover, Cas9:gRNAs can be co-injected with a donor template which, following homology directed repair (HDR), can result in precise gene edits. Because zebrafish develop externally, hundreds of embryos can be injected by a single user in one morning. This allows for efficient creation of induced mutations or replacements at specific genetic loci. Screening phenotypes in injected G0

founder “crispant” animals can further enable rapid and cost-effective assessment of gene function¹. In addition to alleviating the time and resources needed to breed alleles to homozygosity, G0 screens are also amenable to multiplexing strategies in which multiple genes are targeted in the same animal. The ability to detect adult skeletal phenotypes in G0 zebrafish for genes associated with recessive forms of OI (*bmp1a* and *plod2*) and genetic risk for osteoporosis (*wnt16*) was recently demonstrated²⁵.

Zebrafish also provide a versatile system to test gene function through use of transgenesis. This allows for stable or inducible (e.g., heat shock-induced) protein expression, or conditional gene targeting (e.g., Cre-mediated recombination). The Tol2 transposon system is commonly used for introducing transgenes. There exists a large, growing panel of zebrafish fluorescent reporter lines for cell types within the musculoskeletal system (Table 1). As zebrafish are also relatively transparent and develop externally, development can be easily observed in real time. With these two attributes, use of transgenic reporters for particular cell types and proteins have provided an unmatched ability to visualize the dynamics of skeletal patterning and regeneration. These advantages also permit the visualization of cell behaviors in specific genetic contexts to gain mechanistic understanding of disease etiology.

Because of their small size and low cost, zebrafish are also amenable to drug discovery via chemical screens. In such screens, large libraries of small molecules are tested to identify specific compounds that affect gene function or developmental processes. In a typical screen, zebrafish embryos/larvae are dispensed into 48 or 96 well plates, drugs are administered by adding them to the water, and phenotypes are assessed (e.g., via morphological, fluorescent, or behavioral readouts). This strategy can be adapted to adults^{26; 27}. The identification of dorsomorphin as a selective inhibitor of BMP type I receptors was discovered in a large zebrafish chemical screen and led to the development of analogs for treatment of heterotopic ossification²⁸. In another screen, phosphodiesterase (PDE) inhibitors were found to alter phenotypes in a zebrafish model of Duchenne muscular dystrophy²⁹. See³⁰ for a recent review of chemical screens in zebrafish.

3. FORMATION AND INTEGRATION OF THE ZEBRAFISH MUSCULOSKELETAL SYSTEM

3.1. Development and patterning

Fully developed, the zebrafish skeleton comprises several functional groups including the cranial skeleton, axial skeleton, caudal skeleton, unpaired fins (dorsal, anal, caudal fins), paired fins (pectoral, pelvic fins), and elasmoid scales (Fig. 1A–D). As in all vertebrates, the zebrafish cranial skeleton and its associated connective tissues, tendons and ligaments, arise from the cranial neural crest; the fin skeletal elements arise from the lateral plate mesoderm, and the myosepta and axial skeleton from somitic paraxial mesoderm^{31–33}. The cranial musculoskeletal system forms rapidly and can function by 5 days post fertilization (dpf). The pectoral fin cartilage and muscles are also developing at this time. Although the axial skeleton does not form cartilage and bone until later stages, it has the same somitic compartments, sclerotome, syndetome³⁴, and myotome, fated to become skeletal, tendon,

and muscle tissues as in higher vertebrates. Prior to 5 dpf, the axial musculoskeletal structures primarily are composed of muscle and myosepta, a *scleraxis*-expressing myotendinous tissue that links the myomeres^{35; 36}. Bony elements form through direct/intramembranous ossification, or via a cartilage or cartilage-like template (e.g., via perichondral or endochondral ossification)^{37–40}. Modes of ossification can differ in zebrafish and mammals in similar bones. For example, in mouse, the vertebrae form by endochondral ossification⁴¹; in zebrafish, vertebrae form by direct mineralization of the notochord sheath (perichordal ossification), without passing through a cartilaginous stage⁴². In some bones, osteoblasts and osteoclasts act in concert to model bone shape into adulthood⁴³. Although uncommon, osteon-like structures in zebrafish have been reported for lateral ethmoid bone⁴⁰. Notably, these structures contained solely one lamella and no osteocytes. Indeed, most skeletal elements in adult zebrafish skeletons are osteocytic and do not show osteons or hemi-osteons indicative of human-like secondary remodeling. In vertebrae of adult zebrafish, osteocyte lacunar orientation shows a preferred orientation (Fig. 1E)⁴⁴. While the mechanosensing and remodeling characteristics of osteocytic bone in zebrafish remain to be fully understood, lacunae in zebrafish indicate smaller volumes with less numerous canaliculi compared to mice and humans.

Tendons are the tissue interface between muscle and bone⁴⁵. Concurrent to skeletal development, transcripts of *scleraxis a* (*scxa*) are found in the forming tendon cells adjacent to the developing cartilage and muscle by 2 dpf. These cells aggregate and differentiate, turning on expression of tendon matrix genes, *tenomodulin* (*tnmd*), *thrombospondin-4* (*tsp4b*), and type I collagen (*colla1a/b*, *colla2*)^{31; 36}. As in mammals, initiation of the axial tendon program depends on signals from the muscle. Cranial and fin tendons form in the absence of muscle, but require muscle for tendon maintenance^{31; 46}. FGF and TGF β are also important for proper tendon formation³¹, and *cyp26b1* loss of function studies suggest that retinoic acid is required for tendon cell condensation^{47; 48}. Recent studies have shown that mechanical force, through release of TGF β , regulates the formation of tendon cell projections, which are thought to be involved in ECM production⁴⁹. In the adult, the cranial tendons have similar ultrastructure to mammalian tendons with highly ordered type I collagen fibrils observed by TEM³¹. In addition, they can be readily visualized using second harmonic generation (SHG) imaging (Fig. 1F).

Analogous to higher vertebrates, striated muscle of zebrafish contain three main components: contractile proteins, lipids, and connective tissue⁵⁰. Vertebrae are connected by intervertebral ligaments⁵¹. Zebrafish possess both slow- and fast-twitch muscle fibers, which are topographically separated⁵². Together with cellular mineralized bone tissue, muscles, tendon, and other soft tissues, the zebrafish skeleton facilitates locomotion, provides mechanical support, and protects internal organs. In Fig. 2, we compare the intervertebral space in the zebrafish and mouse as a case study of how skeletal structures in each species typically exhibit both morphophysiological similarities and differences.

3.2 Conservation of developmental programs

The molecules that govern zebrafish skeletal development are highly conserved with mammals. Sox9, a transcription factor necessary for chondrogenesis and skeletal

development⁵³, has two co-orthologs in the zebrafish, *sox9a* and *sox9b*. They are expressed in overlapping and complementary patterns during development with *sox9a* in the pharyngeal arches and later restricted to the pre-chondrogenic mesenchyme that will form the jaw cartilage and fin scapulocoracoid, and with *sox9b* in the premigratory neural crest and fin endochondral disc⁷. The *sox9a* expressing chondrocytes also express *col2a1* and are Alcian Blue positive before 3 dpf. These skeletal elements will undergo perichondral or endochondral ossification and later become Alizarin red positive cranial bones. In perichondral ossification, perichondral cells become *runx2a/b*, *osterix (sp7/osx)*, and *collagen 10* positive osteoblasts and initiate ossification⁵⁴. Similar to other vertebrates, *indian hedgehog* co-orthologs (*ihha/b*) are expressed by chondrocytes and are thought to signal to patched, Hh receptors (*ptc1/2*) in the perichondrium and mediate bone formation^{55;56}. Other cranial elements, such as the maxilla undergo direct intramembranous ossification via *osx*-expressing osteoblasts^{40;57}. For many of these genes and cell types reporter and lineage tracing transgenic zebrafish lines have been generated, which, along with the optical access provided by zebrafish, allow unprecedented ability to visualize skeletogenesis (Fig. 3).

3.3. Physiology during development and in homeostasis

The skeleton serves as a key organ which mediates systemic signaling affecting physiology. Although many of these non-structural functions of the skeleton are just being identified, it is clear that many have conservation between humans and zebrafish. One key function of the skeleton is to facilitate mineral homeostasis. The skeleton participates in part by regulating phosphate homeostasis in the kidney through bone-kidney crosstalk. There is evidence that Fgf23, which in humans and mice, is synthesized in osteocytes and regulates kidney phosphate reabsorption, also regulates phosphate homeostasis in zebrafish⁵⁸. In mammals, the skeleton also serves as a calcium and phosphorus reservoir. Because calcium regulation can occur through the gills in fish, compared to humans, the physiological role of the skeleton in calcium homeostasis in fish may differ³⁹. Another function of the mammalian skeleton is acting as a site of hematopoiesis, as well as fat storage, in the marrow cavities. Zebrafish possess bone marrow spaces³⁹, which is evident in endochondral bones, which are filled with fatty tissue⁴⁰. However, unlike in humans, this is never colonized by hematopoietic stem cells (HSC). Thus, zebrafish bone marrow spaces lack hematopoietic tissue³⁹. A number of zebrafish bones possess adipocytes within their marrow spaces⁴⁰, however it is unknown whether this adiposity responds to metabolic demands, as it does in mice⁵⁹. Finally, the skeleton can regulate metabolic processes independent of mineral metabolism. For instance, the bone-derived hormone osteocalcin has been implicated in glucose homeostasis, cognition, and male fertility⁶⁰. Whether the zebrafish skeleton functions as an endocrine organ through osteocalcin secretion requires further investigation.

3.4 Aging

Compared to early development, processes such as homeostasis and aging have not been studied in depth in the zebrafish. As certain debilitating conditions arise in the skeleton as a function of age, such as osteopenia and osteoporosis, the ability of the zebrafish to model components of these processes would be important. Zebrafish typically have a lifespan of approximately 2–3 years (though 5 years or more is possible)⁶¹, and exhibit growth

throughout life. There is evidence that zebrafish skeletal function declines with age. For instance, tendon mechanical properties diminish with age⁶². Moreover, alterations of vertebral bone and disc are observed in aged zebrafish⁶³. Bone dependence on estrogen has been modeled in another small teleost, medaka, and thus basic properties of the etiology are likely present in zebrafish⁶⁴. With more analysis of late developmental stages, it is likely more insight will emerge from the zebrafish into how the skeletal system ages and its consequences.

3.5 Regeneration and repair

Zebrafish have not been used as a common model for understanding human fracture repair. This is in part due to lack of accessible long bones, as well as its high regenerative capacity which may utilize different repair mechanisms than in mammals. Previous studies have examined the repair properties of damaged membranous bones of the skull roof⁶⁵ as well as mandible⁶⁶. Although these are not directly comparable to analysis of long bone fractures studied in mouse and most commonly seen in patients, there were some similarities in terms of the genes and cell types involved. For example, *runx2+* cells in the periosteum were likely involved in new bone formation and proper formation of the cartilage callus relied upon *Indian hedgehog a (ihha)*⁶⁶. In addition to examination of intrinsic regenerative mechanisms, the transparency of the zebrafish permits the analysis of extrinsic cell populations in the healing process. Studies have shown that the immune system plays an important role in mediating tissue regeneration^{67; 68}. Visualization of immune infiltration after injury can be accomplished through the use of transgenic reporter lines that either label all leukocytes (*cd45:DsRed*)⁶⁹ or are specific for neutrophils (*mpx:GFP^{114Tg}*)⁷⁰ or macrophages (*mpeg:eGFP*)⁷¹. There are also several methods to functionally deplete immune cell populations (reviewed in⁶⁷), which can permit temporal control over cell type specific cell ablation to assess the role of immune cell populations at different stages of the regenerative process, as has been performed for tail fin regeneration⁷².

Zebrafish have a significant capacity for epimorphic regeneration [3,14]. One example is the caudal fin, which regenerates following amputation⁷³. Similar to salamander limb regeneration, fin regeneration involves a heterogeneous pool of progenitors called the blastema, which is comprised, at least in part, of mature cells at the amputation stump that dedifferentiated, including osteoblasts⁷⁴. A variety of pathways known to be important for skeletogenesis in mammals are recapitulated during fin redevelopment, as reviewed in⁷³. An intact musculoskeletal system is required for normal regeneration as zebrafish subjected to injection of Botulinum toxin, which inhibits synaptic release at cholinergic nerves, exhibit impaired regeneration⁷⁵. This model has also revealed the existence of mesenchymal progenitor populations within specific regions that robustly respond to injury and generate new *osx+* osteoblasts^{76; 77}. Dedifferentiation of mature osteoblasts also occurs during repair of zebrafish fin fractures and skull injuries⁶⁵. While osteoblast dedifferentiation is more limited in mammals, fin repair after fracture exhibits some similarities to mammalian long bone fracture, including formation of a remodeling callus⁷⁸, and recruitment of osteoclasts⁷⁹. Recently, it was shown that neutrophils dynamically colonize the fracture site. When infected with *Staphylococcus aureus*, neutrophils were retained in the fracture site, and

repair was reduced⁷⁹. Further studies examining the utility of the fin fracture model to study aspects of fracture biology are warranted.

3.6. Musculoskeletal loading

While the zebrafish skeleton has a reduced role in resisting gravitational loads relative to humans, there is evidence that the zebrafish skeleton can respond to exercise, as well as disuse. Swim training routines have been established to force exercise and stimulate natural modes of skeletal loading in zebrafish. In this way, the complex interplay of cellular, structural, and compositional bone characteristics can be assessed using multiscale approaches in zebrafish to study the effects of genetic and environmental interactions on the skeletal system *in vivo*. During early development in zebrafish, swim training alters timing of skeletogenesis⁸⁰. In adult zebrafish, swim training increases vertebral bone formation and alters quality⁴⁴. Moreover, this type of forced exercise also induces muscle adaptations in adult zebrafish⁸¹. This paradigm opens up avenues for genetic and small molecule screens to identify signaling pathways critical for musculoskeletal adaptation to loading and exercise.

4. PHENOTYPING

4.1. MicroCT

3D-high-resolution micro-computed tomography has become established as a powerful method to assess bone morphology and microstructure in zebrafish^{18; 44; 82; 83}. Using a 5 μ m voxel size, bone structure indices as vertebral bone volume, thickness, and eccentricity can be characterized^{18; 44}. Neural arch area, which reflects modeling arising from osteoblast and osteoclast activity, can also be captured⁸². Because of their small size, whole body, high-resolution scans are readily acquired⁸³. Software for semi-automated segmentation enables in-depth phenotyping at a large number of skeletal sites. By quantifying hundreds of measures, this was shown to increase sensitivity in discriminating mutant populations⁸³. Moreover, the osteocyte lacunar network in the vertebral tissue can be imaged at high resolution with lab-based nano-CTs and 3D X-ray Microscopy (3DXRM). Orientation of the osteocyte lacunae in relation to the long and short axis of the vertebral bodies, sphericity, mean lacunar volume and lacunar density can be quantified^{18; 44}. Finally, synchrotron-based X-ray microCT, when combined with tissue contrast stains, can yield whole-organism images suitable for cell-level quantitative histological phenotyping in zebrafish⁸⁴.

4.2. Histomorphometry

In zebrafish, histologic sections stained using von Kossa/van Gieson, Goldner's modified Masson-trichrome and toluidine blue enable static bone histomorphometry, and performed in accordance with standardized nomenclature set forth by the ASBMR nomenclature committee for practitioners of bone histomorphometry⁸⁵. Calcein labeling or double labeling with calcein and Alizarin Red S can be performed and double labels can be evaluated for dynamic bone histomorphometry^{18; 44}. Such an approach was used to quantify increases in mineral apposition rate (MAR), mineralizing surface per bone surface (MS/BS), and bone formation rate (BFR) at the vertebral endplates in zebrafish subjected to swimming exercise⁴⁴.

4.3. Assessment of bone composition, mineral density distribution, and mechanical properties

Recently, *quantitative backscattered electron imaging (qBEI)* has been established as an effective means to measure bone mineral density distribution in zebrafish^{18;44}. Gray value histograms were used to assess the mean calcium content in the mineralized bone tissue, as well as the homogeneity of mineralization^{18;44}. Vibrational spectroscopy methods (e.g. *Fourier transform infrared spectroscopy (FTIR) and Raman spectroscopy*) have also been adapted to zebrafish bone^{18;86}. Parameters such as the mineral-to-matrix-ratio, carbonate-to-phosphate ratio, cross-link-ratio (collagen maturity), and crystallinity (purity, size of mineral crystals) of the bone were shown to provide information about the molecular and compositional bone characteristics¹⁸. Finally, nanoindentation of vertebrae can be performed in zebrafish to assess local mechanical properties material properties such as Young's Modulus (Elastic modulus), hardness, and fracture toughness¹⁸. Biomechanical properties of zebrafish cranial tendons can also be measured. A maxillary tendon was found to have stress-strain nonlinearity and a linear modulus similar to mammalian tendon data⁶².

5. DISEASE APPLICATIONS

5.1. Collagenopathies

OI is a disease of the collagen matrix which results in brittle bones and skeletal deformities. In humans, collagen type I is a heterotrimer composed by two alpha chains, $\alpha 1(I)$ and $\alpha 2(I)$, which trimerize in a 2:1 ratio, respectively, to form a fibril with a triple helix structure. In zebrafish, the collagen type I triple helix is composed of three α chains, $\alpha 1(I)$, $\alpha 2(I)$, and $\alpha 3(I)$, which are encoded for by the genes *colla1a*, *colla2*, and *colla1b*, respectively⁸⁷. Most human patients with OI are attributed to mutations in type I collagens, with the majority of mutations disrupting the conserved Gly-X-Y motifs responsible for fibrillar assembly of the collagen heterotrimers⁸⁸. In zebrafish, several dominant mutants have been identified carrying heterozygous glycine substitution in the $\alpha 1$ chain of collagen type I, and which exhibit severe, pathological features of classical OI. This was demonstrated in the *chihuahua* mutant, which exhibited changes in vertebral tissue composition¹⁸. A large panel of zebrafish mutants of *colla1* genes with qualitative and quantitative defects in collagen type I have been characterized, and found to mirror genotype-phenotype relationships of the range of OI subtypes found in humans¹⁶⁻²⁰. Disease models have also been developed for mutations affecting COL1A2¹⁷ as well as rare recessive forms of OI affecting non-collagenous proteins (e.g., PLOD2^{83;89} and BMP1^{83;90}).

5.2. Spinal curvature

Adolescent idiopathic scoliosis (AIS) is defined as scoliosis without underlying vertebral malformations⁹¹. While the pathogenesis of this disease is still controversial, the zebrafish has made significant advances in our understanding of this disorder. AIS-like scoliosis was demonstrated in *cc2d2a* mutant zebrafish, which has a role in vesicle trafficking and fusion at the transition zone of photoreceptor connecting cilium in the eye⁹². Recently, mutant zebrafish displaying larval or late-onset scoliosis without vertebral malformations, analogous to AIS, have been described for *c21orf59*, *ccdc40*, *ccdc151*, *dyx1c1*, *kif6*, and *ptk7*^{93;94}. A common mechanism has emerged from these studies, where loss of ependymal

cell cilia function lining the ventricles of the brain, leading to reduced cerebrospinal fluid flow can generate AIS in zebrafish^{93; 95}. Interestingly, maternal-zygotic *ptk7* mutant zebrafish display scoliosis with vertebral malformations, while strictly zygotic *ptk7* mutant fish display late-onset AIS without vertebral malformations⁹⁶. This suggests that severity of scoliosis can be on a spectrum based on temporal requirements for gene function, which may explain the strong association of AIS in families of children with CS⁹⁷.

The cellular mechanism of AIS in *ptk7* was demonstrated by a *foxj1:ptk7* transgenic zebrafish, which can completely rescue the onset of scoliosis phenotypes observed in *ptk7* mutant zebrafish⁹³. *Foxj1* is a master transcriptional regulator of motile cilia⁹⁸, which labels motile cilia of the ventricles of the brain and in the pronephros but also labels subset of central canal lining ciliated CSF-fluid contacting neurons. Indeed, disruption of a major signaling receptor, *pdk211*, of CSF-contacting neurons led to mild alterations in spine curvature in zebrafish⁹⁹. While it is still unclear how these disruptions of CSF physiology cause scoliosis, recent studies demonstrated that CSF flow (i) helps to stimulate the proper formation of the extracellular Reissner's fiber which can directly contribute to body straightness during embryonic development, via an unknown mechanisms¹⁰⁰; and (ii) that CSF flow transports adrenergic signals which stimulate the expression of urotensin neuropeptides from CSF-contacting neurons along the spinal cord and mutant zebrafish of the urotensin receptor *uts2ra* display AIS¹⁰¹. How these studies will translate to mammalian physiology is still unclear. However, at least one candidate gene for AIS uncovered in zebrafish *kif6*⁹⁴ does not recapitulate AIS phenotypes when mutated in mouse or human⁹⁵.

Although having early differences in vertebral specification compared to mammals, the zebrafish may also serve as a model for aspects of congenital scoliosis (CS). Disruption of the extracellular sheath through chemical disruption of lysyl oxidases¹⁰² or specific genetic disruptions of the sheath extracellular matrix components of the notochord sheath such as: *col8a1a*, *col27a1a/b*, or *calymmin*^{16; 103; 104} can generate CS-like scoliosis with vertebral malformations in zebrafish suggesting potential underlying components of zebrafish development that can be used to assess gene function in CS etiology.

5.3. Disease loci

Human genome-wide association studies (GWAS) are a powerful means to understand genetic risk factors for chronic diseases such as osteoarthritis¹⁰⁵ and osteoporosis¹⁰⁶. These loci may harbor novel drug targets for orthopedic diseases, as evidenced by the fact that OPG/RANK/RANKL and LRP5/SOST, all genes at BMD loci, are members of pathways targeted by osteoporosis drugs (Denosumab and Romosozumab, respectively). A recent analysis of UK-Biobank data identified 515 loci associated with eBMD¹⁰⁶. The causal genes responsible for most associations have yet to be assigned, and thus gene discovery in animal models is needed to complement GWAS in human populations¹⁰⁷. One limitation with knockout mice is the lack of coverage of genes at BMD loci. For instance, of the >23,000 protein coding genes in the mouse genome, <5% have been rigorously analyzed for bone phenotypes in knockout mice within ancillary bone phenotyping projects of the International Mouse Phenotyping Consortium¹⁰⁷. A recent study demonstrated the potential to rapidly generate mutations in CRISPR-edited G0 zebrafish to attribute functional

contributions of candidate genes at BMD loci²⁵. This study also identified a robust, low bone mass phenotype in zebrafish with mutations in *wnt16*²⁵, a candidate gene with strong ties to osteoporosis-related traits in humans. Skeletal abnormalities have been observed in fish with altered function in other genes at BMD loci, including LRP5, OSX/SP7, and RANKL^{108–110}, further evidence that the identification of genes that contribute to human osteoporosis-related traits in zebrafish is feasible. The utility of zebrafish for human skeletal genomics is reviewed in¹⁰⁷.

5.4 Osteoarthritis

Recent data has shown zebrafish can be used to study the development of synovial joints and osteoarthritis. Mutations in *coll1a2* in zebrafish leads to joint pathologies reminiscent of early-onset osteoarthritis in humans¹¹¹. Further, as in humans and mice, zebrafish lubricin or *proteoglycan-4b* (*prg4b*) expression was found within some joint regions, such as articular chondrocytes in the jaw¹¹². These joint regions also expressed *coll10a1*, *acana*, and *matrilin1*, which together with *prg4b*, show similarities to genes expressed in mammalian synovial joints. Loss of both *prg4* orthologs resulted in synovial hyperplasia and deterioration of the joint surface by 6–12 months of age. This phenotype recapitulates that found in mouse where loss of Prg4 results in joint disease by 2 months¹¹³. Although it is unknown why there is a significant delay in timing of osteoarthritis onset in the zebrafish compared with mouse genetic models, possible explanations are the zebrafish's robust regenerative abilities combined with different mechanical environments. Even with these differences, this work establishes the conservation in synovial joint gene expression and function in the zebrafish.

6. SUMMARY/FUTURE DIRECTIONS

The genetic causes of skeletal disorders are rapidly becoming identified, and it is now clear that many common musculoskeletal disorders are fundamentally complex in their causes^{106; 114}. The field is faced with the need to more fully interrogate the functional consequences of genetic and environmental stresses in how the skeleton and its connecting tissues are formed, how it integrates with broader physiology, and how it repairs damage. There is now a strong rationale to validate newly discovered disease candidate genes in the zebrafish prior to extensive mouse analyses. The refinement of clonal analyses of gene function will expedite combinatorial analysis of gene function and permit a systematic testing platform for genetic association studies and analysis of genetic modifiers. With the ability to visualize cellular dynamics during formation of the skeleton as well as during repair, in different genetic contexts, the zebrafish provides a powerful system to bring functional characterization in line with the rate of genetic discoveries.

In addition to validation, the zebrafish is also a valuable platform for discovery. Due to its small size, low cost, and genetic malleability, the zebrafish has opened new screening methods that have already discovered small molecules efficacious in regulating skeletal phenotypes. Similarly, through unbiased mutational screening, new genes—and new functions for existing genes—have been discovered. These mutations have shown to be

predictive of causes of skeletal disorders in humans^{115; 116}, and opened new areas of musculoskeletal development previously uncharacterized or neglected^{101; 117}.

As the use of zebrafish for orthopaedic research is still in its relative infancy, there are a number of open questions regarding developmental stages, bones, and phenotypic traits in zebrafish that best serve as a model for human skeletal biology¹⁰⁷. Morphophysiological differences can make one-to-one modeling of human skeletal phenotypes in zebrafish challenging. Origins of mammalian bones and their connections to fish bones (e.g., the mammalian middle ear bones, which derive from bones that form the jaw in fish¹¹⁸) can sometimes be revealed through evolutionary analyses, however, such relationships cannot always be made. In this context, a community effort to phenotype zebrafish mutants for orthologs of genes examined in mutant mouse phenotyping consortiums may aid in identifying zebrafish phenotypes that are most consistently associated with phenotypic changes in the orthologous mutant mouse¹⁰⁷.

With the extension of zebrafish work into phenotypes of the skeleton beyond early development, the broad utility of this model has emerged. While there are differences stemming from the use of a non-mammalian vertebrate for modeling human disorders, the zebrafish model provides both genetic and anatomical foundations in which informed analyses can be made concerning the etiology of disorders and a tool in which to refine potential therapeutic strategies.

ACKNOWLEDGEMENTS

This work was supported by the National Institutes of Health under award numbers AR071554 (JLG), AR074541 (JLG), AR072009 (RSG), (DE024434 (MPH), and AR066061 (RYK). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. BB would like to thank Hrishikesh A. Bale, Imke A. K. Fiedler and Tessa Krappa for imaging support. JLG would like to thank Marie Noedl and Xubo Niu for providing the tendon images.

REFERENCES

1. Shah AN, Davey CF, Whitebitch AC, et al. 2015 Rapid reverse genetic screening using CRISPR in zebrafish. *Nat Methods* 12:535–540. [PubMed: 25867848]
2. Keller PJ, Schmidt AD, Wittbrodt J, et al. 2008 Reconstruction of zebrafish early embryonic development by scanned light sheet microscopy. *Science* 322:1065–1069. [PubMed: 18845710]
3. Ahrens MB, Li JM, Orger MB, et al. 2012 Brain-wide neuronal dynamics during motor adaptation in zebrafish. *Nature* 485:471–477. [PubMed: 22622571]
4. Kang J, Hu J, Karra R, et al. 2016 Modulation of tissue repair by regeneration enhancer elements. *Nature* 532:201–206. [PubMed: 27049946]
5. Yan C, Brunson DC, Tang Q, et al. 2019 Visualizing Engrafted Human Cancer and Therapy Responses in Immunodeficient Zebrafish. *Cell*.
6. Howe K, Clark MD, Torroja CF, et al. 2013 The zebrafish reference genome sequence and its relationship to the human genome. *Nature* 496:498–503. [PubMed: 23594743]
7. Yan YL, Willoughby J, Liu D, et al. 2005 A pair of Sox: distinct and overlapping functions of zebrafish sox9 co-orthologs in craniofacial and pectoral fin development. *Development* 132:1069–1083. [PubMed: 15689370]
8. Rohner N, Bercsenyi M, Orban L, et al. 2009 Duplication of fgfr1 permits Fgf signaling to serve as a target for selection during domestication. *Curr Biol* 19:1642–1647. [PubMed: 19733072]
9. Mork L, Crump G. 2015 Zebrafish Craniofacial Development: A Window into Early Patterning. *Curr Top Dev Biol* 115:235–269. [PubMed: 26589928]

10. Bergen DJM, Kague E, Hammond CL. 2019 Zebrafish as an Emerging Model for Osteoporosis: A Primary Testing Platform for Screening New Osteo-Active Compounds. *Front Endocrinol (Lausanne)* 10:6. [PubMed: 30761080]
11. Kwon RY, Watson CJ, Karasik D. 2019 Using zebrafish to study skeletal genomics. *Bone*.
12. Schilling TF, Piotrowski T, Grandel H, et al. 1996 Jaw and branchial arch mutants in zebrafish I: branchial arches. *Development* 123:329–344. [PubMed: 9007253]
13. Piotrowski T, Schilling TF, Brand M, et al. 1996 Jaw and branchial arch mutants in zebrafish II: anterior arches and cartilage differentiation. *Development* 123:345–356. [PubMed: 9007254]
14. Neuhauss SC, Solnica-Krezel L, Schier AF, et al. 1996 Mutations affecting craniofacial development in zebrafish. *Development* 123:357–367. [PubMed: 9007255]
15. Harris MP, Rohner N, Schwarz H, et al. 2008 Zebrafish *eda* and *edar* mutants reveal conserved and ancestral roles of ectodysplasin signaling in vertebrates. *PLoS Genet* 4:e1000206. [PubMed: 18833299]
16. Henke K, Daane JM, Hawkins MB, et al. 2017 Genetic Screen for Postembryonic Development in the Zebrafish (*Danio rerio*): Dominant Mutations Affecting Adult Form. *Genetics* 207:609–623. [PubMed: 28835471]
17. Gistelink C, Kwon RY, Malfait F, et al. 2018 Zebrafish type I collagen mutants faithfully recapitulate human type I collagenopathies. *Proc Natl Acad Sci U S A* 115:E8037–E8046. [PubMed: 30082390]
18. Fiedler IAK, Schmidt FN, Wolfel EM, et al. 2018 Severely Impaired Bone Material Quality in Chihuahua Zebrafish Resembles Classical Dominant Human Osteogenesis Imperfecta. *J Bone Miner Res* 33:1489–1499. [PubMed: 29665086]
19. Gioia R, Tonelli F, Ceppi I, et al. 2017 The chaperone activity of 4PBA ameliorates the skeletal phenotype of Chihuahua, a zebrafish model for dominant osteogenesis imperfecta. *Hum Mol Genet* 26:2897–2911. [PubMed: 28475764]
20. Fisher S, Jagadeeswaran P, Halpern ME. 2003 Radiographic analysis of zebrafish skeletal defects. *Dev Biol* 264:64–76. [PubMed: 14623232]
21. Haffter P, Odenthal J, Mullins MC, et al. 1996 Mutations affecting pigmentation and shape of the adult zebrafish. *Dev Genes Evol* 206:260–276. [PubMed: 24173565]
22. Kettleborough RN, Busch-Nentwich EM, Harvey SA, et al. 2013 A systematic genome-wide analysis of zebrafish protein-coding gene function. *Nature* 496:494–497. [PubMed: 23594742]
23. Bedell VM, Wang Y, Campbell JM, et al. 2012 In vivo genome editing using a high-efficiency TALEN system. *Nature* 491:114–118. [PubMed: 23000899]
24. El-Brolosy MA, Kontarakis Z, Rossi A, et al. 2019 Genetic compensation triggered by mutant mRNA degradation. *Nature* 568:193–197. [PubMed: 30944477]
25. Watson CJ, Monstad-Rios AT, Bhimani RM, et al. 2018 Decoding G0 somatic mutants through deep phenotyping and mosaic pattern analysis in the zebrafish skeleton. *bioRxiv* doi: 10.1101/466185.
26. Oppedal D, Goldsmith MI. 2010 A chemical screen to identify novel inhibitors of fin regeneration in zebrafish. *Zebrafish* 7:53–60. [PubMed: 20384483]
27. Monstad-Rios AT, Watson CJ, Kwon RY. 2018 ScreenCube: A 3D Printed System for Rapid and Cost-Effective Chemical Screening in Adult Zebrafish. *Zebrafish* 15:1–8. [PubMed: 29083959]
28. Yu PB, Hong CC, Sachidanandan C, et al. 2008 Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. *Nat Chem Biol* 4:33–41. [PubMed: 18026094]
29. Kawahara G, Karpf JA, Myers JA, et al. 2011 Drug screening in a zebrafish model of Duchenne muscular dystrophy. *Proc Natl Acad Sci U S A* 108:5331–5336. [PubMed: 21402949]
30. Wiley DS, Redfield SE, Zon LI. 2017 Chemical screening in zebrafish for novel biological and therapeutic discovery. *Methods Cell Biol* 138:651–679. [PubMed: 28129862]
31. Chen JW, Galloway JL. 2014 The development of zebrafish tendon and ligament progenitors. *Development* 141:2035–2045. [PubMed: 24803652]
32. Ma RC, Jacobs CT, Sharma P, et al. 2018 Stereotypic generation of axial tenocytes from bipartite sclerotome domains in zebrafish. *PLoS Genet* 14:e1007775. [PubMed: 30388110]

33. Schilling TF, Kimmel CB. 1997 Musculoskeletal patterning in the pharyngeal segments of the zebrafish embryo. *Development* 124:2945–2960. [PubMed: 9247337]
34. Dubrulle J, Pourquie O. 2003 Welcome to syndetome: a new somitic compartment. *Dev Cell* 4:611–612. [PubMed: 12737797]
35. Charvet B, Malbouyres M, Pagnon-Minot A, et al. 2011 Development of the zebrafish myoseptum with emphasis on the myotendinous junction. *Cell Tissue Res* 346:439–449. [PubMed: 22086205]
36. Subramanian A, Schilling TF. 2014 Thrombospondin-4 controls matrix assembly during development and repair of myotendinous junctions. *Elife* 3.
37. Bird NC, Mabee PM. 2003 Developmental morphology of the axial skeleton of the zebrafish, *Danio rerio* (Ostariophysi: Cyprinidae). *Dev Dyn* 228:337–357. [PubMed: 14579374]
38. Cabbage CC, Mabee PM. 1996 Development of the cranium and paired fins in the zebrafish *Danio rerio* (Ostariophysi, Cyprinidae). *J Morphol* 229:121–160. [PubMed: 29852585]
39. Apschner A, Schulte-Merker S, Witten PE. 2011 Not all bones are created equal - using zebrafish and other teleost species in osteogenesis research. *Methods Cell Biol* 105:239–255. [PubMed: 21951533]
40. Weigle J, Franz-Odenaal TA. 2016 Functional bone histology of zebrafish reveals two types of endochondral ossification, different types of osteoblast clusters and a new bone type. *J Anat* 229:92–103. [PubMed: 27278890]
41. Lefebvre V, Bhattaram P. 2010 Vertebrate skeletogenesis. *Curr Top Dev Biol* 90:291–317. [PubMed: 20691853]
42. Fleming A, Keynes R, Tannahill D. 2004 A central role for the notochord in vertebral patterning. *Development* 131:873–880. [PubMed: 14736741]
43. Chatani M, Takano Y, Kudo A. 2011 Osteoclasts in bone modeling, as revealed by in vivo imaging, are essential for organogenesis in fish. *Dev Biol* 360:96–109. [PubMed: 21963458]
44. Suniaga S, Rolvien T, Vom Scheidt A, et al. 2018 Increased mechanical loading through controlled swimming exercise induces bone formation and mineralization in adult zebrafish. *Sci Rep* 8:3646. [PubMed: 29483529]
45. Chen JW, Galloway JL. 2017 Using the zebrafish to understand tendon development and repair. *Methods Cell Biol* 138:299–320. [PubMed: 28129848]
46. Brent AE, Tabin CJ. 2004 FGF acts directly on the somitic tendon progenitors through the Ets transcription factors *Pea3* and *Erm* to regulate scleraxis expression. *Development* 131:3885–3896. [PubMed: 15253939]
47. Pryce BA, Watson SS, Murchison ND, et al. 2009 Recruitment and maintenance of tendon progenitors by TGFbeta signaling are essential for tendon formation. *Development* 136:1351–1361. [PubMed: 19304887]
48. McGurk PD, Swartz ME, Chen JW, et al. 2017 In vivo zebrafish morphogenesis shows *Cyp26b1* promotes tendon condensation and musculoskeletal patterning in the embryonic jaw. *PLoS Genet* 13:e1007112. [PubMed: 29227993]
49. Subramanian A, Kanzaki LF, Galloway JL, et al. 2018 Mechanical force regulates tendon extracellular matrix organization and tenocyte morphogenesis through TGFbeta signaling. *Elife* 7.
50. Kiessling A, Ruohonen K, Bjørnevik M. 2006 Muscle fibre growth and quality in fish. *Arch Tierz, Dummerstorf* 49:137–146.
51. Inohaya K, Takano Y, Kudo A. 2007 The teleost intervertebral region acts as a growth center of the centrum: in vivo visualization of osteoblasts and their progenitors in transgenic fish. *Dev Dyn* 236:3031–3046. [PubMed: 17907202]
52. Lieschke GJ, Currie PD. 2007 Animal models of human disease: zebrafish swim into view. *Nat Rev Genet* 8:353–367. [PubMed: 17440532]
53. Akiyama H, Chaboissier MC, Martin JF, et al. 2002 The transcription factor *Sox9* has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of *Sox5* and *Sox6*. *Genes Dev* 16:2813–2828. [PubMed: 12414734]
54. Felber K, Croucher P, Roehl HH. 2011 Hedgehog signalling is required for perichondral osteoblast differentiation in zebrafish. *Mech Dev* 128:141–152. [PubMed: 21126582]

55. Avaron F, Hoffman L, Guay D, et al. 2006 Characterization of two new zebrafish members of the hedgehog family: atypical expression of a zebrafish indian hedgehog gene in skeletal elements of both endochondral and dermal origins. *Dev Dyn* 235:478–489. [PubMed: 16292774]
56. Eames BF, Singer A, Smith GA, et al. 2010 UDP xylose synthase 1 is required for morphogenesis and histogenesis of the craniofacial skeleton. *Dev Biol* 341:400–415. [PubMed: 20226781]
57. Li N, Felber K, Elks P, et al. 2009 Tracking gene expression during zebrafish osteoblast differentiation. *Dev Dyn* 238:459–466. [PubMed: 19161246]
58. Elizondo MR, Arduini BL, Paulsen J, et al. 2005 Defective skeletogenesis with kidney stone formation in dwarf zebrafish mutant for *trpm7*. *Curr Biol* 15:667–671. [PubMed: 15823540]
59. de Paula FJ, Rosen CJ. 2013 Bone Remodeling and Energy Metabolism: New Perspectives. *Bone Res* 1:72–84. [PubMed: 26273493]
60. Moser SC, van der Eerden BCJ. 2018 Osteocalcin-A Versatile Bone-Derived Hormone. *Front Endocrinol (Lausanne)* 9:794. [PubMed: 30687236]
61. Gerhard GS, Kauffman EJ, Wang X, et al. 2002 Life spans and senescent phenotypes in two strains of Zebrafish (*Danio rerio*). *Exp Gerontol* 37:1055–1068. [PubMed: 12213556]
62. Shah RR, Nerurkar NL, Wang CC, et al. 2015 Tensile properties of craniofacial tendons in the mature and aged zebrafish. *J Orthop Res* 33:867–873. [PubMed: 25665155]
63. Hayes AJ, Reynolds S, Nowell MA, et al. 2013 Spinal deformity in aged zebrafish is accompanied by degenerative changes to their vertebrae that resemble osteoarthritis. *Plos One* 8:e75787. [PubMed: 24086633]
64. Shanthanagouda AH, Guo BS, Ye RR, et al. 2014 Japanese medaka: a non-mammalian vertebrate model for studying sex and age-related bone metabolism in vivo. *Plos One* 9:e88165. [PubMed: 24523879]
65. Geurtzen K, Knopf F, Wehner D, et al. 2014 Mature osteoblasts dedifferentiate in response to traumatic bone injury in the zebrafish fin and skull. *Development* 141:2225–2234. [PubMed: 24821985]
66. Paul S, Schindler S, Giovannone D, et al. 2016 Ihha induces hybrid cartilage-bone cells during zebrafish jawbone regeneration. *Development* 143:2066–2076. [PubMed: 27122168]
67. Keightley MC, Wang CH, Pazhakh V, et al. 2014 Delineating the roles of neutrophils and macrophages in zebrafish regeneration models. *Int J Biochem Cell Biol* 56:92–106. [PubMed: 25058313]
68. Eming SA, Wynn TA, Martin P. 2017 Inflammation and metabolism in tissue repair and regeneration. *Science* 356:1026–1030. [PubMed: 28596335]
69. Richardson R, Slanchev K, Kraus C, et al. 2013 Adult zebrafish as a model system for cutaneous wound-healing research. *J Invest Dermatol* 133:1655–1665. [PubMed: 23325040]
70. Renshaw SA, Loynes CA, Elworthy S, et al. 2007 Modeling inflammation in the zebrafish: how a fish can help us understand lung disease. *Exp Lung Res* 33:549–554. [PubMed: 18075830]
71. Ellett F, Pase L, Hayman JW, et al. 2011 *mpeg1* promoter transgenes direct macrophage-lineage expression in zebrafish. *Blood* 117:e49–56. [PubMed: 21084707]
72. Petrie TA, Strand NS, Yang CT, et al. 2014 Macrophages modulate adult zebrafish tail fin regeneration. *Development* 141:2581–2591. [PubMed: 24961798]
73. Watson CJ, Kwon RY. 2015 Osteogenic programs during zebrafish fin regeneration. *Bonekey Rep* 4:745. [PubMed: 26421148]
74. Knopf F, Hammond C, Chekuru A, et al. 2011 Bone regenerates via dedifferentiation of osteoblasts in the zebrafish fin. *Dev Cell* 20:713–724. [PubMed: 21571227]
75. Recidoro AM, Roof AC, Schmitt M, et al. 2014 Botulinum toxin induces muscle paralysis and inhibits bone regeneration in zebrafish. *J Bone Miner Res* 29:2346–2356. [PubMed: 24806738]
76. Ando K, Shibata E, Hans S, et al. 2017 Osteoblast Production by Reserved Progenitor Cells in Zebrafish Bone Regeneration and Maintenance. *Dev Cell* 43:643–650 e643. [PubMed: 29103952]
77. Singh SP, Holdway JE, Poss KD. 2012 Regeneration of amputated zebrafish fin rays from de novo osteoblasts. *Dev Cell* 22:879–886. [PubMed: 22516203]
78. Sousa S, Valerio F, Jacinto A. 2012 A new zebrafish bone crush injury model. *Biol Open* 1:915–921. [PubMed: 23213486]

79. Tomecka MJ, Ethiraj LP, Sanchez LM, et al. 2019 Clinical pathologies of bone fracture modelled in zebrafish. *Dis Model Mech* 12.
80. Fiaz AW, Leon-Kloosterziel KM, Gort G, et al. 2012 Swim-training changes the spatio-temporal dynamics of skeletogenesis in zebrafish larvae (*Danio rerio*). *Plos One* 7:e34072. [PubMed: 22529905]
81. Palstra AP, Rovira M, Rizo-Roca D, et al. 2014 Swimming-induced exercise promotes hypertrophy and vascularization of fast skeletal muscle fibres and activation of myogenic and angiogenic transcriptional programs in adult zebrafish. *Bmc Genomics* 15:1136. [PubMed: 25518849]
82. Charles JF, Sury M, Tsang K, et al. 2017 Utility of quantitative micro-computed tomographic analysis in zebrafish to define gene function during skeletogenesis. *Bone* 101:162–171. [PubMed: 28476577]
83. Hur M, Gistelinc CA, Huber P, et al. 2017 MicroCT-based phenomics in the zebrafish skeleton reveals virtues of deep phenotyping in a distributed organ system. *Elife* 6.
84. Ding Y, Vanselow DJ, Yakovlev MA, et al. 2019 Computational 3D histological phenotyping of whole zebrafish by X-ray histotomography. *Elife* 8.
85. Dempster DW, Compston JE, Drezner MK, et al. 2013 Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res* 28:2–17. [PubMed: 23197339]
86. Fiedler IAK, Casanova M, Keplinger T, et al. 2018 Effect of short-term formaldehyde fixation on Raman spectral parameters of bone quality. *J Biomed Opt* 23:1–6.
87. Gistelinc C, Gioia R, Gagliardi A, et al. 2016 Zebrafish Collagen Type I: Molecular and Biochemical Characterization of the Major Structural Protein in Bone and Skin. *Scientific Reports* 6:21540. [PubMed: 26876635]
88. Ricard-Blum S 2011 The collagen family. *Cold Spring Harb Perspect Biol* 3:a004978. [PubMed: 21421911]
89. Gistelinc C, Witten PE, Huysseune A, et al. 2016 Loss of Type I Collagen Telopeptide Lysyl Hydroxylation Causes Musculoskeletal Abnormalities in a Zebrafish Model of Bruck Syndrome. *J Bone Miner Res* 31:1930–1942. [PubMed: 27541483]
90. Asharani PV, Keupp K, Semler O, et al. 2012 Attenuated BMP1 function compromises osteogenesis, leading to bone fragility in humans and zebrafish. *Am J Hum Genet* 90:661–674. [PubMed: 22482805]
91. Cheng JC, Castelein RM, Chu WC, et al. 2015 Adolescent idiopathic scoliosis. *Nat Rev Dis Primers* 1:15030. [PubMed: 27188385]
92. Bachmann-Gagescu R, Phelps IG, Stearns G, et al. 2011 The ciliopathy gene *cc2d2a* controls zebrafish photoreceptor outer segment development through a role in Rab8-dependent vesicle trafficking. *Hum Mol Genet* 20:4041–4055. [PubMed: 21816947]
93. Grimes DT, Boswell CW, Morante NF, et al. 2016 Zebrafish models of idiopathic scoliosis link cerebrospinal fluid flow defects to spine curvature. *Science* 352:1341–1344. [PubMed: 27284198]
94. Buchan JG, Gray RS, Gansner JM, et al. 2014 Kinesin family member 6 (*kif6*) is necessary for spine development in zebrafish. *Dev Dyn* 243:1646–1657. [PubMed: 25283277]
95. Konjikusic MJ, Yeetong P, Boswell CW, et al. 2018 Mutations in Kinesin family member 6 reveal specific role in ependymal cell ciliogenesis and human neurological development. *PLoS Genet* 14:e1007817. [PubMed: 30475797]
96. Hayes M, Gao X, Yu LX, et al. 2014 *ptk7* mutant zebrafish models of congenital and idiopathic scoliosis implicate dysregulated Wnt signalling in disease. *Nat Commun* 5:4777. [PubMed: 25182715]
97. Purkiss SB, Driscoll B, Cole WG, et al. 2002 Idiopathic scoliosis in families of children with congenital scoliosis. *Clin Orthop Relat Res*:27–31.
98. Yu X, Ng CP, Habacher H, et al. 2008 *Foxj1* transcription factors are master regulators of the motile ciliogenic program. *Nat Genet* 40:1445–1453. [PubMed: 19011630]
99. Sternberg JR, Prendergast AE, Brosse L, et al. 2018 *Pkd211* is required for mechanoreception in cerebrospinal fluid-contacting neurons and maintenance of spine curvature. *Nat Commun* 9:3804. [PubMed: 30228263]

100. Cantaut-Belarif Y, Sternberg JR, Thouvenin O, et al. 2018 The Reissner Fiber in the Cerebrospinal Fluid Controls Morphogenesis of the Body Axis. *Curr Biol* 28:2479–2486 e2474. [PubMed: 30057305]
101. Zhang X, Jia S, Chen Z, et al. 2018 Cilia-driven cerebrospinal fluid flow directs expression of urotensin neuropeptides to straighten the vertebrate body axis. *Nat Genet* 50:1666–1673. [PubMed: 30420648]
102. Gansner JM, Mendelsohn BA, Hultman KA, et al. 2007 Essential role of lysyl oxidases in notochord development. *Dev Biol* 307:202–213. [PubMed: 17543297]
103. Gray RS, Wilm TP, Smith J, et al. 2014 Loss of col8a1a function during zebrafish embryogenesis results in congenital vertebral malformations. *Dev Biol* 386:72–85. [PubMed: 24333517]
104. Christiansen HE, Lang MR, Pace JM, et al. 2009 Critical early roles for col27a1a and col27a1b in zebrafish notochord morphogenesis, vertebral mineralization and post-embryonic axial growth. *Plos One* 4:e8481. [PubMed: 20041163]
105. Tachmazidou I, Hatzikotoulas K, Southam L, et al. 2019 Identification of new therapeutic targets for osteoarthritis through genome-wide analyses of UK Biobank data. *Nat Genet* 51:230–236. [PubMed: 30664745]
106. Morris JA, Kemp JP, Youlten SE, et al. 2019 An atlas of genetic influences on osteoporosis in humans and mice. *Nat Genet* 51:258–266. [PubMed: 30598549]
107. Kwon RY, Watson CJ, Karasik D. 2019 Using zebrafish to study skeletal genomics. *Bone* 126:37–50. [PubMed: 30763636]
108. Willems B, Tao S, Yu T, et al. 2015 The Wnt Co-Receptor Lrp5 Is Required for Cranial Neural Crest Cell Migration in Zebrafish. *Plos One* 10:e0131768. [PubMed: 26121341]
109. Kague E, Roy P, Asselin G, et al. 2016 Osterix/Sp7 limits cranial bone initiation sites and is required for formation of sutures. *Dev Biol* 413:160–172. [PubMed: 26992365]
110. To TT, Witten PE, Renn J, et al. 2012 Rankl-induced osteoclastogenesis leads to loss of mineralization in a medaka osteoporosis model. *Development* 139:141–150. [PubMed: 22096076]
111. Lawrence EA, Kague E, Aggleton JA, et al. 2018 The mechanical impact of col11a2 loss on joints; col11a2 mutant zebrafish show changes to joint development and function, which leads to early-onset osteoarthritis. *Philos Trans R Soc Lond B Biol Sci* 373.
112. Askary A, Smeeton J, Paul S, et al. 2016 Ancient origin of lubricated joints in bony vertebrates. *Elife* 5.
113. Hill A, Duran J, Purcell P. 2014 Lubricin protects the temporomandibular joint surfaces from degeneration. *Plos One* 9:e106497. [PubMed: 25188282]
114. Estrada K, Stykarsdottir U, Evangelou E, et al. 2012 Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. *Nat Genet* 44:491–501. [PubMed: 22504420]
115. Clement A, Wiweger M, von der Hardt S, et al. 2008 Regulation of zebrafish skeletogenesis by ext2/dackel and papst1/pinscher. *PLoS Genet* 4:e1000136. [PubMed: 18654627]
116. Wiweger MI, Zhao Z, van Merkesteyn RJ, et al. 2012 HSPG-deficient zebrafish uncovers dental aspect of multiple osteochondromas. *Plos One* 7:e29734. [PubMed: 22253766]
117. Daane JM, Lanni J, Rothenberg I, et al. 2018 Bioelectric-calcineurin signaling module regulates allometric growth and size of the zebrafish fin. *Sci Rep* 8:10391. [PubMed: 29991812]
118. Anthwal N, Joshi L, Tucker AS. 2013 Evolution of the mammalian middle ear and jaw: adaptations and novel structures. *J Anat* 222:147–160. [PubMed: 22686855]
119. Kwak J, Park OK, Jung YJ, et al. 2013 Live image profiling of neural crest lineages in zebrafish transgenic lines. *Mol Cells* 35:255–260. [PubMed: 23456294]
120. Mongera A, Singh AP, Levesque MP, et al. 2013 Genetic lineage labeling in zebrafish uncovers novel neural crest contributions to the head, including gill pillar cells. *Development* 140:916–925. [PubMed: 23362350]
121. Dale RM, Topczewski J. 2011 Identification of an evolutionarily conserved regulatory element of the zebrafish col2a1a gene. *Dev Biol* 357:518–531. [PubMed: 21723274]

122. Askary A, Mork L, Paul S, et al. 2015 Iroquois Proteins Promote Skeletal Joint Formation by Maintaining Chondrocytes in an Immature State. *Dev Cell* 35:358–365. [PubMed: 26555055]
123. Hammond CL, Schulte-Merker S. 2009 Two populations of endochondral osteoblasts with differential sensitivity to Hedgehog signalling. *Development* 136:3991–4000. [PubMed: 19906866]
124. Berger J, Currie PD. 2013 503unc, a small and muscle-specific zebrafish promoter. *Genesis* 51:443–447. [PubMed: 23444339]
125. Kague E, Gallagher M, Burke S, et al. 2012 Skeletogenic fate of zebrafish cranial and trunk neural crest. *Plos One* 7:e47394. [PubMed: 23155370]
126. Mitchell RE, Huitema LF, Skinner RE, et al. 2013 New tools for studying osteoarthritis genetics in zebrafish. *Osteoarthritis Cartilage* 21:269–278. [PubMed: 23159952]
127. DeLaurier A, Eames BF, Blanco-Sanchez B, et al. 2010 Zebrafish sp7:EGFP: a transgenic for studying otic vesicle formation, skeletogenesis, and bone regeneration. *Genesis* 48:505–511. [PubMed: 20506187]
128. Spoorendonk KM, Peterson-Maduro J, Renn J, et al. 2008 Retinoic acid and Cyp26b1 are critical regulators of osteogenesis in the axial skeleton. *Development* 135:3765–3774. [PubMed: 18927155]
129. Bussmann J, Schulte-Merker S. 2011 Rapid BAC selection for tol2-mediated transgenesis in zebrafish. *Development* 138:4327–4332. [PubMed: 21865323]
130. Sharif F, de Bakker MA, Richardson MK. 2014 Osteoclast-like Cells in Early Zebrafish Embryos. *Cell J* 16:211–224. [PubMed: 24567948]

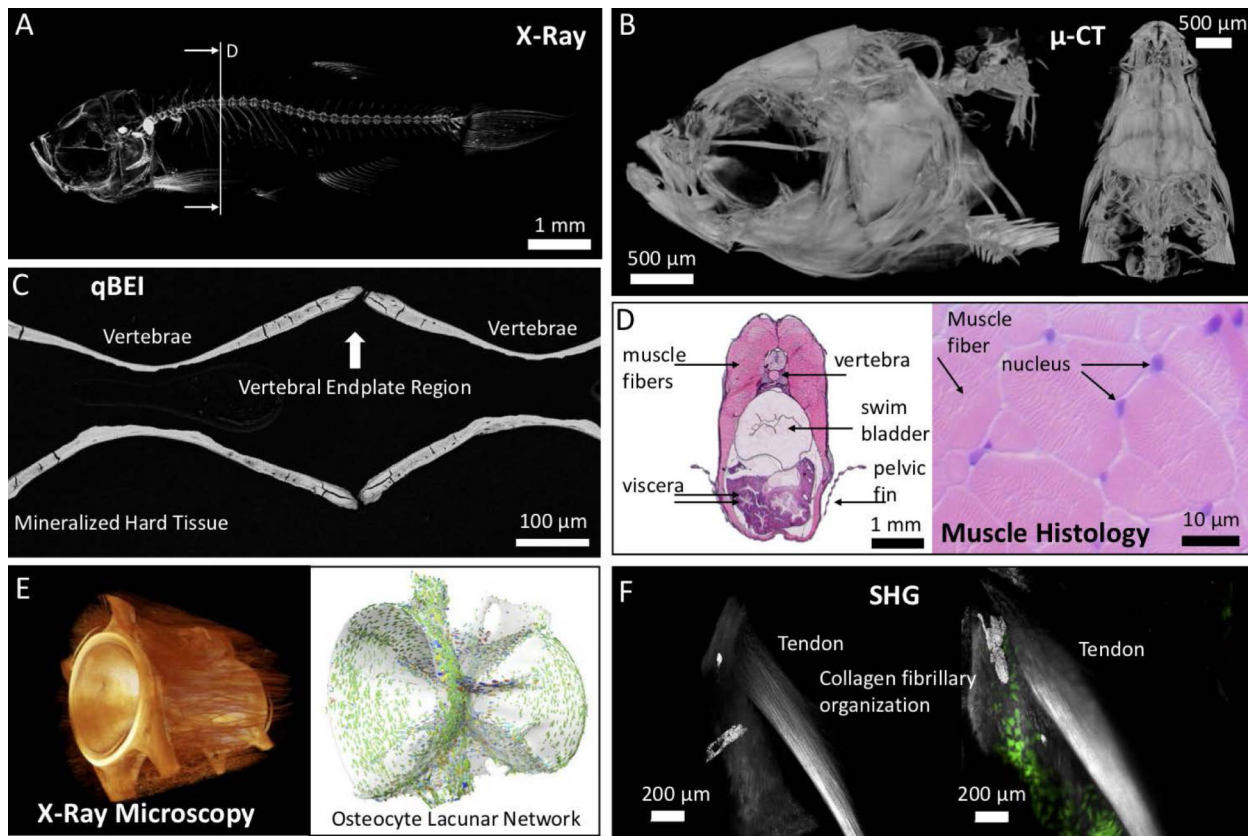


Figure 1: Imaging of tissue structure, composition, and quality.

(A) Contact X-Ray of a juvenile zebrafish. The vertical line shows the histological plane for the image in (D). (B) Microcomputed tomography ($2\mu\text{m}$ isotropic voxel size) of an adult zebrafish skull. (C) Quantitative backscattered scanning electron imaging (qBEI) in the spine of an adult zebrafish. Bone growth occurs at the vertebral endplates. (D) H&E stained section of the zebrafish trunk. Muscle fiber density and cross-sectional muscle fiber area are readily assessed. (E) High-resolution imaging of a vertebral body via X-Ray Microscopy highlighting the osteocyte lacunar network. The osteocyte-lacunar orientation may reflect orientation of collagen fibers, and loading patterns in zebrafish vertebrae. The lacunar orientation follows a specific pattern, i.e. longitudinal orientation in the center of the vertebrae and circumferential orientation near the endplate regions. (F) An adult tendon attached to the maxilla in zebrafish is imaged using *in vivo* Second Harmonic Generation (SHG) imaging, an indicator of type I collagen organization and density. The right panel shows dual imaging of tendon in concert with osteoblasts (green: osteocalcin+ cells expressing the *ocn:GFP* transgene).

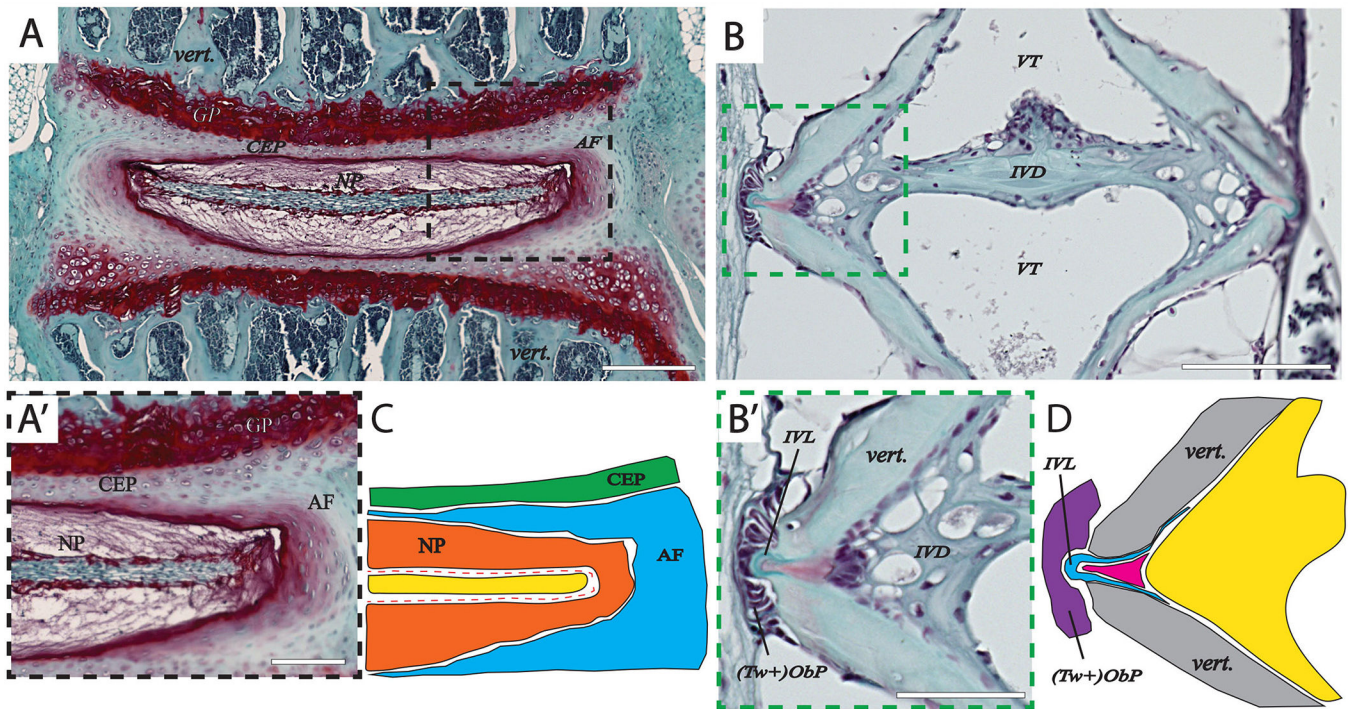


Figure 2: Comparison of the intervertebral disc (IVD) in mouse and zebrafish.

(A-A') and (B-B'): Midline section of a Safranin-O/Fast green staining of an intervertebral disc region in mouse (6-months) (A-A') and zebrafish (1-year) (B-B'). (C) and (D): Cartoon schematic of insets for mouse (C) and zebrafish (D). In mouse, the IVD is composed of a proteoglycan-rich lamellar fibrocartilaginous cartilage called the annulus fibrosus (AF) which surrounds the nucleus pulposus (NP) joins adjacent bony vertebrae at the level of the cartilaginous end plate (CEP). Zebrafish IVD retains notochord-derived vacuolated cells embedded in a fibrocartilaginous matrix, however, there is no NP-like structure observed in zebrafish. An analogous structure to the outer AF layer in is observed as a small acellular intervertebral ligament (IVL). Histologically, the NP in mouse appears to be composed of: an outer tissue layer which stains for Safranin-O (orange in (C)); an inner cell layer (dotted red line in (C)); and an inner tissue layer that does not stain well for Safranin-O (yellow in (C)). In contrast, the zebrafish IVD has only weak Safranin-O staining (magenta in (B', D)) in an interior region adjacent to the intervertebral ligament (IVL) (B', blue in (D)). The zebrafish does not display a true cartilaginous NP tissue, rather the IVD is composed of vacuolated cells and fibrocartilaginous matrix. In contrast to mouse vertebrae which contains bone marrow and trabecular bone filling the vertebrae, zebrafish vertebrae contain bone-shaped vacuolated tissue (VT). Twist positive osteoblast progenitor cells ((Tw+)ObP) are observed adjacent to the IVL. GP-Growth plate; CEP-Cartilaginous endplate; NP- Nucleus pulposus; AF- Annulus fibrosus; IVL-intervertebral ligament; (Tw+)ObP - Twist positive osteoblast progenitors; vert.- vertebrae.

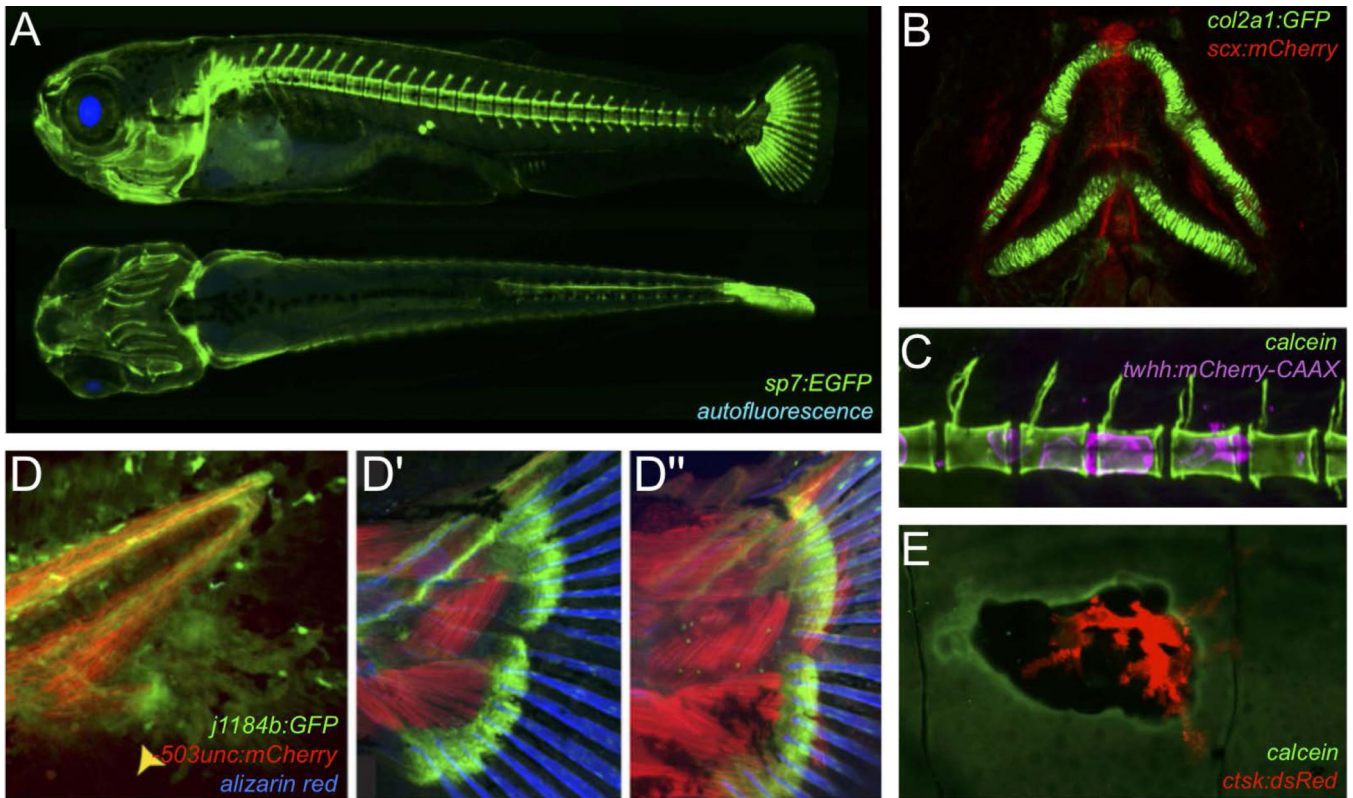


Figure 3: Live imaging of cell dynamics.

(A) Whole-body images of a larval zebrafish (top: lateral view; bottom: ventral view), showing osteoblasts expressing osterix in the cranial skeleton, spine, and fins. (image source: ²⁵; use permitted under the Creative Commons Attribution 4.0 International License; image adapted from original). (B) Ventral view of 3 dpf zebrafish lower jaw showing *scxa:mCherry* expression in the forming tendon cells and *col2a1:eGFP* expression in chondrocytes (C) Tol2-clonal notochordal expressing *twhh-mCherry-CAAX* cells (magenta) and spine marked by calcein (green) in 16 dpf zebrafish. (D) Representative imaging of caudal fin development demonstrating morphogenesis of the connective tissues (Tol2-EGFP^{*j1184bGt*}; green), muscle (*-503unc:mCherry*; red), and bone (Alizarin Red - isolated with a far-red band filter; pseudocolored blue). (E) Osteoclast expressing *ctsk:dsRed* within resorption pit of an adult zebrafish scale stained with calcein to show mineralized matrix.

Table 1:

Transgenic lines for visualizing cells relevant to the musculoskeletal system.

Tissue/cell type	Transgene	Reference	Notes
Early neural crest and CNC-derived cartilages	<i>Tg(sox10:GFP)</i>	119	
Early neural crest and CNC-derived cartilages	<i>Tg(sox10-CreERT2)</i>	120	Tamoxifen-inducible Cre
Cartilage/chondrocytes	<i>Tg(col2a1:eGFP)</i>	121; 122	
Cartilage/chondrocytes	<i>Tg(Col2a1aBAC:mcherry)</i>	123	
Cartilage/chondrocytes	<i>Tg(1.7c2a1a:mEGFP)</i>	121	Membrane-tagged EGFP
Tendon cells	<i>Tg(scx:mCherry)</i>	49	
Muscle cells	<i>Tg(-0.5unc45b:mCherry)</i>	124	
Bone/Pre-osteoblasts	<i>Tg(runx2:GFP)</i>	125	
Bone/Osteoblasts	<i>TgBAC(col10a1a:Citrine)</i>	126	
Bone/Osteoblasts	<i>Tg(sp7:EGFP)</i>	127	
Bone/Osteoblasts	<i>Tg(osx:GFP)</i>	127	
Bone/Osteoblasts	<i>Tg(osx:CreERT2)</i>	74	Tamoxifen-inducible Cre
Bone/Osteoblasts	<i>Tg(Ola.Sp7:NLS-GFP)</i>	128	Nuclear localization signal-tagged GFP
Bone/Osteoblasts	<i>TgBAC(entpd5a:Citrine)</i>	129	
Bone/Mature osteoblasts	<i>Tg(ocn:GFP)</i>	74	
Bone/Osteoclasts	<i>Tg(ctsk:YFP)</i>	130	
Bone/Osteoclasts	<i>Tg(ctsk:DsRed)</i>	Personal communication	