HHS Public Access

Author manuscript

Bone. Author manuscript; available in PMC 2020 September 01.

Published in final edited form as:

Bone. 2019 September ; 126: 37–50. doi:10.1016/j.bone.2019.02.009.

Using Zebrafish to Study Skeletal Genomics

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Abstract

While genome-wide association studies (GWAS) have revolutionized our understanding of the genetic architecture of skeletal diseases, animal models are required to identify causal mechanisms and to translate underlying biology into new therapies. Despite large-scale knockout mouse phenotyping efforts, the skeletal functions of most genes residing at GWAS-identified loci remain unknown, highlighting a need for complementary model systems to accelerate gene discovery. Over the past several decades, zebrafish (Danio rerio) has emerged as a powerful system for modeling the genetics of human diseases. In this review, our goal is to outline evidence supporting the utility of zebrafish for accelerating our understanding of human skeletal genomics, as well as gaps in knowledge that need to be filled for this purpose. We do this by providing a basic foundation of the zebrafish skeletal morphophysiology and phenotypes, and surveying evidence of skeletal gene homology and the use of zebrafish for post-GWAS analysis in other tissues and organs. We also outline challenges in translating zebrafish mutant phenotypes. Finally, we conclude with recommendations of future directions and how to leverage the large body of tools and knowledge of skeletal genetics in zebrafish for the needs of human skeletal genomic exploration. Due to their amenability to rapid genetic approaches, as well as the large number of conserved genetic and phenotypic features, there is a strong rationale supporting the use of zebrafish for human skeletal genomic studies.

Keywords

zebrafish; skeleton; genome; GWAS; bone disease; imaging

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I. INTRODUCTION

GWAS and sequencing studies enable new insights into the genetic architecture of complex skeletal traits

Over the past decade, large-scale genome-wide association studies (GWAS) and sequencing studies have revolutionized our understanding of the genetic architecture of complex skeletal traits. Such studies have enabled deep insights into the number of loci affecting such traits, as well as the distribution of their effects. An archetype of such insights is the use of GWAS to understand the genetic architecture of bone mineral density (BMD), a key indicator for osteoporosis diagnosis and treatment [1]. Understanding genetic risk factors for osteoporosis is important to reduce this massive health burden. Osteoporosis is diagnosed through the measurement of bone mineral density (BMD) utilizing dual-energy X-ray absorptiometry (DXA), which remains the single best predictor of fracture [2, 3]. BMD is characterized by high heritability (h^2), estimated to be 45 – 78% depending upon the skeletal site and age [4, 5]. This heritability provided the rationale to apply methods for genome-wide association to human BMD.

Recent GWAS and sequencing studies [6] have identified hundreds of loci associated with BMD. For instance, a large GWAS meta-analysis of lumbar spine (LS) and femoral neck (FN) BMD involved ~36,000 Caucasian men and women with 2.5 million common SNPs as well as a meta-analysis involving up to 30,000 fracture cases and 100,000 without fracture [7]. This study identified 56 genome-wide significant loci. Of these 56 loci, 14 were also associated with fracture risk [7]. Fifty of these 56 loci were replicated in a recent GWAS using ultrasound-measured BMD of the heel [1]. This study also identified 153 new heel-BMD loci. In a larger study of the same phenotype, in 426,824 individuals from the UK, a total of 518 genome-wide significant loci (301 novel), were identified, explaining 20% of the total variance in BMD of the heel [8]. Finally, a large-scale GWAS meta-analysis for fracture identified 15 genomic loci associated with fracture risk [9], all of which were at, or near, loci previously shown to be associated with BMD. Roughly 90% of BMD-associated variants reside in non-coding regions. Most are believed to harbor cis-regulatory elements that alter the expression of protein-coding genes in proximity.

Animal models are needed to complement GWAS

Because GWAS do not inform causal mechanisms linking genetic variants to their associated phenotypes, gene discovery in animal models is required to complement ongoing GWAS in human populations [10]. A primary example is the International Mouse Phenotyping Consortium (IMPC) [11, 12] and ancillary bone phenotyping projects, which have been used to identify candidate genes residing at BMD loci. The IMPC is a systematic phenotyping program that analyzes knockout mice derived from the International Knockout Mouse Consortium [13]. This pipeline incorporates 20 phenotyping tests that capture measures associated with morphology, metabolism, behavior, sensory function, and others traits. Several aspects of bone are analyzed, including gross skeletal abnormalities assessed via radiography, as well as DXA-measured BMD. In a recent review [10], 79 out of 1820 lines were found to have an outlier phenotype associated with decreased BMD, and 41 lines were

found to have increased BMD. Several ancillary bone phenotyping programs have been established for more comprehensive skeletal analyses [10]. This includes the Bonebase project (bonebase.org), which has generated phenotypes for ~200 lines. This also includes the Origins of Bone and Cartilage Disease (OBCD) project, which currently reports phenotypes for ~600 lines. In several recent BMD GWAS using ultrasound-measured BMD of the heel [1, 8], knockout mouse data from the OBCD was used as a primary basis to prioritize causal gene targets.

The skeletal functions of most genes residing at GWAS-identified loci remain unknown, despite large-scale knockout mouse phenotyping efforts

One limitation with systematic knockout mouse phenotyping efforts is their lack of coverage of genes residing at BMD loci. For instance, of the more than 23,000 protein coding genes in the mouse genome, ~8% (1,820/23,000) have been examined in knockout mice through the IMPC, and ~1% (200/23,000) and ~3% (600/23,000) have been rigorously analyzed for bone phenotypes through the Bonebase and OBCD projects, respectively. Further, knockout lines that are chosen for phenotyping in the IMPC are not necessarily those that reside at loci associated with skeletal traits. As a consequence, the functions of most genes residing at GWAS loci associated with BMD have yet to be tested in knockout animal models, which is essential to understand their roles in skeletal health and disease.

Zebrafish: a vertebrate model amenable to rapid-throughput genetic approaches

Zebrafish (Danio rerio) are a small, tropical freshwater fish. They were established as a genetic model by Streisinger and colleagues [14]. While zebrafish gained prominence as a tractable model of vertebrate development, over the past several decades, their use to model human diseases has rapidly increased [15–17]. Zebrafish are a member of the teleost “infraclass”, which, at over 30,000 named species, comprises more than 95% of all extant fishes and ~50% of all vertebrate species. Another small teleost fish - medaka *(Oryzias latipes)* – is also commonly used to model human diseases [18].

Zebrafish are superbly suited for rapid generation of sequence-targeted mutant lines and generation of human disease models. Due to their small size and low cost, zebrafish are amenable to rapid-throughput studies. Further, their external development and optical transparency enable in vivo fluorescence imaging of bone cell dynamics during skeletogenesis. Finally, there is a powerful toolbox for rapid and precise genome editing in zebrafish. Community efforts have aided in sharing mutant resources and phenotypic data. The Zebrafish Model Organism Database (ZFIN; http://zfin.org) serves as a central hub for sharing genetic, genomic, and phenotypic data [19]. Similarly, the Zebrafish International Resource Center (ZIRC) and European Zebrafish Resource Center (EZRC) serve as central resources to distribute zebrafish strains (both wildtype and mutant strains) and reagents such as zebrafish-specific antibodies. The broad availability of zebrafish mutants arising from efforts to knockout every single protein-coding gene [20], the ability to easily and quickly generate mutant fish using CRISPR/Cas9, and the ability for individual labs to house thousands of adult fish underscores the potential utility of zebrafish to model human skeletal genomics. Further, by virtue of their genetic tractability, optical transparency, and

amenability to high-throughput strategies, zebrafish enable powerful approaches for biomedical research that are challenging in other vertebrate systems (Table 1).

Goals and organization of this review

In this review, we provide evidence that zebrafish have significant potential to address urgent scientific needs in regard to our understanding of human skeletal genomics, though outstanding questions in regard to translation remain. Our goals are to facilitate the use of fish for human skeletal genomic studies by 1) providing a basic foundation of the zebrafish skeletal morphophysiology and phenotypes to non-fish users (Section II); 2) educating the reader about recent advances in understanding the zebrafish genome and surveying evidence of skeletal gene homology (Section III), 3) surveying the use of zebrafish for post-GWAS analysis (Section IV), and outlining important gaps in knowledge (Section V). Finally, we conclude Section V with recommendations of future directions and how to leverage the large body of tools and knowledge of skeletal genetics in zebrafish for the needs of human skeletal genomic exploration.

II. ZEBRAFISH SKELETAL MORPHOPHYSIOLOGY AND PHENOTYPES

Similarities and differences in the zebrafish and mammalian skeletons are scale-dependent

The zebrafish skeleton shares both similarities and differences with mammals in regard to morphophysiology. A substantial portion of morphophysiological differences likely reflect functional and evolutionary demands on the terrestrial mammalian skeleton for quadru- and bipedalism. The bony tissues of the skeleton, together with other connective tissues including muscle, cartilage, ligaments, tendons, and intermediate subtypes of these tissues, comprise the zebrafish musculoskeletal system. Like mammals, the zebrafish skeleton possesses a hierarchical structure that, in conjunction with the cells that reside within it, enables it to carry out its biological functions. By virtue of this hierarchical structure, the extent of similarity in the zebrafish and mammalian skeletons is dependent on scale. Here, we survey such scale-dependent similarities and differences, starting from the nanoscale (molecules), and ending with the macroscale (bone as an organ). We conclude with a survey of phenotypic approaches in the zebrafish skeleton.

While many studies in the zebrafish skeleton have focused on larval fish, given the focus of this review on connecting zebrafish to human adult skeletal phenotypes, including osteoporosis-related traits, here we primarily focus on the adult zebrafish skeleton. Understanding developmental differences can aid in understanding relevant adult phenotypes[21], thus, in some cases we also review aspects of bone development.

Nanoscale: matrix and mineral

At the nanoscale, bone tissue consists of a mineralized organic matrix. The organic matrix is primarily composed of collagen type I. In humans, collagen type I is a heterotrimer composed by two alpha chains, α1(I) and α2(I), encoded for by the *COL1A1* and *COL1A2* genes, respectively. The α1 and α2 chains 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, α1(I), α2(I), and α3(I), which are encoded for by the genes *col1a1a, col1a2,* and *col1a1b,* respectively [22]. In zebrafish, alpha chain stoichiometry within collagen type I fibrils may vary depending on anatomical site [22]. Transmission electron microscopy (TEM) images of adult zebrafish bones have shown collagen fibrils arranged in laterally aligned bundles [23]. TEM studies have also showed plate-shaped mineral crystals residing within the fibrils, with their c-axes aligned with the fibril axes, similar to other vertebrates. Thus, at the nanoscale, zebrafish bone is reminiscent of mammalian bone up to the level of aligned mineralized collagen fibrils [23].

In mammals, the primary inorganic phase in bone is carbonated hydroxyapatite (HA). Raman and Fourier-transform infrared (FTIR) spectroscopy [23] imaging in zebrafish have shown that zebrafish bone contains carbonated HA as well as other mineral phases, similar to mammalian bone. Tissue mineral density (TMD) is a commonly used measure of mineralization that reflects the amount of mineral per unit volume of bone tissue. TMD values of 450–600 mg HA/cm^3 have been reported in the vertebrae of adult zebrafish [24]. Observed zebrafish measures are noticeably less than the TMD values of 800–1000 mg HA/cm^3 in the cortical bone of adult mice [25], but only slightly less than the 600–700 mgHA/cm^3 observed in cancellous bone of the same animals. In human cancellous bone, TMD values of 800–1000 mgHA/cm^3 have been reported [26]. Differences in mineral density have been attributed as a possible reflection of adaptation to mechanical loading and bi- or quadrupedalism in terrestrial mammals [27].

Microscale: Cellular composition and related microstructure

At the cellular level, zebrafish bone comprises several cell types that are distinct in both their morphology and function. A large fraction of the zebrafish skeleton is covered in a single monolayer of osteoblasts [28]. This is similar to mature osteoblasts in rats, which have been reported to appear as a single layer of cuboidal cells [29]. In regard to osteoclasts, there are at least two morphologically identifiable types: mononucleated and multinucleated [30]. Mononucleated osteoclasts appear during early development, and have been found in bones of the craniofacial skeleton. The resorptive function of these mononucleated cells is suggested by their presence at sites of resorption pits, as well as positive staining for TRAP. In zebrafish, multinucleated osteoclasts appear during the larval-to-adult transition [30]. By adulthood, multinucleated osteoclasts are the most predominant, though mononucleated osteoclasts do exist [30]. By contrast, in mammals, mononucleated cells are thought to primarily be a precursor to multinucleated cells, which carry out resorptive function. Osteocytes are present in zebrafish, though not in all bones. In this context, two types of bone are identifiable in zebrafish: cellular bones with osteocytes, and acellular bones that lack osteocytes. Cellular and acellular bones are present in both the cranial and post-cranial skeletons [28]. Cellular bones have also been identified in zebrafish vertebrae; these bones contain osteocytes with a poorly developed lacunocanalicular system [31].

Similar to mice, zebrafish do not (or rarely) form osteons -- concentric layers of compact bone tissue surrounding a Haversian canal -- though such structures have been observed in larger teleosts [32]. In this context, some zebrafish bones have been reported to be avascular [33] as blood vessels do not (or rarely) become embedded within the bone matrix. However,

blood vessels often reside in close proximity to bone tissue, including within bone marrow spaces.

Meso- and macroscale: Identifiable bone types and functional groupings

Mammalian bone is traditionally classified as either cortical (compact) or cancellous (trabecular or spongy) bone. Because most zebrafish bones are dense and homogenous, they are referred to as compact bones [28]. However, zebrafish do not possess cortical bone in a traditional sense, in that their bones do not encapsulate a hematopoietic bone marrow cavity. While much more limited compared to compact bone, zebrafish also possess spongy, trabecular-like bone. For instance, spongy bone has been found in several bones of the skull [28]. A number of these spongy bones form via endochondral ossification [28].

At the organ level, the zebrafish skeleton can be regionalized into functional groups including the craniofacial skeleton, the vertebral column, the median fins, and the paired fins. The developmental morphology of the craniofacial skeleton has been well-described [34–36], and there is a large literature on genetics of craniofacial skeletogenesis in zebrafish [37, 38]. Notably, the number of bones in the adult zebrafish skull greatly exceeds that in adult mammals [34]. The zebrafish vertebral column can be regionalized into three main groups (anterior-posteriorly): Weberian vertebrae, precaudal vertebrae, and caudal vertebrae [39]. The total number of vertebrae in zebrafish can vary slightly, but seems to be around 31 [39], similar to that in mammals. Vertebrae 1–4 contribute to the Weberian apparatus, which is unique for the teleost fish. Vertebrae 5–14 and 15–31 are the precaudal and caudal vertebrae, respectively. Each vertebra possesses a centrum, a neural arch, and a neural spine that extends from the neural arch. Many elements comprising the vertebral column form via intramembranous ossification, while parapophyses, as well as the neural arches on vertebrae 1–5, form via endochondral ossification [39].

The fins include the dorsal, pectoral, anal, and caudal fins. Radial bones such as those at the base of the pectoral fin, adjacent to the dermal fin rays, share a developmental similarity with mammalian long bones in that they form via endochondral ossification. They also share a genetic similarity to mammalian digits as patterning in both are influenced by hox13 genes [40]. The dermal fin rays consist of segmented bone rays, surrounded by nerves, blood vessels, pigment cells and fibroblastic/mesenchymal cells residing within the intra- and inter-ray spaces [41]. Each fin ray is composed of multiple segments joined by fibrous ligaments and lined by a monolayer of osteoblasts. Like other teleosts, following fin amputation, zebrafish can regenerate their fin bone rays through epimorphic regeneration. In adults, newly synthesized bone appears within two to three days, and subsequent nerve, joint, circulatory and mature bone tissue are largely restored by 2 weeks [41]. The regenerative capacity of elements of the fish dermal skeleton (mostly fin rays, but also scales) is widely explored [42–47] [48]. Unlike most other bony elements in zebrafish, fin rays and scales remain optically accessible in adult animals [41].

Phenotypic assays in the zebrafish skeleton

There has been rapid growth in the arsenal of phenotypic assays to assess skeletal biology at the tissue and cellular levels (Fig 1). Here, we survey some of these assays, with particular focus on those with demonstrated potential to enable screening of large numbers of fish.

Histology enables rapid assessment of craniofacial phenotypes—Histological stains enable visualization of cell types such as osteoblasts, osteoclasts, and osteocytes. Osteoblasts can be demarcated by staining for mRNA highly expressed in osteoblasts in zebrafish such as osterix, osteocalcin, or by assessing protein activity in alkaline phosphatase (ALP) [49]. The uncharacterized antigen ZNS-5 is an antibody that stains cell membranes and which can help identify bone lining osteoblasts [50]. In zebrafish, osteocytes can be visualized in hematoxylin-eosin (H&E) stained sections as cells embedded in bone matrix [31]. Osteoclasts can be visualized based on expression of mRNA for CTSK [51], or by TRAP activity [30].

Bone mineral can also be stained to facilitate visualization. A common approach is to use alizarin red or calcein to stain mineralizing surfaces. These fluorochromes can be administered ex vivo or in vivo by immersing live fish in fluorochrome solution [52]. Labels can be monitored in vivo in bones that are optically accessible, such as early developing vertebrae, and adult fin rays. Double fluorochrome labeling has been used to demarcate bone formation between labeling periods [44, 53–55], similar to dynamic histomorphometric approaches in mammals (Fig 1A). Labels are often readily observed in whole-mount, alleviating the time and resources required for tissue sectioning. Fleming et al. demonstrated that fluorochrome labeling can be used for rapid-throughput in vivo chemical screening using larval zebrafish within a 6-day time period [56]. Vitamin D_3 analogs and intermittent parathyroid hormone (PTH) were found to result in bone formation, whereas continuous PTH resulted in decreased bone accumulation [56]. Additionally, cartilages can be stained with alcian blue. This approach was used to identify more than 100 arch mutants in a forward genetic screen [35, 36]. Many of these mutants exhibited defects in posterior pharyngeal arches, or lacked adjacent branchial arches and their associated cartilages.

Fluorescence—Osteoblasts can be demarcated in vivo using fluorescent reporters driven by regulatory sequences for genes highly expressed in osteoblasts (Fig 1B). Examples of transgenic lines include *sp7:EGFP* [47, 57] and *ocn:EGFP* [47], which demarcate early and more mature osteoblasts, respectively. A double transgenic reporter line expressing fluorescent markers for sp7 and ocn was used to gain insights into the effects of calcitriol and cobalt in zebrafish operculum development, with osteoblast maturation shown to be stimulated and inhibited, respectively, by these two agents [58]. Osteoclasts can be demarcated using the zebrafish transgenic line *ctskb:YFP* [51]. In medaka, a similar transgenic line has been used in parallel with heat shock-inducible RANK to screen for drugs that inhibit bone resorption [59, 60], as well as to examine osteoclast-osteoblast interactions [61].

Radiography—Fisher et al. demonstrated radiography is a practical tool to screen for skeletal abnormalities in adult zebrafish[62] In a mutagenesis screen for skeletal dysplasias

in adult zebrafish, these authors isolated the dominant mutant allele *chihuahua* (chi). Zebrafish with the *chi*/+ genotype carry a heterozygous glycine substitution in the al chain of collagen type I, resembling similar glycine substitutions in more severe phenotypes of classical Osteogenesis Imperfecta (OI types II, III, and IV).

microCT—Due to the three dimensional architecture of bone, as well as the fact that many bones in zebrafish are relatively small in size, many aspects of bone morphology, microarchitecture, and mineralization, are not apparent in radiographic analysis. Several recent studies have demonstrated the utility of microCT as a practical tool for the detection of skeletal abnormalities in zebrafish [24, 63–65]. Charles et al. assessed skeletal shape and density measures in the C1 and C2 vertebrae [63]. Measured phenotypes included centrum radius, length, volume, and TMD. These authors found modest strain, but not sex differences, in skeletal measures They also found altered centrum morphology in zebrafish mutants for *bmpla* (Fig 1C), whose ortholog is associated with Osteogenesis Imperfecta. Gistelinck et al. measured centrum TMD, length, and thickness in almost the whole vertebral column (24 individual vertebrae) in zebrafish plod2−/− mutants, a model of Bruck syndrome [66]. Using high-resolution (1um voxel size) microCT, Suniaga et al. found that zebrafish subjected to swim exercise exhibited increased centrum volume and length [55]. They also demonstrated the potential for high-resolution microCT to resolve aspects of osteocyte lacunar orientation, shape, and size in the centrum[55] (Fig 1D).

In addition to centrum measurements, phenotyping of the neural arch has also been performed including measurements of neural arch area [63], angle [63], volume [24], thickness [24], and TMD [24]. In zebrafish and medaka, neural arch area is influenced by bone modeling activity, due to the fact that the neural arch undergoes continual modeling during growth, with osteoclasts on the inner surface, and osteoblasts on the outer surface [67]. Thus, altered neural arch phenotypes may indicate changes in bone modeling mediated by coupling between osteoblasts and osteoclasts.

MicroCT has been used to analyze aspects of zebrafish skull morphology in zebrafish both qualitatively [33, 68] as well as quantitatively [63]. In regard to the latter, Charles et al. measured volume and TMD of the parasphenoid bone, which resides at the skull base, and forms through endochondral ossification [63]. Homologous bones to the zebrafish parasphenoid are present in mouse and chicken, however unlike in these species, in zebrafish, the parasphenoid is not neural crest-derived [69]. Semi-automated methods for 3D segmentation of bones in the fish skull remain nascent. The development of such methods may facilitate the detection of subtle morphological changes in the zebrafish skull. They may also be useful for craniofacial analysis in other fish species for studies of evolutionary development, where the skull is often used to study adaptations associated with evolutionary transitions.

One limitation of microCT is that in the absence of contrast agents, the zebrafish skeleton is insufficiently ossified at early developmental stages for mineralized tissue to be resolved. Generally, when sexual maturity is achieved by 2–3 months post fertilization, the skeleton is sufficiently mineralized in wildtype zebrafish to be imaged via microCT. Because experimental throughput and scalability is generally increased in younger fish, the

identification of experimental ‘windows’ in which animals are still relatively young, yet their skeletons are sufficiently mineralized to be detected by quantitative approaches, may be beneficial. It has been shown that contrast staining using $AgNO_3$ can allow for pCT analysis of the zebrafish skeleton at early stages of development [70], and resolution of mineralized tissue in unstained fish is sometimes possible for fish between 30–50 dpf (unpublished).

Skeletal phenomics—The ability to characterize phenomes—that is, to acquire in-depth phenotypic profiles at the scale of the whole organism—holds promise to enhance our functional understanding of genetic variation in the skeleton [71]. The skeleton is a spatially distributed organ comprised of bones of different developmental origins (i.e., neural crest vs. mesoderm derived), modes of ossification (intramembranous vs. endochondral), and cellular compositions [24]. Since different mutations often affect different skeletal compartments, it is common practice to perform skeletal phenotyping at multiple skeletal sites [24]. By virtue of their small size, it is possible to perform whole-body imaging of zebrafish at high-resolution, and with relatively high throughput [24, 72]. This provides a unique opportunity to integrate rapid genetic methods with skeletal phenomic assays to rapidly map gene-to-phenome relationships in the skeleton.

Pardo-Martin et al. demonstrated the use of automated sample handling and high-throughput optical projection tomography to perform extensive skeletal phenotyping in the skull of Alcian blue-stained zebrafish larvae [72]. Hundreds of morphological features were captured from 9 different skeletal elements. As proof-of-concept, these authors showed the potential to cluster phenotypic signatures into similar pathway perturbations, as well as to identify effects of teratogens on cartilage formation. One limitation of this method is that is limited to early developmental stages when zebrafish remain semi-transparent. While zebrafish mutant lines have been developed that lack pigmentation, skeletal imaging in adults is difficult to scattering of light in deep tissue. Thus, this method is not readily extendable to bones outside of the craniofacial skeleton, or to adults.

A microCT-based approach for skeletal phenomics in adult zebrafish was recently developed by Hur et al [24, 68]. A supervised segmentation algorithm, FishCuT, was developed, enabling profiling of hundreds of phenotypic measures at a large number of anatomical sites in the axial skeleton (Fig 1E). This approach allowed for segmentation of up to 24 vertebrae in the spine, of which each vertebra was automatically segmented into the neural arch, centrum, and haemal arch/ribs. For each skeletal element, quantities such as TMD, volume, thickness, and length were measured (up to 600 measures per fish). These authors also showed the potential to employ multiplexed scanning methods to acquire whole-body, medium resolution scans in ~5min/fish. These authors found that vertebral patterns conferred heightened sensitivity, with similar specificity, in discriminating mutant populations compared with analyzing individual vertebrae in isolation. Allometric modeling-based methods were developed to aid in the discrimination of mutant phenotypes masked by altered growth. This approach has been used to characterize complex phenotypes in mutant models of brittle bone disease [24, 68], as well as thyroid stimulating hormone receptor hyperactivity [24].

Synchrotron radiation microcomputed tomography—Synchrotron radiation microcomputed tomography has been used in zebrafish and medaka to generate extremely high resolution reconstructions [73, 74]. Ding et al. recently used this approach to generate whole-animal reconstructions in larval and juvenile fish at sub-cellular resolution [74]. However, at present, the practicality of this method as a broadly employed approach is limited due to the high cost and limited access for such beam sources [63].

Bone quality—Recent studies by Busse and coworkers [55, 65] showed the potential to adapt assays of bone quality in mammals to zebrafish. This was recently demonstrated in the *chihuahua* mutant, which detailed previously, carries a heterozygous glycine substitution in the al chain of collagen type I. Quantitative backscattered electron microscopy and Fourier-transform infrared spectroscopy were used to show changes in vertebral tissue composition including changes in mean calcium content, matrix porosity, mineral crystallinity, and collagen maturity [65]. By showing that this mutant exhibits bone characteristics associated with human classical dominant osteogenesis imperfecta, this study further validated *chihuahua* as an important disease model. This study also suggests that efforts to examine the utility of zebrafish to model genetic regulators of human bone tissue quality are warranted.

III. ZEBRAFISH SKELETAL GENETICS

Zebrafish and humans exhibit high conservation in coding DNA

Zebrafish are in the teleost infraclass, which relative to modern-day vertebrates, arose from a common ancestor that underwent an additional round of whole-genome duplication called the teleost-specific genome duplication (TGD) [19, 75]. It has been suggested that the TGD occurred before the divergence of ray-finned and lobe-finned fishes 450 million years ago [76]. Duplicated genes arising from genome duplication are called ohnologs. Analysis of a high-quality reference genome indicated that zebrafish possess 26,206 protein-coding genes, with 71% of human genes having at least one zebrafish ortholog [19]. Further, 47% of these human genes possessed a one-to-one relationship with a zebrafish ortholog [19] (i.e., there is only one gene in humans that maps to one gene zebrafish). Such similarity is consistent with studies suggesting that transcription factors (TFs) and the protein coding genes they modulate are predominantly conserved among vertebrates.

Because 71% of human genes have at least one zebrafish ortholog, one can surmise that a similar proportion of GWAS loci are mediated by causal genes testable in mutant zebrafish. For instance, assuming 518 loci associated with eBMD [8], this would predict that 0.71*518=368 BMD loci are mediated by target genes whose functions are testable in mutant zebrafish. This number is substantial, when viewed in the context of the limited coverage of genes at GWAS loci afforded by international knockout mouse phenotyping consortiums (8%). Similar to the fish, ~16,000 mouse protein coding genes have been identified with one-to-one correspondence with human genes (http://www.informatics.jax.org, search: 01/11/19). Assuming ~21,000 human protein coding genes, 16,000/21,000=76% of human genes have a mouse ortholog with one-to-one correspondence. Ignoring genes without one-to-one correspondence, this would predict that

0.76*518=393 BMD loci are mediated by target genes whose functions are testable in mutant mice, which is comparable to 368 BMD loci in zebrafish.

Divergence is observed in non-coding DNA

While conservation in coding DNA among vertebrates is high, conservation in non-coding DNA is less prominent. Indeed, it has been postulated that cis-regulatory sequences and other non-coding elements may be a primary substrate for evolutionary divergence [77, 78]. Conserved non-protein-coding DNA elements (CNEs) often encode cis-regulatory elements; ancient CNEs that arose in the vertebrate ancestor usually have only a small core of sequence conservation in fish species, while mammals often exhibit much broader conserved flanks. High levels of divergence are observed in UTRs, introns, and intergenic DNA [79]; such genomic regions often harbor trait-associated SNPs. Further, less than 1% of non-coding sequences are conserved with more distant vertebrates, such as teleosts [77]. Similarly, only 29 of 550 (5.3%) distinct fish lincRNAs were conserved between fish and mammals (human and mouse) [80]. In regard to sequence similarity, only <4% of non-coding sequences are highly conserved among mammals [77, 81]. Of note, <3% of lincRNAs conserved between human and at least one non-primate mammal [80] could be traced to the last common ancestor of tetrapods and ray-finned fish, compared to >70% of protein-coding genes and >20% of small RNA primary transcripts [82].

While divergence in non-coding DNA makes modeling of human cis-regulatory function less amenable in animal models, the identification of such CNEs is important both to facilitate modeling of noncoding variants in zebrafish, as well to elucidate those of high biological importance: for instance, it has been proposed that the evolutionary distance between human and zebrafish can reveal functional non-coding SNPs of importance for transcription factors binding and gene c/s-regulation [83]. Further, identification of CNEs facilitates annotation of sequences identified in human genome-wide association studies. For instance, in an analysis of ~2,400,000 human GWAS-SNPs, ~25,000 were located in human CNEs [76].

In some cases, human CNEs can be directly connected to zebrafish. For example, Hiller et al. identified ~12,000 CNEs in zebrafish conserved in human or mouse [83]. More recently, Braasch et al. [76] identified ~34,000 CNEs in humans conserved in zebrafish, based solely on a direct whole genome alignment. Moreover, advances in genomic alignment approaches, the assembly of higher quality reference genomes, as well as the publication of new genomes may help to identify cryptic CNEs. An example of the latter was demonstrated by Braasch et al., who sequenced the genome of spotted gar, a species whose lineage diverged from teleosts before the TGD[76]. These authors found that using the gar genome, orthology from human to zebrafish could be inferred for more than 30% of human CNEs that were not previously directly connected from human to zebrafish in the absence of gar[76].

For human non-coding variation which is not conserved in zebrafish, variant function can best tested through humanized transgenic reporters. For example, Praetorius et al. [84] assayed the sequence containing rs12203592 using transgenic zebrafish by engineering a vector containing an intronic sequence for human *IRF4* upstream of a minimal promoter and the GFP gene. Based on differential GFP expression for vectors with either the T or C allele

at rs12203592, it was concluded that this intronic sequence contains a melanocyte enhancer, and that the rs12203592-T allele reduces the activity of this enhancer. While this example shows the potential to test non-conserved GWAS SNPs in fish, since most GWAS SNPs are non-coding and only 25k of 2.4M GWAS SNPs are in CNEs, it can be surmised that most translation from zebrafish to humans, at least via non-transgenic genome modification, will likely occur through shared coding variation.

The teleost-specific genome duplication (TGD) complicates ortholog identification and testing

Connecting teleost genes to human biology requires understanding the functional implications of the TGD, which resulted in duplicates for many human genes. The TGD was followed by reciprocal loss of some ohnologs in teleosts and tetrapods, including humans, which can hinder ortholog identification [76]. Duplicate genes experience several non-exclusive fates after genome duplication: loss of one copy (the most common fate, with a probability of approximately 80%), evolution of new expression domains or protein functions, and partitioning of ancestral functions [76]. In addition, the fate of ohnologs includes subfunctionalization (when each ohnolog retains a subset of its original ancestral function), neofunctionalization (when one or both of the ohnologs develops a new function), or retention of two “similar” copies [85]. Neofunctionalized ohnologs tend to show new expression in other than original (ancestral) organs [85]. As an example of high likelihood of loss of one gene copy after duplication, teleosts have far fewer *hox* cluster genes than the 82 expected after genome duplication (for example, zebrafish has 49 genes), demonstrating massive *hox* gene loss after the TGD [76]. Pasquier et al. [85] showed that most TGD duplicates gained their current status (loss of one duplicate gene or retention of both duplicates) relatively rapidly after TGD (i.e., prior to the divergence of medaka and zebrafish lineages).

Sox and SCPP genes are prototypes of functional changes in bone-related genes arising from duplication

Among bone-related genes, one classic example of ohnolog subfunctionalization and neofunctionalization is the sox gene family. Sox genes encode for transcription factors, and have an essential role in morphology, physiology and behavior of vertebrates. Concerning teleosts, the analysis indicates enrichment in *sox* genes compared to other vertebrates. Phylogenetic and synteny analyses confirmed a noticeable expansion of the *sox* family: 58% (11/19) of *sox* genes are duplicated in teleost genomes, compared to tetrapods. Of note, the elephant shark, a cartilaginous fish, seems to have lost *sox19* [75]. By analyzing the evolution of coding and non-coding sequences, as well as the expression patterns in fish embryos and adult tissues, it can be concluded that the probability for retention of an extra copy is correlated with gene function; retained duplicates are enriched for function in signaling, transcription, calcium ion transport, and metabolism[75].

Another classic example of bone-related genes exhibiting functional changes after duplication involves the secretory calcium-binding phosphoprotein (SCPP) family. This family of genes has attracted special attention, mainly due to their crucial functions in the endochondral ossification and mineralization of dentin and enamel [86]. It is postulated that

duplication of osteonectin (a.k.a. secreted protein acidic and rich in cysteine, SPARC) in teleosts initially gave rise to Sparcl1, and the subsequent tandem duplication of Sparcl1 to the rest of SCPP genes [87]. Subsequently, SCPP genes evolved along with the course of speciation and certain SCPP genes arose in lineage-specific ways. Some duplicated SCPP genes changed into pseudogenes, whereas others were retained [86].

There is one group of SCPP genes that clusters on human chromosome 4; these genes *(SPP1, MEPE, IBSP, DMP1* and *DSPP)* encode acidic proteins and are collectively known as SIBLING genes [87]. This cluster on 4q22.1 was associated with BMD in a large genome-wide association study [7]. There is a single SIBLING gene, *spp1* (also known as osteopontin), in zebrafish and medaka. Zebrafish *spp1* is expressed specifically in osteoblasts and has therefore been proposed to have a primary function in bone formation similar to its mammalian ortholog [87]. Indeed, both the knockdown of *spp1* using morpholinos and insertion/deletion mutations by the CRISPR/Cas9 resulted in a significant reduction in endochondral and dermal bone formation in embryos 5 days post-fertilization [87].

Zebrafish models of human skeletal disorders show functional conservation in bone-related genes

Broad functional conservation in bone-related genes in zebrafish and human is suggested by a large number of zebrafish models of human diseases that affect the skeleton. Several reviews have summarized such models and we point readers to these reviews for more information [38, 88]. Zebrafish models have been generated for a number of genes associated with human genetic disorders that affect the skeleton. These human disorders include OI, craniosynostoses, as well as general dysplasias. Genes affecting mineral metabolism include transcription factors, collagen-associated genes and matrix proteins, signaling molecules. Reported phenotypes have largely been severely dysmorphic, with delayed mineralization, hypermineralization, or ectopic mineralization. For example, the *enpp1*$^{-/-}$ zebrafish *(dragonfish)* features extensive hypermineralization of the axial skeleton [89]. In Table 2, we list select zebrafish models for genes that have been implicated by BMD GWAS.

In zebrafish models of human disease, there is not always a one-to-one relationship in regard to the anatomical compartment affected, or the degree of phenotypic severity. For instance, *lrp5* knock-down in fish resulted in severe craniofacial defects [37] compared to the milder defects described in mouse models or human patients. Willems et al. thus point out there might be modification or possible neo-functionalization of *lrp5's* role in teleosts and other non-mammalian vertebrates [37].

Zebrafish models of brittle bone disease provide evidence of potential for functional conservation in coding variants

The use of the zebrafish skeleton to directly test common human coding variants, i.e. to introduce human sequence into fish chromosomes, remains relatively rare. However, zebrafish mutants with coding mutations similar to those in humans with brittle bone disease have been shown to manifest disease-related phenotypes. As mentioned previously, the

mutant *chihuahua* (chi/+) is a model of more severe phenotypes of classical OI, in which type I collagen exhibits a structural defect arising from substitution of a glycine residue. More recently, severely dysmorphic skeletal phenotypes were found in four other zebrafish mutants with glycine substitutions in the a1 chain of collagen type I [22].

IV. FUNCTIONAL TESTING OF GENES AT GWAS LOCI IN ZEBRAFISH

Prior uses of zebrafish to test genes at GWAS loci provide a prototype for bone-related traits

There are several instances in which zebrafish have been used to functionally test genes at GWAS loci for non-skeletal human diseases and phenotypes. These include studies in the liver, skin, heart, kidney, and brain [90–94]. Studies for bone-related traits are relatively lacking. However, studies in non-skeletal tissues and organs serve as a prototype for the conduction of similar studies in zebrafish skeleton, and thus it is useful to survey them.

The golden *mutation identifies a key role for* SLC24A5 *in human pigmentation*

One of the defining features of zebrafish is the amenability of this model system to unbiased, forward genetic approaches. One of the first examples of a forward genetic screen in zebrafish to isolate mutants that reveal insights into human traits was demonstrated by Cheng and colleagues [91]. The zebrafish “golden” mutant with a pigmentation phenotype was found to possess a mutation in the gene encoding a putative cation exchanger *slc24a5 (nckx5).* The gene product localizes to an intracellular membrane, thus affecting the melanosome or its precursor. The human ortholog *SLC24A5* is highly similar in sequence and function. The evolutionarily conserved ancestral allele of a human coding polymorphism predominates in African and East Asian populations, while the variant allele is nearly fixed in European populations and correlates with lighter skin pigmentation. Thus, by similarity with zebrafish ortholog, a key role for the *SLC24A5* gene was found in human pigmentation [91].

Reverse genetic screens in zebrafish identify novel trait-related genes at GWAS loci

While the above studies demonstrate the utility of unbiased screens in zebrafish in elucidating the genetic basis of a human trait (i.e., zebrafish to human), an alternate paradigm is to identify candidate genes or genetic variants at GWAS loci, and functionally test these genes in zebrafish (i.e., human to zebrafish). Several studies have demonstrated the utility of zebrafish in functionally testing large panels of candidate genes or genetic variants that have been prioritized by GWAS.

One such study involved the identification of genes involved in heart rate regulation. In meta-analysis of GWAS in up to 181,171 individuals, den Hoed et al.[92] identified 21 loci associated with heart rate. To downregulate gene expression in orthologs of the positional candidate genes, morpholino oligonucleotides were injected at the single-cell stage. Differences in heart rate and fractional shortening of the ventricular chamber were compared between control embryos and those injected with morpholino 48 h later [92]. By this, the authors identified 20 genes at 11 loci that are relevant for heart rate regulation; they showed

that zebrafish embryos with downregulated expression of orthologs of these genes have edema, an unlooped heart and atrioventricular canal malformation.

Similarly, GWAS of chronic kidney disease (CKD)[93] discovered 6 new loci in association with estimated glomerular filtration rate, the primary clinical measure of CKD. Morpholino knockdown of *mpped2* and *casp9* genes in zebrafish embryos revealed podocyte and tubular abnormalities with altered dextran clearance, suggesting a role for these genes in renal function. They compared the number of abnormal morphant embryos to control embryos and documented the development of gross edema at 4 and 6 days post-fertilization,[93] thusly matching the human phenotype.

A GWAS for adolescent idiopathic scoliosis (AIS) in Japanese and Chinese populations identified a susceptibility locus on chromosome 9p22.2, with the most significantly associated SNP in intron of a gene which encodes a zinc finger transcription factor, basonuclin-2 *(BNC2)* [95]. In humans, *BNC2* is highly expressed in the spinal cord, bone, cartilage, and in muscle. Overexpression of *BNC2* in zebrafish embryos resulted in variations in body curvature and embryonic lethality in a gene-dosage-dependent manner. Some of the abnormal embryos that were injected with the *BNC2* transgene exhibited malformation of the somite, resulting in larval death within one week [95].

Post-GWAS testing in zebrafish identifies mpp7 as a novel skeletal gene

Among the early attempts to validate human skeletal GWASs with zebrafish phenotypes is the work by Xiao et al. [96]. GWAS in a Hong Kong population revealed association with *MPP7* (further confirmed by the GEFOS consortium [7]). Xiao et al. compared morphology and “bone mass” between the embryonic wildtype and partial *mpp7* knock-down ($mpp7^{MO}$) by morpholino. A previous study in zebrafish demonstrated that mutations of *mpp7* could lead to the *humpback* phenotype of larvae with malformed and ankylotic vertebrae in adult fish. The investigators found that bone mineralization was affected in $mpp7^{MO}$ zebrafish. The relative bone mass, quantified by measuring the fluorescent intensity of the alizarin red stain in vertebrae 3–5, was significantly lower in $mpp7^{MO}$ than in wildtype zebrafish. The attempted rescue of $mpp7^{MO}$ zebrafish by addition of *mpp7* mRNA partially reversed the bent tail phenotype.

Zebrafish mutants derived using CRISPR-based gene editing defines functions for genes associated with schizophrenia

In the above studies, one limitation is that functional testing was employed using transient knockdown (e.g., using morpholinos) or overexpression methods. In this context, the advent of new methods for gene editing such as CRISRP-Cas9 has quickly changed the accessibility and ease by which the scientific community can approach reverse genetics in zebrafish. The potential to perform large-scale screening of genes at GWAS loci in zebrafish mutants generated via CRISPR-based gene editing was recently demonstrated by Thyme et al.[94]. To define candidate causal targets and their functions, these authors mutated zebrafish orthologs of 132 human schizophrenia-associated genes derived from 108 genomic loci at which common variants exhibited genome-wide significant associations with schizophrenia. A systematic phenotyping pipeline was employed, including behavioral tests,

brain activity (as measured by ERK phosphorylation), and brain morphology. A relatively small number of mutants exhibited lethality prior to adulthood. More than half of the mutants showed brain activity and/or behavioral phenotypes, and a number of mutants exhibited changes in multiple phenotypes. These studies prioritized more than 30 candidates for further study (e.g., the magnesium transporter cnnm2 and the translational repressor gigyf2), some of which are located at gene-rich loci.

Identification and testing of conserved non-coding elements in GWAS

As mentioned previously, divergence in non-coding DNA makes modeling of human cis-regulatory function less amenable in animal models. However, human CNEs can be directly connected to zebrafish. It has been demonstrated that CRISPR editing can be used to create loss-of-function mutations in non-coding DNA to examine the function of CNEs with connections to GWAS-SNPs. For instance, Madelaine et al. screened for GWAS SNPs (not for a specific phenotype) embedded in loci with deep non-coding sequence conservation [97]. This allowed for the identification of a set of 45 non-coding SNPs located in deeply conserved CNEs, known for their potential functional role in cis-regulation. Using multiple gRNAs with *Cas9* mRNA, they deleted ~770 bp encompassing a highly conserved core within the CNE, which included the SNP position. Deletion of a CNE led to retinal vasculature defects and to downregulation of miR-9, rather than MEF2C as predicted by the original GWAS [97]. Further, miR-9 depletion affected retinal vasculature formation, suggesting a causal role for this gene in the human variant associated with this trait [97]. By this, they validated that some CNEs act as transcriptional enhancers that can be disrupted by conserved non-coding SNPs [97].

V. GAPS IN KNOWLEDGE AND FUTURE APPROACHES

There are a number of open questions regarding bones, phenotypic traits, and developmental stages in zebrafish that best serve as a model for human skeletal biology, and their fidelity in detecting genes underlying biological risk factors for osteoporosis and related diseases.

What is the utility of zebrafish in modeling monogenic versus polygenic bone disorders?

One potential concern is the relative utility of zebrafish for studying common bone disease-related genes, versus those governing Mendelian traits. As detailed in Section III, mutations in zebrafish genes often manifest as phenotypes resembling those associated with monogenic bone disorders. However, whether similar fidelity may be observed for mutations in genes underlying a complex, multigenic bone disorder, like osteoporosis, is undetermined. Indeed, BMD (or fracture, bone geometry etc.) loci from GWAS usually each make small contributions to the overall heritability of the disease. Thus, GWAS-identified variants are typically of very small effect size.

One argument in support of the fidelity of zebrafish mutants to yield detectable skeletal phenotypes for mutations in genes residing at BMD loci is that null alleles are likely to be of larger effect than GWAS-associated variants themselves. Most GWAS variants are likely regulatory alleles that cause modest changes in gene expression. The fact that they manifest as detectable phenotypic differences in humans implies that null alleles will be of even larger

effect. In support of this, genes whose null alleles confer large effect sizes in mice (e.g., SOST, OPG, RANKL [10]) are known to reside at BMD loci of very small effect sizes [98].

What is the utility of developmental versus adult phenotypes?

Another question is whether mutant phenotypes in development can reflect the biology of an aging skeleton. Skeletal biology in embryonic and larval fish is better characterized than that in juveniles and adults. This is exemplified in Table 2, in which most of the mutants (and all morphants) were examined at very early developmental stages. This is for several reasons, including the historical origins of zebrafish as a model of vertebrate development, the fact that in wildtype zebrafish most of the body remains optically accessible through larval development (which can facilitate the development and use of rapid-throughput phenotypic assays that exploit *in vivo* fluorochrome labeling and/or transgenic fluorescent reporter lines), and the reduced resource requirements associated with housing young animals.

Support for the notion that development can reflect aspects of biology underlying genetic risk for osteoporosis lies in the fact that there is an empiric overlap in GWAS loci found for older-age BMD and fracture risk, and childhood BMD [99–102]. One explanation is existing studies do not have sufficient statistical power to discover children-specific BMD-associated loci. However, an alternative explanation is that the biology underlying genetic risk factors for osteoporosis begin at least in childhood, and are associated with lifelong accrual and maintenance, rather than age-induced onset of disease. The notion that BMD-associated loci are not late-onset suggests that an aging skeleton is not absolutely mandatory to identify causal variants, nor the genes underlying them, at least in some cases.

However, there are also arguments in support of the notion that a threshold amount of aging--in particular, development through adulthood--may be important. For instance, zebrafish mutant models with severe adult skeletal phenotypes can show no obvious skeletal phenotypes, either incompletely penetrant or relatively mild phenotypes, at larval stage. Increase in phenotypic severity with age was demonstrated in zebrafish mutants for *plod2* [66], as well as in medaka and zebrafish mutants for *csfr1a,* which caused deficits in osteoclast formation [67]. In the latter, mutation-causing deficits in neural arch were more obvious in adults rather than larval bone morphology, since neural arch modeling continues throughout life. Finally, biological processes at later stages of development (e.g., juvenile-to-adult transition and adult stage) may be regulated by unique programs that are not active earlier in life. An example of this is the formation of multinucleated osteoclasts, which as described earlier, become predominant only after the juvenile-to-adult transition. Ultimately, a better understanding of the extent to which zebrafish mutants exhibit adult-onset skeletal phenotypes, particularly in the context of genes residing at BMD loci, is needed.

Can zebrafish model environmental influences?

One of the challenges in studying BMD is that osteoporosis is perceived to be a disease of aging, associated with environmental exposures and comorbidities, which are difficult to mimic *in vivo.* It has been shown that in some cases, human mutations with bone phenotypic associations become apparent under stress due to loading or nutrients [103, 104]. The zebrafish skeleton has a reduced requirement for resisting mechanical loads due to residing

within an aquatic environment. However, several studies have demonstrated that swim training can influence timing of skeletogenesis in zebrafish larvae [105], as well as increase bone formation in the centra of adults [55]. Moreover, Botulinum toxin-induced muscle paralysis in zebrafish has been shown to inhibit osteogenesis during fin regeneration [44]. In this context, one question is to what extent adaptation of the zebrafish skeleton in these models is rooted biology similar to that regulating bone adaptation to exercise, spinal cord injury, microgravity, or other modes of mechanical loading/unloading in humans.

What are the skeletal phenotypes in zebrafish most correlated with mammalian bone traits, when the same gene is perturbed?

One-to-one modeling of human skeletal phenotypes in zebrafish can be challenging due to morphophysiological differences, some of which we previously described. For instance, whereas the majority of human bones are endochondral bones, outside of the zebrafish craniofacial skeleton, the proportion of cartilage bones is relatively low. Further, zebrafish do not possess cortical bone in a traditional sense and their bones do not encapsulate a hematopoietic bone marrow cavity. Finally, trabecular-like bone is rarer compared to mammals, particularly outside of the skull.

In some cases, the evolutionary origins of mammalian bones and their connections to the fish skeleton can be identified based on anatomical, developmental, and evolutionary analyses. One classic example is the mammalian middle ear bones. These bones derive from bones that form the jaw in fish, and which connect the ear to the viscerocranium (reviewed by [106]). However, such connections cannot always be made unambiguously. For instance, a recent study examined the evolutionary origins of tetrapod digits [40]. Previous paleontological and developmental studies had indicated that the fin-to-limb transition was associated with two major events: 1) the expansion of distal endochondral bones, and 2) the reduction of the dermal fin skeleton, including the bony fin rays [40]. Based on these studies, it had been suggested that the digital elements of modern tetrapods evolved from distal radial bones of tetrapodomorph fish [107]. Nakamura et al. showed that loss of *hox13* in zebrafish, which results in the loss of the autopod in mice, did not reduce the number of distal radials, but rather increased their numbers. Further, loss of *hox13* resulted in loss of the bony fin rays [40]. This study revealed a surprising developmental connection between fish fin rays and tetrapod digits.

Despite these anatomical differences, several studies have shown a fair degree of correspondence in bone density and/or bone quality between human and zebrafish vertebrae in response to genetic [108][109] and environmental [110] perturbations, suggesting zebrafish vertebral BMD may be useful as a comparable measure to human BMD. For instance, in certain rare models of OI, an increase in vertebral BMD and TMD has been reported for zebrafish with mutations in *bmpla* [111][24] and *plod2* [112][24]. While these findings may seem incongruent with the more common clinical presentation of OI (manifesting as reduced bone mass and increased fragility) in humans, there is evidence for increased BMD in rarer forms of OI resulting from mutations in *BMP1* [113]. A reduction in BMD, however, has been shown for human patients with mutations in *PLOD2* [114]. Although increased TMD has been reported for this zebrafish model [112][24], changes in

the bone microarchitecture are consistent with brittle/fragile bone as seen in humans. Such findings may be due more to inherent differences in quantitative methodologies (BMD in humans vs TMD in zebrafish) rather than the underlying physiologies of the bone itself, as shown for a classical model of OI in *Chi/+* zebrafish where high mineral density at the tissue level still resulted in low bone mass and impaired structural integrity at the whole bone level [115].

A more objective determination of which skeletal phenotypes in zebrafish are most correlated with mammalian bone traits, when the same gene is perturbed, would require to search for cross-species associations in phenotypic databases. Mutations affecting bone phenotypes in mammals are likely to alter non-equivalent structures in orthologous zebrafish mutants if they possess conserved activity in the orthologous gene. Associative methods to discover phenologs -- orthologous phenotypes between organisms based upon overlapping sets of orthologous genes associated with each phenotype -- has been demonstrated [116]. In this context, a community effort to phenotype zebrafish mutants for orthologs of genes examined in mutant mouse phenotyping consortiums such as the IMPC, Bonebase, or OBDC may aid in identifying zebrafish phenotypes that are most consistently associated with phenotypic changes in the orthologous mutant mouse. Similarly, the application of associative methods to phenotypic databases may help identify skeletal phenologs in zebrafish and mammals.

Future approaches

There are a number of avenues in which rapid-throughput, tractable *in vivo* assays may enable a deeper understanding of the genetic basis underlying both monogenic and multigenic bone disorders.

Large-scale functional annotation of genes at BMD loci to identify candidate causal targets—An important step in understanding genetic risk factors for osteoporosis is to define the roles of genes that may underlie them. Reverse genetic screens of candidate genes in model organisms provide a mechanism to attribute functional skeletal contributions of these genes to osteoporosis-related traits. For instance, the target gene approach applied in [8] identified a set of genes that were enriched up to 58-fold for known causal genes. This gives a high yield set of genes that can be explored through animal models, such as zebrafish.

Several prototypes for CRISPR-based reverse genetic screens in zebrafish have been developed [94, 117, 118]. In these screens, phenotyping can be performed in stable germline mutants [94], or in first generation (G0) somatic mutant animals [117, 118]. GO analysis eliminates the need for germline transmission of mutations; this is significant because the time and resources needed to breed mutant alleles through multiple generations is often the primary factor limiting screen throughput. G0 screens also enable multiplexed approaches to increase throughput, and enable knockdown of clusters of genes with functional redundancy [117]. While one limitation of G0 screens is genetic mosaicism, it has recently been shown that the use of multiple gRNAs directed at different target sites within the same gene can result in a high proportion of null phenotypes [118].

The pursuit of reverse genetic screens in zebrafish directed at genes residing at BMD loci would overcome several limitations associated with systematic knockout mouse phenotyping projects. For instance, because such screens would not be limited to pre-existing mouse lines selected for phenotyping, they would enable better coverage of genes at BMD loci. Further, phenotypes may be attained in as little as a few weeks to a few months, reducing costs and resources that can often act as a barrier to pursuing genes that may have unpredictable effects. This may be critical in overcoming a shortcoming in genetic exploration post-sequencing of the human genome: the fact that most biomedical research tends to focus on the same genes, thus, most genes remain under- or unexplored [117]. Finally, zebrafish screens may enable more effective use of resources designated for knockout mouse phenotyping, as hits can be used to prioritize genes for testing in mouse models. In regard to screen throughput, it is difficult to provide “universal” estimates in regard to time frame and costs as they depend highly on genetic strategy (e.g., somatic vs germline mutant analysis) and phenotypic assay (e.g., larval vs. adult), amongst other variables. As one example, Moens and colleagues performed a G0 CRISPR screen of 48 genes involved in electrical synapse formation in zebrafish larvae. These authors reported a total time of three weeks (including sgRNA synthesis, pool injections, and de-multiplexing) [117].

Multigenic interactions—A large body of studies in model organisms have demonstrated that epistasis--interactions between multiple genes--is an important genetic component underlying phenotypic variation [119]. In humans, it has long been hypothesized that epistasis may contribute to a large proportion of phenotypic variation [119], in that the interaction of genetic variants may produce a larger (or smaller) effect than would be expected from additive effects [120]. This hypothesis has long been disputed and is difficult to test, in part because of the large number of pairwise interactions, which results in low statistical power [120].

Evidence in animal models in support of the importance of multigenic interactions in complex human diseases was recently demonstrated by Liu et al. [121]. Using mouse forward genetics, they recovered the first mutant mice with hypoplastic left heart syndrome (HLHS), a severe congenital heart disease with a complex, multigenic etiology. These mice had multiple mutations. In considering the multigenic etiology of HLHS, this likely accounted for the previous failure to obtain HLHS mutant mice [121]. Compound mutations in Sap130 and Pcdha9 were shown to be a digenic cause of HLHS in mutant mice, and one human subject with HLHS with mutations in SAP130 and PCDHA13 was identified [121]. These studies highlight the virtues of identifying multigenic interactions in animal models as an avenue to reveal their contributions to complex diseases.

Because of the sheer number of combinations gene-gene interactions, rapid-throughput studies in zebrafish may be helpful for this purpose. CRISPR-based G0 screens in zebrafish are amenable to multiplexed approaches, thus they can facilitate combinatorial studies of gene-gene interactions within moderately sized panels of genes. This includes genes that may reside on the same chromosome, and which are tightly linked. Interactions in tightly linked genes are underexplored, due in part to difficulties in generating compound mutants, as they cannot be generated by crossing individual mutant lines. Finally, CRISPR editing in zebrafish could facilitate the generation of “additive” series of mutants where mutations are

serially added to generate increasingly mutated genomes. Of note, genome-wide polygenic scores for several common diseases identified individuals with risk equivalent to monogenic mutations[122]. For instance, Kuchenbaecker et al. [123] evaluated polygenic risk scores for breast and ovarian cancer risk prediction in *BRCA1* and *BRCA2* mutation carriers, and made the case for the combined testing of monogenic and polygenic disease risk factors.

Examinations of epistasis in zebrafish may help understand Mendelian skeletal diseases. Many human diseases are not solely caused by a single variant but rather a combination of multiple common variants exerting a weak affect alongside more severe or stronger effect variants [124]; the latter variant often lies within a single Mendelian disease-causing locus [98]. In a recent study, Gistelinck et al. systematically analyzed skeletal phenotypes in a large set of zebrafish with diverse mutations in the genes encoding type I collagen, representing different genetic forms of human OI [68]. These studies revealed select mutations to give rise to intra-genotype variability in phenotypic expressivity and penetrance, mirroring the clinical variability associated with human disease pathology. This suggests the potential for zebrafish to aid understanding the roles of modifier genes in the genetic architecture of OI, and their contribution to variable phenotypic penetrance underlying its clinical variability.

Functional annotation of skeletal CNEs—Rapid genetic approaches in zebrafish may also facilitate large-scale examination of noncoding elements important for skeletal function. Even if not explicitly conserved in humans, the identification of noncoding elements in zebrafish that regulate the function of genes whose orthologs are known to be important for human skeletal function (e.g., RUNX2, SP7, OPG, RANKL, SOST, etc.) may reveal general principles underlying their regulation in humans. As mentioned previously, CRISPR editing can be used to create loss-of-function mutations in non-coding DNA to examine the function of CNEs in zebrafish with connections to GWAS-SNPs in humans [97].

Conclusions

A survey of the literature suggests that zebrafish are a promising aid in post-genomic testing of gene function. This promise derives from high conservation in coding sequence and gene function, emerging technologies for rapid gene editing and skeletal phenotyping in zebrafish, and demonstrated productivity of zebrafish in identifying trait-associated gene functions at loci associated with non-skeletal polygenic disorders. Several gaps in knowledge need to be addressed to facilitate the use of zebrafish as a model of human skeletal biology. This includes the identification of developmental stages and phenotypes in mutant zebrafish that are the most predictive of mammalian phenotypes, following orthologous mutations. Further, while zebrafish models can be rapidly and inexpensively generated to mimic deleterious variations in human genes, compared to mice, examples are still scarce at present. In this context, a community-wide effort to phenotype zebrafish mutants for orthologs of genes similar to mutant mouse phenotyping consortia may be useful. Finally, there are a number of avenues in which rapid-throughput, tractable *in vivo* assays may enable a deeper understanding of the genetic basis underlying both monogenic and multigenic bone disorders. Partnerships between human, mouse, and zebrafish geneticists where bone-

centered human, mouse, and zebrafish studies inform each other is likely to be beneficial for both basic and translational researchers.

Drug targets supported by genetic evidence such as GWAS are 2–3× more likely to pass through clinical development [125]. To translate into a clinical setting, both human discovery and animal-model validation should parallel one another in quality and rigor. Our hope is that introducing the strengths and challenges of zebrafish model is important step to propel the field.

ACKNOWLEDGEM ENTS

This publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) of the National Institutes of Health (NIH) under Award Number AR066061 and AR072199. This publication was also supported by Israel Science Foundation grant #1283/14. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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HIGHLIGHTS

1. The zebrafish and mammalian skeletons exhibit morphophysiological similarities and differences, which are scale-dependent (i.e., whether studied from the nano-, micro-, or macroscale)
2. There is evidence of broad conservation in bone-related genes in zebrafish, despite a teleost-specific genome duplication that complicates analysis of some human orthologs
3. There has been rapid growth in the arsenal of phenotypic assays to assess skeletal biology in zebrafish at the tissue and cellular levels
4. There are open questions regarding the skeletal phenotypes in zebrafish that best serve as a model for human skeletal biology
5. There are promising avenues in which the experimental attributes of zebrafish can be leveraged to address urgent needs in regard to human skeletal genomic exploration

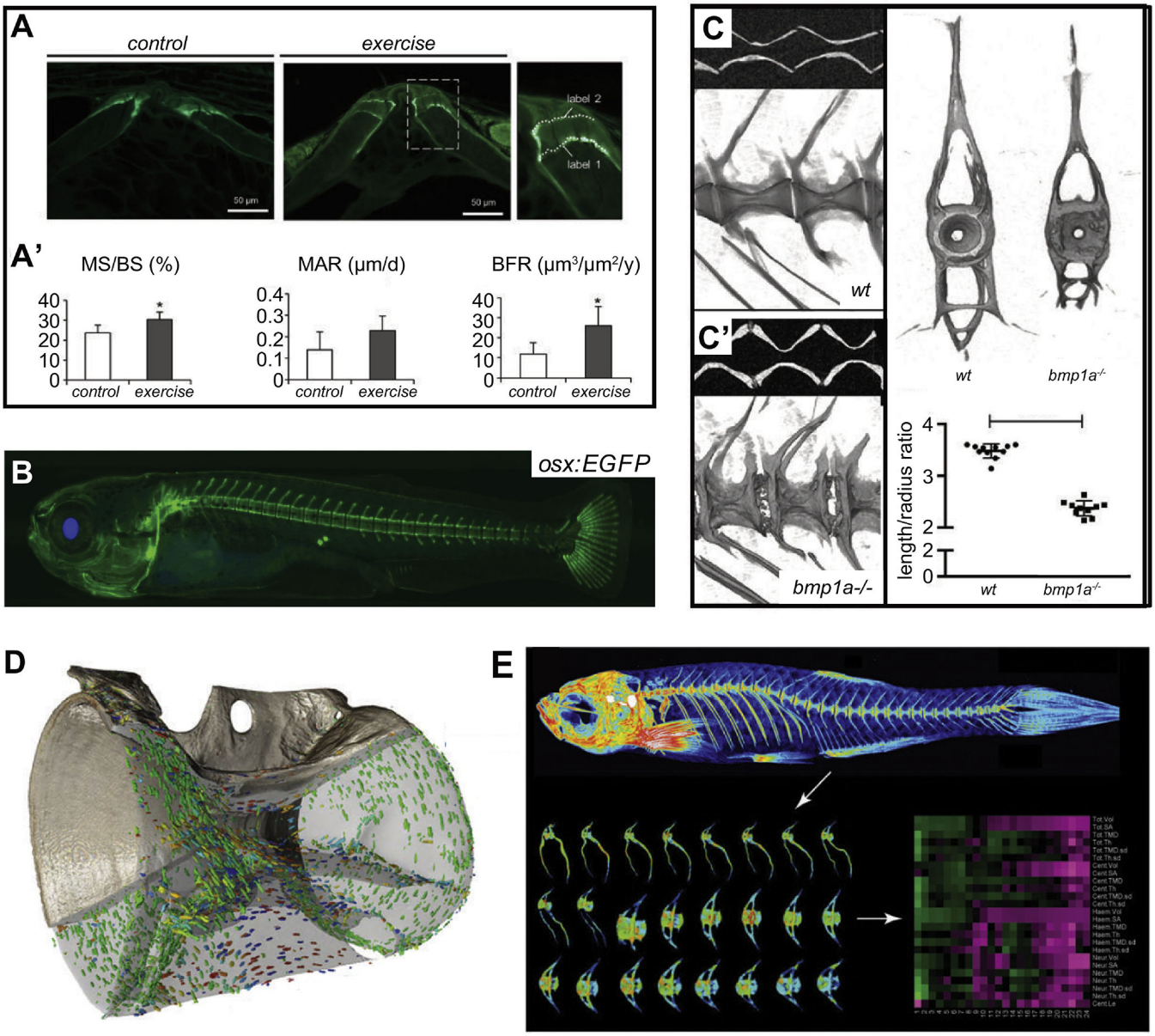

Fig 1. Skeletal phenotyping in zebrafish.

(A) Dynamic histomorphometry (reproduced from [55]). Zebrafish were subjected to swimming exercise, labeled with calcein pre- and post-training, and the vertebral body end plates were analyzed. (A') Quantification of mineralized surface per bone surface (MS/BS), mineral apposition rate (MAR), and bone formation rate (BFR) in (A). (B) *In vivo* visualization of gene expression using transgenic reporter fish. Shown is a larval *osx:EGFP* zebrafish, which enables visualization of cells expressing osterix. (C) microCT (reproduced from [63]). Vertebrae of wildtype (wt) and *bmpla−/−* siblings were examined at 6 months of age. (C') Representative sagittal sections. (C") Comparison of centrum length to radius ratio. (D) Characterization of lacunar properties in zebrafish vertebrae using high-resolution microCT (reproduced from [55]). (E) microCT-based skeletal phenomics (reproduced from [71]). For each fish, a whole body microCT scan was acquired (top), and 24 vertebrae in the

same fish were segmented (bottom left; colors represent local bone thickness). Each vertebra was segmented into three elements, and each element was analyzed for measures such as volume, thickness, and TMD, resulting in 600 measures per fish. Data were organized into “skeletal barcodes” (bottom right), where each column represents a vertebra, and each row represents a measure.

Table 1:

Comparison of experimental attributes in zebrafish and mouse models.

Attribute	Zebrafish	Mouse
Husbandry		
Costs for animal care	Low	High
Space required for housing	Low	High
Age of sexual maturity	~10–12 weeks	~6–8 weeks
Route of fertilization	External	Internal
Number of offspring per parental pair	~50–200	~2–12
Life span	~3 years	~2 years
Genome		
Number of protein coding genes	~26,000	~23,000
Percentage of human protein coding genes with orthologs in each species	~71%	~76% (ignoring genes without one-to-one correspondence)
Number of orthologous genes per human gene	Often, more than one	Usually one
Experimental Genetics		
High-quality reference genome sequence available?	Yes	Yes
Amenable to targeted gene modification?	Yes	Yes
Amenable to transgenesis?	Yes	Yes
Ease and affordability of F3 reverse genetic screens?	Medium	Extremely low
Ease and affordability of F0 reverse genetic screens?	High	Low
Skeletal Phenotyping		
Visibility of skeletal development	High, due to external development and transparency during developmental stages	Low, due to in utero development
Amenability to *in vivo* fluorescence imaging	High	Low
Amenability to rapid-throughput skeletal phenotyping	High	Medium
Amenability to one-to-one modeling of human skeletal phenotypes?	Low	High

Table 2:

Selection of genetic models in zebrafish for human genes associated with BMD GWAS

Human gene	Human GWAS or Disease/trait	Human Reference	Zebrafish Homolog	Type of Model (mutants or morphants)	Fish phenotype (age)	Zebrafish Reference
COL11A 1	Stickler/Marshall syndrome; eBMD*	[8]	*col11a1 a/b*	Morpholino	abnormally thick and sparse fibrils in the cartilage extracellular matrix/ perichordal sheath	[126]
CYP26	Coronal craniosynostosis; eBMD	[127] [8]	*cyp26b1*	Mutants: null (*dolphin*) and hypomorphic (*stocksteif*)	fusions of the vertebral centra, cartilaginous outgrowths of the endochondral fin elements (*dolphin*; larvae); Coronal Craniosynostosis (*stocksteif*, juvenile)	[127]
ENPP1	hypermineralizatio n of the axial skeleton; BMD	[128]	*enpp1*	mutant *dgf*^{hu4581} (*dragonfish*)	ectopic calcifications; patchy mineralization of craniofacial bones; overexpress *spp1* (Osteopontin; 6 – 9 dpf); fusion in vertebral bodies, neural and haemal arches (juvenile)	[89]
JAG1	Alagille syndrome; BMD	[129, 130]	*jag1b*	loss-of-function mutation	facial defects; dorsal hyoid and mandibular arch fusion (5 dpf)	[131]
LRP5	Osteoporosis- pseudoglioma (OPPG), craniosynostosis; BMD	[104, 132, 133]	*Lrp5*	Morpholino; CRISPR/Cas9 (exon 2, exon 3)	severe defects in the ventral craniofacial skeleton (7 dpf)	[37]
MEF2C	Auriculocondylar syndrome; BMD	[130, 134]	*mef2ca*	Mutant (ENU mutagenesis)	malformed faces; ventrally displaced jaws. Dorsal/ventral joints missing; ectopic cartilage; enlarged ventral hyoid bone (5 dpf)	[135]
RUNX2	Cleidocranial dysplasia; eBMD	[8]	*runx2b*	Morpholino	Morphants displayed no overt abnormalities (3.5 – 5dpf)	[136]
SPP1	BMD	[7]	*spp1*	Morpholino, mutant (CRISPR, exons 6 and 7)	reduction in the formation of endochondral and dermal bone (5–15-dpf)	[87]
SOX9	Campomelic dysplasia; BMD	[7]	*sox9a, sox9b*	null mutants (*sox9*^{hi1134} or *jellyfish*; *sox9b*^{b971} + double *sox9* muta nts	curly-down body axis (*sox9b*^{b971}; 4–5-dpf); actinotrichia missing (double mutant)	[137, 138]
TGFB2	eBMD	UK BB	*tgfb2, fgf10a*	Morpholino	shortening and misshaping of the jaw, ethmoid plate, and parasphenoid	[139]

*estimated BMD (heel bone)