



Review Article

Zebrafish models for glucocorticoid-induced osteoporosis

Wen-Ying Lin^{a†}, Kameshwara Kumar Dharini^{b†}, Cheng-Huan Peng^{a,b,c}, Chung-Yen Lin^d, Kuang-Ting Yeh^{a,c}, Wen-Chih Lee^{e*}, Ming-Der Lin^{b,f*}

^aDepartment of Orthopedics, Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Hualien, Taiwan, ^bInstitute of Medical Science, Tzu Chi University, Hualien, Taiwan, ^cSchool of Medicine, Tzu Chi University, Hualien, Taiwan, ^dInstitute of Information Science, Academia Sinica, Taipei, Taiwan, ^eResearch Center for Global SDGs Challenges, Office of Research and Development, Tzu Chi University, Hualien, Taiwan, ^fDepartment of Molecular Biology and Human Genetics, Tzu Chi University, Hualien, Taiwan

[†]Both authors contributed equally to this work.

Submission : 29-Mar-2022
Revision : 10-May-2022
Acceptance : 07-Jun-2022
Web Publication : 23-Aug-2022

ABSTRACT

Glucocorticoid-induced osteoporosis (GIOP) is the most common form of secondary osteoporosis due to excessive or long-term glucocorticoid administration, disturbing the homeostasis between bone formation and bone resorption. The bone biology of zebrafish shares a high degree of similarities with mammals. In terms of molecular level, genes and signaling pathways related to skeletogenesis are also highly correlated between zebrafish and humans. Therefore, zebrafish have been utilized to develop multiple GIOP models. Taking advantage of the transparency of zebrafish larvae, their skeletal development and bone mineralization can be readily visualized through *in vivo* staining without invasive experimental handlings. Moreover, the feasibility of using scales or fin rays to study bone remodeling makes adult zebrafish an ideal model for GIOP research. Here, we reviewed current zebrafish models for GIOP research, focused on the tools and methods established for examining bone homeostasis. As an *in vivo*, convenient, and robust model, zebrafish have an advantage in performing high-throughput drug screening and could be used to investigate the action mechanisms of therapeutic drugs.

KEYWORDS: *Glucocorticoid-induced osteoporosis, Osteoporosis, Zebrafish*

INTRODUCTION

Glucocorticoids are frequently used to treat inflammatory or immune disorders. However, a common but influential complication termed glucocorticoid-induced osteoporosis (GIOP) occurs in many patients receiving high-dose or long-term glucocorticoid therapy. Excessive glucocorticoid disrupts the balance between bone formation and bone resorption, leading to osteoporosis. Glucocorticoids have been demonstrated to promote bone resorption at the initial stage (in the 1st year of treatment) by increasing osteoclastogenesis and inhibiting the apoptosis of osteoclast precursor cells. In addition, glucocorticoids inhibit the differentiation and functions of osteoblasts and induce apoptosis of osteoblasts and osteocytes, leading to a decrease in bone formation [1]. During these processes, epigenetic modifications and mitochondrial bioenergetic alteration have been reported [2]. One research indicates that glucocorticoids induce the Lys-9 acetylation at the histone 3 and thus suppress *Runt-related transcription factor*

2 (*Runx2*) expression and osteoblast differentiation [3]. Besides, a prolonged glucocorticoid treatment usually triggers apoptosis of osteoblasts and osteocytes due to the disruption of mitochondrial bioenergetics that accumulates oxidative stress [4,5] or the induction of endoplasmic reticulum stress [6,7].

Zebrafish larvae are transparent and develop externally, allowing the progression of skeletal development and bone mineralization to be visualized after *in vivo* staining without invasive handling and animal sacrifice [8]. These advantages

***Address for correspondence:** Dr. Wen-Chih Lee, Research Center for Global SDGs Challenges, Office of Research and Development, Tzu Chi University, 701, Zhongyang Road, Section 3, Hualien, Taiwan.

E-mail: mosquito1213@gms.tcu.edu.tw

Prof. Ming-Der Lin,

Department of Molecular Biology and Human Genetics, Tzu Chi University, 701, Zhongyang Road, Section 3, Hualien, Taiwan.

E-mail: mingder@gms.tcu.edu.tw

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: WKHLRPMedknow_reprints@wolterskluwer.com

How to cite this article: Lin WY, Dharini KK, Peng CH, Lin CY, Yeh KT, Lee WC, et al. Zebrafish models for glucocorticoid-induced osteoporosis. Tzu Chi Med J 2022;34(4):373-80.

Access this article online	
Quick Response Code: 	Website: www.tcmjmed.com
	DOI: 10.4103/tcmj.tcmj_80_22

make zebrafish larvae suitable for studying skeletogenesis, building disease models, and screening anti-osteoporotic compounds. On the other hand, adult zebrafish's scales or fin rays are ideal for studying bone remodelling – the homeostasis maintained by osteoclast-mediated mineral resorption and osteoblast-mediated mineral deposition. Moreover, the quantity available and the live-imaging possibility highlight the *ex vivo* scale culture as an invaluable model for the high-throughput drug screen. The feasibility of tagging bone cells with fluorescent proteins through transgenesis makes zebrafish an ideal model for studying bone biology. In this review, we focus on the bone physiology and methodologies for building zebrafish as GIOP models.

ZEBRAFISH SHARE SIMILAR MOLECULAR TOOL KITS WITH MAMMALS IN SKELETAL DEVELOPMENT

Bone is a dynamic tissue that routinely undergoes remodeling to maintain skeletal integrity [9]. The remodeling process can be divided into five major phases: (1) the activation phase – mono-nucleated osteoclasts are recruited to the damaged bone surface; (2) the resorption phase – activated multi-nucleated osteoclasts start to remove the damaged bone; (3) the reversal phase – osteoclasts undergo apoptosis or leave the bone surface, and preosteoblasts are recruited to the digested bone surface; (4) the formation phase – mature osteoblasts lay down the nonmineralized organic matrix (osteoid), gradually embedding themselves into the osteoid, and undergo terminal differentiation into osteocyte; and (5) the mineralization phase – the osteoblasts and the osteocytes mineralize the osteoid, and the osteoblasts on the mineralized surface enter a quiescence state, which named as bone lining cells.

Although the entire bone remodeling processes have not been thoroughly documented in zebrafish, the essential cellular and molecular machinery are evolutionally conserved. The skeletal cells, such as bone-forming osteoblast and osteocyte, bone-resorbing osteoclast, and cartilage-forming chondroblast and chondrocyte, are all identified in zebrafish [10]. Signal pathways and genes involved in the differentiation of these skeletal cells also show similarities between zebrafish and mammals [Table 1]. For instance, Wingless-type MMTV integration site (Wnt) signaling is evolutionarily conserved in promoting osteoblastogenesis. Knockout of zebrafish *wnt16*, an

ortholog of human *WNT16*, resulted in severe deformities and reduced bone mineral density [21]; similar phenotypes were also found in *Wnt16*^{-/-} mice [22]. Although epigenetic control of zebrafish genes responding to glucocorticoids has not been explored, zebrafish genes involved in the differentiation and functions of osteoblast, osteocyte, or osteoclast are mostly conserved to their human counterparts [Tables 2 and 3]. These similarities make zebrafish a reasonable model for studying bone-related disorders.

MODELING GLUCOCORTICOID-INDUCED OSTEOPOROSIS IN ZEBRAFISH LARVAE

The GIOP model using zebrafish larvae was first established in 2006 by Barrett *et al.* The authors demonstrated a significant mineral loss in the head bone after treating 25 μ M prednisolone to the zebrafish larvae from 5 to 10 days post fertilization (dpf) [40]. Subsequent studies use either prednisolone (25 μ M) or dexamethasone (10 or 15 μ M) as the glucocorticoids to establish the GIOP models. The timing of glucocorticoid treatment in different studies could start from 3 to 5 dpf and stop at 9–10 dpf [41,42]. To evaluate the therapeutic effect of candidate drugs against GIOP, colorimetric or fluorescent staining methods in conjunction with quantifying methods such as the staining area of head bones, integrated optical density of bones, or the numbers of calcified vertebrae could be adopted [Figure 1A] [40,42,43]. Using the quantitative reverse-transcription polymerase chain reaction, the expression of genes related to the extracellular matrix, osteoblast, and osteoclast could be quantified in zebrafish larvae to investigate the molecular mechanism of anti-osteoporotic compounds [42,44]. The possible impacts of prednisolone on zebrafish larvae have been demonstrated through upregulating the osteoclast-activity markers such as *matrix metalloproteinase 9 (mmp9)* and downregulating the preosteoblast and osteoblast markers such as *sp7 transcription factor (sp7)* and *secreted phosphoprotein 1/osteopontin (spp1/opn)*, respectively [44]. Besides, prednisolone also suppresses the expression of various collagen genes, such as *collagen, type XI, alpha 1a (coll11a1a)*, resulting in cartilage defects [45], which is similar to that observed in osteoarthritis patients administrated with glucocorticoids [46].

MODELING GLUCOCORTICOID-INDUCED OSTEOPOROSIS IN ADULT ZEBRAFISH

Scales as a model for glucocorticoid-induced osteoporosis studies

The zebrafish skin is covered by a layer of calcified elasmoid scales, which belong to the dermal bone. The scales are stacked and attached by collagen fibrils comprising mainly two layers. The hyposquamal side (internal layer) is incompletely mineralized and composed of multiple layers of collagen fibrils. The episquamal side (external layer) is mineralized, containing a network of collagen fibrils [47]. Scleroblasts, the scale-forming osteoblasts derived from mesenchymal cells, are highly functionally similar to mammalian osteoblasts and express canonical osteoblastic markers [33,48,49]. The scale also contains osteoclasts required to maintain the homeostasis of scale growth [39]. The

Table 1: Signaling pathways involved in zebrafish bone homeostasis

Pathways	Roles	References
Wnt	Promoting osteoblast differentiation and chondrogenic development; beneficial to fracture healing and bone repair	[11-13]
Bmp	Promoting osteoblast differentiation and functions; required for bone repair	[14-16]
Hh	Promoting osteoblast differentiation; required for bone repair	[17,18]
Fgf	Promoting osteoblast differentiation and chondrogenic development; required for bone repair	[11,19,20]

Wnt: Wingless-type MMTV integration site, Bmp: Bone morphogenetic protein, Hh: Hedgehog, Fgf: Fibroblast growth factor

Table 2: Zebrafish-human orthologs implicated in osteoblast and osteocyte differentiation and function

Cell type	Function	Human		Zebrafish		Reference	
		Gene name	Gene ID*	Gene name	Gene ID*		
Osteoblast	Promoting proliferation and differentiation of osteoblast	<i>RUNX2</i>	860	<i>runx2a</i>	405784	[23]	
				<i>runx2b</i>	405788	[23]	
		<i>SP7/OSX</i>	121340	<i>sp7/osx</i>	405789	[24]	
		<i>ATF4</i>	468	<i>atf4a</i>	406514	N/A	
				<i>atf4b</i>	556410	N/A	
	Related to bone matrix mineralization [†]	<i>COL1A1</i>	1277	<i>coll1a1a</i>	337158	[25]	
		<i>ALPL</i>	249	<i>alpl</i>	393982	[26]	
		<i>SPP1/OPN</i>	6696	<i>spp1/opn</i>	432385	[27]	
		<i>BGLAP/OCN</i>	632	<i>bglap/ocn</i>	792433	[24]	
		<i>MMP13</i>	4322	<i>mmp13a</i>	387293	[28]	
		<i>mmp13b</i>	100006896	[29]			
		<i>PHOSPHO1</i>	162466	<i>phospho1</i>	100002812	[30]	
		<i>SPARC/ON</i>	6678	<i>sparc</i>	321357	[31]	
Osteoblast and osteocyte	Regulating differentiation of osteoclast [‡]	<i>CSF1</i>	1435	<i>csf1a</i>	100004617	N/A	
				<i>csf1b</i>	790931	N/A	
		<i>TNFSF11/RANKL</i>	8600	<i>tnfsf11/rankl</i>	100331628	[32]	
		<i>TNFRSF11B/OPG</i>	4982	<i>tnfrsf11b/opg</i>	407674	[32]	
Osteocyte	Inhibiting differentiation of osteoblast	<i>DKK1</i>	22943	<i>dkk1a</i>	799377	[33]	
				<i>dkk1b</i>	30197	[34]	
			<i>SOST</i>	50964	<i>sost</i>	100000500	[35]
	Promoting bone matrix mineralization	<i>PHEX</i>	5251	<i>phex</i>	386969	[33]	

*NCBI gene identifier, [†]All these genes play positive roles in bone mineralization, except *MMP13*, [‡]*CSF1* and *TNFSF11/RANKL* play positive roles in osteoclast differentiation, except that *TNFRSF11B/OPG* encodes an inhibitor of RANKL. *RUNX2*: *Runt-related transcription factor 2*, *SP7/OSX*: *Sp7 transcription factor/Osterix*, *ATF4*: *Activating transcription factor 4*, *COL1A1*: *Collagen type 1 alpha 1 chain*, *ALPL*: *Alkaline phosphatase, biomineralization associated*, *SPP1/OPN*: *Secreted phosphoprotein 1/Osteopontin*, *BGLAP/OCN*: *Bone gamma-carboxyglutamate protein/Osteocalcin*, *MMP13*: *Matrix metalloproteinase 13*, *PHOSPHO1*: *Phosphoethanolamine/phosphocholine phosphatase 1*, *SPARC/ON*: *Secreted protein acidic and cysteine rich/Osteonectin*, *CSF1*: *Colony stimulating factor 1*, *TNFSF11/RANKL*: *TNF superfamily member 11/Receptor activator of nuclear factor kappa-B ligand*, *TNFRSF11B/OPG*: *TNF receptor superfamily member 11b/Osteoprotegerin*, *DKK1*, *Dickkopf WNT signaling pathway inhibitor 1*, *SOST*: *Sclerostin*, *PHEX*: *Phosphate regulating endopeptidase homolog X-linked*, N/A: Not available, NCBI: National Center for Biotechnology Information

Table 3: Zebrafish-human orthologs implicated in osteoclast differentiation and function

Cell type	Function	Human		Zebrafish		Reference
		Gene name	Gene ID*	Gene name	Gene ID*	
Osteoclast	Promoting differentiation of osteoclast	<i>SPI1</i>	6688	<i>spi1a</i>	751704	N/A
				<i>spi1b</i>	30117	N/A
		<i>CSF1R</i>	1436	<i>csf1ra</i>	64274	[36]
				<i>csf1rb</i>	568405	[36]
		<i>TNFRSF11A/RANK</i>	8792	<i>tnfrsf11a/rank</i>	100037357	[37]
	Promoting bone matrix degradation	<i>NFATC1</i>	4772	<i>nfatc1</i>	568315	[38]
		<i>CTSK</i>	1513	<i>ctsk</i>	550475	[37]
		<i>ACP5/TRAP</i>	54	<i>acp5a/trap</i>	406801	[37]
				<i>acp5b/trap</i>	436725	[37]
		<i>MMP9</i>	4318	<i>mmp9</i>	406397	[39]
		<i>CA2</i>	760	<i>ca2</i>	387526	N/A

*NCBI gene identifier. *SPI1*: *Spi-1 proto-oncogene*, *CSF1R*: *Colony stimulating factor 1 receptor*, *TNFRSF11A/RANK*: *TNF receptor superfamily member 11a/Receptor activator of nuclear factor kappa-B*, *NFATC1*: *Nuclear factor of activated T cells 1*, *CTSK*: *Cathepsin K*, *ACP5/TRAP*: *Acid phosphatase 5/ Tartrate-resistant acid phosphatase*, *MMP9*: *Matrix metalloproteinase 9*, *CA2*: *Carbonic anhydrase 2*, N/A: Not available, NCBI: National Center for Biotechnology Information

zebrafish scale has a regenerative capacity and can completely regenerate within 21 days of removal [50].

The zebrafish scales are readily available and suitable for *ex vivo* and *in vivo* GIOP-related studies [48,50,51]. Pasqualetti *et al.* established an *ex vivo* zebrafish scale culture model in 2012 for evaluating the osteoblast and osteoclast behavior [Figure 1B] [48]. They explanted scales from 6 months old zebrafish and placed them in a 96-well plate

in Dulbecco's Modified Eagle Medium. They also observed apoptotic cells on the scale cultured for 72 h; accordingly, they concluded that assays for determining osteoblast and osteoclast activities, such as measuring alkaline phosphatase (Alp) and tartrate-resistant acid phosphatase (Trap) activities, should be performed within 72 h after explantation [48]. The scale culture could be considered an explanted organ and used to study the interaction among osteoblast, osteoclast, and the

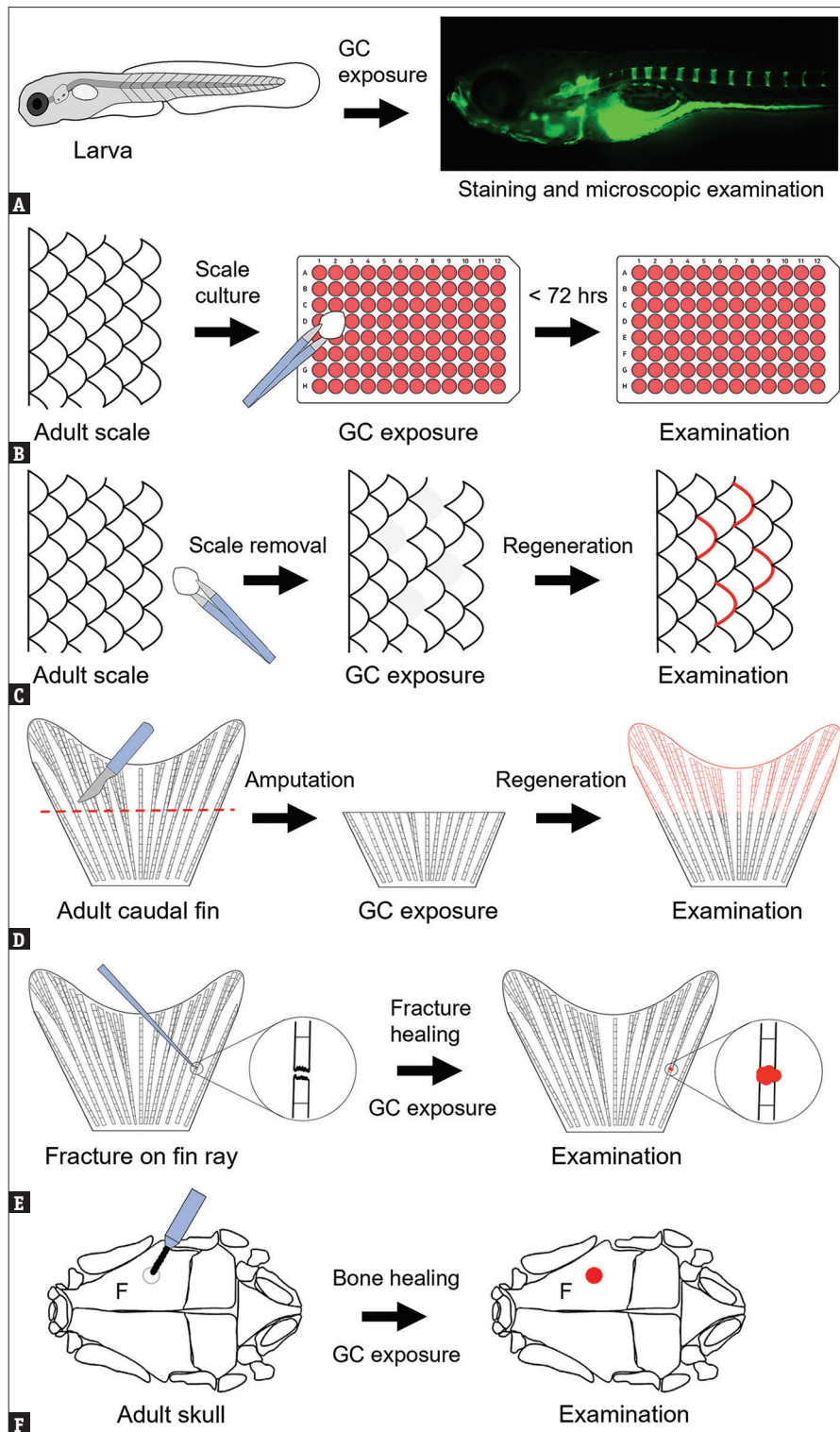


Figure 1: Zebrafish models for glucocorticoid-induced osteoporosis. (A) The larval model for GIOP. After the glucocorticoid exposure, larvae could be stained for the examination of bone calcification. Inset on the right: calcein staining showing bone calcification. (B) The *ex vivo* scale-culture model for GIOP. The scales collected from adult zebrafish could be cultured in 96-well plates with glucocorticoids. The numbers and activity of osteoblast and osteoclast can be examined within 72 h post *ex vivo* culture. (C) The *in vivo* scale GIOP model. Scales are removed from the adult zebrafish using forceps. Then, the zebrafish will be exposed to the glucocorticoid to study the impact of glucocorticoids on scale regeneration. The numbers and activity of osteoblast and osteoclast could be examined. (D) Caudal fin amputation model for GIOP. Caudal-fin amputation is performed by surgical removal using a scalpel. The caudal fin amputated zebrafish is then exposed to glucocorticoids for weeks. The impact of glucocorticoids on bone regeneration could be addressed by examining the numbers and activity of osteoblast and osteoclast. (E) Fin-ray fracture model for GIOP. The fracture could be generated by using a needle or a pair of fin-tip forceps to crush the hemiray. After the fracture is generated, the zebrafish will be exposed to glucocorticoids to study the impact of glucocorticoids on fracture healing. (F) Skull trepanation model for GIOP. For the skull trepanation, a micro-drill is used to damage the os frontale of the calvarial bone. After the surgical intervention, the zebrafish will be exposed to glucocorticoids, and the bone healing process could be monitored. Abbreviations: F, os frontale; GC, glucocorticoid

matrix, which may provide insights into bone regeneration and remodeling. This cost-efficient scale culture model has been used in a primary screen for anti-osteoporotic compounds [52]. The authors created a transgenic zebrafish expressing luciferase under the control of *sp7*, which is actively expressed in preosteoblast and osteoblast [52]. They explanted the scale from the transgenic fish and cultured them with selected compounds for 48 h, and then the corresponding luciferase activity was measured to quantify the changes in osteoblast differentiation [52]. This *ex vivo* zebrafish scale culture model preserves a natural environment for bone cells, which is more adequate to study the cell-cell interaction in a spacious manner compared to the traditional culture system.

As for the *in vivo* GIOP scale model, the adult zebrafish has to be exposed to 50 μ M prednisolone for 15 days and the scales can be collected for further assays [51]. After the prednisolone exposure, the mineralization of scales is reduced, and the resorption of the scale lacunae is increased. The treatment of alendronate, an FDA-approved bisphosphonate anti-resorptive drug, alleviates the impact of prednisolone on the scale by decreasing the Trap activity and increasing the Alp activity [51]. Recent studies have adopted this *in vivo* scale GIOP model to evaluate the curative effect of the herbal mixture or compounds on the 9 months old zebrafish by treating them with 80 μ M prednisolone for 14 days [53,54].

The regenerating scales could also be used as an *in vivo* model to investigate the impact of glucocorticoids on bone mineralization [Figure 1C] [50]. In the regenerating scale GIOP model, the adult zebrafish are treated with prednisolone for 1 day prior to scale removal. After scale removal, the zebrafish are continually exposed to prednisolone, and the regenerated scales can be collected on the 8th or 21st days after prednisolone exposure [50]. The size and mineralization reduction, the activity of bone cells, and the matrix resorption of the scales from the prednisolone-treated zebrafish can be then measured [50]. This zebrafish scale model has been used to demonstrate the therapeutic effect of anti-osteoporotic compounds in treating GIOP [55]. A recent study showed that the intraperitoneal injection of dexamethasone to the adult zebrafish could shorten the administration time to less than 5 days, making this GIOP model more efficient and beneficial for a large-scale drug screening [56].

ESTABLISHMENT OF GLUCOCORTICOID-INDUCED OSTEOPOROSIS MODELS USING THE CAUDAL FIN

Zebrafish caudal fin is a nonmuscularized organ covered by the epidermis with strong regenerating capability and is supported by 16–18 principal bony rays. The fin ray is composed of bilateral parenthetical bones and is regularly divided into multiple bone segments [57]. A single layer of osteoblasts lines the inner and outer surfaces of the rays to secrete bone matrix [58]. In uninjured fins, osteoclasts may not be present in zebrafish caudal fins as the osteoclast-specific Trap activity is undetectable. However, Trap activity can first be detected 24 h after fin amputation, suggesting the recruitment of osteoclasts for the regeneration of fin rays [59].

The impact of glucocorticoids on caudal fin regeneration could be accessed by exposing the caudal-fin amputated adult zebrafish to 50 μ M prednisolone for 4–6 weeks [Figure 1D] [60,61]. Significant reductions in osteoblast proliferation and maturation and bone matrix mineralization were detected in prednisolone-treated zebrafish compared to control zebrafish after the caudal fin amputation, but osteoblast apoptosis was not observed. Prednisolone treatment reduces the number of osteoclasts and affects the migration of osteoclasts from the neighboring stump tissue to the wound site, thereby impairing bone resorption and then delaying bone regeneration [60]. Such an inhibitory effect of prednisolone against the osteoclast cells could be due to the high dosage of prednisolone treatment or the lack of the initial phase of enhancing osteoclastogenesis as reported in mammals. Continued prednisolone exposure could impair the expression of genes encoding extracellular matrix components and disrupt the machinery of macromolecule and vesicular transport [62]. A related study indicates that alendronate can restore the negative impact of glucocorticoids on caudal fin regeneration [63].

While GIOP increases the incidence of fractures [64], a bone crush model can also be established on the caudal fin rays to model fracture healing in patients with GIOP. The fracture can be easily generated on the hemiray segment using an injection needle or a pair of forceps [Figure 1E] [65]. Then, fractured rays undergo the healing process, including inflammation, chondrogenesis, ossification, and remodeling [66]. In the crush region, the elevated expression of *spp1/opn* could be recognized as an indicator for bone repairing [65]. It has also been found that excessive alendronate treatment impedes the process of bone healing and the removal of bone debris on the crush site, indicating the necessity of maintaining osteoclast activity for bone repair [67]. The fractured GIOP model could be readily established by treating the zebrafish with fractured fin-rays with 50 μ M prednisolone for weeks [61]. On this basis, potential pro-healing compounds for GIOP-related fracture could be screened, and their mechanism of action could be analyzed.

CALVARIAE OF THE SKULL COULD BE USED FOR MODELING GLUCOCORTICOID-INDUCED OSTEOPOROSIS

The zebrafish calvariae, comprising the roof part of the skull, is suitable for studying bone healing under the condition of GIOP. The trepanation procedure could be used to create a wound by drilling a small hole in the os frontale [Figure 1F]. After the injury, the adult zebrafish are exposed to 50 μ M prednisolone for 14 days. In the prednisolone-treated zebrafish, osteoblast number in the injured site is reduced, and the bone healing is delayed compared to the untreated control [60]. It is noteworthy that osteoblasts in the wound site undergo a distinct dedifferentiation process and become an essential source for bone healing [68]. This skull injury model could be helpful in studying the osteoblast dedifferentiation in zebrafish in the presence of glucocorticoids which can help in better understanding of bone healing mechanism under GIOP conditions.

IMAGING TECHNIQUES FOR EXAMINING BONE PHYSIOLOGY IN ZEBRAFISH

Techniques used to quantify bone mineralization have been extensively reviewed in zebrafish [69,70]. Here, we summarize the tools applicable in zebrafish GIOP models. Whole-mount staining techniques are generally implemented to reveal the effect of glucocorticoids on bone mineralization and morphological changes in the larval or adult stages. Both alizarin red and calcein could be used to stain mineralized tissues in the head, axial skeleton, and fins in larval and adult stages. Alizarin red is mainly used as a chromogenic agent, whereas calcein stain is a fluorescent dye [8]. Both of which could be applied for live stain and observed by fluorescent microscopy [71,72]. Cartilage could be stained by alcian blue, a polyvalent basic dye that could bind to the proteoglycan of cartilage at low pH [73]. Alcian blue staining is mainly applied in larval stages to assist the study of bone biology [74]. The dual stain of alizarin red and alcian blue could be performed to examine the overall morphology of both the hard bone and cartilaginous bones, respectively [75]. In adult zebrafish, making the sample transparent before performing cartilage and mineralization staining is critical. In addition to the whole-mount staining, transgenic fishes expressing fluorescent protein reporters of bone-related genes could be immensely helpful for speeding up the examination of bone cell differentiation by directly assessing under fluorescence microscopy [76]. For instance, transgenic zebrafish carrying a GFP reporter under the control of the *sp7* promoter could be used to monitor the number of osteoblasts *in vivo* [60,77,78].

Clinically, dual-energy X-ray absorptiometry is used to detect the bone mineral density of GIOP patients. Similarly, the X-ray skeletal images could be adopted as a primary tool to assess the deformity of fish bones [79]. For imaging the skeleton of zebrafish, a Faxitron MX-20 cabinet X-ray machine could be used [80]. Unfortunately, this technique is not suitable for larvae or juvenile zebrafish because of the resolution limitation [81]. To obtain much clearer and quantifiable images of zebrafish skeletons, micro-computed tomography (microCT) is a better option compared to X-ray photography. The 3-dimensional microCT imaging allows one to quantify the mineral density and bone morphology [82]. Besides, microCT imaging is demonstrated to be sensitive enough to quantify skeletal defects in the zebrafish model of skeletal dysplasia [83]. However, it is still difficult to detect the hypo mineralized bone of juvenile zebrafish under 30 dpf. For juvenile zebrafish older than 30 dpf, the bone structure could be seen by enhancing the detection sensitivity of microCT using a selective contrast agent, silver nitrate [83]. By contrast, transmission electron microscopy could also be applied to investigate the ultrastructure of bone matrix or cells at all stages [84]. Lastly, nano-indentation is used to assess the local mechanical properties of zebrafish vertebrae, including elastic modulus and hardness [85]. However, this technique is only suitable for extracted bone samples or biopsies.

CONCLUSIONS

To enhance the development of therapeutic drugs, it is essential to establish a fast, convenient, and reliable *in vivo*

model. Current mammalian models used in GIOP research include mice, rats, rabbits, beagles, and ewes. One of the drawbacks of these mammalian models is that they are time-consuming to be established. For example, it takes 3 months to establish GIOP in rats through continuous oral administration [86] or subcutaneous injection of glucocorticoids [87]. As a result, it might take a long period of time to confirm the therapeutic effect of the candidate compounds. According to the genetic, physical, and physiological similarities between zebrafish and mammals in bone biology, zebrafish are suitable for modeling GIOP. The zebrafish models allow us to perform fast and efficient GIOP-related research with a relatively low experimental cost. The research result from zebrafish could be translated and applied to mammals well. For example, anti-GIOP compounds identified through zebrafish models [42,77], such as tanshinol and salvianolic acid B, are also effective in mammalian models [88,89]. Furthermore, using the zebrafish model for a preliminary drug screening could reduce the usage of mammalian models and comply with the three Rs – reduction, replacement, and refinement – ethical guidelines.

Financial support and sponsorship

This study was supported by the grants from Tzu Chi University (610400239-13) to M.D.L.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

- Peng CH, Lin WY, Yeh KT, Chen IH, Wu WT, Lin MD. The molecular etiology and treatment of glucocorticoid-induced osteoporosis. *Tzu Chi Med J* 2021;33:212-23.
- Xu F, Li W, Yang X, Na L, Chen L, Liu G. The roles of epigenetics regulation in bone metabolism and osteoporosis. *Front Cell Dev Biol* 2020;8:619301.
- Wang FS, Chen YS, Ko JY, Kuo CW, Ke HJ, Hsieh CK, et al. Bromodomain protein BRD4 accelerates glucocorticoid dysregulation of bone mass and marrow adiposis by modulating H3K9 and foxp1. *Cells* 2020;9:1500.
- Hsu CN, Jen CY, Chen YH, Peng SY, Wu SC, Yao CL. Glucocorticoid transiently upregulates mitochondrial biogenesis in the osteoblast. *Chin J Physiol* 2020;63:286-93.
- Wang FS, Wu RW, Chen YS, Ko JY, Jahr H, Lian WS. Biophysical modulation of the mitochondrial metabolism and rdox in Bbone homeostasis and osteoporosis: How biophysics converts into bioenergetics. *Antioxidants (Basel)* 2021;10:1394.
- Sato AY, Tu X, McAndrews KA, Plotkin LI, Bellido T. Prevention of glucocorticoid induced-apoptosis of osteoblasts and osteocytes by protecting against endoplasmic reticulum (ER) stress *in vitro* and *in vivo* in female mice. *Bone* 2015;73:60-8.
- Yang J, Wu Q, Lv J, Nie H. 4-Phenyl butyric acid prevents glucocorticoid-induced osteoblast apoptosis by attenuating endoplasmic reticulum stress. *J Bone Miner Metab* 2017;35:366-74.
- Du SJ, Frenkel V, Kindschi G, Zohar Y. Visualizing normal and defective bone development in zebrafish embryos using the fluorescent chromophore calcein. *Dev Biol* 2001;238:239-46.
- Truesdell SL, Saunders MM. Bone remodeling platforms: Understanding the need for multicellular lab-on-a-chip systems and predictive agent-based models. *Math Biosci Eng* 2019;17:1233-52.
- Witten PE, Harris MP, Huysseune A, Winkler C. Small teleost fish

- provide new insights into human skeletal diseases. *Methods Cell Biol* 2017;138:321-46.
11. Felber K, Elks PM, Lecca M, Roehl HH. Expression of osterix is regulated by FGF and Wnt/ β -catenin signalling during osteoblast differentiation. *PLoS One* 2015;10:e0144982.
 12. Curtin E, Hickey G, Kamel G, Davidson AJ, Liao EC. Zebrafish *wnt9a* is expressed in pharyngeal ectoderm and is required for palate and lower jaw development. *Mech Dev* 2011;128:104-15.
 13. McGowan LM, Kague E, Vorster A, Newham E, Cross S, Hammond CL. Wnt16 elicits a protective effect against fractures and supports bone repair in zebrafish. *JBMR Plus* 2021;5:e10461.
 14. Windhausen T, Squifflet S, Renn J, Muller M. BMP signaling regulates bone morphogenesis in zebrafish through promoting osteoblast function as assessed by their nitric oxide production. *Molecules* 2015;20:7586-601.
 15. Stewart S, Gomez AW, Armstrong BE, Henner A, Stankunas K. Sequential and opposing activities of Wnt and BMP coordinate zebrafish bone regeneration. *Cell Rep* 2014;6:482-98.
 16. Smith A, Avaron F, Guay D, Padhi BK, Akimenko MA. Inhibition of BMP signaling during zebrafish fin regeneration disrupts fin growth and scleroblasts differentiation and function. *Dev Biol* 2006;299:438-54.
 17. Hu Z, Chen B, Zhao Q. Hedgehog signaling regulates osteoblast differentiation in zebrafish larvae through modulation of autophagy. *Biol Open* 2019;8:bio040840.
 18. Paul S, Schindler S, Giovannone D, de Millo Terrazzani A, Mariani FV, Crump JG. Ihha induces hybrid cartilage-bone cells during zebrafish jawbone regeneration. *Development* 2016;143:2066-76.
 19. Sun X, Zhang R, Chen H, Du X, Chen S, Huang J, et al. *Fgfr3* mutation disrupts chondrogenesis and bone ossification in zebrafish model mimicking CATSHL syndrome partially via enhanced Wnt/ β -catenin signaling. *Theranostics* 2020;10:7111-30.
 20. Knopf F, Hammond C, Chekuru A, Kurth T, Hans S, Weber CW, et al. Bone regenerates via dedifferentiation of osteoblasts in the zebrafish fin. *Dev Cell* 2011;20:713-24.
 21. Qu X, Liao M, Liu W, Cai Y, Yi Q, Long J, et al. Loss of Wnt16 leads to skeletal deformities and downregulation of bone developmental pathway in zebrafish. *Int J Mol Sci* 2021;22:6673.
 22. Movérare-Skrtic S, Henning P, Liu X, Nagano K, Saito H, Börjesson AE, et al. Osteoblast-derived WNT16 represses osteoclastogenesis and prevents cortical bone fragility fractures. *Nat Med* 2014;20:1279-88.
 23. Flores MV, Tsang VW, Hu W, Kaley-Zylinska M, Postlethwait J, Crosier P, et al. Duplicate zebrafish *runx2* orthologues are expressed in developing skeletal elements. *Gene Expr Patterns* 2004;4:573-81.
 24. Chen Z, Song Z, Yang J, Huang J, Jiang H. *Sp7/osterix* positively regulates *dlx2b* and *bglap* to affect tooth development and bone mineralization in zebrafish larvae. *J Biosci* 2019;44:127.
 25. Gistelinc C, Gioia R, Gagliardi A, Tonelli F, Marchese L, Bianchi L, et al. Zebrafish collagen Type I: Molecular and biochemical characterization of the major structural protein in bone and skin. *Sci Rep* 2016;6:21540.
 26. Ohlebusch B, Borst A, Frankenbach T, Klopocki E, Jakob F, Liedtke D, et al. Investigation of *alpl* expression and *Tnap*-activity in zebrafish implies conserved functions during skeletal and neuronal development. *Sci Rep* 2020;10:13321.
 27. Topczewska JM, Shoela RA, Tomaszewski JP, Mirmira RB, Gosain AK. The Morphogenesis of cranial sutures in zebrafish. *PLoS One* 2016;11:e0165775.
 28. Li L, Zhang J, Akimenko MA. Inhibition of *mmp13a* during zebrafish fin regeneration disrupts fin growth, osteoblasts differentiation, and Laminin organization. *Dev Dyn* 2020;249:187-98.
 29. Kessels MY, Huitema LF, Boeren S, Kranenbarg S, Schulte-Merker S, van Leeuwen JL, et al. Proteomic analysis of the zebrafish skeletal extracellular matrix. *PLoS One* 2014;9:e90568.
 30. Suarez-Bregua P, Saxena A, Bronner ME, Rotllant J. Targeted Pth4-expressing cell ablation impairs skeletal mineralization in zebrafish. *PLoS One* 2017;12:e0186444.
 31. Rotllant J, Liu D, Yan YL, Postlethwait JH, Westerfield M, Du SJ. Sparc (Osteonectin) functions in morphogenesis of the pharyngeal skeleton and inner ear. *Matrix Biol* 2008;27:561-72.
 32. Zhao Y, Wang HL, Li TT, Yang F, Tzeng CM. Baicalin ameliorates dexamethasone-induced osteoporosis by regulation of the RANK/RANKL/OPG signaling pathway. *Drug Des Devel Ther* 2020;14:195-206.
 33. Bergen DJ, Tong Q, Shukla A, Newham E, Zethof J, Lundberg M, et al. Regenerating zebrafish scales express a subset of evolutionary conserved genes involved in human skeletal disease. *BMC Biol* 2022;20:21.
 34. Stoick-Cooper CL, Weidinger G, Riehle KJ, Hubbert C, Major MB, Fausto N, et al. Distinct Wnt signaling pathways have opposing roles in appendage regeneration. *Development* 2007;134:479-89.
 35. McNulty MS, Bedell VM, Greenwood TM, Craig TA, Ekker SC, Kumar R. Expression of sclerostin in the developing zebrafish (*Danio rerio*) brain and skeleton. *Gene Expr Patterns* 2012;12:228-35.
 36. Caetano-Lopes J, Henke K, Urso K, Duryea J, Charles JF, Warman ML, et al. Unique and non-redundant function of *csflr* paralogues in regulation and evolution of post-embryonic development of the zebrafish. *Development* 2020;147:dev181834.
 37. Sharif F, de Bakker MA, Richardson MK. Osteoclast-like cells in early zebrafish embryos. *Cell J* 2014;16:211-24.
 38. Kim HM, He L, Lee S, Park C, Kim DH, Han HJ, et al. Inhibition of osteoclasts differentiation by CDC2-induced NFATc1 phosphorylation. *Bone* 2020;131:115153.
 39. de Vrieze E, Sharif F, Metz JR, Flik G, Richardson MK. Matrix metalloproteinases in osteoclasts of ontogenetic and regenerating zebrafish scales. *Bone* 2011;48:704-12.
 40. Barrett R, Chappell C, Quick M, Fleming A. A rapid, high content, *in vivo* model of glucocorticoid-induced osteoporosis. *Biotechnol J* 2006;1:651-5.
 41. Huo L, Wang L, Yang Z, Li P, Geng D, Xu Y. Prednisolone induces osteoporosis-like phenotypes via focal adhesion signaling pathway in zebrafish larvae. *Biol Open* 2018;7:bio029405.
 42. Luo S, Yang Y, Chen J, Zhong Z, Huang H, Zhang J, et al. Tanshinol stimulates bone formation and attenuates dexamethasone-induced inhibition of osteogenesis in larval zebrafish. *J Orthop Translat* 2016;4:35-45.
 43. Molagoda IM, Kang CH, Lee MH, Choi YH, Lee CM, Lee S, et al. Fisetin promotes osteoblast differentiation and osteogenesis through GSK-3 β phosphorylation at Ser9 and consequent β -catenin activation, inhibiting osteoporosis. *Biochem Pharmacol* 2021;192:114676.
 44. He H, Wang C, Tang Q, Yang F, Xu Y. Possible mechanisms of prednisolone-induced osteoporosis in zebrafish larva. *Biomed Pharmacother* 2018;101:981-7.
 45. Jiang Y, Xin N, Yang J, Wu W, Wang M, Feng N, et al. Prednisolone suppresses collagen-encoding gene expression causing cartilage defects in zebrafish larvae. *Environ Toxicol Pharmacol* 2021;87:103719.
 46. Pemmari A, Leppänen T, Hämäläinen M, Moilanen T, Vuolteenaho K, Moilanen E. Widespread regulation of gene expression by glucocorticoids in chondrocytes from patients with osteoarthritis as determined by RNA-Seq. *Arthritis Res Ther* 2020;22:271.
 47. Sire JY, Akimenko MA. Scale development in fish: A review, with description of sonic hedgehog (*shh*) expression in the zebrafish (*Danio rerio*). *Int J Dev Biol* 2004;48:233-47.
 48. Pasqualetti S, Banfi G, Mariotti M. Osteoblast and osteoclast behavior in zebrafish cultured scales. *Cell Tissue Res* 2012;350:69-75.
 49. Suzuki N, Hayakawa K, Kameda T, Triba A, Tang N, Tabata MJ, et al. Monohydroxylated polycyclic aromatic hydrocarbons inhibit both osteoclastic and osteoblastic activities in teleost scales. *Life Sci* 2009;84:482-8.
 50. de Vrieze E, van Kessel MA, Peters HM, Spanings FA, Flik G, Metz JR. Prednisolone induces osteoporosis-like phenotype in regenerating

- zebrafish scales. *Osteoporos Int* 2014;25:567-78.
51. Pasqualetti S, Congiu T, Banfi G, Mariotti M. Alendronate rescued osteoporotic phenotype in a model of glucocorticoid-induced osteoporosis in adult zebrafish scale. *Int J Exp Pathol* 2015;96:11-20.
 52. de Vrieze E, Zethof J, Schulte-Merker S, Flik G, Metz JR. Identification of novel osteogenic compounds by an *ex-vivo* sp7: luciferase zebrafish scale assay. *Bone* 2015;74:106-13.
 53. Carnovali M, Ramoni G, Banfi G, Mariotti M. Herbal preparation (bromelain, papain, curcuma, black pepper) enhances mineralization and reduces glucocorticoid-induced osteoporosis in zebrafish. *Antioxidants (Basel)* 2021;10:1987.
 54. Carnovali M, Banfi G, Mariotti M. Liquiritigenin reduces osteoclast activity in zebrafish model of glucocorticoid-induced osteoporosis. *J Pharmacol Sci* 2020;143:300-6.
 55. Saito Y, Nakamura S, Chinen N, Shimazawa M, Hara H. Effects of anti-osteoporosis drugs against dexamethasone-induced osteoporosis-like phenotype using a zebrafish scale-regeneration model. *J Pharmacol Sci* 2020;143:117-21.
 56. Chaichit S, Sato T, Yu H, Tanaka YK, Ogra Y, Mizoguchi T, et al. Evaluation of dexamethasone-induced osteoporosis *in vivo* using zebrafish scales. *Pharmaceuticals (Basel)* 2021;14:536.
 57. König D, Dagenais P, Senk A, Djonov V, Aegerter CM, Jaźwińska A. Distribution and restoration of serotonin-immunoreactive paraneuronal cells during caudal fin regeneration in zebrafish. *Front Mol Neurosci* 2019;12:227.
 58. Johnson SL, Bennett P. Growth control in the ontogenetic and regenerating zebrafish fin. *Methods Cell Biol* 1999;59:301-11.
 59. Blum N, Begemann G. Osteoblast de- and redifferentiation are controlled by a dynamic response to retinoic acid during zebrafish fin regeneration. *Development* 2015;142:2894-903.
 60. Geurtzen K, Vernet A, Freidin A, Rauner M, Hofbauer LC, Schneider JE, et al. Immune suppressive and bone inhibitory effects of prednisolone in growing and regenerating zebrafish tissues. *J Bone Miner Res* 2017;32:2476-88.
 61. Geurtzen K, Knopf F. Adult zebrafish injury models to study the effects of prednisolone in regenerating bone tissue. *J Vis Exp* 2018;140:58429.
 62. Schmidt JR, Geurtzen K, von Bergen M, Schubert K, Knopf F. Glucocorticoid treatment leads to aberrant ion and macromolecular transport in regenerating zebrafish fins. *Front Endocrinol (Lausanne)* 2019;10:674.
 63. Bohns FR, Shih YR, Chuang YJ, Akhtar R, Chen PY. Influence of prednisolone and alendronate on the *de novo* mineralization of zebrafish caudal fin. *JBMR Plus* 2021;5:e10435.
 64. Chotiyarnwong P, McCloskey EV. Pathogenesis of glucocorticoid-induced osteoporosis and options for treatment. *Nat Rev Endocrinol* 2020;16:437-47.
 65. Sousa S, Valerio F, Jacinto A. A new zebrafish bone crush injury model. *Biol Open* 2012;1:915-21.
 66. Shimizu T, Fujita N, Tsuji-Tamura K, Kitagawa Y, Fujisawa T, Tamura M, et al. Osteocytes as main responders to low-intensity pulsed ultrasound treatment during fracture healing. *Sci Rep* 2021;11:10298.
 67. Tomecka MJ, Ethiraj LP, Sánchez LM, Roehl HH, Carney TJ. Clinical pathologies of bone fracture modelled in zebrafish. *Dis Model Mech* 2019;12:dmm037630.
 68. Geurtzen K, Knopf F, Wehner D, Huitema LF, Schulte-Merker S, Weidinger G. Mature osteoblasts dedifferentiate in response to traumatic bone injury in the zebrafish fin and skull. *Development* 2014;141:2225-34.
 69. Tonelli F, Bek JW, Besio R, De Clercq A, Leoni L, Salmon P, et al. Zebrafish: A resourceful vertebrate model to investigate skeletal disorders. *Front Endocrinol (Lausanne)* 2020;11:489.
 70. Foessel I, Bassett JH, Bjørnerem Å, Busse B, Calado Â, Chavassieux P, et al. Bone phenotyping approaches in human, mice and zebrafish – Expert overview of the EU cost action GEMSTONE (“Genomics of MusculoSkeletal traits Translational Network”). *Front Endocrinol (Lausanne)* 2021;12:720728.
 71. Bensimon-Brito A, Cardeira J, Dionisio G, Huysseune A, Cancela ML, Witten PE. Revisiting *in vivo* staining with alizarin red S – A valuable approach to analyse zebrafish skeletal mineralization during development and regeneration. *BMC Dev Biol* 2016;16:2.
 72. Pasqualetti S, Banfi G, Mariotti M. The zebrafish scale as model to study the bone mineralization process. *J Mol Histol* 2012;43:589-95.
 73. Kiernan JA. *Histological and Histochemical Methods: Theory and Practice*. 5th ed. Banbury: Scion Publishing; 2015.
 74. Cabbage CC, Mabee PM. Development of the cranium and paired fins in the zebrafish *Danio rerio* (Ostariophysi, Cyprinidae). *J Morphol* 1996;229:121-60.
 75. Hammond CL, Schulte-Merker S. Two populations of endochondral osteoblasts with differential sensitivity to Hedgehog signalling. *Development* 2009;136:3991-4000.
 76. Hammond CL, Moro E. Using transgenic reporters to visualize bone and cartilage signaling during development *in vivo*. *Front Endocrinol (Lausanne)* 2012;3:91.
 77. Luo SY, Chen JF, Zhong ZG, Lv XH, Yang YJ, Zhang JJ, et al. Salvianolic acid B stimulates osteogenesis in dexamethasone-treated zebrafish larvae. *Acta Pharmacol Sin* 2016;37:1370-80.
 78. Huang HX, Lin H, Lan F, Wu YF, Yang ZG, Zhang JJ. Application of bone transgenic zebrafish in anti-osteoporosis chemical screening. *Animal Model Exp Med* 2018;1:53-61.
 79. Witten PE. Towards a classification and an understanding of developmental relationships of vertebral body malformations in Atlantic salmon (*Salmo salar* L.). *Aquaculture* 2009;295:6-14.
 80. Fisher S, Jagadeeswaran P, Halpern ME. Radiographic analysis of zebrafish skeletal defects. *Dev Biol* 2003;264:64-76.
 81. Bruneel B, Witten PE. Power and challenges of using zebrafish as a model for skeletal tissue imaging. *Connect Tissue Res* 2015;56:161-73.
 82. du Plessis A, Broeckhoven C, Guelpa A, le Roux SG. Laboratory x-ray micro-computed tomography: A user guideline for biological samples. *Gigascience* 2017;6:1-11.
 83. Charles JF, Sury M, Tsang K, Urso K, Henke K, Huang Y, et al. Utility of quantitative micro-computed tomographic analysis in zebrafish to define gene function during skeletogenesis. *Bone* 2017;101:162-71.
 84. Tonelli F, Cotti S, Leoni L, Besio R, Gioia R, Marchese L, et al. Crtp and p3h1 knock out zebrafish support defective collagen chaperoning as the cause of their osteogenesis imperfecta phenotype. *Matrix Biol* 2020;90:40-60.
 85. Cotti S, Huysseune A, Koppe W, Rücklin M, Marone F, Wölfel EM, et al. More bone with less minerals? The effects of dietary phosphorus on the post-cranial skeleton in zebrafish. *Int J Mol Sci* 2020;21:E5429.
 86. Lin S, Huang J, Zheng L, Liu Y, Liu G, Li N, et al. Glucocorticoid-induced osteoporosis in growing rats. *Calcif Tissue Int* 2014;95:362-73.
 87. Jiang Y, Gou H, Wang S, Zhu J, Tian S, Yu L. Effect of pulsed electromagnetic field on bone formation and lipid metabolism of glucocorticoid-induced osteoporosis rats through canonical wnt signaling pathway. *Evid Based Complement Alternat Med* 2016;2016:4927035.
 88. Yang YJ, Zhu Z, Wang DT, Zhang XL, Liu YY, Lai WX, et al. Tanshinol alleviates impaired bone formation by inhibiting adipogenesis via KLF15/PPAR γ 2 signaling in GIO rats. *Acta Pharmacol Sin* 2018;39:633-41.
 89. Cui L, Li T, Liu Y, Zhou L, Li P, Xu B, et al. Salvianolic acid B prevents bone loss in prednisone-treated rats through stimulation of osteogenesis and bone marrow angiogenesis. *PLoS One* 2012;7:e34647.