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An Adult Zebrafish Model of Fibrodysplasia Ossificans Progressiva

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Abstract

Fibrodysplasia ossificans progressiva (FOP) is a rare human skeletal disease caused by constitutively activating mutations in the gene *ACVR1*, which encodes a type I BMP/TGF β family member receptor. FOP is characterized by progressive heterotopic ossification (HO) of fibrous tissues, including skeletal muscle, tendons, and ligaments, as well as malformation of the big toes, vertebral fusions, and osteochondromas. Surgical interventions in patients often result in enhanced HO, which can exacerbate rather than improve diagnostic outcomes. As a result of these difficulties, a variety of animal models are needed to study human FOP. Here we describe the methods for creating and characterizing zebrafish conditionally expressing *Acvr1l*^{Q204D}, the first adult zebrafish model for FOP.

Keywords

Fibrodysplasia ossificans progressive; Disease model; Zebrafish; *Acvr1l*

1 Introduction

The TGF β /BMP signaling pathways are involved in many cellular and developmental processes, from early embryonic axis formation, to left-right asymmetry, to organogenesis (Reviewed in [1]). Endochondral bone formation is one such process that is uniquely regulated by the BMP and TGF β signaling pathways [2]. The type I TGF β /BMP receptor family member *ACVR1* is a key player in the BMP signaling that promotes endochondral ossification. Constitutively activating mutations in *ACVR1* cause the rare human skeletal disease, fibrodysplasia ossificans progressiva (FOP) [3, 4]. Patients with FOP develop heterotopic ossification (HO) throughout skeletal muscle, tendons, and ligaments, and also display a number of other bone-related defects, including digit malformations, vertebral fusions, and osteochondromas [4, 5]. Overactive BMP signaling caused by constitutive activation of *ACVR1* leads to the formation of endochondral bone in HO lesions in FOP [6–8].

In the decade since *ACVR1* was named as the causative gene for human FOP, researchers have developed a number of animal models to learn more about FOP disease etiology and progression [6, 8–13]. Each of these models has provided critical insight in the FOP field, including altered ligand affinity and signaling activation resulting from FOP-associated human *ACVR1* mutations [9, 10, 12], the importance of injury and inflammation in promoting HO formation [6, 11], the identification of an abnormal mechanism of activation of *ACVR1*^{R206H} by the ligand activin A [8], and the identification of progenitor cell

populations that contribute to HO formation in FOP patients [13]. While each of these studies has made significant contributions to our understanding of FOP, many questions remain.

The zebrafish model also offers numerous advantages to study FOP (reviewed in [14]). Their small size, rapid and transparent embryonic development, and high fecundity make them ideal for numerous large-scale screening methods, including in vivo drug and genetic modifier screening. In addition, a large number of transgenic reporter lines are readily available to perform cell lineage tracing experiments. Finally, zebrafish exhibit strong conservation of key signaling pathways that drive the development of human tissues and organ systems, an important consideration for accurately modeling FOP etiology and progression.

The functional ortholog of human *ACVR1* in zebrafish is called *acvr1l* or *alk8* [15]. Embryonic overexpression of constitutively active *Acvr1l* results in ventralization of the embryo due to upregulated BMP signaling, resulting in early embryonic lethality [16–18]. Therefore, to study roles for activated *Acvr1l* in adult FOP zebrafish models, we created heat-shock inducible expression constructs for *acvr1l^{Q204D}*, which we used to express *Acvr1l^{Q204D}* after early embryonic patterning was completed. Here we describe the establishment of long-term heat-shock experiments in adult *Tg(hsp70l:acvr1l_Q204D-mCherry)* zebrafish as a method for modeling FOP. We also describe imaging and staining techniques used to analyze the development of FOP-like phenotypes in *Acvr1l^{Q204D}*-expressing zebrafish.

2 Materials

For all methods, use deionized water unless otherwise noted.

2.1 Creating a Zebrafish Model for Human FOP

1. Zebrafish carrying *Tg(Bre:GFP)*, a reporter construct containing five tandem BMP response elements driving GFP expression [19].
2. Heat-shock-inducible *Acvr1l^{Q204D}*-expressing zebrafish: zebrafish carrying *Tg(Bre:GFP)* and *Tg(hsp70l:acvr1l_Q204D-mCherry)* were described in LaBonty et al. 2017 [20]. *Acvr1l^{Q204D}* is expressed in response to heat shock for 1 h at 38 °C.

2.2 Heat-Shock System

1. Pro-Heat IC 50 W (Won Brothers, Fredericksburg, VA, USA): a submersible heater-controller system recalibrated to heat to 38 °C (see Note 1).
2. Four-channel temperature logger with digital display (UX120–006 M, Onset Computer, Bourne, MA, USA).
3. Four air/water/soil temperature sensors with a one-foot long cable (TMC1-HD, Onset Computer, Bourne, MA, USA) attached to the four-channel temperature logger.

4. Power strip with GFCI plug (*see* Note 2).
5. Outlet timer (TN111, Intermatic, Spring Grove, IL, USA).

2.3 Fixation of Whole Zebrafish

1. 250 mg/L tricaine methanesulfonate (Sigma Aldrich) in water.
2. Modified Davidson's fixative (MDF): 22 mL 37% formaldehyde, 11.5 mL glacial acetic acid, 33 mL 95% ethanol, 33.5 mL water.
3. 10% formalin in phosphate-buffered saline (PBS).
4. PBS.

2.4 MicroCT Analyses

1. Fixed zebrafish stored in PBS at 4 °C.
2. Skyscan 1176 high-resolution microCT scanner (Bruker, Allentown, PA, USA).
3. Plastic wrap (important as does not interfere with imaging).
4. Plastic spoon.

2.5 Paraffin Embedding and Sectioning of Fixed Zebrafish

1. CalEx decalcifying solution (Sigma).
2. Graded ethanol series.
3. Graded tert-butyl alcohol series.
4. Xylene.
5. Shaker.
6. Paraplast Plus paraffin.
7. Disposable plastic base molds (*see* Note 3).
8. Embedding cassettes.
9. Microtome.
10. SuperFrost Plus charged glass microscope slides (Fisher Scientific).
11. Water bath.
12. Slide warmer.
13. Blunt scalpel.
14. Forceps.

2.6 Histological Reagents

1. Hematoxylin.

2. Dilute hydrochloric acid: 40 drops of concentrated (~37%) hydrochloric acid in 250 mL of water.
3. Ammonium water: 4 mL of ammonium hydroxide in 250 mL of water.
4. Eosin.
5. 1% Safranin O in water.
6. 0.02% Fast Green in 0.2% acetic acid.
7. Celestine Blue stock solution: Mix 25 g ferric ammonium sulfate and 2.5 g Celestine Blue (C7143, Sigma-Aldrich, St. Louis, MO, USA) in 250 mL water, stirring over gentle heat, until powder is fully dissolved. Cover with aluminum foil, boil for 3 min, cool, and filter. Add water to up to 500 mL, then add 70 mL of glycerol.
8. Celestine Blue working solution: add 50 mL of Celestine Blue stock solution to 450 mL of water. Make fresh every month.
9. Mayer's hematoxylin.
10. Acid alcohol: 0.5% glacial acetic acid in 80% ethanol.
11. Alcian Blue solution: Mix equal parts of filtered 1% Alcian Blue (05500, Sigma-Aldrich, St. Louis, MO, USA) in water and 1% glacial acetic acid in water. Make fresh every month.
12. 1% phosphomolybdic acid in water, filtered. Make fresh for every use.
13. 0.5% Direct Red (CI 28160, 195,251, Sigma-Aldrich, St. Louis, MO, USA) in water, filtered. Make fresh every month.
14. Permount mounting media.
15. Glass cover slips.

3 Methods

3.1 Heat-Shock Induction of *Acvr1*^{Q204D} in Adult Zebrafish

1. Cross adult zebrafish pairs of *Tg(Bre:GFP)* and *Tg(Bre:GFP); Tg(hsp70l:acvr1l_Q204D-mCherry)* lines and raise progeny to 14 days post fertilization (dpf).
2. While raising embryonic zebrafish, set up long-term automated heat-shock systems [20, 21]. Submerge calibrated heaters in system tanks filled with system water and plug into power strip. Connect power strip to outlet timer and set timer to turn on for 1 h a day (see Note 4). Install four-channel temperature logger near tanks with heaters and extend temperature sensor into each individual tank to allow for daily temperature monitoring.
3. When zebrafish reach 14 dpf, carefully transfer 10 *Tg(Bre:GFP)* or *Tg(Bre:GFP) or Tg(hsp70l:acvr1l_Q204D-mCherry)* zebrafish to their respective heat-shock system tanks (see Note 5). Establish a low flow rate into each heat-shock tank of

10–20 mL/min, which is needed to allow the heaters to reach and maintain optimal heat-shock temperature. After 24 h, check the logger to confirm that the temperature in each tank was successfully raised to 38 °C.

4. At desired collection time points, lethally anesthetize zebrafish using 250 mg/L tricaine methanesulfonate in water and then fix. Fixation and processing methods are described in further detail in Subheading 3.3 below.

3.2 MicroCT to Visualize FOP-Like Phenotypes

1. Euthanized, fixed adult zebrafish can be analyzed by microCT prior to processing for paraffin embedding and sectioning. The easiest way to get clear images of fixed zebrafish by microCT is to mount them in the machine as follows: tightly wrap a piece of plastic wrap around the machine's specimen base to create a flat surface for the fish to rest upon. Transfer the fish in PBS to the plastic wrap surface using a plastic spoon (*see* Note 6).
2. Image each fish using the following parameters: 200 µm aluminum filter, 9 µm resolution, 360 views at 0.5° increments, 2 frame averages. We have used these parameters with success on a Skyscan 1176 high-resolution microCT scanner.
3. Reconstruct 3D data using NRecon (Bruker, Allentown, PA, USA) and generate 3D volume renderings using CTVox (Bruker, Allentown, PA, USA). Keep all reconstruction and rendering settings constant between samples to allow for comparison.

3.3 Paraffin Embedding and Sectioning

1. MDF fixation and processing method [20] (*see* Note 7):
 - (a) Fixation: Immerse animals in MDF for 2 days at roomtemperature, then rinse with PBS 3–4 times and store in PBS at 4 °C.
 - (b) Processing: Immerse samples for 1 h each in graded ethanol (25% and 50% in PBS, 75% in water, 100%), followed by xylene.
2. Fixed and processed samples can be embedded in fresh molten paraffin in disposable plastic base molds, with an embedding cassette placed on top and filled with molten paraffin to serve as a secure adaptor for future sectioning. Allow samples to harden at room temperature.
3. Serially section each paraffin block at 7 µm using a microtome and collect paraffin ribbons in order of cutting. Carefully label rows of paraffin ribbons in numerical order to keep track of position within sample.
4. To mount sections, transfer a series of 2–3 paraffin sections using forceps or a blunt scalpel to the surface of a 48 °C water bath. Wick sections off of the surface of the water using SuperFrost Plus charged glass microscope slides. Let sections dry on slide for at least 10 min, move to a slide warmer at 45 °C for 1 h, and then turn the slide warmer up to 54 °C overnight.

3.4 Histological Analysis of FOP-Like Phenotypes

1. For all staining methods, begin with a standard protocol to deparaffinize and rehydrate the sections: immerse slides in xylene for 5 min twice, then for 2 min each in 100% ethanol, 95% ethanol, 75% ethanol, and 50% ethanol. Immerse in water for at least 2 min.
2. H&E staining: Stain with hematoxylin for 1 min, then rinse gently with tap water for 2.5 min. Dip once in dilute hydrochloric acid and three times in ammonia water. Immerse in water for 2 min, then transfer to eosin for 20 s. Proceed to dehydration and cover slipping (**step 5**) (*see Note 8*).
3. Safranin O staining: Stain with 0.02% Fast Green in 0.2% acetic acid for 15 s, then stain in 1% Safranin O in water for 30 s (*see Note 9*).
4. Hall's and Brunt's Quadruple (HBQ) staining [22]: stain in the following solutions, in order: Celestine Blue working solution for 30 s; Mayer's hematoxylin for 1 min; acid alcohol for 2 min; Alcian Blue solution for 5 min; phosphomolybdic acid for 5 min; and Direct Red for 4 min (*see Note 10*).
5. All stained samples should be dehydrated after staining is complete: 6 dips each in 95% ethanol #1, 95% ethanol #2, 100% ethanol #1, and 100% ethanol #2. Immerse slides in xylene for 5 min twice. Use Permount mounting media to secure a cover slip over top of the sections and dry overnight.

4 Notes

1. To recalibrate the Pro-Heat IC 50 W submersible heaters, unscrew the controller box to open and turn the capacitor between 90 and 180° clockwise. Next, manually calibrate each individual heater to the desired temperature by mounting the heater in a fish tank filled with system water, turning on the heater, and using a thermometer to monitor the point at which the heater reaches 38 °C. Mark this point on the heater using a permanent marker. This procedure is described in further detail with useful images by Duszynski et al. 2011 [21].
2. It is imperative to use power strips with GFCI plugs in aquatic facilities to reduce the risk of electric shock when in contact with water.
3. We recommend 37 × 24 × 10 mm molds for adult zebrafish ranging in size from 15 to 25 mm in length (27147–6, Ted Pella, Inc., Redding, CA, USA).
4. We recommend setting the heat-shock outlet timer to turn on during the regular facility light cycle so that proper heating can be monitored daily. We heat shock from 11 am to 12 pm daily to avoid interfering with our fish feeding schedule. Be sure to make adjustments as needed for annual Daylight Savings time changes.
5. In our preliminary experiments to establish our long-term heat-shock systems, we found that zebrafish that were heat-shocked starting at 14 dpf showed good survival to adulthood. Starting heat shock at 7 dpf resulted in a loss of at least 50% of animals by 14 dpf.

6. When performing microCT, we found that placing the zebrafish in a small pool of PBS helped to avoid any movements that could occur if the animal began to dry out while imaging. PBS introduced only minimal background noise during microCT imaging that was easily removed during post-processing.
7. We have used two different methods for fixation and processing of adult zebrafish and have identified certain pros and cons with each of these methods. When using the MDF method, we found that zebrafish sections stained well with common histological stains including Safranin O and HBQ, but that some tissue integrity could be lost due to chatter encountered during paraffin sectioning. When using the Webb lab method, we maintained good tissue integrity while sectioning, as needed for performing immunohistochemistry (IHC) or immunofluorescence (IF), but noticed that some histological stains did not stain as well as compared to the MDF method. Given these findings, we recommend testing each method on your samples first to determine the ideal conditions for you based on your desired outcomes. We have provided the Webb lab method here.

Webb lab method [23]:

- (a) Fixation: Immerse animals in 10% formalin in PBS for 2 days at room temperature, then rinse with PBS 3–4 times, and store in PBS at 4 °C.
- (b) Processing: Decalcify samples in CalEx and then rinse in PBS for 2 h. Immerse samples for 1 h in 50% ethanol, overnight in 75% ethanol, and then in a graded ethanol/ water/t-butyl alcohol series for 1 h each: (1) 20:20:10, (2) 25:7.5:17.5, (3) 22.5:0:27.5, (4) 12.5:0:37.5, and (5) 100% t-butyl alcohol twice. Transfer samples to molten Paraplast Plus paraffin overnight. t-Butyl alcohol freezes at 20 °C but also has a flash point of 38 °C. We recommend setting a water bath to 25 °C and storing the t-butyl alcohol in it throughout the processing steps to keep the alcohol in liquid form.
8. Hematoxylin provides a dark blue-purple nuclear stain, while eosin provides a bright pink cytoplasmic stain.
9. Safranin O stains cartilaginous proteoglycans a dark red color, while Fast Green provides a light green-blue cytoplasmic counter stain.
10. Celestine Blue and hematoxylin both create a dark blue nuclear stain, Alcian Blue stains acidic mucopolysaccharides found in mucus and cartilage a bright blue, and Direct Red stains the collagen in the bone and basement membranes a bright pink-red.

All experimental procedures on zebrafish were approved by the Tufts University Institutional Animal Care and Use Committee (IACUC) and Ethics Committee.

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