Injury of Adult Zebrafish Expressing *Acvr1l*^{Q204D} Does Not Result in Heterotopic Ossification

Melissa LaBonty,^{1,2} Nicholas Pray,² and Pamela C. Yelick^{1,2}

Abstract

Fibrodysplasia Ossificans Progressiva (FOP) is a rare, autosomal dominant genetic disorder in humans characterized by the gradual ossification of fibrous tissues, including skeletal muscle, tendons, and ligaments. In humans, mutations in the Type I BMP/TGF β family member receptor gene, *ACVR1*, are associated with FOP. Zebrafish *acvr1l*, previously known as *alk8*, is the functional ortholog of human *ACVR1*. We previously created and characterized the first adult zebrafish model for FOP by generating animals harboring heat shock-inducible mCherry-tagged constitutively active *Acvr1l* (Q204D). Since injury is a known trigger for heterotopic ossification (HO) development in human FOP patients, in this study, we investigated several injury models in *Acvr1l*^{Q204D}-expressing zebrafish and the subsequent formation of HO. We performed studies of Activin A injection, cardiotoxin (CTX) injection, and caudal fin clip injury. We found that none of these methods resulted in HO formation at the site of injury. However, some of the cardiotoxin-injected and caudal fin-clipped animals did exhibit HO at distant sites, including the body cavity and along the spine. We describe these results in the context of new and exciting reports on FOP, and discuss future studies to better understand the etiology and progression of this disease.

Keywords: fibrodysplasia ossificans progressiva, heterotopic ossification, activating *ACVR1* mutations

Introduction

FIBRODYSPLASIA OSSIFICANS PROGRESSIVA (FOP) is a rare human disease, affecting only 1 in 2 million individuals, which is characterized by big toe malformations evident at birth, and the progressive development of heterotopic ossification (HO) within skeletal muscle, tendons, and ligaments.^{1–5} The formation of HO, and/or FOP lesions, is typically observed at about 5–7 years of age. As the disease progresses, patients experience a progressive loss of mobility due to the ossification of the spine, joints, and limbs. The cumulative formation of HO in FOP patients results in morbidity by ~40 years of age.⁴ FOP is the result of spontaneous germline mutations that result in causative amino acid substitutions in the Type I TGF β /BMP receptor family member, *ACVR1*.⁶

In general, the progression of an FOP lesion is currently thought to follow a two-phase process.⁷ In the first phase, inflammation results from a triggering event, which can include a physical injury, a surgical procedure, or a systemic

infection.^{4,8,9} Inflammatory cells overwhelm the lesion site and promote muscle tissue degradation, angiogenesis, and fibroproliferation.^{10–12} In the second phase, a recruited population of fibro/adipogenic progenitor cells (FAPs) then undergoes the process of normal endochondral ossification to generate HO at the site of the FOP lesion.^{7,13–15}

Given the intriguing finding that immunosuppression was able to mitigate the development of HO in a human FOP patient,¹⁶ it is possible that the immune system may play a greater role in driving early stage FOP lesion and HO formation than currently understood. Thus, a better understanding of the inflammatory response in FOP patients is needed and may help in developing therapeutic strategies to reduce and/or abolish FOP lesion formation. To address this and other questions in the FOP field, researchers have developed a number of FOP animal injury models that induce HO in a consistent and site-directed manner.^{13–15,17–24} These valuable models have provided significant insight into the mechanism of FOP-induced HO, including the important finding that *ACVR1*^{R206H} is activated by activin A rather than

¹Program in Cell, Molecular, and Developmental Biology, Sackler School of Graduate Biomedical Sciences, Tufts University School of Medicine, Boston, Massachusetts.

²Division of Craniofacial and Molecular Genetics, Department of Orthodontics, Tufts University School of Dental Medicine, Boston, Massachusetts.

inhibited by it, as in the wild-type ACVR1,^{20,22} that ACVR1^{R206H} expressing FAPs are cells contributing to HO in FOP patients,^{13–15} and that enhanced mTOR signaling promotes endochondral ossification in FOP.²¹ These models have also been invaluable in validating possible new therapeutic strategies to treat FOP.^{17,18,21} In light of these advances, we felt that the generation of an adult zebrafish model of FOP, with all of the advantages that the zebrafish system provides (reviewed in Ref.²⁵), would be an excellent tool in which to further probe open questions in the field.

In our prior work, we developed the first adult zebrafish model for FOP.²⁶ We used Tol2 transgenesis to generate transgenic zebrafish carrying a heat shock-inducible, mCherry-tagged, constitutively active form of *acvr1l*, the functional zebrafish ortholog of human *ACVR1*. We exposed these Acvr1l^{Q204D}-expressing animals to several months of daily heat shock to investigate whether constitutively active Acvr1l would promote the formation of FOP-like phenotypes, including HO. We found that adult zebrafish ubiquitously express Acvr1l^{Q204D} when exposed to long-term daily heat shock and develop FOP-like characteristics, including fully penetrant abnormal spinal curvature, and partially penetrant vertebral fusions, pelvic fin malformations, and HO.

In this study, we expand upon our work with the *Tg(acvr1l_Q204D)* zebrafish using a series of injury models to investigate whether Acvr1l^{Q204D}-expressing zebrafish develop reliable site-directed HO lesions. We found that injury of Acvr1l^{Q204D}-expressing animals by any one of three methods, (1) injection of recombinant human (rh) activin A, (2) injection of cardiotoxin (CTX), or (3) clipping of the caudal fin, resulted in early tissue damage that resolved normally and did not result in HO at the site of injury. Interestingly, several Acvr1l^{Q204D}-expressing animals, injured either by CTX injection or caudal fin clip, did develop HO lesions at sites distal to the injury site, including the body cavity or along the spine. In this study, we describe the development and characterization of each of these injury models and discuss our future plans to create more robust zebrafish models for human FOP.

Materials and Methods

Zebrafish husbandry

Zebrafish (*Danio rerio*) were raised in the Tufts Yelick Lab Zebrafish Facility at 28.5°C in a controlled, automated recirculating environment, with 14/10 h light/dark cycle, as previously described.²⁷ Zebrafish of both sexes were used in this study, at ages indicated in the article. We used the following mutant and transgenic strains: *Tg(Bre:GFP)*²⁸ and *Tg(Bre:GFP); Tg(hsp70l:acvr1l_Q204D-mCherry)*.²⁶ The following shorthand names are used in this article: *Tg(hsp70l:acvr1l_Q204D-mCherry)* is referred to as *Tg(acvr1l_Q204D)*, protein product as Acvr1l^{Q204D}.

Ethics statement

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

Heat shock procedures

Adult *Tg(Bre:GFP)* and *Tg(Bre:GFP); Tg(acvr1l_Q204D)* zebrafish were heat-shocked once daily for 1 h at 38°C, as previously described.²⁶ Animals were maintained on heat shock systems throughout the duration of each injury experiment. At collection time points, animals were euthanized using 250 mg/L tricaine methanesulfonate (Argent Aquaculture, Redmond, WA) and fixed before microcomputed tomography (Micro-CT) analysis or processing for histological and/or immunohistochemical analyses.

Injury methods

Zebrafish were injured using one of the following three methods: (1) rh activin A injection; (2) CTX injection; or (3) caudal fin clip. For the rh activin A injury experiments, zebrafish were anesthetized with 150 mg/L tricaine methanesulfonate. Blunt forceps were used to gently remove scales at the injury site (on the right side, above the spine, just anterior to the dorsal fin). The rh activin A (0.1 mg/mL in dimethyl sulfoxide [DMSO], 2 µL per injection; 338-AC, R&D Systems, Minneapolis, MN) was then injected at the site of scale removal using a 100 µm diameter microcapillary needle and FemtoJet microinjection station (Eppendorf, Hamburg, Germany). Control animals were injected with DMSO alone. After injection, animals were allowed to recover in fresh system water and then returned to their respective heat-shocked tanks for continued daily heat shock. *Tg(Bre:GFP)* and *Tg(Bre:GFP); Tg(acvr1l_Q204D)* zebrafish were heat-shocked starting at 14 days post-fertilization (dpf) for 8 months before rh activin A injections and harvested at 1 h postinjury (hpi), 2 days postinjury (dpi), and 4 weeks postinjury (wpi). All specimens were prepared and analyzed using histology and immunohistochemistry (IHC).

The CTX injury experiments were carried out essentially as described above for the rh activin A injury experiments. The injury site was descaled and injected with CTX from *naja pallida* (0.3 mg/mL in phosphate-buffered saline [PBS], 1 µL per injection; 217503, Millipore, Burlington, MA) as per prior published reports in zebrafish.²⁹ Control animals were injected with PBS alone. *Tg(Bre:GFP)* and *Tg(Bre:GFP); Tg(acvr1l_Q204D)* zebrafish were heat-shocked starting at 14 dpf for 10 months before CTX injections. CTX-injected zebrafish were collected at 2 dpi, and at 4 and 8 wpi. Collected specimens were analyzed by micro-CT (8 wpi only), histology, and IHC.

For caudal fin clip injury, ~50% of the caudal fin was removed from anesthetized zebrafish using a sterile razor blade. Bright field images before and after fin clip tails were captured using an AxioCam 503 color camera (Zeiss, Oberkochen, Germany) attached to an M2-Bio dissecting microscope (Zeiss) and processed using AxioVision SE64 microscopy software (Zeiss). *Tg(Bre:GFP)* and *Tg(Bre:GFP); Tg(acvr1l_Q204D)* zebrafish were heat-shocked starting at 14 dpf for 7 months before fin clip injury. Fin clip-injured zebrafish were all collected at 2 wpi and analyzed by Micro-CT, histology, and IHC.

Histology and immunohistochemistry

Two different methods of fixation, tissue processing, and paraffin embedding were used for zebrafish injury experiments. For the rh-activin A and CTX injury experiments, zebrafish were processed as previously described by us.²⁵

For the tail clip injury experiments, animals were processed as previously described with the following modifications²⁹: briefly, zebrafish were fixed in 10% formalin in PBS for 48 h at room temperature (RT), and then rinsed and stored in PBS at 4°C. For paraffin embedding, samples were decalcified in CalEx (Fisher Scientific, Pittsburgh, PA) for 24 h and rinsed in PBS for 2 h, before dehydration in EtOH series. Samples were transferred to 65°C molten Paraplast Plus paraffin (McCormick Scientific, St. Louis, MO) overnight, transferred to fresh molten paraffin, and allowed to harden at RT. Paraffin blocks were serially sectioned at 7 μ m and mounted on SuperFrost Plus charged glass microscope slides (Fisher Scientific).

For histological analyses, mounted sections were stained with H&E or with Safranin O and Fast Green counter stain. For Safranin O staining, slides were submerged in 0.02% Fast Green in 0.2% acetic acid for 15 s, followed directly by submersion in 1% Safranin O for 30 s. IHC analyses were performed as previously described²⁶ using the following antibodies: 1:200 goat anti-activin A (AF338; R&D Systems); 1:100 goat anti-Collagen I (AB758; Millipore); 1:200 rabbit anti-Collagen II (ab34712; Abcam, Cambridge, MA); 1:250 rabbit anti-pSmad2 (AB3849-I; Millipore); 1:50 rabbit anti-von Willebrand factor (vWF) (HPA001815; Sigma-Aldrich, St. Louis, MO); 1:500 donkey anti-rabbit and 1:500 donkey anti-goat (Jackson ImmunoResearch Laboratories, West Grove, PA). Stained sections were analyzed and imaged using a Zeiss AxioPhot epifluorescence microscope (Zeiss) and AxioCam 503 color camera (Zeiss) and processed using AxioVision SE64 microscopy software (Zeiss).

Microcomputed tomography

Euthanized zebrafish were imaged using a Skyscan 1176 high-resolution micro-CT Scanner (Bruker, Allentown, PA) as previously described by us.²⁶ Scans were performed at 9 μ m resolution, every 0.5° over 180°. Reconstructions were performed using NRecon (Bruker) and 3D renderings were generating using CTVox (Bruker). All reconstruction and rendering settings were kept constant between samples to allow for comparison.

Methodology and statistics

All animals established on a given heat shock system that were part of injury experiments were included in analyses described below. Animal numbers are indicated as per figure legends. Animals that died of natural causes before the desired collection times were excluded from analyses.

Results

rh activin A injection of heat-shocked

Tg(acvr1l_Q204D) zebrafish does not induce HO at the injury site

To study the progression of HO formation in the context of FOP, researchers have created a variety of FOP mouse injury models.^{13,14,17–22,24,30} Based on these reports, we used similar approaches to create Acvr1l^{Q204D} zebrafish injury models, to induce and study HO formation in these animals.

Activin A was recently identified as a critical ligand for ACVR1^{R206H}-driven HO formation in an FOP mouse model.²⁰ We therefore chose to test whether an injection injury

model that also delivers rh activin A resulted in HO formation in our Acvr1l^{Q204D}-expressing zebrafish. As there are no existing protocols for injecting into adult zebrafish, we developed the following approach.

Briefly, adult zebrafish were anesthetized and scales were gently removed from the injection site (Fig. 1A). We then injected 2 μ L of either 0.1 mg/mL rh activin A or DMSO vehicle control into the muscle tissue using a microcapillary needle (Fig. 1B). Successful delivery of rh activin A was confirmed by IHC analyses of injected animals at 1 hpi (Fig. 1C–H), which showed rh activin A protein in the muscle tissue of rh activin A-injected animals (Fig. 1E, H), but not in DMSO-injected animals (Fig. 1D, G), or no primary antibody negative controls (Fig. 1C, F).

After confirming the effective delivery of rh activin A to zebrafish muscle tissue, we proceeded to perform rh activin A injections on adult heat-shocked Tg(Bre:GFP) and Tg(Bre:GFP); Tg(acvr1l_Q204D) zebrafish. Animals were collected at 2 dpi and 4 wpi and each injection site was analyzed using H&E staining and IHC (Fig. 2). The 2 dpi time point was chosen to assess muscle tissue damage at the site of injury, while the 4 wpi time point was intended to assess HO formation, based on the fact that HO typically develops within 3 weeks of injury in FOP mouse models.^{20,31,32} At 2 dpi, all animals displayed clear tissue damage at the injury site in response to either rh activin A or DMSO injection (Fig. 2A–D, damage denoted by dashed lines). Nuclear pSmad2 expression was used to demonstrate activated TGF β signaling in all injected zebrafish (Fig. 2E–H), as expected in response to injury.^{33–35} By 4 wpi, heat-shocked Tg(Bre:GFP) and Tg(Bre:GFP); Tg(acvr1l_Q204D) zebrafish showed normal healing and resolution of the tissue damage (Fig. 2I–L), regardless of the injected compound. These results indicated that injury and delivery of rh activin A did not promote HO formation in Acvr1l^{Q204D}-expressing zebrafish, consistent with recently published reports of the lack of responsiveness of the Q204D variant to activin A.

CTX injection does not result HO formation at the injury site in heat-shocked Tg(acvr1l_Q204D) zebrafish

We next tested whether the CTX injection injury model could induce HO formation in Acvr1l^{Q204D}-expressing zebrafish. CTX injection was previously used to study muscle regeneration in zebrafish²⁹ and in mouse models of FOP.^{13,14,18,19,21,24,30} CTX is a myonecrotic agent that induces severe muscle fiber contraction leading to cell death.³⁶ We performed CTX injections as described for rh activin A injections, in both heat-shocked Tg(Bre:GFP) and Tg(Bre:GFP); Tg(acvr1l_Q204D) zebrafish, using 1 μ L of 0.3 mg/mL CTX or PBS vehicle control. Injected zebrafish were collected at 2 dpi and 4 wpi, and each injection site was analyzed using histological and IHC analyses (Fig. 3A–L). At 2 dpi, all injected zebrafish exhibited tissue damage at the injection site (Fig. 3A–D, damage denoted by dashed lines), similar to that observed in rh activin A injection injuries (Fig. 2). Notably, the extent of tissue damage in CTX-injected Tg(Bre:GFP) and Tg(Bre:GFP); Tg(acvr1l_Q204D) zebrafish (Fig. 3B, D) was larger than that observed in PBS control-injected zebrafish (Fig. 3A, C). pSmad2 expression confirmed activation of TGF β signaling in all injected animals, with no obvious differences in pSmad2 levels between

FIG. 1. rh activin A is detectable in zebrafish muscle tissue at 1 hpi. Before injection injury, adult zebrafish were anesthetized, and scales were gently removed from the right, dorsal side, just anterior to the dorsal fin (A). Zebrafish were injected at the descaled site with the indicated treatment using a microcapillary needle (B). To first test this model, adult *Tg(Bre:GFP)* zebrafish were injected with rh activin A (E: 5×, H: 20×) or DMSO (D: 5×, G: 20×), collected at 1 hpi, and analyzed for rh activin A expression by IHC. Act A was clearly detected at the Act A-injected injury site, and not in control DMSO or primary controls (C: 5×, F: 20×). (F–H) Enlarged views of areas indicated by boxes in (C–E), respectively. $n=2$ zebrafish per treatment group. (C–E) 5× scale bar is 200 μm . (F–H) 20× scale bar is 50 μm . Act A, activin A; DMSO, dimethyl sulfoxide; hpi, hours postinjury; IHC, immunohistochemistry; rh, recombinant human. Color images available online at www.liebertpub.com/zeb

treatment groups (Fig. 3E–H). By 4 wpi, CTX injection-induced tissue damage appeared to be resolved, as normal muscle tissue was observed in injured *Tg(Bre:GFP)* and *Tg(Bre:GFP); Tg(acvr1l_Q204D)* zebrafish (Fig. 3I, J). Safranin O staining revealed no indication of cartilaginous proteoglycans, a hallmark of FOP-associated HO lesions, at the injury sites of zebrafish of either genotype (Fig. 3K, L). These results suggested that *Acvr1l^{Q204D}*-expressing zebrafish did not form HO at the site of CTX injury.

CTX-injured *Acvr1l^{Q204D}*-expressing zebrafish exhibited body cavity HO

A cohort of CTX-injured *Tg(Bre:GFP)* and *Tg(Bre:GFP); Tg(acvr1l_Q204D)* zebrafish was collected at 8 wpi and analyzed by Micro-CT to detect skeletal defects and HO (Fig. 4A–D). Of the three *Acvr1l^{Q204D}*-expressing zebrafish analyzed at 8 wpi, one displayed densely mineralized HO lesions within the body cavity (arrows in Fig. 4C, D), similar to the HO lesions observed in uninjured *Tg(Bre:GFP); Tg(acvr1l_Q204D)* zebrafish.²⁶ None of the heat-shocked *Tg(Bre:GFP)* zebrafish examined exhibited any detectable HO (Fig. 4A, B). Histological analyses of the mineralized masses in this *Acvr1l^{Q204D}*-expressing animal revealed a heterogeneous HO lesion (Fig. 4E, F), and Safranin O staining was used to confirm large areas of cartilage matrix deposition within the lesion (Fig. 4G, H).

Caudal fin clip injury did not induce HO formation at the injury site of *Acvr1l^{Q204D}*-expressing zebrafish

For our third and final injury model, we turned to the well-established zebrafish injury method, the caudal fin clip.²⁷ First, anesthetized *Tg(Bre:GFP)* and *Tg(Bre:GFP); Tg(acvr1l_Q204D)* zebrafish were imaged using bright field microscopy before caudal fin clip (Fig. 5A, B). Next, a sterile razorblade was used to remove ~50% of the caudal fin of each animal (Fig. 5C, D). All zebrafish were subsequently monitored for fin regeneration at 1 and 2 wpi (Fig. 5E, F). Gross inspection by bright field imaging showed that all of the tail clipped *Tg(Bre:GFP)* and *Tg(Bre:GFP); Tg(acvr1l_Q204D)* zebrafish exhibited normal caudal fin regeneration by 2 wpi (Fig. 5G, H). Micro-CT analyses of zebrafish at 2 wpi revealed no signs of HO formation at the injury site (Fig. 5I, J).

HO formation was observed along the spine and in the body cavity of caudal fin clip-injured *Acvr1l^{Q204D}*-expressing zebrafish

Careful inspection of micro-CT reconstructions of caudal fin clip-injured, heat-shocked *Tg(Bre:GFP)* and *Tg(Bre:GFP); Tg(acvr1l_Q204D)* zebrafish revealed the presence of numerous HO lesions in two of the three *Acvr1l^{Q204D}*-expressing zebrafish (Fig. 6D–I). One *Acvr1l^{Q204D}*-expressing zebrafish exhibited small HO lesions both within the body cavity and in

FIG. 2. rh activin A injection caused tissue damage apparent at 2 dpi that resolved by 4 wpi. Adult heat-shocked *Tg(Bre:GFP)* and *Tg(Bre:GFP);Tg(HS-acvr1l_Q204D-mCherry)* zebrafish were injected with rh Activin A (B, D, F, H, J, L) or DMSO (A, C, E, G, I, K), collected at 2 dpi (A–H) and 4 wpi (I–L), paraffin sectioned, and analyzed by H&E staining (A–E, I–L) and anti-pSmad2 IHC (E–H). At 2 dpi, tissue damage was apparent, as indicated by a dashed line (A–H). In contrast, no tissue damage was apparent at 4 wpi (I–L). $n = 2$ zebrafish per treatment group, per time point. The $5\times$ scale bar is $200\ \mu\text{m}$ (A–H), and the $20\times$ scale bar is $100\ \mu\text{m}$. (I–L). dpi, days postinjury; wpi, weeks postinjury. Color images available online at www.liebertpub.com/zeb

rod-like HO structures that had formed adjacent to the spine (arrows in Fig. 6D–F). The other *Acvr1l^{Q204D}*-expressing zebrafish exhibited a single large HO lesion within the body cavity (arrows in Fig. 6G–I). None of the *Tg(Bre:GFP)* zebrafish showed any signs of HO formation (Fig. 6A–C).

The *Acvr1l^{Q204D}*-expressing zebrafish that displayed HO lesions, which were identified by micro-CT, were subsequently paraffin sectioned and analyzed by H&E staining (Fig. 7A, B, F, G) and IHC (Fig. 7C–E, H–J). H&E staining revealed that the HO lesions in *Acvr1l^{Q204D}*-expressing

FIG. 3. CTX injection caused tissue damage apparent at 2 dpi that resolved by 4 wpi. Adult heat-shocked *Tg(Bre:GFP)* and *Tg(Bre:GFP);Tg(HS-acvr1l_Q204D-mCherry)* zebrafish injected with CTX (B, D, F, H, I–L) or PBS (A, C, E, G) were collected at 2 dpi (A–H) and 4 wpi (I–L). Paraffin sections of the lesion site were analyzed by H&E staining (A–D, I, J), Safranin O staining (K, L), and anti-pSmad2 IHC (E–H). Tissue damage is indicated by the dashed outline (A–H). The injury lesion and pSmad2 expression were apparent at 2 dpi (A–H), and not at 4 wpi (I–L). $n = 1$ zebrafish per PBS treatment group, $n = 3$ zebrafish per CTX treatment group at 2 dpi. $n = 1$ zebrafish per CTX treatment group at 4 wpi. (A–L) $5\times$ scale bar is $400\ \mu\text{m}$. CTX, cardiotoxin; PBS, phosphate-buffered saline. Color images available online at www.liebertpub.com/zeb

FIG. 4. CTX -injected heat-shocked *Tg(acvr1l_Q204D)* zebrafish exhibited body cavity HO at 8 wpi. Micro-CT imaging of CTX-injected heat-shocked *Tg(Bre:GFP)*; *Tg(acvr1l_Q204D-mCherry)* zebrafish (C, D) at 8 wpi revealed the presence of HO in the body cavity (arrows in C, D). Control CTX-injected heat-shocked *Tg(Bre:GFP)* zebrafish did not exhibit detectable HO (A, B). H&E (E, F) and Safranin O (G, H) staining of HO in heat-shocked *Tg(Bre:GFP)*; *Tg(acvr1l_Q204D-mCherry)* zebrafish revealed heterogeneity of the HO lesion, including numerous proteoglycan-dense regions (G, H, strong red Safranin O stain). (F, H) Enlarged views boxed areas in (E, G), respectively. $n=3$ *Tg(Bre:GFP)*; *Tg(acvr1l_Q204D-mCherry)* zebrafish with CTX treatment. $n=1$ *Tg(Bre:GFP)* zebrafish with CTX treatment. (E, G) $10\times$ scale bar is $200\ \mu\text{m}$. (F, H) $20\times$ scale bar is $100\ \mu\text{m}$. HO, heterotopic ossification; micro-CT, microcomputed tomography. Color images available online at www.liebertpub.com/zeb

FIG. 5. Caudal fin clip did not result in HO formation at injury site in heat-shocked *Tg(acvr1l_Q204D)* zebrafish by 2 wpi. Caudal fin clip-injured adult heat-shocked *Tg(Bre:GFP)* and *Tg(Bre:GFP)*; *Tg(acvr1l_Q204D-mCherry)* zebrafish were imaged with bright field microscopy before fin clip (A, B), immediately after fin clip (C, D), and at 1 wpi (E, F) and 2 wpi (G, H). The original site of the fin clip is marked by dashed line (E–H). Zebrafish were imaged by micro-CT (I, J) at 2 wpi before euthanization. $n=3$ zebrafish per genotype. Color images available online at www.liebertpub.com/zeb

FIG. 6. Caudal fin clip-injured heat-shocked *Tg(acvr1l_Q204D)* zebrafish exhibited spinal and body cavity HO at 2 wpi. Micro-CT image of tail fin clip-injured HS *Tg(Bre:GFP); Tg(acvr1l_Q204D-mCherry)* zebrafish (**D–I**) at 2 wpi revealed the presence of HO in the body cavity and along the spine (**D–I**, arrows). Control fin clip-injured HS *Tg(Bre:GFP)* zebrafish did not exhibit detectable HO (**A–C**). $n = 3$ *Tg(Bre:GFP); Tg(acvr1l_Q204D-mCherry)* zebrafish. $n = 1$ *Tg(Bre:GFP)* zebrafish. HS, heat shock.

animals were quite large (Fig. 7A, F, lesion denoted by dashed lines) and heterogeneous (Fig. 7B, G). IHC analyses of the HO lesions confirmed the presence of several cell types commonly found in HO, including vWF-expressing endothelial cells (Fig. 7C, H), collagen II-expressing cartilage (Fig. 7D, I), and collagen I-expressing osteoblasts (Fig. 7E, J). Interestingly, each marker exhibited different expression

patterns throughout the HO lesion, indicative of various stages of HO progression within the lesion.

Discussion

In this study, we describe the development and characterization of three injury methods used to induce HO in the

FIG. 7. Cartilage and bone formation in body cavity HO in *Acvr1l^{Q204D}*-expressing zebrafish. HO lesions in tail fin clip-injured HS *Tg(Bre:GFP); Tg(acvr1l_Q204D-mCherry)* zebrafish at 2 wpi, paraffin sectioned and H&E stained (**A**, **B**, **F**, **G**), and analyzed by IHC for vWF (**C**, **H**), Col II (**D**, **I**), and Col I (**E**, **J**). The HO lesion is outlined with a dashed line (**A**, **F**). (**B**, **G**) Enlarged views of boxed areas in (**A**, **F**), respectively. (**A**, **F**) $5\times$ scale bar is $40\mu\text{m}$. (**B–E**, **G–J**) $20\times$ scale bar is $100\mu\text{m}$. Col I, collagen I; Col II, collagen II; vWF, von Willebrand factor. Color images available online at www.liebertpub.com/zeb

previously characterized *Tg(acvr1l_Q204D)* zebrafish FOP model.²⁶ Prior characterization of this zebrafish FOP model showed that 1 h/day heat shock of *Tg(hsp70l:acvr1l_Q204D-mCherry)* zebrafish was sufficient to induce ubiquitous expression of Acvr1l Q204D for up to 3 days.

Acvr1l^{Q204D}-expressing zebrafish injured by rh activin A injection, CTX injection, or caudal fin clip showed early signs of tissue damage, but none exhibited HO at the site of injury. Acvr1l^{Q204D}-expressing zebrafish did exhibit HO at sites distal to the injury, either within the body cavity or along the spine. These HO lesions observed in injured Acvr1l^{Q204D}-expressing zebrafish resembled those observed in uninjured Acvr1l^{Q204D}-expressing zebrafish, although they were larger in size. It is important to note that our sample sizes for each experiment were small due to the complexity and duration of each experiment. Nonetheless, these results suggest that while Acvr1l^{Q204D}-expressing zebrafish acquire phenotypes consistent with human FOP, they do not exhibit reliable HO formation at the site of injury. Additional animals can be tested in future experiments to provide further support for these findings.

We first determined that Acvr1l^{Q204D}-expressing zebrafish did not form HO at the injury site in response to rh activin A injection. There are several possible explanations for this finding. It is possible that rh activin A cannot bind to the zebrafish Acvr1l receptor as it does to mouse and human ACVR1. Full-length zebrafish Acvr1l is 69% identical to human ACVR1 at the amino acid level, including an intracellular serine/threonine kinase domain that is 85% identical, strongly suggesting the conservation of receptor function in intracellular signaling cascades.^{25,37} However, the extracellular ligand binding domain of zebrafish Acvr1l is only 30.3% conserved compared to human ACVR1,³⁷ indicating that human Activin A may not bind to zebrafish Acvr1l (the mouse and human extracellular domains are 96% identical). In addition, the zebrafish inhibin β_A subunits that dimerize to form activin A are only 55% identical to the human inhibin β_A subunits. Consistent with this, we did not detect any endogenous zebrafish activin A expression when we performed IHC using the anti-rh activin A antibody (Fig. 1D, G). It is therefore possible that zebrafish Acvr1l is not capable of binding and responding to injected rh activin A. Another possible explanation is that zebrafish Acvr1l^{Q204D} is not responsive to activin A in the same manner as is human ACVR1^{R206H}. Previous work has shown that the ACVR1 p.Q207D mutation is ligand independent.³⁸ Indeed, recent work has suggested that the p.Q207D mutation is unaffected by activin A binding, in that activin A can neither suppress nor stimulate further activity of ACVR1^{Q207D}.³⁹ Therefore, it is also possible that zebrafish Acvr1l^{Q204D} is similarly unresponsive to rh activin A.

We next observed that neither muscular injection of CTX nor caudal fin clip injury of *Tg(acvr1l_Q204D)* zebrafish resulted in HO formation at the site of injury. Despite observing clear tissue damage at early time points in each model, none of these injuries resulted in HO at later time points. It is possible that the efficient resolution of the injury and lack of HO formation may reflect unique properties of the regenerative abilities of zebrafish, which belong to the small group of vertebrate organisms capable of regenerating many adult tissues. Adult zebrafish have well-studied capacities to regenerate fins, heart, retina, and spinal cord (reviewed in Ref.⁴⁰). In contrast, adult mammals, including humans, have

very limited regenerative capacity.⁴¹ However, adult mammals do possess a robust capacity to regenerate muscle tissue. Therefore, we believe that there may be additional aspects of tissue regeneration in Acvr1l^{Q204D}-expressing zebrafish, which may protect them from HO. For example, it has been determined that the RA signaling pathway is upregulated in early stages of zebrafish fin regeneration, where it plays a key role in establishing the blastema.⁴² In models of HO development, RA has been shown to act through RA receptor gamma (RAR γ) to inhibit BMP signaling through Smad1/5/8, so that infiltrating fibroproliferative cells do not undergo chondrogenesis and osteogenesis.^{24,43,44} In fact, the RAR γ agonist palovarotene has been demonstrated to prevent HO formation in FOP mouse models,^{24,45} and has recently shown potential in clinical trials with human FOP patients (www.prnewswire.com/news-releases/phase-2-part-a-open-label-extension-trial-of-palovarotene-for-treatment-of-patients-with-fibrodysplasia-ossificans-progressive-continues-positive-trends-300429975.html). Future studies may investigate whether Acvr1l^{Q204D}-expressing zebrafish avoid injury-induced HO formation, as seen in these studies, by activating RA signaling in response to injury.

Although CTX and caudal fin clip injury of Acvr1l^{Q204D}-expressing zebrafish did not result in HO at the injury sites, both of these injury models were found to exhibit HO in areas other than the sites of injury. Since these animals were harvested and analyzed 2–4 weeks after injury, it is likely that the HO lesions had already formed in these animals before the time of injury. However, it is possible that injury may have contributed to a heightened level of systemic inflammation in these zebrafish, thereby promoting increased HO lesion size compared to uninjured Acvr1l^{Q204D}-expressing zebrafish. Although not examined in the study described here, this possibility is supported by recent work describing an increased inflammatory response directly preceding mineralization and that existing HO begets more HO.³¹ Since systemic inflammation status was not monitored in these experiments, future injury experiments in zebrafish could incorporate the use of MRI to visualize inflammation and edema to more accurately correlate inflammation and HO formation in *Tg(acvr1l_Q204D)* zebrafish and other zebrafish FOP models as they are created.

The locations of the HO that formed in Acvr1l^{Q204D}-expressing zebrafish are both curious and somewhat puzzling. Since the zebrafish body cavity is not expected to experience greater mechanical stimulation from swimming compared with the tail and skeletal tissues, mechanical stimulation is likely not a stimulating factor for these HO lesions. One possible explanation is that the locations where HO forms in Acvr1l^{Q204D}-expressing zebrafish are where FAPs normally reside within the zebrafish. FAPs have been found to be a cell source contributing to HO in mouse FOP models.^{13–15} Future studies will explore the locations, characteristics, and activities of FAPs in zebrafish FOP models. We do not believe that the Safranin O-stained tissues are degenerated oocytes, for the following reasons: the Safranin O staining color in oocytes is a deep purple, while that of the putative HO masses is bright red, and distinct from the cartilaginous tissues in the zebrafish. In addition, we never observed these HO masses in female BRE-GFP heat-shocked or non-heat-shocked control animals. In addition, the IHC staining patterns observed in these putative HO masses were never observed in the ovaries of BRE-GFP control animals.

While the p.Q204D/Q207D variant has been studied in FOP mouse^{24,46–49} and zebrafish²⁶ models, it is not a naturally occurring mutation in human FOP patients. In addition, this mutation results in constitutive activation of ACVR1 in a ligand-independent manner and causes significantly more severe phenotypes than the human FOP-associated mutations. The p.Q207E variant is ligand-dependent, while the p.R206H variant can respond to Activin A with increased signaling, but can also signal independent of BMP ligand or Activin A.^{36,51,52} Therefore, our future work will focus on generating and characterizing zebrafish expressing a p.R203H variant, which is equivalent to the p.R206H variant present in more than 90% of human FOP patients.^{6,50} We anticipate that Acvr1l^{R203H}-expressing zebrafish may more faithfully exhibit features observed in FOP patients, including the formation of HO in response to injury.

In summary, this study demonstrated that although Acvr1l^{Q204D}-expressing zebrafish do form HO resembling that observed in FOP patients, they do not do so at the site of injury. These results are consistent with recently characterized functional differences between the artificial p.Q204D Acvr1l variant and FOP patient-associated ACVR1 variants, and may also reflect a fundamental difference in the biology of wound healing between zebrafish and mammals. Future studies will address these and other questions, to determine whether novel mechanisms of healing in zebrafish could be exploited to benefit human FOP patients.

Acknowledgments

The authors sincerely thank John Lyons, Patricia Hare, Shaina Colón, Shirin Shabahang, Caroline Banevicius, Anabel Azziz, Michael Chen, and Andrade Hendricks for expert technical support and helpful discussions. This work was supported by NIH/NIDCR R01DE018043 (PCY) and R21AR065761 (PCY), and NSF GRFP NS9344 (ML). The funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the article.

Authors' Contributions

Conceived and designed the experiments: M.L. and P.C.Y. Performed the experiments: M.L. and N.P. Analyzed the data: M.L., N.P., and P.C.Y. Contributed reagents/materials/analysis tools: M.L. and P.C.Y. Wrote the article: M.L. and P.C.Y.

Disclosure Statement

No competing financial interests exist.

References

- Kaplan FS, Tabas JA, Gannon FH, Finkel G, Hahn GV, Zasloff MA. The histopathology of fibrodysplasia ossificans progressiva. An endochondral process. *J Bone Joint Surg Am* 1993;75:220–230.
- Kaplan FS, Le Merrer M, Glaser DL, Pignolo RJ, Goldsby RE, Kitterman JA, *et al.* Fibrodysplasia ossificans progressiva. *Best Pract Res Clin Rheumatol* 2008;22:191–205.
- Kartal-Kaess M, Shore EM, Xu M, Schwering L, Uhl M, Korinthenberg R, *et al.* Fibrodysplasia ossificans progressiva (FOP): watch the great toes! *Eur J Pediatr* 2010;169:1417–1421.
- Pignolo RJ, Shore EM, Kaplan FS. Fibrodysplasia ossificans progressiva: clinical and genetic aspects. *Orphanet J Rare Dis* 2011;6:80.
- Kaplan FS, Chakkalakal SA, Shore EM. Fibrodysplasia ossificans progressiva: mechanisms and models of skeletal metamorphosis. *Dis Model Mech* 2012;5:756–762.
- Kaplan FS, Xu M, Seemann P, Connor JM, Glaser DL, Carroll L, *et al.* Classic and atypical fibrodysplasia ossificans progressiva (FOP) phenotypes are caused by mutations in the bone morphogenetic protein (BMP) type I receptor ACVR1. *Hum Mutat* 2009;30:379–390.
- Shore EM, Kaplan FS. Inherited human diseases of heterotopic bone formation. *Nat Rev Rheumatol* 2010;6:518–527.
- Lanchoney TF, Cohen RB, Rocke DM, Zasloff MA, Kaplan FS. Permanent heterotopic ossification at the injection site after diphtheria-tetanus-pertussis immunizations in children who have fibrodysplasia ossificans progressiva. *J Pediatr* 1995;126:762–764.
- Scarlett RF, Rocke DM, Kantanie S, Patel JB, Shore EM, Kaplan FS. Influenza-like viral illnesses and flare-ups of fibrodysplasia ossificans progressiva. *Clin Orthop Relat Res* 2004;423:275–279.
- Gannon FH, Glaser D, Caron R, Thompson LD, Shore EM, Kaplan FS. Mast cell involvement in fibrodysplasia ossificans progressiva. *Hum Pathol* 2001;32:842–848.
- Gannon FH, Valentine BA, Shore EM, Zasloff MA, Kaplan FS. Acute lymphocytic infiltration in an extremely early lesion of fibrodysplasia ossificans progressiva. *Clin Orthop Relat Res* 1998;346:19–25.
- Kaplan FS, Shore EM, Gupta R, Billings PC, Glaser DL, Pignolo RJ, *et al.* Immunological features of fibrodysplasia ossificans progressiva and the dysregulated BMP4 pathway. *Clinic Rev Bone Miner Metab* 2005;3:189–193.
- Agarwal S, Loder SJ, Cholok D, Peterson J, Li J, Breuler C, *et al.* Scleraxis-lineage cells contribute to ectopic bone formation in muscle and tendon. *Stem Cells* 2017;35:705–710.
- Dey D, Bagarova J, Hatsell SJ, Armstrong KA, Huang L, Ermann J, *et al.* Two tissue-resident progenitor lineages drive distinct phenotypes of heterotopic ossification. *Sci Transl Med* 2016;8:366ra163.
- Lees-Shepard JB, Yamamoto M, Biswas AA, Stoessel SJ, Nicholas SE, Cogswell CA, *et al.* Activin-dependent signaling in fibro/adipogenic progenitors causes fibrodysplasia ossificans progressiva. *Nat Commun* 2018;9:471.
- Kaplan FS, Glaser DL, Shore EM, Pignolo RJ, Xu M, Zhang Y, *et al.* Hematopoietic stem-cell contribution to ectopic skeletogenesis. *J Bone Joint Surg Am* 2007;89:347–357.
- Agarwal S, Loder SJ, Breuler C, Li J, Cholok D, Brownley C, *et al.* Strategic targeting of multiple BMP receptors prevents trauma-induced heterotopic ossification. *Mol Ther* 2017;25:1974–1987.
- Brennan TA, Lindborg CM, Bergbauer CR, Wang H, Kaplan FS, Pignolo RJ. Mast cell inhibition as a therapeutic approach in fibrodysplasia ossificans progressiva (FOP). *Bone* 2018;109:259–266.
- Chakkalakal SA, Zhang D, Culbert AL, Convente MR, Caron RJ, Wright AC, *et al.* An Acvr1 R206H knock-in mouse has fibrodysplasia ossificans progressiva. *J Bone Miner Res* 2012;27:1746–1756.
- Hatsell SJ, Idone V, Wolken DM, Huang L, Kim HJ, Wang L, *et al.* ACVR1R206H receptor mutation causes fibrodysplasia ossificans progressiva by imparting responsiveness to activin A. *Sci Transl Med* 2015;7:303ra137.

21. Hino K, Horigome K, Nishio M, Komura S, Nagata S, Zhao C, *et al.* Activin-A enhances mTOR signaling to promote aberrant chondrogenesis in fibrodysplasia ossificans progressiva. *J Clin Invest* 2017;127:3339–3352.
22. Hino K, Ikeya M, Horigome K, Matsumoto Y, Ebise H, Nishio M, *et al.* Neofunction of ACVR1 in fibrodysplasia ossificans progressiva. *Proc Natl Acad Sci U S A* 2015;112:15438–15443.
23. Kan L, Liu Y, McGuire TL, Berger DM, Awatramani RB, Dymecki SM, *et al.* Dysregulation of local stem/progenitor cells as a common cellular mechanism for heterotopic ossification. *Stem Cells* 2009;27:150–156.
24. Shimono K, Tung WE, Macolino C, Chi AH, Didizian JH, Mundy C, *et al.* Potent inhibition of heterotopic ossification by nuclear retinoic acid receptor-gamma agonists. *Nat Med* 2011;17:454–460.
25. LaBonty M, Yelick PC. Animal models of fibrodysplasia ossificans progressiva. *Dev Dyn* 2018;247:279–288.
26. LaBonty M, Pray N, Yelick PC. A zebrafish model of human fibrodysplasia ossificans progressiva. *Zebrafish* 2017;14:293–304.
27. Westerfield M: The Zebrafish Book. University of Oregon Press, Eugene, OR, 1995.
28. Alexander C, Zuniga E, Blitz IL, Wada N, Le Pabic P, Javidan Y, *et al.* Combinatorial roles for BMPs and Endothelin 1 in patterning the dorsal-ventral axis of the craniofacial skeleton. *Development* 2011;138:5135–5146.
29. Seger C, Hargrave M, Wang X, Chai RJ, Elworthy S, Ingham PW. Analysis of Pax7 expressing myogenic cells in zebrafish muscle development, injury, and models of disease. *Dev Dyn* 2011;240:2440–2451.
30. Kan L, Lounev VY, Pignolo RJ, Duan L, Liu Y, Stock SR, *et al.* Substance P signaling mediates BMP-dependent heterotopic ossification. *J Cell Biochem* 2011;112:2759–2772.
31. Upadhyay J, Xie L, Huang L, Das N, Stewart RC, Lyon MC, *et al.* The expansion of heterotopic bone in fibrodysplasia ossificans progressiva is activin A-dependent. *J Bone Miner Res* 2017;32:2489–2499.
32. Wosczyzna MN, Biswas AA, Cogswell CA, Goldhamer DJ. Multipotent progenitors resident in the skeletal muscle interstitium exhibit robust BMP-dependent osteogenic activity and mediate heterotopic ossification. *J Bone Miner Res* 2012;27:1004–1017.
33. Gabbiani G. The myofibroblast in wound healing and fibrocontractive diseases. *J Pathol* 2003;200:500–503.
34. Jazwinska A, Badakov R, Keating MT. Activin-betaA signaling is required for zebrafish fin regeneration. *Curr Biol* 2007;17:1390–1395.
35. Penn JW, Grobbelaar AO, Rolfe KJ. The role of the TGF-beta family in wound healing, burns and scarring: a review. *Int J Burns Trauma* 2012;2:18–28.
36. Lin Shiau SY, Huang MC, Lee CY. Mechanism of action of cobra cardiotoxin in the skeletal muscle. *J Pharmacol Exp Ther* 1976;196:758–770.
37. Yelick PC, Abduljabbar TS, Stashenko P. zALK-8, a novel type I serine/threonine kinase receptor, is expressed throughout early zebrafish development. *Dev Dyn* 1998;211:352–361.
38. Haupt J, Deichsel A, Stange K, Ast C, Boccardi R, Ravazzolo R, *et al.* ACVR1 p.Q207E causes classic fibrodysplasia ossificans progressiva and is functionally distinct from the engineered constitutively active ACVR1 p.Q207D variant. *Hum Mol Genet* 2014;23:5364–5377.
39. Alessi Wolken DM, Idone V, Hatsell SJ, Yu PB, Economides AN. The obligatory role of Activin A in the formation of heterotopic bone in Fibrodysplasia Ossificans Progressiva. *Bone* 2018;109:210–217.
40. Gemberling M, Bailey TJ, Hyde DR, Poss KD. The zebrafish as a model for complex tissue regeneration. *Trends Genet* 2013;29:611–620.
41. Godwin J. The promise of perfect adult tissue repair and regeneration in mammals: learning from regenerative amphibians and fish. *Bioessays* 2014;36:861–871.
42. Blum N, Begemann G. Retinoic acid signaling controls the formation, proliferation and survival of the blastema during adult zebrafish fin regeneration. *Development* 2012;139:107–116.
43. Pacifici M, Cossu G, Molinaro M, Tato F. Vitamin A inhibits chondrogenesis but not myogenesis. *Exp Cell Res* 1980;129:469–474.
44. Weston AD, Hoffman LM, Underhill TM. Revisiting the role of retinoid signaling in skeletal development. *Birth Defects Res C Embryo Today* 2003;69:156–173.
45. Chakkalakal SA, Uchibe K, Convente MR, Zhang D, Economides AN, Kaplan FS, *et al.* Palovarotene inhibits heterotopic ossification and maintains limb mobility and growth in mice with the human ACVR1(R206H) fibrodysplasia ossificans progressiva (FOP) mutation. *J Bone Miner Res* 2016;31:1666–1675.
46. Bagarova J, Vonner AJ, Armstrong KA, Borgermann J, Lai CS, Deng DY, *et al.* Constitutively active ALK2 receptor mutants require type II receptor cooperation. *Mol Cell Biol* 2013;33:2413–2424.
47. Fukuda T, Scott G, Komatsu Y, Araya R, Kawano M, Ray MK, *et al.* Generation of a mouse with conditionally activated signaling through the BMP receptor, ALK2. *Genesis* 2006;44:159–167.
48. Wang H, Lindborg C, Lounev V, Kim JH, McCarrick-Walmsley R, Xu M, *et al.* Cellular hypoxia promotes heterotopic ossification by amplifying BMP signaling. *J Bone Miner Res* 2016;31:1652–1665.
49. Yu PB, Deng DY, Lai CS, Hong CC, Cuny GD, Bouxsein ML, *et al.* BMP type I receptor inhibition reduces heterotopic [corrected] ossification. *Nat Med* 2008;14:1363–1369.
50. Hildebrand L, Stange K, Deichsel A, Gossen M, Seemann P. The fibrodysplasia ossificans progressiva (FOP) mutation p.R206H in ACVR1 confers an altered ligand response. *Cell Signal* 2017;29:23–30.
51. Shen Q, Little SC, Xu M, Haupt J, Ast C, Katagiri T, *et al.* The fibrodysplasia ossificans progressiva R206H ACVR1 mutation activates BMP-independent chondrogenesis and zebrafish embryo ventralization. *J Clin Invest* 2009;119:3462–3472.
52. Mucha BE, Hashiguchi M, Zinski J, Shore EM, Mullins MC. Variant BMP receptor mutations causing fibrodysplasia ossificans progressiva (FOP) in humans show BMP ligand-independent receptor activation in zebrafish. *Bone* 2018;109:225–231.

Address correspondence to:

Pamela C. Yelick, PhD

Division of Craniofacial and Molecular Genetics

Department of Orthodontics

Tufts University School of Dental Medicine

136 Harrison Avenue

Boston, MA 02111

E-mail: pamela.yelick@tufts.edu