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An *Acvr1* R206H Knock-in Mouse Has Fibrodysplasia Ossificans Progressiva

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ABSTRACT

Fibrodysplasia ossificans progressiva (FOP; MIM #135100) is a debilitating genetic disorder of dysregulated cellular differentiation characterized by malformation of the great toes during embryonic skeletal development and by progressive heterotopic endochondral ossification postnatally. Patients with these classic clinical features of FOP have the identical heterozygous single nucleotide substitution (c.617G > A; R206H) in the gene encoding ACVR1/ALK2, a bone morphogenetic protein (BMP) type I receptor. Gene targeting was used to develop an *Acvr1* knock-in model for FOP ($Acvr1^{R206H/+}$). Radiographic analysis of $Acvr1^{R206H/+}$ chimeric mice revealed that this mutation induced malformed first digits in the hind limbs and postnatal extraskeletal bone formation, recapitulating the human disease. Histological analysis of murine lesions showed inflammatory infiltration and apoptosis of skeletal muscle followed by robust formation of heterotopic bone through an endochondral pathway, identical to that seen in patients. Progenitor cells of a $Tie2^{+}$ lineage participated in each stage of endochondral osteogenesis. We further determined that both wild-type (WT) and mutant cells are present within the ectopic bone tissue, an unexpected finding that indicates that although the mutation is necessary to induce the bone formation process, the mutation is not required for progenitor cell contribution to bone and cartilage. This unique knock-in mouse model provides novel insight into the genetic regulation of heterotopic ossification and establishes the first direct in vivo evidence that the R206H mutation in *ACVR1* causes FOP. © 2012 American Society for Bone and Mineral Research.

KEY WORDS: *ACVR1*; ALK2; FIBRODYSPLASIA OSSIFICANS PROGRESSIVA; HETEROTOPIC OSSIFICATION; ENDOCHONDRAL BONE

Introduction

Heterotopic ossification (HO), the formation of ectopic (extraskeletal) bone in soft tissues, is most commonly associated with acute tissue damage such as severe burns, spinal cord and head injuries, and high-impact trauma including war-induced injuries.[1–5] Heterotopic ossification also occurs commonly in patients after total hip replacement surgery and is a complication of aging, occurring in association with atherosclerosis, valvular heart disease, and pressure ulcers. Heterotopic bone is qualitatively normal bone tissue that is induced to form at extraskeletal sites. Little is known about the cellular events or molecular mechanisms that induce and promote heterotopic ossification.

In addition to trauma-induced heterotopic ossification, two rare inherited human diseases of heterotopic ossification, fibrodysplasia ossificans progressiva (FOP) and progressive osseous heteroplasia (POH), have also been described.[6] The genetic mutations that cause these disorders have been identified, providing the opportunity to understand the cellular and molecular regulation of bone formation.

Genetic analyses identified heterozygous mutations in ACVR1 (also known as ALK2), a bone morphogenetic protein (BMP) type I receptor, in all FOP patients.[7,8] FOP is diagnosed clinically on the basis of two defining, or classic, FOP features: congenital malformation of the great toes and progressive heterotopic endochondral ossification that develops in characteristic anatomic patterns.[6]

Spontaneous and episodic flare-ups (episodes of heterotopic ossification) in FOP typically begin during childhood, however, flare-ups can also be activated by soft tissue trauma and injury. The subsequent extensive bone formation causes extra-articular

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ankylosis of the joints of the axial and appendicular skeleton leading to progressive immobility. In addition to postnatal soft tissue ossification, patients with FOP also have developmental skeletal malformations, including characteristic great toe malformations and other common but variable skeletal features such as proximal medial tibial osteochondromas; cervical spine malformations; short, broad femoral necks; costovertebral malformations; scoliosis; and fusion of vertebrae and diarthrodial joints.[8,9]

All patients with classic FOP features share a single nucleotide substitution (c.617G > A) in the glycine-serine (GS) activation domain of the ACVR1/ALK2 receptor that replaces arginine with histidine in codon 206 (R206H). Protein modeling predicts that this amino acid substitution alters receptor signaling activity[10] and functional analyses show that the ACVR1 R206H mutation induces increased BMP signaling that is both ligand independent and BMP responsive.[11–14]

Signaling through bone morphogenetic proteins (BMPs) and their receptors is a key mechanism regulating chondrogenesis and endochondral bone formation.[15–18] BMP receptor signaling is essential for the mesenchymal cell condensations that precede skeletal element formation. BMP signaling also participates in proliferation, differentiation, and maturation of chondrocytes during the development of cartilage and bone. These effects of BMP signal transduction occur through both canonical SMAD signaling and noncanonical MAPK pathways.[16,19,20]

Initial investigations of the cellular events that lead to heterotopic ossification used in vivo BMP protein implants to induce bone formation.[21,22] More recently, transgenic mouse lines that express BMP4 under the control of the neuron-specific enolase promoter or that conditionally overexpress constitutively active *Acvr1/Alk2*^{Q207D} were shown to develop progressive heterotopic ossification within skeletal muscle.[23–26] However, none of these models fully reproduce the phenotype and progression of FOP, and more faithful disease models are required to understand the molecular and cellular mechanisms that direct heterotopic bone formation as well as to serve as in vivo systems to test potential therapies.

In this study, we describe the development and characterization of an *Acvr1* R206H (c.617G > A) knock-in mouse (*Acvr1*$^{R206H/+}$). Although germline transmission of this mutation is perinatal lethal, mice that are chimeric for *Acvr1*$^{R206H/+}$ cells recapitulate every clinical feature of patients with classic FOP including embryonic skeletal malformations and postnatal heterotopic endochondral bone formation. In addition, histological analyses of developing heterotopic ossification show the same progression of cellular events including inflammation-induced catabolism of connective tissues followed by a robust anabolic tissue replacement by cartilage and bone.

Material and Methods

Generation of R206H *Acvr1* knock-in mice

Acvr1 sequences from a mouse C57BL/6 BAC library were inserted into the retroviral vector pL253 by a BAC recombineering strategy. *Acvr1* codon 206 was engineered with the FOP mutation (CGC > CAC) in murine exon 5, and a *neo*^{r} marker gene under the regulation of the Pgk promoter[27] was added (Fig. 1*A*). Correctly targeted homologous recombination was determined by *Bgl*II digestion and Southern analysis; a 14.4 kb fragment indicated the presence of the mutant allele, and a 12.4 kb fragment corresponded to the endogenous allele (Fig. 1*B*, *C*). From 300 G418 resistant ES colonies, we identified 16 positive ES clones showing homologous recombination; positive clones were karyotyped, verified by sequencing, and used for blastocyst injection into CD-1/BALB/c mice. Six different clones were injected to generate chimeras. The proportion of mutant cells in the resulting mutant/wild-type cell chimeric progeny was estimated by coat color; mutant cells from C57BL/6J ES cells generate black coat and wild-type (WT) cells from CD-1/BALB/c are white. Additional details are provided in Supplemental Information.

All animal studies were approved by the Institutional Animal Use and Care Committee of the University of Pennsylvania.

Imaging analyses

Microcomputed tomography (μCT): Paraformaldehyde (PFA)-fixed whole mouse specimens were imaged using an eXplore Locus SP μCT specimen scanner (GE Healthcare, London, ON, Canada) at the Small Animal Imaging Facility of the University of Pennsylvania. Volumetric data were acquired using the following parameters: 80 kVp and 80 μA X-ray tube voltage and current, 250 μm aluminum filter, 1.7 s integration time, 400 views at 0.5° increments, 2 × 2 detector bin mode, four averages, 1 hour scan time. Image data were reconstructed at a resolution of 40.5 μm isotropic voxels using a Feldkamp cone beam algorithm. The reconstructed 3D data were analyzed and volume rendered using OsiriX software (www.osirix-viewer.com).

X-ray imaging: Whole-body radiographic images of PFA-fixed mice were performed with a prototype digital breast imaging system (Selenia Dimensions, Hologic, Bedford, MA, USA) located at the Hospital of the University of Pennsylvania. Both projection radiographic and tomosynthesis images were acquired. Radiographic images were acquired using a ×2.0 geometric magnification at 25 kVp and 90 mAs, with a 0.1 mm nominal focal spot and a W/Rh target/filter combination. Image processing by the manufacturer was restricted to flat-field corrections. The resultant linear projection images have a 35 μm pixel size in the plane of the mice. The images were analyzed using ImageJ software (http://rsbweb.nih.gov/ij/).

Histological analyses

Heterotopic lesions were dissected based on imaging analyses. Fixed tissues (4% PFA) were decalcified using Immunocal (Decal Chemical Corporation, Tallman, NY, USA), embedded in paraffin, and sectioned serially at 7 microns. Control sections were from age-matched WT mice. Sections were stained with Harris Modified hematoxylin and eosin Y solution, safranin O, or Alcian blue, and mast cells were detected by CEM staining (American Master Tech Scientific Inc., Lodi, CA, USA).

Deparaffinized sections were treated for antigen retrieval with 10 mM sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide solution.

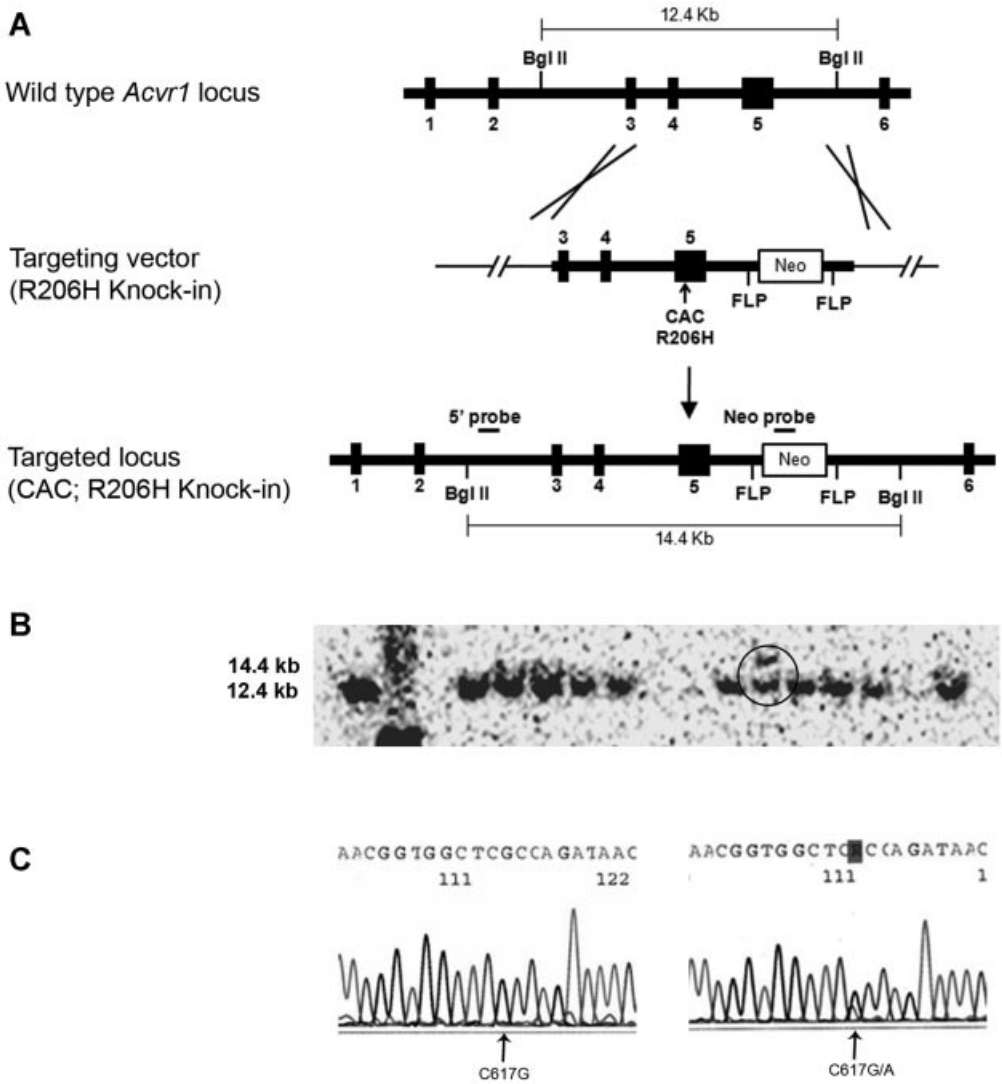

Fig. 1. Generation of *Acvr1*$^{R206H/+}$ knock-in mouse model for FOP. (*A*) Targeting strategy. Schematic representation of the WT *Acvr1* locus, the targeting construct with the R206H substitution in exon 5, and the inserted mutant allele after homologous recombination. Exons (black boxes), *Bgl*II restriction sites, probes for Southern detection, and the neomycin selection cassette flanked by FLP sites are shown. (*B*) Homologous recombination detection by Southern analysis of *Bgl*II-cleaved genomic DNA from ES cell clones. Homologous recombination was indicated by detection of mutant (14.4 kb) and WT (12.4 kb) alleles (example circled). (*C*) DNA sequence analysis of heterozygous *Acvr1* alleles in recombined ES cells (right panel); WT sequence (left panel). Position c.617 is indicated by arrows. (A color version of this figure is available as online Supplemental Information (Supplemental Figure 4).)

Sections were blocked (Background Buster; American Master Tech Scientific Inc.), then incubated with primary antibody overnight at 4°C, followed by incubation with appropriate HRP-linked secondary antibody and development of color using DAB (SuperPicture Polymer Detection Kit, Invitrogen, Carlsbad, CA, USA). Primary antibodies detected: phosphorylated-Smad1/5/8 and phosphorylated-p38-MAPK (Cell Signaling Technology Inc., Danvers, MA, USA); collagen II, collagen X, myeloperoxidase, and TGFβ (Abcam Inc., Cambridge, MA, USA); proliferative cell nuclear antigen (PCNA) and CD45 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); F4/80 (AbD Serotec, Raleigh, NC, USA); Tie2, Cleaved Caspase-3, and Neomycin phosphotransferase II (EMD Millipore, Billerica, MA, USA). Sections were counterstained by hematoxylin. TUNEL staining used the In Situ Cell Death Detection Kit (Roche, Indianapolis, IN, USA).

Double immunohistochemical staining used the PicTure-Double Staining Kit (Invitrogen). Processed sections were incubated overnight at 4°C with rabbit pSmad1/5/8 and mouse Neomycin phosphotransferase II (Abcam) antibodies, followed by incubation with alkaline phosphatase–conjugated anti-rabbit and HRP linked anti-mouse secondary antibodies. DAB (SuperPicture Polymer Detection Kit, Invitrogen) was used for HRP color development (brown), followed by Vector Blue (Vector Lab, Burlingame, CA, USA) staining for alkaline phosphatase.

Skeletal muscle injury by cardiotoxin

Quadriceps muscle injury was induced with 100 μL of 10 μM cardiotoxin (Calbiochem, San Diego, CA, USA).[28] Contralateral injections of saline were used as controls. R206H chimeric mice developed immobility in the cardiotoxin-injected hind limb by 30 days postinjection and were analyzed by X-ray, μCT, and histology ($n = 3$).

Results

Gene targeting to generate an R206H *Acvr1* allele

The *ACVR1/ALK2* gene is highly conserved between human and mouse and identical at codon 206.[29] *Acvr1* from a mouse C57BL/6 BAC clone was modified to replace codon 206 in exon 5 with the FOP R206H mutation (CGC > CAC) and add a *neo*^{r} marker gene (Fig. 1 and Supplemental Information). After blastocyst injection of ES cells positive for homologous recombination, the resulting chimeric mice (Supplemental Fig. 1) were bred with C57BL/6 or CD-1 mice, but viable progeny with germline transmission of the mutant allele were not recovered (see Supplemental Information). Chimeras with estimated 70% to 90% mutant cells were used for phenotypic analysis of effects of the heterozygous *Acvr1*^{R206H} knock-in allele.

Acvr1$^{R206H/+}$ chimeric mice develop characteristic clinical features of classic FOP patients

In addition to postnatal heterotopic ossification, FOP patients with the ACVR1 R206H mutation have congenital malformation of the great toes (a shortened first metatarsal with a single or delta-shaped proximal phalanx) (Fig. 2*A*). Patients can be diagnosed with FOP solely on the basis of digit malformations, even before the appearance of preosseous soft tissue lesions.[30]

At birth, 13 of 27 *Acvr1*$^{R206H/+}$ knock-in chimeric mice displayed shortened first digits in the hind limbs (Fig. 2*B* and Supplemental Fig. 2). The absence of the great toe malformation in some chimeric mice is expected because of variable distributions of mutant cells (also see Supplemental Information). Consistent with patients who have ACVR1 R206H mutations, first digit malformations were observed in hind limbs but not fore limbs. X-ray and μCT analyses revealed shortened or absent proximal and distal phalanges that were comparable to the unique and characteristic malformations in FOP patients (Fig. 2*B*).

Movement and activity of the mice appeared normal during the first several weeks after birth. However, by 6 to 8 weeks of age, most chimeras with a high proportion of mutant cells displayed severe physical disability, evidenced by soft tissue swelling, ankylosed joints, limited mobility, and difficulty in movement. μCT and X-ray analyses of five *Acvr1*$^{R206H/+}$ chimeras revealed extensive heterotopic ossification in skeletal muscle, causing ankylosis of major joints of the axial and appendicular skeleton, as is often observed in FOP patients (Fig. 2*C*, Table 1).

Other common but more variable FOP features[8] were observed in the chimeric mice, including fusion of the posterior facet joints of the subaxial cervical vertebrae (Fig. 2*D*) and variable rib fusions and costovertebral malformations with secondary scoliosis (Fig. 2*E*). Osteochondromas, cartilage-capped bony outgrowths that typically form in metaphyseal regions, occur in most patients with FOP[31] and in *Acvr1*$^{R206H/+}$ chimeras (Fig. 2*F*), most commonly at the proximal medial tibia, but also in other bones, notably the humerus and femur. Chimeras with lower percentages of mutant cells (based on coat color) were also examined and found to display subsets of the FOP-associated phenotypes (Supplemental Information and Supplemental Fig. 1).

Distinct cellular events are associated with heterotopic endochondral ossification

Cellular events associated with FOP lesion progression have been defined through histological analyses that showed formation of heterotopic ossification through a complex process that involves an initial cell and tissue catabolic phase followed by an anabolic phase.[22,32] Destruction of connective tissues, such as skeletal muscle, is accompanied by a robust inflammatory response. This phase is followed by proliferation of fibroblast-like cells that are subsequently replaced through chondrogenesis, angiogenesis, and osteogenesis to form endochondral bone tissue with mature marrow elements. Histological analyses showed these same stages of tissue metamorphosis in the *Acvr1*$^{R206H/+}$ mice (Fig. 3).

Apoptosis of connective tissue and abundant immune cell infiltration are initial events in lesion formation

Early evidence of skeletal muscle degeneration in *Acvr1*$^{R206H/+}$ mouse lesions was indicated by loss of peripheral nuclei and many myofibers with central nuclei (Fig. 3*A*, *B*). These degenerating cells were positively labeled by nuclear DNA fragmentation (TUNEL) assays (Fig. 3*A*) and activated caspase-3 (Fig. 3*B*). Although some necrosis of the degenerating muscle tissue cannot be excluded, the data show that apoptosis occurs at the earliest stages of HO lesion formation and that mature muscle cells and other connective tissue cells are actively lost through apoptosis.

A strong inflammatory response was also observed within the degenerating tissues. Tissues containing enucleated cells (dead) and ghost bodies were infiltrated with CD45$^+$ lymphocytes (Fig. 3*C*). Large numbers of polymorphonuclear cells that were positive for the neutrophil marker myeloperoxidase surrounded the dead and degenerating myofibers and indicate neutrophil participation in scavenging degrading cells/tissues (Fig. 3*D*).

Fibroproliferation follows the inflammatory response and skeletal muscle apoptosis

Apoptosis of skeletal muscle is accompanied by expanding islands of spindle-shaped, nucleated fibroblastic cells, indicated by proliferative cell nuclear antigen (PCNA) staining (Fig. 3*E*), as tissue is cleared of dead muscle cells and the lesion transitions from a catabolic to an anabolic phase. Such tissue fibrosis has been associated with secreted growth factors and collagens that provide a tissue environment that supports tissue remodeling and replacement.[33,34]

Activated macrophages and cells of monocyte origin are present within degenerating muscle tissue and in regions of newly forming fibroproliferative cells (Fig. 3*F*) suggesting an active role of macrophages in this tissue remodeling. Activated, granular mast cells (Fig. 3*G*) were present at every stage of lesion formation with the most pronounced presence during the highly vascular fibroproliferative stage, as well as at sites initiating chondrogenesis, identical to that seen in human lesions.[35] Control skeletal muscle from WT mice showed only rare scattered mast cells, which were nongranular and smaller in size than those in the Acvr1 R206H-induced lesions.

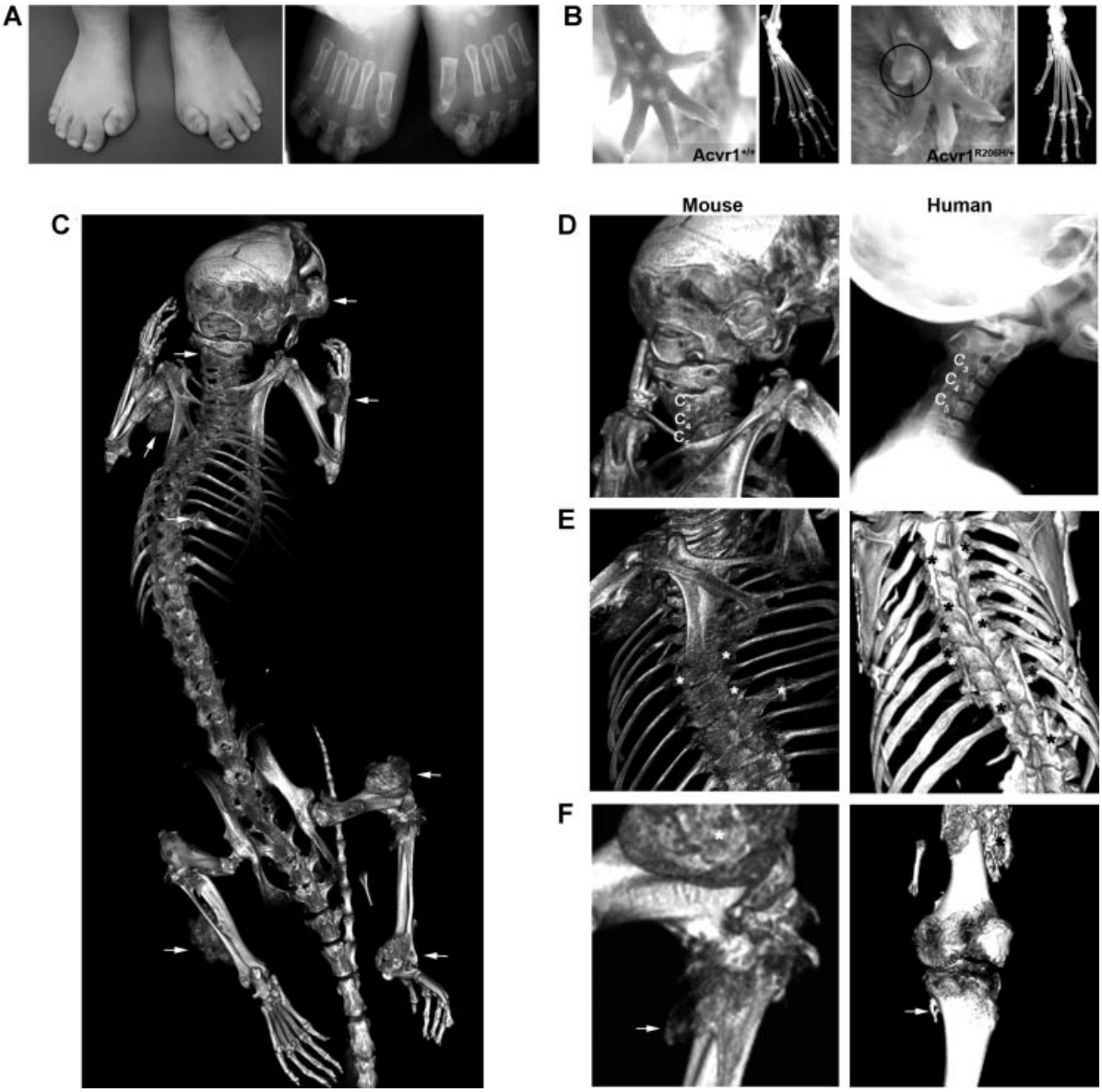

Fig. 2. *Acvr1*$^{R206H/+}$ mice display classic FOP phenotypes. (*A*) Characteristic great toe malformation in FOP. Gross anatomical (left panel) and X-ray (right panel) images from a patient with the classic *Acvr1*$^{R206H/+}$ FOP mutation. (*B*) *Acvr1*$^{R206H/+}$ chimeric mice (right panels; representative 3-month-old mouse) displayed malformation of the first digits of the hind limbs (circled) at birth. X-ray analyses revealed shortened or absent proximal and distal phalanges, which are comparable to the malformations observed in FOP patients. (*C–F*) μCT shows extraskeletal bone formation and skeletal malformations associated with FOP (a representative *Acvr1*$^{R206H/+}$ mouse at 8 weeks old). Whole body image (*C*) with arrows to indicate extraskeletal bone formation is shown. Fusion of cervical vertebrae (C_3, C_4, C_5) (*D*), costovertebral malformations and fusion of vertebrae (asterisks) (*E*), and osteochondromas (arrows) (*F*) are observed in the mouse and FOP patients. (A color version of this figure is available as online Supplemental Information (Supplemental Figure 5).)

Table 1. Comparison of FOP Clinical Features With *Acvr1*$^{R206H/+}$ Chimeric Mice

	Human R206H	Chimeras ($n = 5$)
Heterotopic endochondral ossification	+	+
Malformed great toes	+	+
Orthotopic fusion of subaxial cervical vertebrae	+	+
Fusion of thoracic and lumbar vertebrae	+	+
Malformation of costoverterbral joints + ribs	+	+
Osteochondromas	+	+
Short broad femoral necks	+	+
Early degenerative joint disease	+	+
Injury and Inflammation preceding heterotopic bone formation	+	+

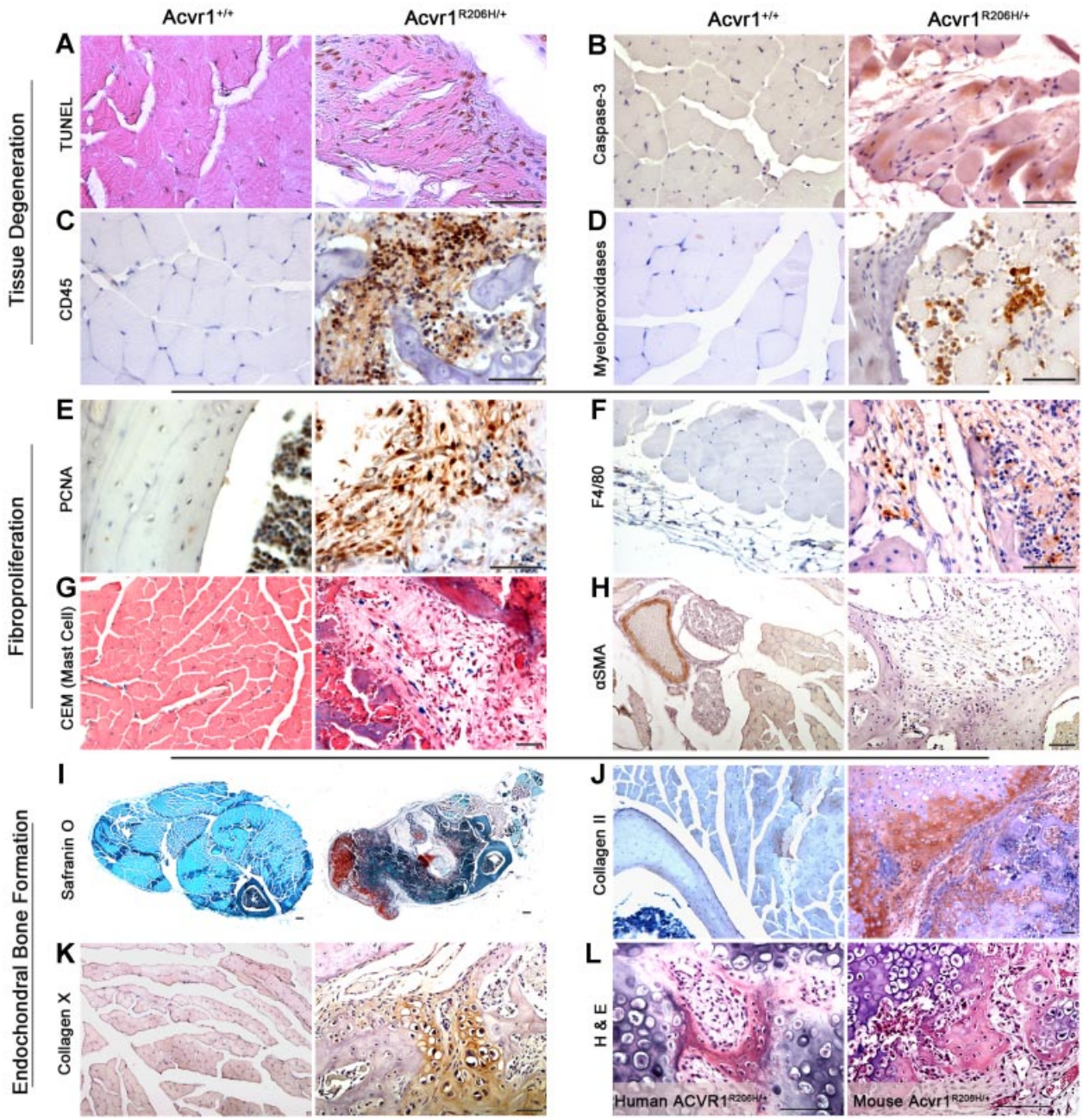

Fig. 3. Histological analysis of cellular events during heterotopic bone formation in *Acvr1*$^{R206H/+}$ knock-in chimeric mice. Skeletal muscle tissue sections from chimeric *Acvr1*$^{R206H/+}$ mice (at 2 months of age) are shown in comparison with control tissues from WT mice. (*A–D*) Initiation of heterotopic bone formation is associated with degeneration of soft connective tissues. (*A*) TUNEL staining revealed DNA fragmentation (brown staining) that is characteristic of apoptosis. (*B*) Apoptosis was confirmed by the positive staining for activated caspase-3; myocentric nuclei (arrow) in degenerating myofibers are observed. (*C*) Immunohistochemical staining for CD45 (lymphocyte marker) supports immune system cell participation in early stages of lesion formation. (*D*) Immunohistochemical staining for myeloperoxidase, a marker for neutrophils, was detected around degenerating myofibers. (*E–H*) A fibroproliferative stage follows tissue degeneration. (*E*) Regions containing fibroblasts stained positively for PCNA, indicating robust proliferation. In control tissues, abundant PCNA positive cells were located within the bone marrow cavity. (*F*) Immunostaining with F4/80 antibodies detected monocytes/macrophages in the fibroproliferative areas; these cells were also observed in degenerating tissues. (*G*) Activated granular mast cells (blue CEM staining) were abundant in fibroproliferative and degenerative stages. Occasional nonactivated agranular mast cells were detected in control sections. (*H*) Immunostaining for alpha smooth muscle actin (αSMA) indicated angiogenesis and new blood vessel formation in fibroproliferative areas. (*I–L*) Heterotopic endochondral bone formation in *Acvr1*$^{R206H/+}$ mice. Low power images of a cross section through heterotopic lesion were stained with safranin O (*I*) to show chondrocytes and cartilage. (*J*) Cartilage is positive for collagen II, and (*K*) hypertrophic chondrocytes were detected by collagen X. (*L*) High-power images (H&E) of late-stage lesions from an FOP patient (left panel) and mouse (right panel) show mature chondrocytes and bone formation. Scale bar: 50 μm for all images (except panel I bar = 200 μm).

In addition to abundant monocytes/macrophages and activated mast cells, fibroproliferative regions were angiogenic, detected by staining for alpha smooth muscle actin (Fig. 3*H*) and von Willebrand factor, throughout this transition from a catabolic lesion to an intensely anabolic lesion.

Extraskeletal bone formation occurs by an endochondral process

The robust fibroproliferative response in developing lesions was followed by chondrogenesis as detected by cell morphology and safranin O staining (Fig. 3*I*). These areas of newly formed cartilage express characteristic markers of early chondrocyte differentiation (collagen II; Fig. 3*J*) and maturation (collagen X; Fig. 3*K*) with the characteristic changes in cell morphology from early- to late-stage chondrogenesis and hypertrophy, chondrocyte apoptosis, and replacement by osteoblasts. Subsequent bone formation is coordinated with angiogenesis, the appearance of bone marrow, and mature bone formation as in FOP heterotopic ossification (Fig. 3*L*).

Skeletal muscle injury in *Acvr1*$^{R206H/+}$ chimeric mice triggers heterotopic ossification

Although HO lesions can form spontaneously in FOP patients, tissue injury can also induce HO.[9] In order to determine whether the *Acvr1*$^{R206H/+}$ knock-in mouse similarly responds to tissue trauma by forming heterotopic bone, we used an established skeletal muscle injury model of intramuscular injection of cardiotoxin.[28,36] By 6 weeks postcardiotoxin injection, *Acvr1*$^{R206H/+}$ mice showed progressive immobility in response to cardiotoxin-induced injury, and X-ray (Fig. 4*A*), and μCT analysis revealed substantial heterotopic ossification at the site of cardiotoxin injection and in the surrounding soft tissues by 6 weeks. Histological analysis (Fig. 4*B*) showed endochondral bone formation with all the previously described stages of HO lesion formation, including apoptotic muscle tissue degeneration, mononuclear infiltration, inflammation with acute fibroproliferative response, chondrogenesis, and bone formation with marrow elements (Fig. 4*B*). No heterotopic ossification or earlier stages of lesion formation were observed in PBS-treated contralateral limbs of *Acvr1*$^{R206H/+}$ knock-in (Fig. 4*A*) or in WT mice. Although relatively minor injury by PBS injection is not sufficient to stimulate heterotopic ossification, our results show that more severe connective tissue injury in the context of the R206H mutation triggers a severe inflammatory response that is followed by development of heterotopic ossification.

Tie2$^+$ progenitor cells contribute to heterotopic ossification

Lineage tracing studies in mouse models of heterotopic ossification previously showed that Tek/Tie2$^+$ lineage cells are a progenitor cell population recruited to differentiate to cartilage and bone cells in HO lesions.[28,37,38] Consistent with these studies, abundant Tie2$^+$ cells were detected in regions of skeletal muscle tissue degradation and fibroproliferation in *Acvr1*$^{R206H/+}$ lesions (Fig. 5*A*). At later stages, fibroproliferative cells and many newly formed chondrocytes were Tie2$^+$ (Fig. 5*A*). By contrast, Tie2$^+$ cells were not observed in skeletal muscle tissue from WT mice (Fig. 5*A*), except in vessels. These data indicate that a Tie2$^+$ cell lineage contributes to the progenitor cells that form all stages of R206H Acvr1-induced heterotopic endochondral bone.

Acvr1 R206H is not required by progenitor cells that form ectopic cartilage and bone

Given that both Acvr1 R206H mutant and WT cells are present in the *Acvr1*$^{R206H/+}$ chimeric mice, we investigated the contributions of both cell types to ectopic endochondral bone formation. Mutant *Acvr1*$^{R206H/+}$ cells were detected by their expression of neomycin phosphotransferase II (neo) (Fig. 1*A*). Both neo-positive (neo$^+$) and neo-negative (neo$^-$) cells were present in fibroproliferative areas of the developing ectopic lesions (Fig. 5*B*), as well as in heterotopic bone, indicating that both mutant and WT progenitor cells participate in this process (Fig. 5*B*). Both neo$^+$ and neo$^-$ cells were also detected in histologic sections of malformed toes (Supplemental Fig. 2).

Areas of heterotopic ossification showing tissue degradation, fibroproliferative, and endochondral ossification stages indicated that neo$^+$ cells were present in greater numbers than neo$^-$ cells (Fig. 5*B, C*), and cell counts for fibroproliferative and

Fig. 4. Skeletal muscle injury induces heterotopic ossification in *Acvr1*$^{R206H/+}$ mice. Skeletal muscle was injured by cardiotoxin injection into the hind limbs of *Acvr1*$^{R206H/+}$ mice and examined 6 weeks after injury. (*A*) X-ray images revealed severe heterotopic bone formation in response to cardiotoxin but not PBS. (*B*) Histological analysis (H&E) of a limb cross-section showed the range of stages of lesion formation including newly formed heterotopic bone. Scale bar: 50 μm.

Fig. 5. Cells participating in heterotopic lesion formation. Skeletal muscle and areas of heterotopic ossification, similar to Fig. 3, are shown. (*A*) Tie2-positive lineage cells contribute to heterotopic lesion formation. Cells positive for Tie2, an endothelial cell marker, are present throughout lesion formation, as shown in tissues from the degeneration, fibroproliferative, and endochondral bone formation stages in *Acvr1*$^{R206H/+}$ mice. Tie2$^+$ cells were undetected in skeletal muscle from WT mice. Scale bar: 50 μm. (*B*) The *Acvr1*$^{R206H/+}$ mutation is not required in cells that differentiate into ectopic cartilage and bone. In chimeric *Acvr1*$^{R206H/+}$ mice, mutant cells are distinguished from WT by neomycin phosphotransferase II. Representative images from *Acvr1*$^{R206H/+}$ mice show both neomycin positive and neomycin negative (WT) cells in the degenerative, fibroproliferative, and chondrogenesis stages, indicating the participation of both mutant and WT cells in the progression of heterotopic ossification lesion formation. Tissues from WT mice (*Acvr1*$^{+/+}$; right panel) showed no staining for neomycin phosphotransferase II. Scale bar: 50 μm. (*C*) Correlation of neo-expressing cells (*Acvr1*$^{R206H/+}$) with BMP signaling. Lesional areas of tissue degradation, fibroproliferative, and endochondral ossification stages were detected for neomycin (brown, cytoplasmic staining) and pSmad1/5/8 (blue, nuclear staining) by double immunohistochemistry. Double positive cells are observed in all stages of lesion development and were the most abundant cells present (also see Supplemental Fig. 3). Scale bar: 50 μm.

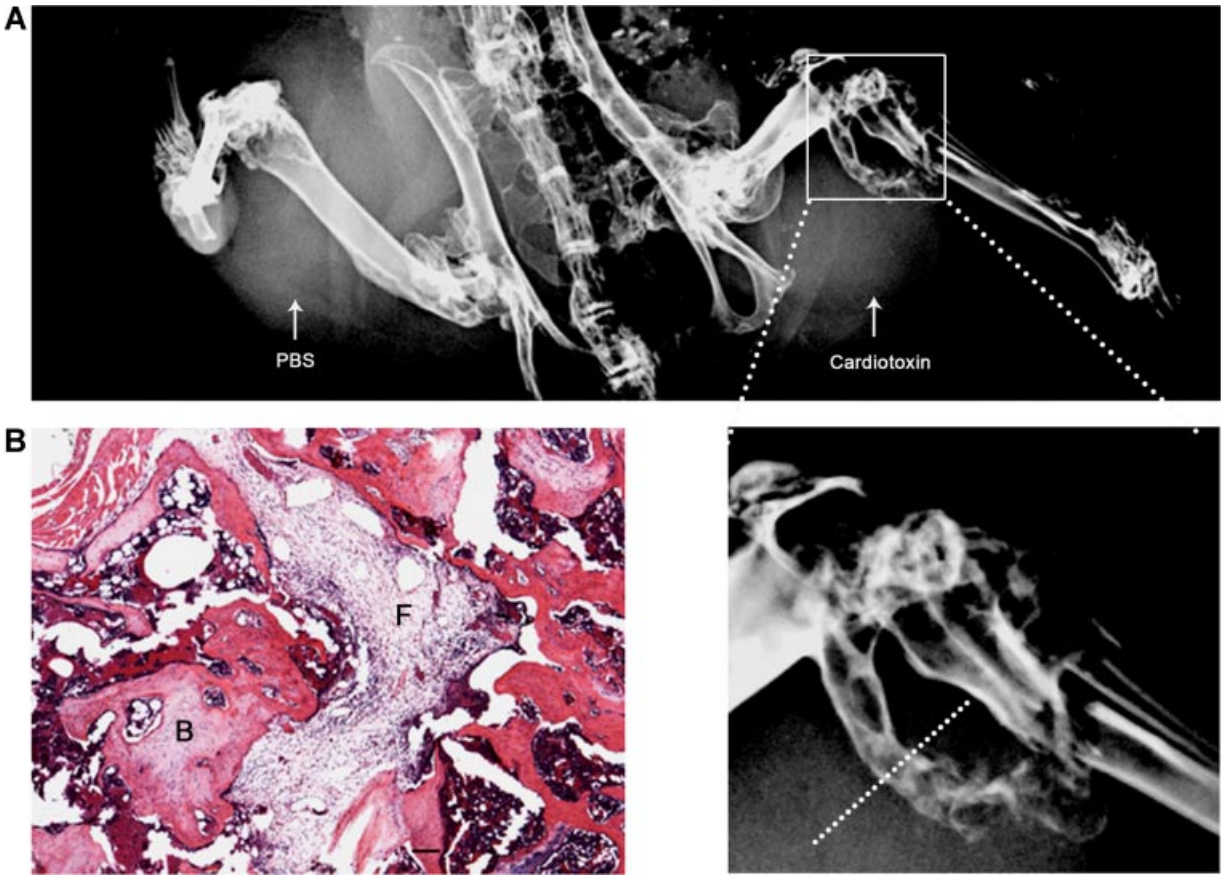
A
PBS
Cardiotoxin
B
F
B

Fig. 4.

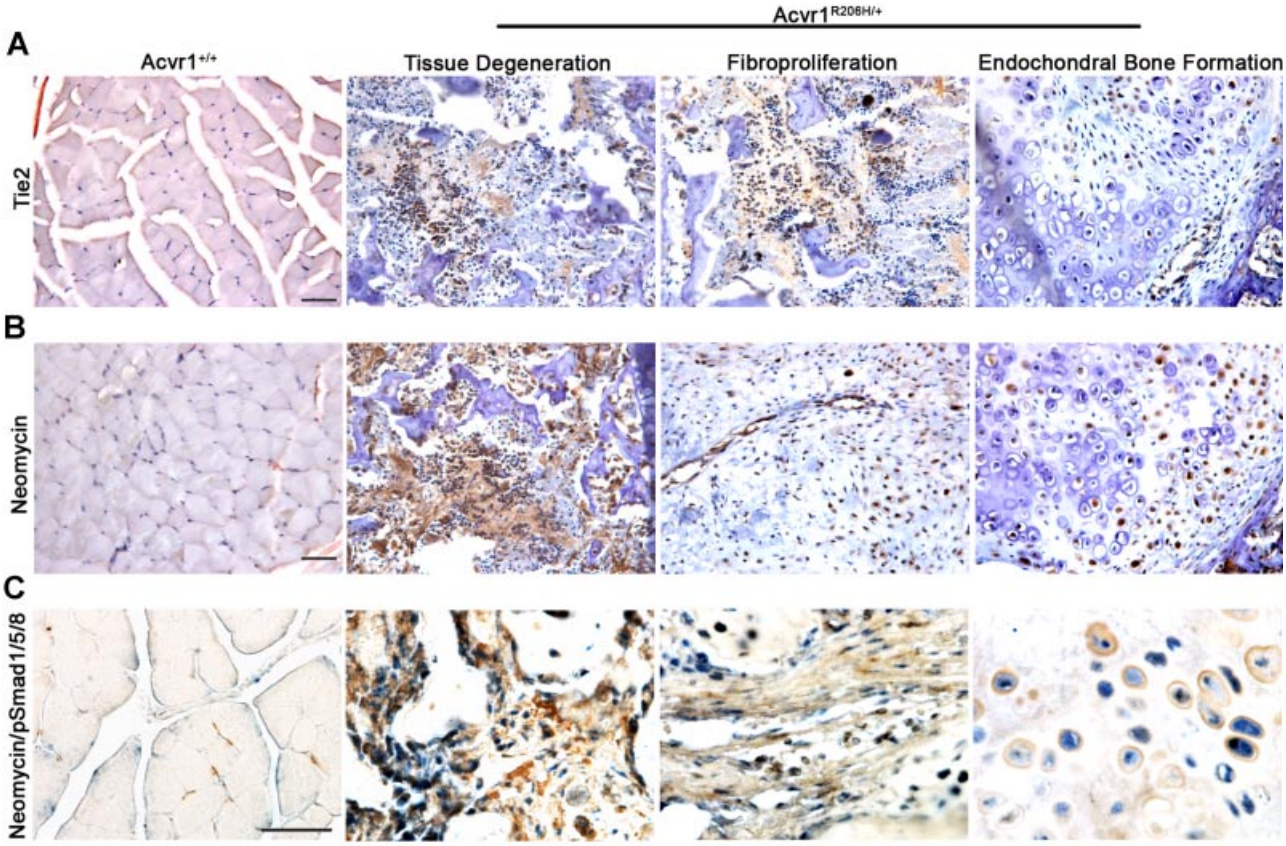
Acvr1R206H/+
A
Acvr1+/+
Tissue Degeneration
Fibroproliferation
Endochondral Bone Formation
Tie2
B
Neomycin
C
Neomycin/pSmad1/5/8

Fig. 5.

endochondral stages show that both contain ~65% neo$^+$ cells (Supplemental Information and Supplemental Fig. 3). To investigate whether BMP signaling is activated in neo-expressing cells, as expected in the presence of the *Acvr1*^{R206H} mutation, we conducted double immunohistochemical staining for neo and phosphorylated Smad1/5/8 (pSmad1/5/8) (Fig. 5*C*). At both fibroproliferative and endochondral stages, ~80% of neo$^+$ cells were also positive for pSmad1/5/8 (Supplemental Fig. 3*C, G*) supporting a strong correlation of BMP signaling with the *Acvr1* mutation. A small population of neo$^-$/pSmad1/5/8$^+$ cells (~17% of total cells) may represent WT cells that are recruited to heterotopic ossification, a process that involves activation of BMP signaling.

Discussion

Animal models of human genetic disease are vital for validating the exact genetic cause of a condition, for understanding the cellular and molecular mechanisms of disease pathology, and for developing translational strategies to prevent and treat affected individuals. The described chimeric knock-in animal model of the rare and disabling human genetic disorder fibrodysplasia ossificans progressiva displays all of the embryonic and postnatal features of FOP that are present in the human condition. These mice validate that the recurrent mildly activating mutation of the BMP type I receptor ACVR1/ALK2 (c.617G > A; R206H) that occurs in all individuals with classic clinical features of the disease[6] is the direct genetic cause of FOP and of all of its resulting pathology.

Along with progressive heterotopic endochondral ossification, malformation of the great toes is a hallmark of classic FOP. *Acvr1*$^{R206H/+}$ murine knock-in chimeras had malformations of hind limb first-digits, nearly identical to those seen in patients with classic FOP. No malformations were seen in the fore limb digits of any of the chimeras. *Acvr1*$^{R206H/+}$ chimeras also showed the full spectrum of congenital malformations observed in patients with FOP: fusion of subaxial cervical facet joints, costovertebral malformations, and osteochondromas of the proximal tibias and scattered other sites. Importantly, the knock-in mice also developed spontaneous and injury-induced FOP lesions that differentiated into mature heterotopic endochondral bone as described in patients with FOP. The study reveals that heterotopic ossification in FOP is not simply a process of ectopic bone formation within skeletal muscle. Rather, the original skeletal muscle tissue is replaced with heterotopic bone in a complex multistage process characterized by an inflammatory and apoptotic catabolic phase, followed by an anabolic endochondral phase. These findings establish that heterozygous substitution of c.617G > A in *Acvr1* causes all of the congenital and postnatal features of FOP, and further establishes the first knock-in mouse model of classic human FOP.

This work sheds light on many important questions about the cellular targets of the FOP mutation, including those that could only be addressed in viable chimeras exhibiting the classic FOP phenotype. This mouse model is consistent with recent findings that cells of Tie2$^+$ origin differentiate to form mature heterotopic bone through an endochondral pathway.[28,37,38] Importantly, both WT and mutant Tie2$^+$ mesenchymal progenitor cells comprise much of the early anabolic fibroproliferative lesion in the chimeric mice and are capable of differentiating to heterotopic bone. This unexpected finding strongly suggests that the *Acvr1*^{R206H} mutation is not required in precursor cells, and that once formed, WT precursor cells can receive instructive signals in a cell nonautonomous manner to guide their differentiation through an endochondral pathway. We confirmed that the majority of mutant cells within lesions, as well as some WT cells, show activated BMP signaling, supporting their differentiation along a chondro/osseous pathway. This finding supports the physiological importance of designing preventions and treatments that target both cell autonomous and cell nonautonomous responses to BMP signaling.

In addition to the postnatal cellular events and targets of *Acvr1*^{R206H} activity that lead to heterotopic ossification, this work also provides important insight into the prenatal developmental targets of the FOP mutation. As in patients with FOP, the *Acvr1*$^{R206H/+}$ chimeric mice develop malformations and subsequent ankylosis in a wide array of small joints of the axial and appendicular skeleton, including, but not limited to, the great toes, the intervertebral joints, and the costovertebral joints, identical to those seen in individuals affected with FOP. These findings suggest that articular chondrocytes or prechondrocytes have a lower threshold and higher sensitivity for the activation of BMP signaling caused by *Acvr1*^{R206H} compared with other cells of the developing skeleton. This observation is supported by earlier findings on the sensitivity of diarthrodial joint development to BMP morphogenetic gradients.[15,39–42] Further, the presence of widespread osteochondromas underscores that cells of the perichondrium are highly sensitive to the direct effects of BMP signaling and its interacting pathways.

Given that patients with the ACVR1 R206H mutation have relatively few major effects on development, the severe consequences of this mutation that lead to perinatal lethality on germline transmission in our knock-in mouse model were unexpected. The precise defects leading to early lethality remain under investigation, however, this mouse model was developed in an isogenic C57BL/6 background; it is possible that the *Acvr1*^{R206H} mutation would provoke a less severe phenotype in an alternate genetic context. The extensive genomic heterozygosity in humans may support viability, as well as explain the variations in disease severity that we observe among patients with the R206H mutation. Alternatively, because BMP signaling has been implicated in the development and function of many tissues and cells, including germ cells, early mouse embryonic development may be more sensitive to perturbations in the level of BMP signaling, and/or the expression pattern of Alk2 may be different in mice compared with humans.

Many additional questions remain unanswered by this work, including the cause of the extremely robust inflammatory infiltration that occurs in early spontaneous FOP lesions, whether the *ACVR1* mutation in FOP influences the immunosuppressive phenotype that has been associated with apoptosis,[43,44] the basis for the distinct anatomic progression of lesions, and

the identity of the factors that direct the episodic progression of the disease. This FOP chimeric knock-in mouse model is novel, and it is rare among animal models by its recapitulation of all of the features of a complex human disease with complete fidelity, and thus provides a valuable tool to address important physiological questions and therapeutic strategies that can be applied to treat heterotopic ossification.

Disclosures

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