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Palovarotene inhibits heterotopic ossification and maintains limb mobility and growth in mice with the human *ACVR1*^{R206H} Fibrodysplasia Ossificans Progressiva (FOP) mutation

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Abstract

Fibrodysplasia Ossificans Progressiva (FOP) is a rare and as yet untreatable, genetic disorder of progressive extraskeletal ossification, is the most disabling form of heterotopic ossification (HO) in humans and causes skeletal deformities, movement impairment and premature death. Most FOP patients carry an activating mutation in a BMP type I receptor gene, *ACVR1*^{R206H}, that promotes ectopic chondrogenesis and osteogenesis and in turn HO. We showed previously that the retinoic acid receptor γ (RARγ) agonist Palovarotene effectively inhibited HO in injury-induced and genetic mouse models of the disease. Here we report that the drug additionally prevents spontaneous HO, using a novel conditional-on knock-in mouse line carrying the human *ACVR1*^{R206H} mutation for classic FOP. In addition, Palovarotene restored long bone growth, maintained growth plate function, and protected growing mutant neonates when given to lactating mothers. Importantly, Palovarotene maintained joint, limb and body motion, providing clear evidence for its encompassing therapeutic potential as a treatment for FOP.

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Disclosures

MI and MP are consultants of Clementia Pharmaceuticals. ANE is an employee of Regeneron Pharmaceuticals, Inc. and holds stock in the company. The other authors have no disclosures related to this work.

Keywords

Fibrodysplasia ossificans progressiva (FOP); retinoic acid receptor (RAR); Palovarotene; heterotopic ossification; ACVR1

Introduction

Fibrodysplasia Ossificans Progressiva (FOP; MIM 135100) is an extremely disabling genetic disorder in which excess bone tissue forms episodically and postnatally at multiple ectopic sites around the skeletal bones and joints and within soft connective tissues such as tendons, ligaments, and skeletal muscles, leading to progressive joint ankylosis, skeletal deformities, growth impairment, breathing difficulty, and premature death[1]. Analyses of surgical retrieval specimens showed that the extra-skeletal bone tissue that forms in FOP patients – a process described as heterotopic ossification (HO) – is invariably endochondral in nature[2]. As such, its formation involves recruitment and proliferation of progenitor mesenchymal cells, their differentiation into chondrocytes, maturation and hypertrophy of cartilage, and transition to endochondral bone and mature heterotopic ossification. An anatomical site where HO initiates is recognized by FOP patients as a local 'flare-up' and is characterized by swelling, pain, erythema, and stiffness preceding overt bone formation[3]. Such symptoms indicate local inflammation, and early stage lesions are often characterized by the presence and accumulation of innate immune cells, including mast cells that are thought to have an important role in inducing and initiating the HO formation process[4]. As a consequence, the current standard of care for FOP patients includes systemic treatment with corticosteroids within 24 hours of the onset of a flare-up, with treatment continued for several days to reduce inflammation and pain[5]. However, corticosteroids, or any other current drug treatments, are unable to reliably prevent HO[6].

Patients with a classic clinical presentation of FOP carry a specific germline heterozygous gain-of-function mutation – R206H – in the bone morphogenetic protein (BMP) type I receptor *ACVR1* (also known as *ALK2*)[7]. BMP signaling plays a major role in skeletogenesis and promotes chondrogenesis[8, 9]. ACVR1/ALK2 acts as a BMP receptor and a constitutively active Alk2 mouse mutant induces ectopic endochondral ossification[10, 11]. Importantly, the activating $ACVR1^{R206H}$ mutation promotes excess chondrogenic differentiation of mesenchymal progenitor cells recruited to the flare-up site, eliciting a skeletogenic response that leads to formation of heterotopic bone through an endochondral process[3, 12].

Previous findings showed that endogenous retinoid signaling is normally attenuated during chondrogenesis, that this attenuation is required for chondrogenic differentiation[13, 14], and that exogenous retinoid agonists can block chondrogenesis effectively and rapidly[15]. We therefore targeted the RA signaling pathway to inhibit the obligate chondrogenic phase of HO in FOP and in turn reduce or prevent HO[16, 17]. Synthetic retinoid agonists, each selective for different nuclear retinoic acid receptors (RARα or RARγ)[18, 19] were tested in mouse models of subcutaneous and intramuscular injury-induced HO as well as mice bearing an inducible and constitutive active $Acvr1^{Q207D}$ transgene[20], the only model of

genetically-driven HO available at the time, but one that is not fully representative of the human FOP genotype[21]. We found that agonists for RARα and RARγ were indeed successful in inhibiting chondrogenesis and preventing HO, but the RARγ agonists were far more effective[16, 17]. This is likely due to the fact that RARγ is highly and selectively expressed in chondrogenic cells and chondrocytes compared to the other RAR members [22, 23]. One of the RARγ class drugs was Palovarotene, a highly-specific RARγ agonist that was previously tested in a two-year Phase 2 clinical trial for another condition[24].

Although the previous studies were quite promising, many questions remained unanswered and most importantly whether Palovarotene would be as effective in preventing HO triggered by the human *ACVR1*^{R206H} mutation as it did for *Acvr1*^{Q207D} and whether this treatment could ameliorate overall skeletal function and restore skeletal growth. The *Acvr1*^{Q207D} mutation is a constitutively activating Acvr1 mutation that induces robust downstream BMP pathway activation in the absence of ligand; this mutation does not occur in FOP patients. In contrast, the *Acvr1*^{R206H} mutation is the most common mutation in FOP, occurring in >97% of FOP patients; it is a mildly-activating mutation in the absence of exogenous ligand, but is also very highly ligand responsive. The data in the present study, using a novel conditional-on knock-in *ACVR1*$^{[R206H]FlEx}$ mouse model that faithfully phenocopies classic FOP, provide strong and clear support for Palovarotene as an effective treatment to prevent FOP heterotopic ossification and protect skeletal function.

Materials and Methods

Mice

A conditional-on knock-in mouse model *Acvr1*$^{[R206H]FlEx}$ was developed to encode the common R206H mutant allele in FOP following recombination by Cre[25]. For doxycycline-inducible global allele expression, *Acvr1*$^{[R206H]FlEx/+}$ mice were mated with mice double transgenic for R26-rtTA and tetO-Cre (heterozygous Gt(ROSA)26Sor$^{tm1(rtTA*M2)Jae}$ and hemizygous Tg(tetO-Cre)1Jaw; Jackson Laboratories) to generate *Acvr1*$^{[R206H]FlEx/+}$;Gt(ROSA)26Sor$^{tm1(rtTA*M2)Jae}$; Tg(tetO-Cre)1Jaw mice (which we refer to as *Acvr1*$^{cR206H/+}$). For expression in *Prrx1*$^{+}$ progenitor cells, *Acvr1*$^{[R206H]FlEx/+}$ mice were mated with heterozygous B6.Cg-Tg(Prrx1-Cre)1Cjt (Jackson Laboratories) to generate *Acvr1*$^{[R206H]FlEx/+}$;Prrx1-Cre$^{+/-}$ mice (*Prrx1-R206H*). *Acvr1*$^{+/+}$ and *Acvr1*$^{[R206H]FlEx/+}$(without Cre) littermates were used as controls. To confirm the distribution of *Prrx1*-expressing cells in mouse limbs, Prrx1-Cre mice were crossed with Gt(ROSA)26Sor$^{tm4(ACTB-tdTomato-EGFP)Luo}$ reporter mice (mT/mG; Jackson Laboratories). To detect PTHrP expression in growth plates, *Prrx1-R206H* and control mice in a heterozygous PTHrP-lacZ reporter background[26] (a gift from Arthur Broadus, Yale) were used. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee at University of Pennsylvania.

Injury-induced heterotopic ossification in *Acvr1*$^{R206H/+}$;R26-rtTA;tetO-Cre mice and treatment with Palovarotene

One-month-old *Acvr1*$^{cR206H/+}$ mice were provided doxycycline chow (Harlan Laboratories, Madison, WI) for 3 days to induce mutant gene expression globally. Mouse quadriceps muscles were injured by injection with 50 μl of 10 μM cardiotoxin (Sigma). Beginning on the day of injury, Palovarotene or vehicle (1:4 DMSO in corn oil) was administered daily for 14 days by oral gavage (100μg/mouse from day 1–3 and 15μg/mouse from day 4–14) using a 20-gauge gavage needle (Fine Science Tools). Palovarotene (R667, Atomax Chemicals, China) solution in DMSO was stored at −20°C under argon and diluted (1:4) with corn oil for administration.

Induction of HO in *Prrx1-R206H* mice and treatment with Palovarotene

Heterotopic ossification in *Prrx1-R206H* mice occurs spontaneously in the absence of injury. Palovarotene treatment of *Prrx1-R206H* and control mice was administered orally to lactating mice (50μg/mouse/day) beginning on day of delivery and continuing for 15 days. Treatment to pups was continued by oral gavage (20μg/pup) on alternate days from P16 to P30.

Imaging analyses

Heterotopic ossification was detected and quantified by micro-computed tomography (μCT) of paraformaldehyde (PFA)-fixed whole mouse specimens (eXplore Locus SP μCT Specimen Scanner; GE Healthcare). 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, 4 averages, 1 hr scan time. Image data were reconstructed at a resolution of 40.5 μm isotropic voxels using a Feldkamp cone beam algorithm. Reconstructed 3D data were analyzed and volumes rendered using OsiriX software (www.osirix-viewer.com). High-resolution, cross-sectional images of hindlimbs were obtained using a VivaCT 40 μCT (Scanco Medical AG, Brüttisellen, Switzerland) at a source voltage of 55kV, a source current of 145 μA, and an isotropic voxel size of 10.5 μm. Reconstructed 3-D images and Scanco μCT software were used for measuring lengths of femurs and tibia.

Histology and immunohistochemistry

Fixed tissues (4% PFA) were decalcified using Immunocal™ (Decal Chemical Corporation, Tallman, NY) for 3 days, embedded in paraffin, and sectioned serially at 7 μm. Deparaffinized sections were stained with hematoxylin/eosin (Sigma) or Alcian blue/ hematoxylin/Orange G for cartilage and bone. Mast cells were detected by modified CEM staining (American MasterTech, KTCEM).

For immunohistochemical staining, deparaffinized sections were treated for antigen retrieval with 10 mM Na citrate buffer (pH 6.0) or 1% hyaluronidase at 37°C for 60 min. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide solution. Sections were blocked (Background Buster; American MasterTech), and incubated with primary antibody overnight at 4°C, followed by appropriate HRP linked secondary antibody and DAB detection (*SuperPicTure*™ Polymer, Invitrogen). Primary antibodies used were for: Collagen

II (Abcam, ab21291), Sox9(Abcam, ab26414), PTHrP (Abcam, ab93121), phosphorylated-Smad1/5/8 (Cell Signaling, 9511S),. Hematoxylin (Vector Labs) was the counterstain. For β-galactosidase detection, fixed tissues were processed with a LacZ staining kit (Invivogen, rep-lz-t), decalcified in EDTA (pH6.5), transferred to 30% sucrose (Fisher Scientific) and embedded in OTC (American MasterTech) before serial sectioning at 14 μm.

To detect proliferating cells *in vivo*, mice were injected intraperitoneally with 5-bromo-2′-deoxyuridine (BrdU) solution (Thermo Fisher Scientific) at 10 μl/g mouse[27]. Tissues were harvested after 3 or 36 hr, fixed in 4% PFA, processed as above, and detected by BrdU immunohistochemical staining (Thermo Fisher Scientific).

Microscopy imaging and quantification

Histological images were captured using Eclipse 90i microscope (Nikon) and processed using NIS-Elements microscope imaging software (Nikon). Measurements of the lengths of growth plate zones were performed using the NIS-Elements microscope imaging software and data obtained was analyzed statistically. Stained sections were analyzed by independent investigators without bias and with random blinding methods.

Whole mount skeletal staining

For skeletal staining, skin and internal organs were removed from neonates and adult mice and skeletons were processed for differential staining using Alcian blue and Alizarin red as described[28]. Cleared and stained skeleton images were captured and lengths of long bones were measured using a Leica DFC 450c instrument and software.

Statistical analysis

Data obtained were analyzed statistically using Graph Pad Prism software (t-test and two-way Anova); values are expressed as the mean ± SEM in bar graphs. All data are from a minimum of three independent experiments.

Results

Palovarotene inhibits heterotopic ossification (HO) in a mouse model of FOP

To create a mouse model of FOP, we used a knock-in mouse line in which exon 5 containing the human R206H mutation replaces a wild-type exon 5 within the *Acvr1* locus upon Cre recombinase activity (Supplemental Fig. 1; see Methods). In a first set of studies, 1-month-old heterozygous *Acvr1*$^{[R206H]FlEx/+}$;Gt(ROSA)26Sor$^{tm1(rtTA*M2)Jae}$;Tg(tetO-Cre)1Jaw mice (briefly referred to as *Acvr1*$^{cR206H/+}$) were treated with doxycycline to globally induce Cre recombinase and concomitant recombination of the *Acvr1*$^{[R206H]FlEx/+}$ allele leading to expression of *Acvr1*^{R206H} in the tissues where *Acvr1* is normally expressed. Subsequently, the mice were injected with cardiotoxin into the quadriceps muscle of one leg to provoke local inflammation, muscle damage and HO[17]. Starting from day 1 of injury, half of the *Acvr1*$^{cR206H/+}$ mice were treated with Palovarotene by daily gavage for 14 days and the other half received vehicle as control. Analysis by μCT and 3D image reconstruction at day 14 showed that large HO tissue masses had formed in the targeted leg of *Acvr1*$^{cR206H/+}$ mutant mice receiving vehicle (Fig. 1A, arrow, left panel), but HO formation was greatly

diminished in Palovarotene-treated companions (Fig. 1A, arrow, right panel) by over 80% based on bone volume/total volume quantification (Fig. 1B). Histochemical analysis confirmed that newly formed cartilaginous tissue and endochondral bone were present in untreated mutants (Fig. 1C, circled area), but not in treated *Acvr1*$^{cR206H/+}$ mutant mice (Fig. 1C, right panel). This analysis revealed also that untreated mutants showed a strong local fibroproliferative response and numerous mast cells within the muscle tissues at and around the site of incipient HO formation (Fig. 1D, F; left panels), but both responses were greatly reduced in Palovarotene-treated animals (Fig. 1D, F; right panels and Supplemental Fig. 2) by 60 to 80% (Fig. 1E, G).

A severe functional consequence of HO is that it can impair or completely block skeletal movement and joint function[29]. Indeed, video of live, untreated *ACVR1*$^{cR206H/+}$ mutant mice at ~14 days after HO induction by injury showed that movement of HO-affected legs was overtly impaired, but in Palovarotene-treated mice the cardiotoxin-injured legs appeared to move and function well and similarly to the contralateral uninjured leg (Supplemental Video 1).

Prenatal expression of *Acvr1*^{R206H} causes limb skeletal malformations and HO

FOP patients are characterized by a great toe (first digit) malformation that is present at birth and reflects aberrant embryonic skeletal development[29]. Thus, we investigated whether prenatal expression of *Acvr1*^{R206H} would affect skeletal development, growth and morphogenesis. Since unrestricted endogenous activation of *Acvr1*^{R206H} during early embryonic development leads to perinatal lethality[30], we limited expression to a population of skeletal progenitor cells (*Prrx1*$^{+}$) in order to investigate effects on local skeletal development. *Acvr1*$^{[R206H]FlEx/+}$ mice were mated with *Prrx1-Cre* mice to generate mutant embryos expressing *Acvr1*^{R206H} throughout the lateral plate mesoderm-derived limb mesenchyme[31]. *Acvr1*$^{[R206H]FlEx/+}$*; Prrx1-Cre* mice (which will be referred to as *Prrx1-R206H*) were born at a normal Mendelian frequency and were not grossly different from companion control littermates (*Acvr1*$^{+/+}$ and *Acvr1*$^{[R206H]FlEx/+}$ without Cre) (Fig. 2A). However, when mice were examined after birth, the first digits of the hindlimbs were observed to be malformed as clearly revealed by radiographic imaging (Fig. 2C, arrows, right panel). While no obvious HO was present within the limbs themselves at these early ages (Fig. 2B), spontaneous HO became extensive by 1 month of age in mutants (Fig. 2D, E, arrows, right panels) and, and was detected first in the hindlimbs at ~P7 and then in the forelimbs beginning at ~P14. HO in both hind and forelimbs progressively increased over time (Supplemental Fig. 3) but was absent in control littermates. These phenotypes were fully penetrant, and the sites of HO formation were consistent with the locations of Prrx1^{+} lineage cells. The appearance of HO in this Prrx1-R206H mouse model was also consistent with the appearance of HO in patients with FOP. Patients do, however, develop HO in other areas of their body, including the back and neck, indicating that HO in FOP can involve cell types belonging to lineages other than those descending from *Prrx1*-expressing cells.

Long bone elongation was significantly impaired in *Prrx1-R206H* mice (Fig. 3A). Lengths of humerus, radius, femur and tibia were reduced in 5 day-old mutants (Fig. 3B), and growth retardation persisted at 1 month (Supplemental Fig. 4). Of note, lengths of bones did not

appear to be proportionally reduced. Clinical observations have suggested that disproportionate limb lengths may occur in some FOP patients, however this remains to be verified in detailed studies. Histological analysis showed that the epiphyseal area was not grossly altered in the mutants, retaining a largely normal shape and size, however there was a significant reduction in the height of the growth plate hypertrophic zone (Fig. 3C, right panel; and Fig. 3D), a region of the growth plate primarily responsible for long bone elongation[32, 33].

To further characterize the phenotype of the growth plate, longitudinal sections of tibias from day 14 mutant and control mice were processed for cartilage marker analysis by immunohistochemistry. Overall growth plate homeostasis and functioning were found to be defective in mutants as indicated by: (i) increased levels of phosphorylated SMAD1/5/8 and Sox9 in mutant hypertrophic zone (Fig. 4A, B, right panels), both of which are normally expressed at barely detectable levels in that area of the elongating growth plate[34, 35] (Fig. 4A, B, left panels); and (ii) higher levels of collagen II and PTHrP throughout the mutant growth plates (Fig. 4C, D, right panels) compared to the more restricted patterns in controls (Fig. 4C, D, left panels). To verify cells transcribing *PTHrP*, we used *PTHrP-lacZ* mice[26] to create reporter;*Prrx1-R206H* mice. We found that *PTHrP* expression was expanded in mutants (Fig. 4E, right panel), correlating well with the expanded distribution of PTHrP protein (Fig. 4D), while reporter activity was present more narrowly in control growth plates (Fig. 4E, left panel). Together, dysregulated *PTHrP* expression and high levels of phosphorylated SMAD1/5/8 and Sox9 may have overwhelmed the growth plate, disturbing hypertrophic differentiation of chondrocytes and delaying the cartilage to bone transition in the growth-plates of $Acvr1^{R206H/+}$ mutant mice.

In addition to hypertrophic zone elongation, physiologic activity of the growth plate is also influenced by chondrocyte proliferation, a process that is restricted to the distal end of the growth plate where commitment of proliferating chondrocytes to mature and differentiate occurs. To determine whether this process was altered in mutants, 2-week-old *Prrx1-R206H* mice and control littermates were injected with the cell proliferation marker BrdU, sacrificed at 3 or 36 hours after injection, and processed for immunohistochemical detection of BrdU-labeled chondrocytes in long bone growth plates. At the 3-hour time point, both control and mutant long bones displayed labeled chondrocytes in the upper portion of their growth plates that includes the proliferative and prehypertrophic zones (Fig. 5A). Interestingly, mutant growth plates contained a consistently higher number of labeled chondrocytes (Fig. 5B, upper zone), indicating higher cell proliferation rates compared to controls. At 36 hours, substantial numbers of BrdU+ cells in control growth plates have transitioned to the hypertrophic zone (Fig. 5C, left panel); however, mutant growth plates retained a considerable number of labeled cells in the upper zone and only a few in the hypertrophic zone (Fig. 5C, right panel; and Fig. 5D), indicating that mutant cells did not advance through the growth plate zones at normal rates.

Palovarotene reduces spontaneous HO and improves skeletal malformations and growth in *Acvr1*$^{R206H/+}$ mutants

To determine whether Palovarotene could prevent or alleviate the severe neonatal and postnatal skeletal defects in *Prrx1-R206H* mice, treatment was initiated from a neonatal stage. Palovarotene was administered to nursing transgenic females (that had been mated to *Prrx1-Cre* males) by daily gavage with the expectation that the drug would be passed to the pups through breastfeeding. Companion nursing females received vehicle. Litters included both mutants and control littermates, and drug or vehicle administration to the nursing mothers was continued until day 14. Thereafter, the drug (or vehicle) was administered directly to the young mice by oral gavage on alternate days until 1 month of age. Whole body skeletal examination by μCT showed that compared to controls (Fig. 6A, first panel), extensive HO had developed in the limbs of mutants, in particular their hindlimbs (Fig. 6A, third panel, arrows). Treatment of control mice with Palovarotene caused reduction in skeletal growth (Fig. 6A, second panel; and Fig. 6B), as previously observed in response to other retinoid agonists[36, 37], but no other major obvious phenotype was noted. Palovarotene-treated mutants showed markedly reduced HO that was nearly absent in their forelimbs and much reduced in hindlimbs (Fig. 6A, fourth panel). Of particular note, long bone length was considerably protected in Palovarotene-treated mutants (Fig. 6B) in contrast to the negative effects of Palovarotene on control long bone growth (Fig. 6B). Furthermore, while vehicle-treated mutants had severe movement impediment and difficulties, drug-treated mutants were mobile and appeared to function well (Supplemental Video 2). This was consistent with the beneficial effects of Palovarotene on mobility of injury-induced older mutants shown above (Supplemental Video 1). When Palovarotene treatment was delayed until day 14 and given only to the young mice, it was not as effective at inhibiting HO and maintaining mobility (not shown), suggesting importance of early treatment.

Control mice receiving Palovarotene from birth were not only stunted (Fig. 6A) but exhibited also a significant reduction in safranin-O positive cartilage matrix in their growth plates (Fig. 6C, second panel) compared to untreated controls (Fig. 6C, first panel), likely the result of decreased matrix production or enhanced catabolism[38, 39]. In sharp contrast, Palovarotene treatment not only protected long bone growth of the mutant mice (Fig. 6B), but also preserved the organization of their growth plates and matrix deposition (Fig. 6C, third panel; and Fig. 6D). These data suggest that interactions between Palovarotene and *Acvr1*^{R206H} compensated for the negative effects of each on skeletal growth and phenotype, and that the effects of, and responses to, drug treatment differ in control and mutant cells and tissues.

Discussion

This study demonstrates that the highly specific RARγ agonist Palovarotene is a potent inhibitor of both trauma-induced and spontaneous HO elicited by the human *ACVR1*^{R206H} FOP mutation in a novel mouse model of classic FOP. We further show that Palovarotene can inhibit HO that was triggered by post-natal tissue trauma or provoked systemically from an early age in the absence of overt trauma. Our data additionally demonstrate that Palovarotene can counteract the broader ravages of HO, including deleterious, often

debilitating, effects on skeletal growth and mobility. Previous studies conducted through a phase 2 clinical trial for emphysema showed that Palovarotene is well tolerated by patients with relatively few and minor side effects[24]. Those studies and the data here provide further support for Palovarotene as a safe and effective drug for the treatment and prevention of HO in FOP patients and potentially also for non-FOP patients who can form endochondral HO following severe injury or invasive surgeries[40, 41].

Acvr1^{R206H} knock-in mice mimic the defects seen in FOP patients[29, 30], including skeletal growth retardation which is likely a result of (i) the extensive HO within muscles and connective tissues surrounding the skeletal elements that indirectly hampers skeletal growth and (ii) direct effects of the *Acvr1*^{R206H} mutation and enhanced BMP signaling on growth plate physiology. Normally, BMP signaling - mainly via Bmpr1a and 1b - is active in the proliferative and prehypertrophic zones where it promotes and sustains *Sox9* expression and chondrocyte proliferation, and is markedly reduced in the hypertrophic zone in order to facilitate transition to bone[34, 42]. Previous *in vitro* data indicate that the ACVR1 receptor plays a key role in early steps of chondrocyte differentiation and commitment[12]. *Acvr1* was recently shown to be expressed in growth plates, and its conditional ablation in cartilage was found to cause growth plate dysfunction and reduced chondrocyte proliferation[43]. Consistently, we observe greater proliferation in the *Acvr1*^{R206H} growth plates, with failure or significant delay in hypertrophic chondrocyte differentiation. As a result, the overall effects of enhanced activation of *Acvr1*^{R206H} led to compromised bone growth, supporting the notion that the appropriate magnitude and distribution of BMP receptor signaling are critical for normal longitudinal bone growth[9]. Because Palovarotene inhibits BMP signaling[16, 17], it may restore skeletal growth in mutant mice by reducing BMP signaling to re-establish more physiologic signaling levels. Importantly, patients with FOP have multiple, variable and often severe skeletal anomalies affecting long bone growth predominantly in the lower limbs, and the skeletal effects noted in the mouse model emphasize the necessity for a more robust clinical characterization of this often overlooked feature in patients[44, 45]. Such studies are ongoing.

In this study, we observed that Palovarotene counteracted multiple soft tissue and skeletal pathologies in *Acvr1*$^{R206H/+}$ mutant mice. In their damaged muscle tissue, Palovarotene treatment was associated with fewer mast cells and reductions in fibroproliferation, cartilage formation, and the amount of HO. Within the skeletons of mutant mice, Palovarotene treatment led to restoration of proliferative and hypertrophic zones of growth plates and improvement of compromised bone growth. The articular malformations in the great toe, the heterotopic endochondral ossification and the severe skeletal dysplasia observed in our knock-in mouse model of classic FOP confirm the widespread effects of the *Acvr1*^{R206H} mutation on a broad population of cartilage cells and precursor cells in the skeleton and soft connective tissues. Furthermore, in conjunction with our previous studies showing a role of retinoid signaling in hypertrophic cartilage to bone transition[22, 39], the abrogation of heterotopic ossification and reversal of skeletal defects by Palovarotene provide robust *in vivo* support for the idea that a fine balance between BMP and retinoid signaling, and in particular between Acvr1 and RARγ, is normally critical for growth plate function and skeletal growth, as occurs in other biological processes[46]. For example, one role for endogenous retinoid signaling in hypertrophic cartilage would be to reduce BMP signaling

and facilitate transition to bone. Thus, interactions between retinoid and BMP signaling are likely to be context dependent and/or receptor isoform specific, and to be greatly modulated by altered activity of mutant BMP receptors. Further characterization of Acvr1 and RARγ interactions will be useful not only for understanding the action and mechanisms of such key regulators, but also for identifying additional druggable targets and even more effective treatment regimens.

HO is highly damaging to the well being of FOP patients because it progressively interferes with, and limits, multiple body functions including walking, bending, breathing, mastication, and swallowing. Since Palovarotene inhibits the chondrogenic stage of heterotopic endochondral ossification, to be effective Palovarotene treatment would be needed at each flare-up to reduce or possibly prevent each new round of HO, possibly starting from a young age. Once formed, HO is permanent in FOP patients; the ectopic bone cannot be removed by surgery because the resulting tissue damage triggers additional episodes of HO[29]. Given the potency of Palovarotene to prevent HO, this drug has the potential to suppress HO initiation following surgery in FOP patients, a therapeutic feat if indeed possible. Notably, the complete recovery of growth plate structure and function that we observe in Palovarotene-treated mutants supports the possibility that drug treatment of skeletally-immature patients might enable suppression of HO during childhood while restoring skeletal growth, an attainment that originally seemed counter-intuitive with an RARγ agonist.

The goal of the present study was to test whether Palovarotene can block HO elicited by the human Acvr1 R206H mutation and whether this treatment could also correct skeletal defects caused by this mutation in FOP patients. The data presented demonstrate that Palovarotene is in fact able to do so and, quite significantly, appears to be able to act on neonates via the mother's milk. While the cellular mechanisms through which Palovarotene and Acvr1 R206H operate remain to be elucidated in further detail, these findings are a major step in establishing an effective and preventative treatment for FOP, possibly administrable from infancy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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SC, KU, MC, DZ performed experiments. All authors analyzed data and/or participated in experimental design. A.E. developed the conditional *Acvr1* mouse model. MP and EMS wrote the manuscript with contributions by FSK, MI and AE. All authors contributed and approved of the final version of the manuscript. EMS, MP, and MI directed the project and take responsibility for the integrity of the data.

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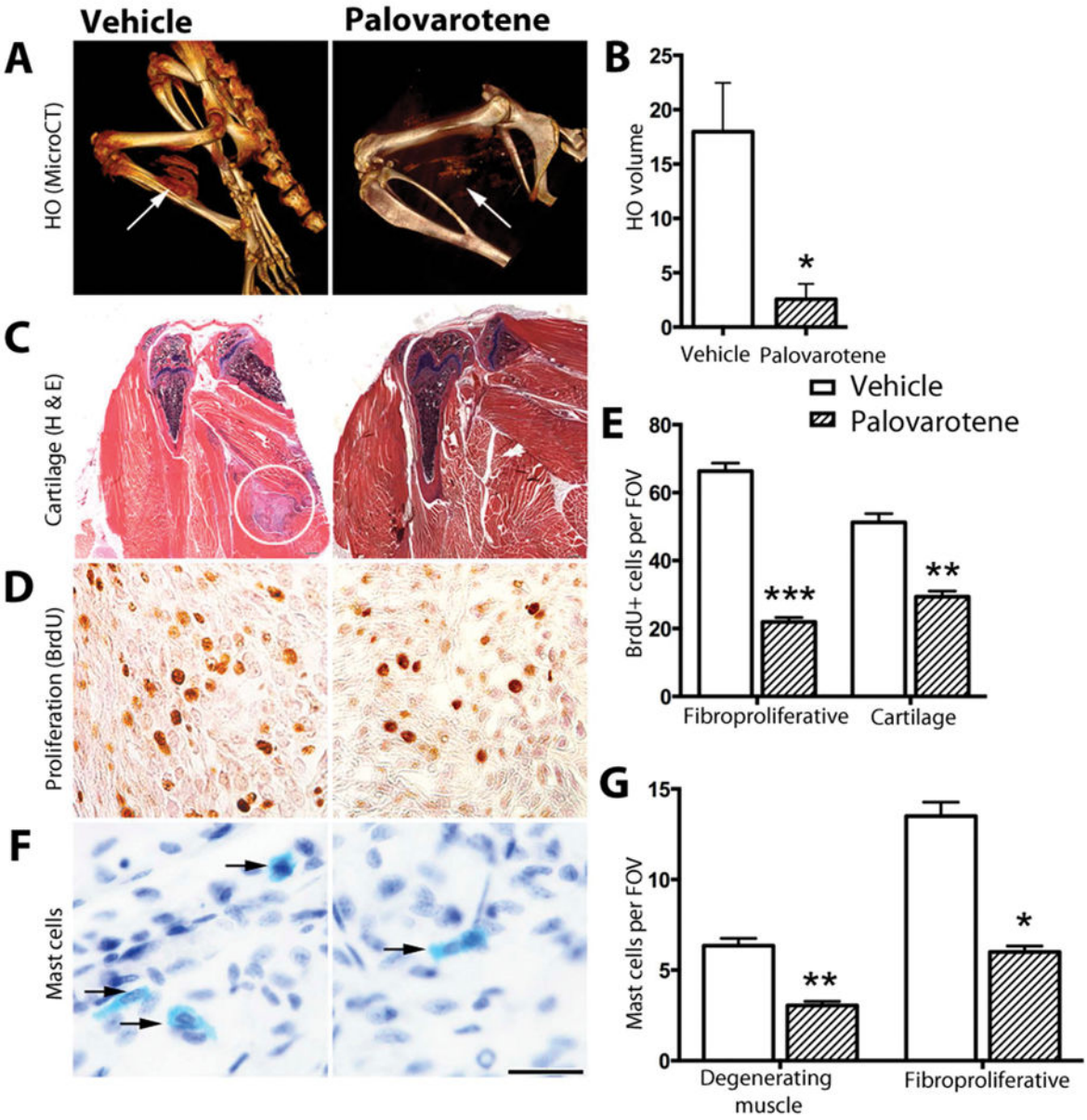

Fig. 1.

Injury-induced heterotopic ossification in *Acvr1*$^{cR206H/+}$ mutant mice is inhibited by the RARγ agonist Palovarotene. (A) *Acvr1*$^{cR206H/+}$;R26-rtTA;tetO-cre mice treated with doxycycline to induce global expression of *Acvr1*$^{cR206H/+}$ were subjected to hindlimb muscle injury with cardiotoxin and examined by μCT imaging 14 days after treatment with Palovarotene (right panel) or vehicle (left panel) to detect heterotopic ossification (HO; arrows) (vehicle, n= 4; Palovarotene, n=6). (B) Heterotopic bone (arrows in A) was quantified as total mineralized tissue volume (vehicle, n= 4; Palovarotene, n=6). (C) Tissue sections of hindlimbs were stained with hematoxylin and eosin. Areas of cartilage and bone were detected in untreated (left panel, circle) but not in Palovarotene-treated samples (right panel) (n=3 mice per group). (D) Proliferating cells were detected by BrdU incorporation at

the site of lesion formation in untreated (left panel) and treated (right panel) samples, and (E) quantified in the early stage fibroproliferative region following injury and in ectopic cartilage at later stages. Positive cells in 3 fields of view (FOV) in each of 4 sections were counted (n=3 mice per group). (F) Activated mast cells were detected by histochemical staining (bright blue; indicated by arrows) in early stage lesions (untreated, left panel and treated, right panel), and (G) quantified in FOV of degenerating muscle and fibroproliferative regions (n=3 mice per group). Scale bar (D and F), 25 μm. In B, E, G, hatched bars represent data from Palovarotene-treated mice and white bars from vehicle-treated. Data shown are mean±SEM; statistics compared Palovarotene-treated vs. vehicle-treated using an unpaired t-test; ***$p<0.001$, **$p<0.01$, *$p<0.05$.

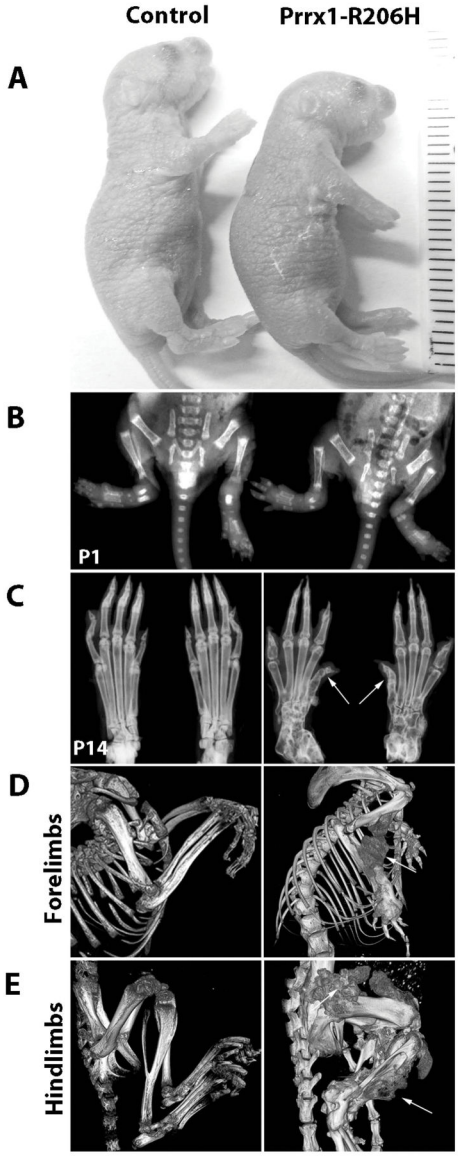

Fig. 2.

Acvr1^{cR206H} expression in *Prrx1*$^{+}$ skeletal progenitor cells induces malformation of hindlimb first digits and heterotopic ossification. (A) At birth (P1), *Prrx1-R206H* mice were shorter compared to control mice (n=12 mice per group). (B) Radiography of control and mutant mice at P1; no heterotopic ossification was detected (n=3 mice per group). (C) Radiography of hindlimbs at two weeks (P14) showed FOP-like malformation of the first digits (arrows) in mutant mice (n=3 mice per group). (D, E) At 4 weeks of age, μCT imaging showed spontaneous (non-injury-induced) heterotopic ossification (arrows) in forelimbs (D, right panel) and hindlimbs (E, right panel) of mutant mice. Control mice are *Acvr1*$^{cR206H/+}$ without Prrx1 activation of cre (n=3 mice per group).

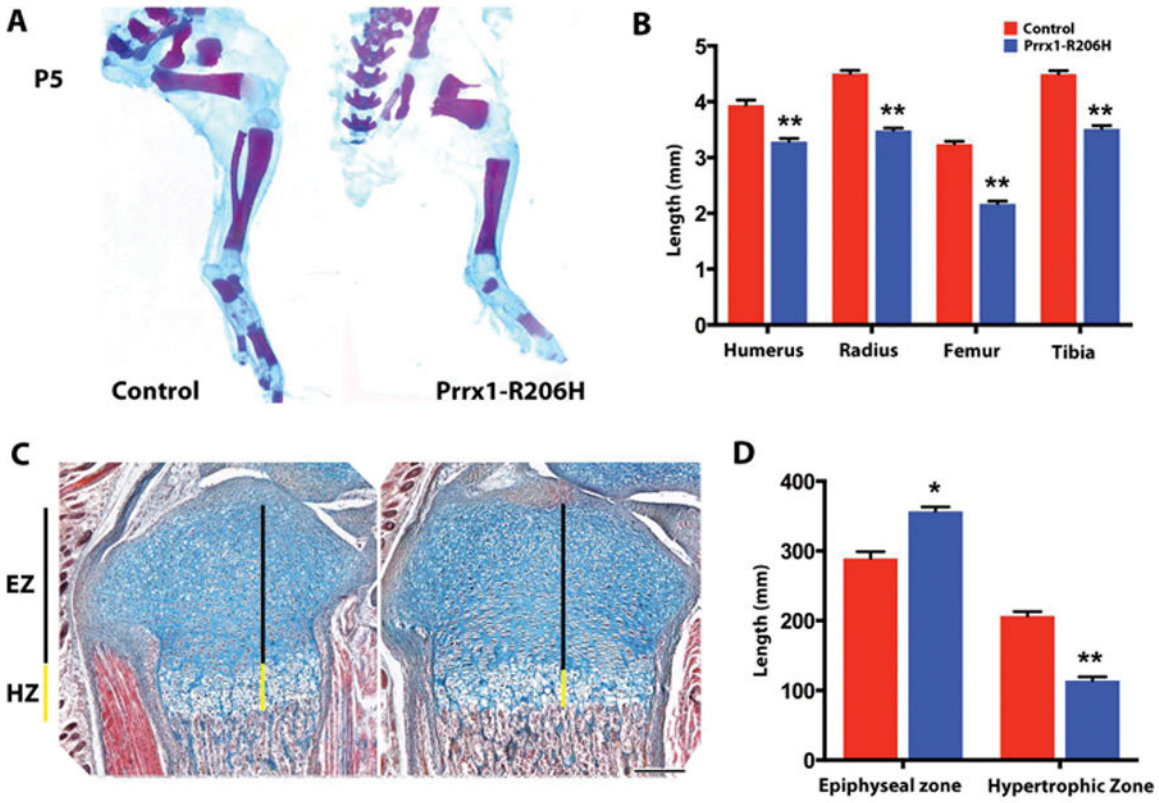

Fig. 3.

Long bone elongation is impaired by expression of *Acvr1*^{cR206H} in *Prrx1*$^{+}$ cells. (A) Hindlimb skeletons from 5-day-old (P5) control and mutant mice were stained to detect cartilage (Alcian blue) and bone (Alizarin red) (n=3 mice per group). (B) Lengths of bones in forelimbs (humerus, radius) and hindlimbs (femur, tibia) were quantified in control (red bars) and mutant (blue bars) mice (n=3 mice per group). (C, D) Proximal region of P7 tibias from control (C, right panel) and mutant (C, left panel) mice were stained with Alcian blue, Orange G, and eosin. Epiphyseal zones (EZ; black line) and hypertrophic zones (HZ; yellow line) are indicated in (C) and quantified in (D) (n=3 mice per group). Scale bar, 200 μm. Data shown are mean±SEM; statistics compared control vs. mutant using an unpaired t-test; **p<0.01, *p<0.05.

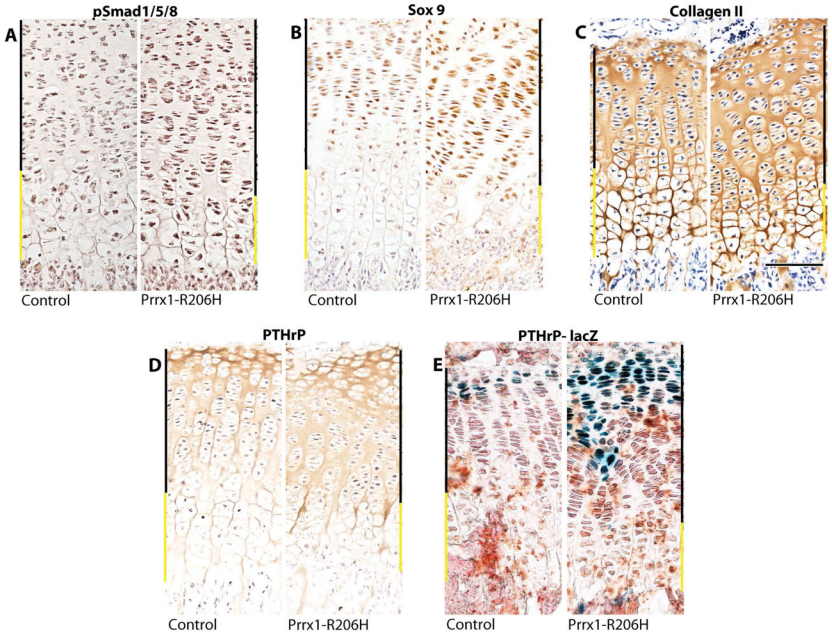

Fig. 4.

Prrx1-Acvr1$^{cR206H/+}$ expression alters growth plate homeostasis. Proximal tibial growth plate sections from 14 day old mutant (*Prrx1-Acvr1*$^{cR206H/+}$) mice (left panels) show altered distributions of growth plate markers and regulatory proteins compared to controls (*Acvr1*$^{cR206H/+}$) (right panels) as revealed by immunohistochemical staining for: (A) pSmad 1/5/8; (B) Sox 9; (C) Collagen II; and (D) PTHrP. (E) Cells expressing PTHrP were identified in control and mutant mice in a *PTHrP-LacZ* background by staining for β-galactosidase. Upper zones (black line) and hypertrophic zones (yellow line) are indicated. Scale bar, 100 μm. (n=3 mice per group, similar data was observed at P1, and P7 (data not shown)).

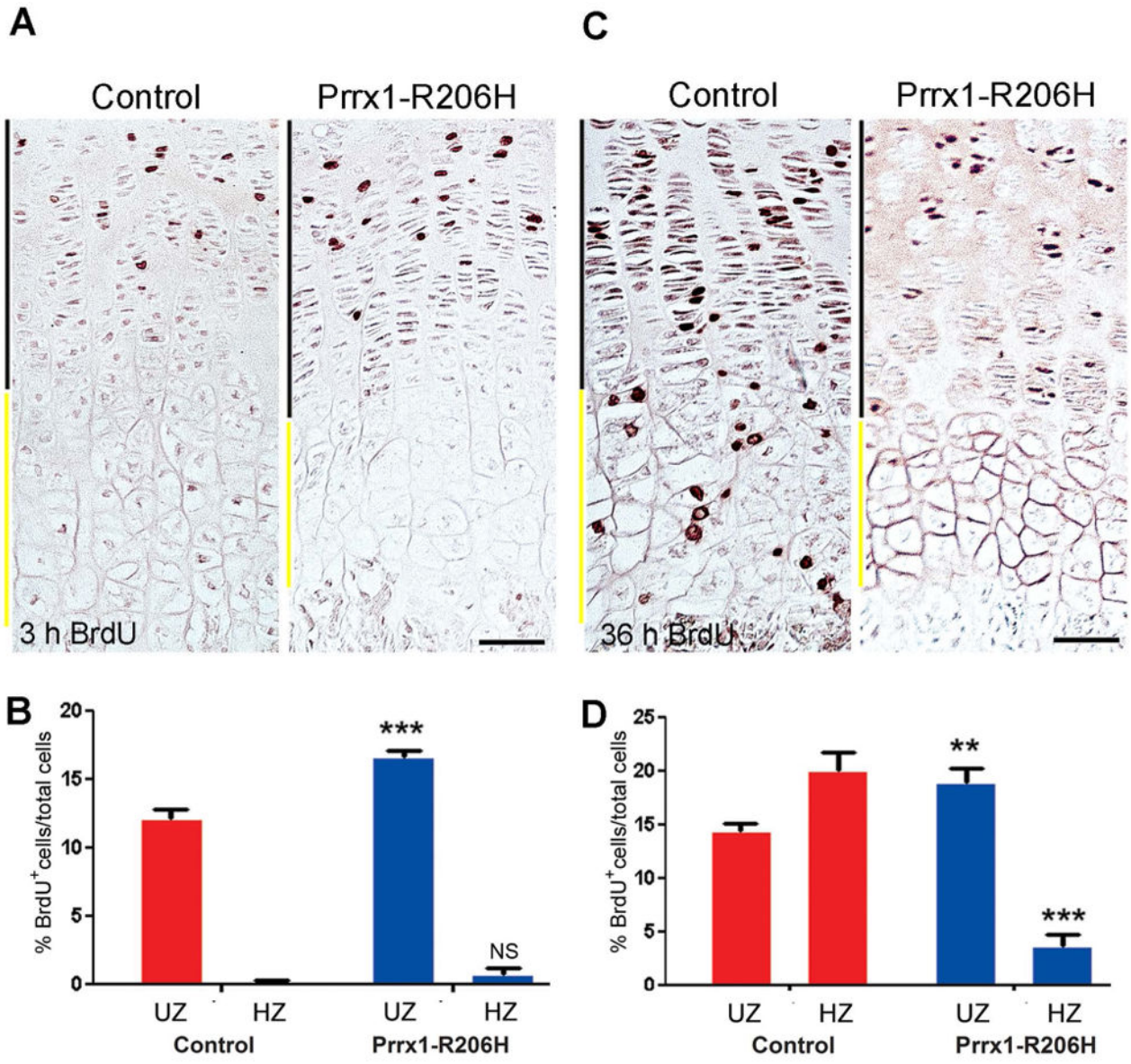

Fig. 5.

Chondrocyte proliferation and progression are altered in *Prrx1-R206H* growth plates. (A) Proximal tibial growth plates of control (left panel) and mutant (right panel) 14-day-old mice were labeled for 3 hours with BrdU and then processed for BrdU immunostaining to identify proliferating cells. Sections were counterstained with haemotoxylin. Upper zones (UZ; black line) and hypertrophic zones (HZ; yellow line) are indicated. Scale bar, 50 um. (B) In each of 3 sections per mouse (n=3 mice per group), BrdU positive cells in UZ and HZ regions were counted. The percentage of $BrdU^+$ cells was determined relative to the total number of cells with haemotyoxylin-stained nuclei in each zone. (C) After 36-hour of BrdU labeling the fate of labeled cells in control (left panel) and mutant (right panel) over time was determined. Upper zones (UZ; black line) and hypertrophic zones (HZ; yellow line) are indicated. Scale bar, 50 um. (D) In each of 3 sections per mouse (n=3 mice per group), BrdU

positive cells in UZ and HZ regions were counted and percentage of $BrdU^+$ per total cells calculated. In B and D, red bars indicate data from control and blue bars from mutant mice. Data shown are mean±SEM; in each graph, statistics compared control vs. mutant in UZ or HZ using an unpaired t-test; **p<0.01, ***p<0.001, NS=no significant difference.

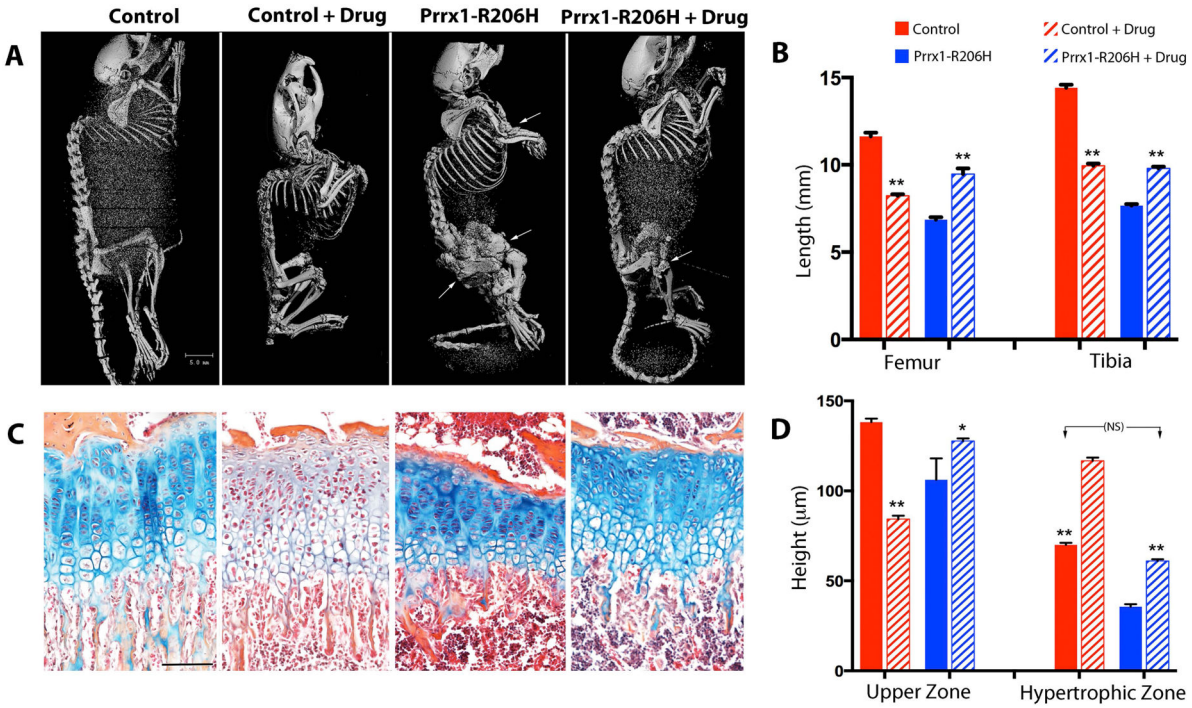

Fig. 6.

Palovarotene preserves long bone growth and growth plate organization in *Prrx1-R206H* mice. (A) Control and mutant mice were treated with Palovarotene (drug) or vehicle from birth (P1) to 30 days of age and then examined by μCT analysis to visualize skeletons and heterotopic bone (arrows) (n= 6 mice per group). The observed spinal curvatures are an artifact of sample position in μCT tubes, not the result of defects in the spine. (B) Quantification of femur and tibia lengths by μCT imaging (n=6 mice per group). (C) Proximal tibial growth plates were stained with Alcian blue, Orange G, and eosin. Note that Alcian blue staining cartilage matrix is markedly reduced in drug-treated controls (second panel from right) but is preserved in drug-treated mutants (leftmost panel) (n=4 mice per group). Scale bar, 100 μm. (D) Quantification of upper zones and hypertrophic zones from histological analysis. In B and D, red bars indicate data from control and blue bars from mutant mice; hatched bars represent data from palovarotene-treated mice. Data shown are mean±SEM; statistics compared control vs. control+drug or mutant vs. mutant+drug using a two-way Anova; **p<0.01, *p<0.05. Note that (in panel D), lengths of hypertrophic zones in mutants treated with drug (blue hatched bar) are protected and not significantly (NS) different from untreated controls (red bar).