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Original Article

Methods for the reliable induction of heterotopic ossification in the conditional *Alk2^{Q207D}* mouse

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Abstract

Objectives: Conditional *ALK2$^{Q207D\text{-}floxed}$* (*caALK2^{fl}*) mice have previously been used as a model of heterotopic ossification (HO). However, HO formation in this model can be highly variable, and it is unclear which methods reliably induce HO. Hence, these studies report validated methods for reproducibly inducing HO in *caALK2^{fl}* mice. **Methods:** Varying doses of Adex-cre and cardiotoxin (CTX) were injected into the calf muscles of 9, 14, or 28-day-old *caALK2$^{fl/-}$* or *caALK2$^{fl/fl}$* mice. HO was measured by planar radiography or microCT at 14-28 days post-injury. **Results:** In 9-day-old *caALK2$^{fl/-}$* or *caALK2$^{fl/fl}$* mice, single injections of 10^9 PFU Adex-cre and 0.3 µg of CTX were sufficient to induce extensive HO within 14 days post-injury. In 28-day-old mice, the doses were increased to 5 x 10^9 PFU Adex-cre and 3.0 µg of CTX to achieve similar consistency, but at a slower rate versus younger mice. Using a crush injury, instead of CTX, also provided consistent induction of HO. Finally, the Type 1 BMPR inhibitor, DMH1, significantly reduced HO formation in 28-day-old *caALK2$^{fl/fl}$* mice. **Conclusions:** These data illustrate multiple methods for reliable induction of localized HO in the *caALK2^{fl}* mouse that can serve as a starting point for new laboratories utilizing this model.

Keywords: Fibrodysplasia Ossificans Progressiva, BMP, Acvr1/ALK2, Muscle Injury, Mouse Model

Introduction

Heterotopic ossification (HO) of skeletal muscle is among the most common adverse events following major orthopaedic surgeries such as spine fusion and total joint arthroplasty[1,2] and major musculoskeletal injuries[3-5]. There are currently no approved treatments for HO, regardless of the cause. For decades, progress in HO research was stymied by the lack of appropriate and reliable animal models because of the poor incidence of HO in trauma-induced models[6] and the unknown cause of HO in congenital diseases.

A mouse carrying a cre-inducible transgene encoding ALK2^{Q207D} (a human gene with Q207D mutation, *caALK2^{fl}*), a constitutively active mutant, had previously been created to study the developmental role of ALK2[7], and was later adapted for use as an early model of HO[8]. The *CAG-Z-EGFP-ALK2^{Q207D}* transgene (*caALK2^{fl}*) contains a *lox-lacZ-poly A sites-lox* sequence between the promoter and *ALK2^{Q207D}* coding sequence to prevent translation of ALK2^{Q207D} and eGFP prior to cre-mediated recombination[7]. Cre-mediated recombination of the lox sites excises the lacz and polyA sequences leading to overexpression of ALK2^{Q207D} and eGFP driven by the CAG promoter[7]. Intramuscular injection of Ad5-CMV-cre (Adex-cre) can be used to induce permanent recombination and ectopic overexpression of caALK2 in

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all recombined cells that is restricted to the injection site[8]. Yu, et al. first reported the induction of HO in this model via intramuscular injection of 10^8 PFU Adex-cre alone in neonatal, 7 day-old mice[8]. Using this system, we reported that retinoic acid antagonists can mitigate formation of HO through destabilization of Smad1/5[9]. Safety and efficacy of palovarotene, one of the derivatives of the retinoic acid antagonists, is now being tested in a Phase 3 clinical trial (NCT03312634) for the treatment of HO in fibrodysplasia ossificans progressiva (FOP) patients (see below). This system was also used to explore a mechanism to develop trauma-induced intramuscular fibrosis which is associated with HO. We found increased mTOR signaling is critical for fibrosis and rapamycin treatment significantly reduces formation of fibrosis and HO[10]. Similar results have been reported using induced pluripotent stem (iPS) cells from FOP patients[11,12]. These and other studies demonstrate the utility of the *caALK2*^{fl} mice for studies that are applicable to both genetic and acquired forms of HO.

HO also occurs in FOP, where it often leads to complete immobilization[13]. FOP is a rare congenital genetic disorder characterized by malformations of the great toes (hallux valgus) and tumor-like fibrous swellings that famously progress to extensive HO in skeletal muscle and adjacent connective tissues[14]. In 2006, Shore et al., reported that FOP is caused by a point mutation in the Type 1 BMP Receptor, ALK2, with the most common mutation being *ALK2*^{R206H} [15]. This and other ALK2 mutations that cause FOP result in aberrant ALK2 activation, phosphorylation of Smad 1/5, and inappropriate transcription of BMP target genes[16].

The discovery of *ALK2* mutations as the cause of FOP led to the creation of multiple mouse models carrying analogous mutations in *ALK2*[7,8,17-19]. Chimeric mice carrying an *ALK2*^{R206H} knock-in allele recapitulated many of the phenotypes seen in FOP patients including malformation of the great toes, injury-induced intramuscular HO and spontaneous formation of osteochondromas[18]. Use of a mouse model that accurately mimics the clinical phenotype led to a major breakthrough in the pathology of FOP, i.e. FOP-causing mutations in ACVR1 cause aberrantly increased BMP-Smad signaling by widening ligand sensitivity towards activin A[11,17,19-21]. An antibody treatment against activin A is now in a clinical trial for FOP patients (NCT03188666).

The Q207D mutation has not been found in nature and should not be confused with FOP-causing mutations in ACVR1. Unlike the most common cause of FOP (ALK2^{R206H}), caALK2 (ALK2^{Q207D}) displays constitutive ligand-independent kinase activity[22]. Another important difference between the caALK2 mouse and murine models of FOP carrying inducible *ALK2*^{R206H} mutations is the mode of mutant ALK2 expression. The two reported mouse models of inducible *ALK2*^{R206H} are knock-ins, created by inserting floxed constructs into the endogenous murine Alk2 locus, enabling cre-mediated expression of *ALK2*^{R206H}, presumably, under the control of endogenous regulatory elements, leading to near-normal physiological expression patterns. In contrast, the caALK2 mouse is a transgenic in which an entirely synthetic caALK2 floxed gene driven by a constitutively active promoted was randomly inserted into the mouse genomic DNA. Thus, cre-mediated recombination of the caALK2 gene results in overexpression of the protein in all cells that transiently express cre. Still both mutations result in aberrant, excessive ALK2 signaling that can lead to HO in injured skeletal muscle[8,15,17,19,23]. While newer mouse models with conditionally inducible *ALK2*^{R206H} mutations are now used as more accurate models of FOP[17,19], *caALK2*^{fl} mice remain a valuable model for the study of both FOP-related HO and trauma-induced HO[10,24-30].

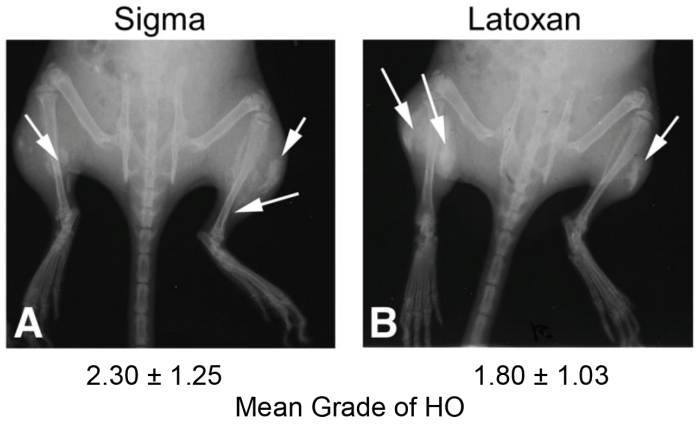
Yu, et al. first reported the induction of HO in this model via intramuscular injection of 10^8 PFU Adex-cre alone in neonatal, 7 day-old mice[8]. However, this method gave inconsistent results in other labs (personal communications) and the developmental state of 7-day-old mice may not accurately reproduce the mechanisms of HO formation in adolescent and adult patients, necessitating refinement of the model. Muscle injury via co-injection of cardiotoxin (CTX), an established model for muscle injury and repair[9,31], may increase the consistency of intramuscular endochondral HO[9], but the optimal timing, doses, and age of the mice was not fully established. Hence, we initiated a series of experiments to improve the induction of intramuscular HO in *caALK2* mice using a combination of intramuscular injections of Adex-cre for recombination and cardiotoxin (CTX) for muscle injury. Here, we present the effects of varying genotype, PFUs of Adex-cre, amount and frequency of CTX, injection volume, and age on the formation of HO in *caALK2*^{fl} mice leading to the establishment of multiple protocols that, in our hands, consistently produce HO in hindlimb muscles.

Methods

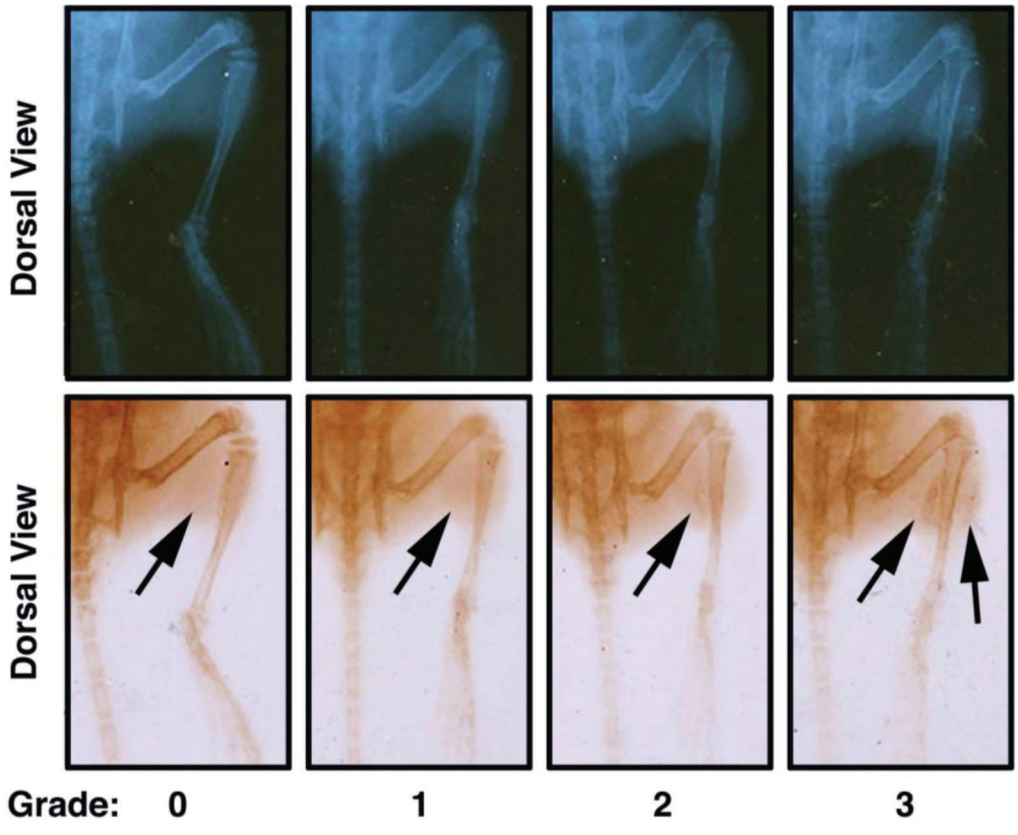
All procedures involving live animals were performed in accordance with the policy and federal law of judicious use of vertebrate animals as approved by the Institutional Animal Care and Use Committee at Vanderbilt University or the University of Michigan. Mice carrying the inducible *caALK2*^{fl} transgene were described previously[7]. Importantly, homozygous mice (*caALK2*$^{fl/fl}$) are healthy and fertile, allowing the line to be maintained as homozygotes.

Toxin-induced muscle injury

Local expression of caALK2 and skeletal muscle injury were induced by respective intramuscular injections of *Ad5-CMV-cre* (*Adex-cre*) (Baylor University Vector Development Laboratory, Houston TX) and CTX (Sigma, Product Number C9759) in the lower hind limb muscles at the times, doses, and volumes indicated in Results. Prior to submission of this paper, the CTX product from Sigma was discontinued by the supplier. Since then, we have used CTX



Supplemental Figure 1. Cardiotoxin from a new source produces a comparable amount of HO. Bilateral co-injection of 7x10^9 PFU Adex-cre and 0.3 µg of CTX in the calf muscles of 21-day-old caALK2$^{fl/fl}$ mice resulted in HO detectable at 14 days post-injury. (A) CTX from SIGMA was used. (B) CTX from Latoxan was used. n=10 per group. The amount of HO was not quantified; rather, we qualitatively assess levels of HO from the X-ray radiographs as summarized in Supplemental Figure 2. As shown, we divided HO samples into 4 groups (0 as no signs of ossification, 3 as full of HO in a leg, and set 2 additional grades between). We set up two groups using 0.7x10^9 PFU of Adex-Cre (Vector Development Lab) per site to compare the influence of CTX from two suppliers, (1) SIGMA, 0.3 µg/site or (2) Latoxan, 0.3 µg/site. Typical examples from group 1 and 2 are shown in Supplemental Figure 1. There was not a statistically significance difference between the groups (p=0.343 by Student's t-test).



Supplemental Figure 2. HO was visualized by X-ray radiographs and the degree of HO was classified to 4 groups: 0 as no signs of HO, 1 as lightly mineralized HO, 2 as heavily mineralized but restricted to one area of the lower limb or 3 as extensive HO throughout the lower leg muscles. An example of a right leg in each category is shown. Top, native films. Bottom, inverted color pallete with Photoshop.

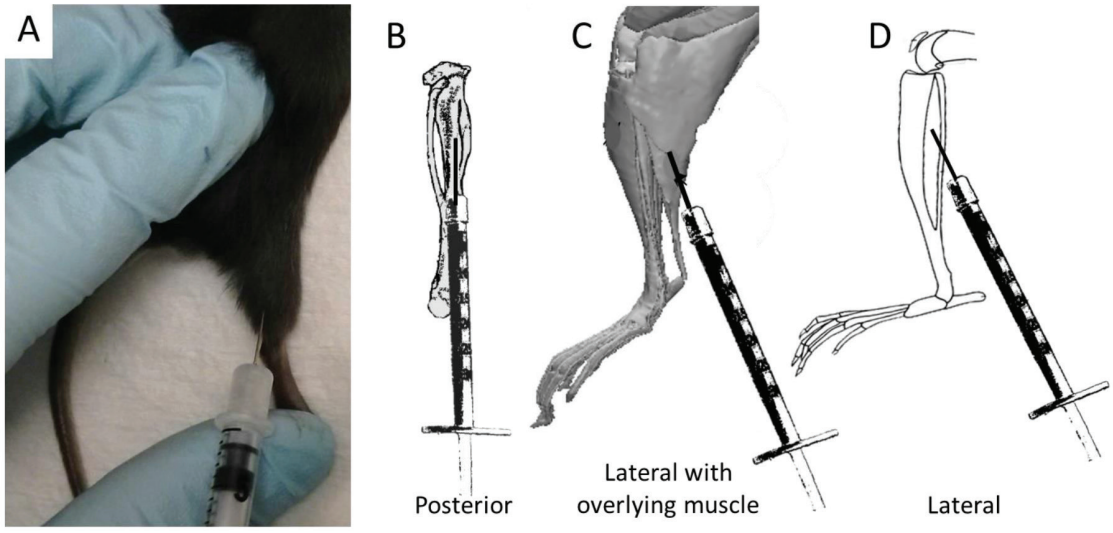


Figure 1. Positioning of the mouse and needle during injection of the lower hindlimb muscles. (A) The thumb and forefinger are placed on the foot and pelvis to gently hold the leg in near-full extension. (B) With the syringe and needle aligned with the long axis of the lower limb, (C) the needle is inserted just proximal to the junction of the Achilles tendon with the gastrocnemius until it very gently touches the poster surface of the tibia. (D) The needle is then retracted approximately 1 mm before injecting the solution.

Table 1. Summary of experimental conditions used in Figures 2-6.

	Age @ Injury (days)	Genotype(s)	Adex-cre (PFU)	CTX (µg)	Injection Volume (µl)
Figure 2	10-13	*caALK2*$^{fl/fl}$;*mTmG*$^{+/-}$ or *caALK2*$^{fl/fl}$	0.7x10^9	0.3	10
Figure 3 A-C	9	*caALK2*$^{fl/-}$	1.0x10^9 or 1.9x10^9	0.3	10 or 40
Figure 3 D-F	9	*caALK2*$^{fl/-}$ or *caALK2*$^{fl/fl}$	1.9x10^9	0.3 @ P7-9 or only P9	10
Figure 4	7	*caALK2*$^{fl/-}$	1.0x10^9	none	10
Figure 5	21	*caALK2*$^{fl/fl}$	5.0x10^9	3.0	20
Figure 6	28	*caALK2*$^{fl/fl}$	4.44x10^9	1.5	20

from Latoxan (Product Number L8102, Latoxan, Valence, France), which produces similar results with the same dose as the previous supplier (Supplemental Figure 1).

Mice were anesthetized with 1-4% isoflurane in oxygen. For mice greater than 20 days old, the lower hind limbs were shaved and gently scrubbed with betadine. The mice were placed prone and the hindlimb extended. A 29G 0.5 in. needle was inserted in the area of the mid-gastrocnemius with a posterior approach, holding the needle at an approximate 20-degree angle to the leg (Figure 1). The needle was inserted into the muscles until gently contacting the posterior surface of the tibia, taking care not to scratch or substantially injure the periosteum, then retracted slightly before administering the injection. All animals were monitored at least twice weekly from the time of injection until being humanely sacrificed, 10-28 days after injection of CTX.

Crush Injury

In one experiment, 7-day-old mice received i.m. injections of 10^9 PFU of Adex-cre followed by the creation of a crush injury at the same location the following day. Adex-cre was injected into the lower hindlimb as above and returned to their cages. The following day, and under deep anesthesia, hemostats were used to create a severe crush injury of the lower hindlimb muscles, taking care to avoid breaking the fibula. With mice under deep anesthesia, the mid-belly of the gastrocnemius and overlying skin was grasped in the jaws of 3.5 mm wide, smooth surfaced hemostats which were then closed to the third rachet position and held for 2-3 seconds before release. *Table 1 summarizes variables in each experiment*. Mice received 0.1 mg/kg buprenorphine s.c. while under anesthesia and twice daily for the next 4 days.

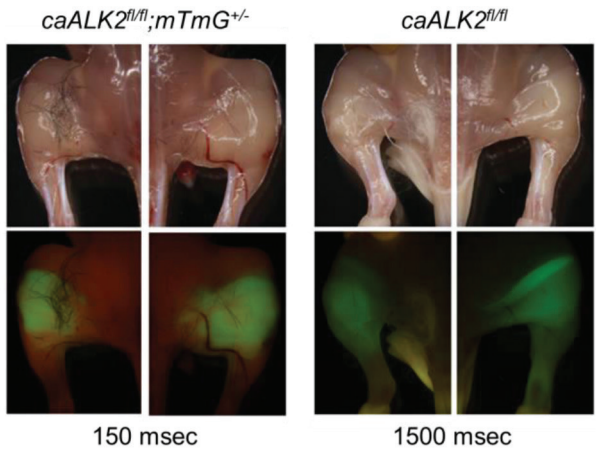


Figure 2. Weak green fluorescence was detected from the transgene construct. Both calf muscles of 10-13-day-old *caALK2*$^{fl/fl}$ mice were co-injected with 0.7x10^9 PFU Adex-cre and 0.3 µg of CTX. Fluorescence was examined with the skin removed 10 days after injection. Left, mice also carrying one copy of the *mTmG* cre-reporter (*caALK2*$^{fl/fl}$*;mTmG*$^{+/-}$, red before cre activity and green after cre activity). Right, *caALK2*$^{fl/fl}$ mouse without *mTmG* cre-reporter (no fluorescence without cre activity and green with cre activity). Dorsal views of left and right legs in bright field and GFP channel are shown with the exposure time for GFP imaging indicated. In these conditions, 1500 msec exposure was required to achieve similar levels of GFP signal from *caALK2*$^{fl/fl}$ mice without mTmG cre-reporter as seen in *caALK2*$^{fl/fl}$*;mTmG*$^{+/-}$ mice with only 150 msec of exposure.

ALK Inhibitor Treatment

To determine whether inhibition of Type 1 BMP Receptors was capable of preventing HO formation when using the refined methodology, *caALK2*$^{fl/fl}$ mice were treated twice daily with DMH1 or vehicle. Intramuscular injections of 4.44x10^9 PFU Adex-cre and 1.5 µg CTX were administered at p27. DMH1 was dissolved at 2.5 mg/ml in 44% w/v aqueous (2-hydroxypropyl-β)-cyclodextrin (Sigma-Aldrich, Product H5784), and mice received intraperitoneal injections of 10 mg/kg DMH1 (4 µl/g of bodyweight) or vehicle alone twice daily (total dose of 20 mg/kg/day) from the time of injury until sacrifice.

Analyses of HO formation

The expression of eGFP from the *CAG-Z-EGFP-ALK2*^{Q207D} transgene or mTmG Cre-reporter in mouse hindlimb muscles was examined under a fluorescence stereoscope (Leica Microsystems GmbH., Wetzlar, Germany) immediately after sacrifice and removal of the skin. Image exposures were either 150 or 1500 ms.

HO formation was monitored via plain radiographs at weekly intervals after administration of CTX. Radiographs were acquired at 35kV for 4 seconds using a LX-60 digital closed cabinet x-ray unit (Faxitron Bioptics, LLC, Tucson, AZ) at VUMC or an MX20 X-ray unit (Faxitron Bioptics, LLC, Tucson, AZ) with BioBlue-MR film (ALKali Scientific, Pompano Beach, FL) at UM. Film x-rays were subsequently digitized on a flatbed scanner. Within each study all radiographs were acquired at the same magnification. The area of HO in the hindlimbs was semi-quantified from the radiographs using ImageJ. All images were viewed with same brightness and contrast. The area of each mineralized lesion was outlined, and the total HO area recorded in arbitrary units.

In some studies, HO was analyzed by micro computed tomography (µCT) of the excised hindlimbs. Briefly, microCT images of the entire hindlimb were acquired in a µCT50 (Scanco Medical AG, Switzerland) at 55 kVp, 200 µA, projected through a 0.5 mm Al filter, 750 projections/180° rotation, an integration time of 500 ms/projection, with a nominal isotropic resolution of 10 µm in a 15.2 mm field of view. The manufacturer's 1200 mgHA/ccm beam hardening correction was applied during image reconstruction. The volume of HO was determined by selecting the entire limb as the volume of interest, but excluding the tibia, femur, fibula, and patella then applying a threshold of 225 mgHA/ccm with noise filter settings of Support 1 and Sigma 0.3. The volume of bone in the resulting 3D structure was calculated by direct voxel counting.

Statistical analyses

Within each experiment, the area or volume of HO was compared among treatments using Student's t-test, Two-way ANOVA, or Repeated Measures ANOVA with Tukey's post-hoc test. All data are reported as mean ± SEM and $p<0.05$ was considered statistically significant.

Results

Global overexpression of the *caALK2* transgene in the current mouse model has dramatic effects in multiple tissues which preclude such an approach[7]. Hence, efficient

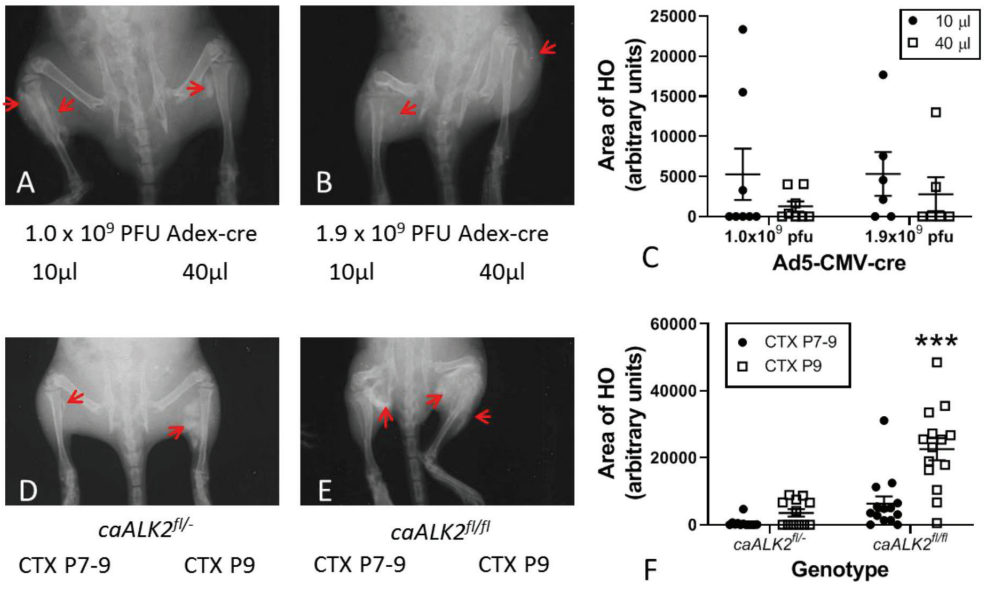


Figure 3. The volume of HO is increased by gene dose and inhibited by multiple injections of CTX prior to Adex-cre injection. (A-C) Both calf muscles of *caALK2*$^{fl/-}$ mice were injected with 0.3 µg of CTX on P7, P8, and P9. On P9, the mice also received intramuscular injections of either 1.0 x 10^9 pfu or 1.9 x 10^9 pfu of Adex-Cre. Both legs of each mouse received the same dose of virus, but the virus was delivered in 10 µL in the left leg or 40 µL in the right leg. (*N=6 legs per group*) Mice were sacrificed at P23, 14 days after injury. (D-F) Both legs of *caALK2*$^{fl/-}$ or *caALK2*$^{fl/fl}$ mice (*N=14 per genotype*) received 1.9 x 10^9 pfu of Adex-Cre in 10 µL PBS at P9. Right legs received a single injection of 0.3 µg of CTX at P9. Left legs were injected with 0.3 µg of CTX at P7, P8, and P9. *p<0.01 vs all other * by two-way ANOVA and Tukey's post-hoc test.

local recombination/induction of caALK2 transgene expression in HO studies must be limited to skeletal muscle which was achieved previously via intramuscular injection of Adex-cre virus in the hindlimb at P9[8]. However, the effect of variations in viral load on the extent of HO was not established. Previous reports also used i.m. injection of CTX to induce injury at the site of Adex-cre injection[9]. However, influences of the amount of CTX, volume of the bolus injection, and/or frequency of CTX injections on the reliability of HO formation were not previously reported.

Cre-mediated recombination of the *CAG-Z-EGFP-ALK2*^{Q207D} transgene induces *permanent recombination and overexpression* of both caALK2 and eGFP[7] enabling direct imaging of eGFP fluorescence (Figure 2) as a marker of recombination and surrogate for caALK2 expression[7]. Although fluorescence levels from the transgene are approximately 1/10th of those from the mTmG cre reporter, the GFP signal in *caALK2*$^{fl/fl}$ mice could be used to confirm efficient recombination limited to the upper and lower hindlimbs after i.m. injection of 5x10^9 PFU of Adex-cre in 11-13-day-old mice (Figure 2).

The effects of moderate changes in the viral load of Adex-cre and the volume of the injection were examined by injecting the hindlimbs of 9-day-old *caALK2*$^{fl/-}$ mice with either 1.0x10^9 or 1.9x10^9 PFU of Adex-cre in 10 or 40 µl of PBS containing 1% BSA on post-natal day 9. In this study, all hindlimbs were injected with 0.3 µg of CTX once daily on P7-P9. Radiographic analysis of HO 14 days after Adex-cre injection demonstrated significant HO in all hindlimbs (Figure 3A and B). The radiographic area of HO was not statistically different regardless of viral dose or injection volume, although there was a trend toward decreased HO with the larger, 40 µl injection (Figure 3C).

The effects of genetic dose of *caALK2* and repeated CTX injections were examined in a second study in which the lower hindlimbs of *caALK2*$^{fl/-}$ and *caALK2*$^{fl/fl}$ were injected with 0.3 µg of CTX only on P9 or on P7-P9 followed by injection of 1.9 x 10^9 PFU Adex-cre on P9. Again, HO was radiographically detectable in all hindlimbs at 14 days after injury (Figure 3D and E). Analysis revealed that *caALK2*$^{fl/fl}$ mice formed significantly more HO than *caALK2*$^{fl/-}$ mice regardless of the number of CTX injections (p<0.01), while multiple CTX injections surprisingly resulted in significantly less HO than the single injection at P9 (Figure 3F).

While CTX provides reliable muscle injury and induction of HO in the *caALK2* mice, it is not clear how well this mimics contusions or crush injuries that are commonly experienced by FOP patients. Further, developing a method that reduces the variance of the amount of HO formed compared to CTX-induced injury is desirable. To address both of these concerns, hemostats were used to create a muscular crush injury immediately after injection of 1.0 x 10^9 PFU Adex-cre in the lower hindlimbs of *caALK2*$^{fl/-}$ mice

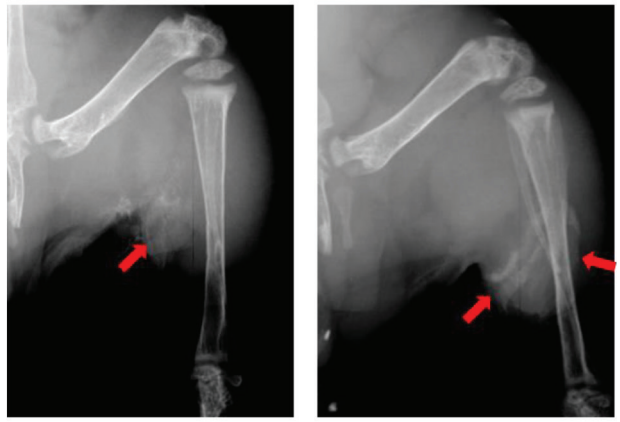
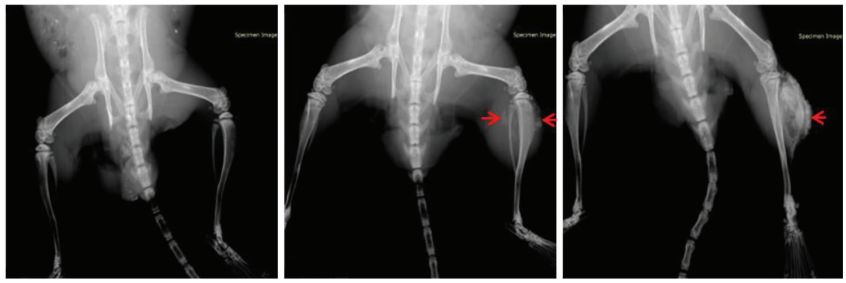


Figure 4. HO can be induced by physical crush injury in *caALK2*$^{fl/-}$ mice. Mice received bilateral injections of 10^9 PFU Adex-cre in the lower hindlimb muscles at 7 days of age. Hemostats were used to create a crush injury in the left tibialis muscles the following day. HO was evident in all crush injured legs (red arrows), on day 14 post injury.



Days Post Injury	7	14	21
Area	12.8 ± 11.1	63.0 ± 24.9	143.7 ± 56.3*
Incidence	2/7	6/7	7/7

Figure 5. HO can be reliably induced in 21-day-old *caALK2*$^{fl/fl}$ mice. Co-injection of $5x10^9$ PFU Adex-cre and 3.0 µg of CTX in the calf muscle resulted in HO detectable at 2 wk and maturing by 3 wk. Area is mean arbitrary units ± SEM. *p<0.05 vs Day 7 by RM-ANOVA and Tukey's post-hoc test. N=7 per time point

at P7. At 14 days post-injury, mineralized intramuscular HO was visible on all radiographs (Figure 4). In contrast to the diffuse localization of CTX-induced HO, the crush injury resulted in HO that was highly localized. However, the amount of HO formed was considerably less than that seen with a single injection of 0.3 µg CTX (Figure 3D) and still variable.

These studies established reliable methods to induce HO in young, 7-9-day-old mice, which is consistent with the developmental stage at which many FOP patients experience their first flares[32]. However, FOP patients experience flares throughout their lives[14,32], and acquired trauma induced-HO is more common in adults[33-35]. Concerns were raised that age-related physiological differences between neonates and adults could alter the disease process and affect the accuracy of the model. Hence, methods to induce HO in older mice are needed to address these questions.

Early attempts to induce HO in 21-day-old adolescent *caALK2*$^{fl/-}$ mice using the protocol above resulted in the formation of little, if any, HO (data not shown). Considering the dramatic increase in muscle volume of the 21-day-old mice, we reasoned that the larger muscle volume might require more CTX and Adex-cre to achieve the same relative rate of injury and infection/recombination, respectively, as in the younger mice. The amount of Adex-cre was increased from $1x10^9$ to $5x10^9$ PFU and CTX was increased from 0.3 µg to 3.0 µg, and a 10 µl mixture of the

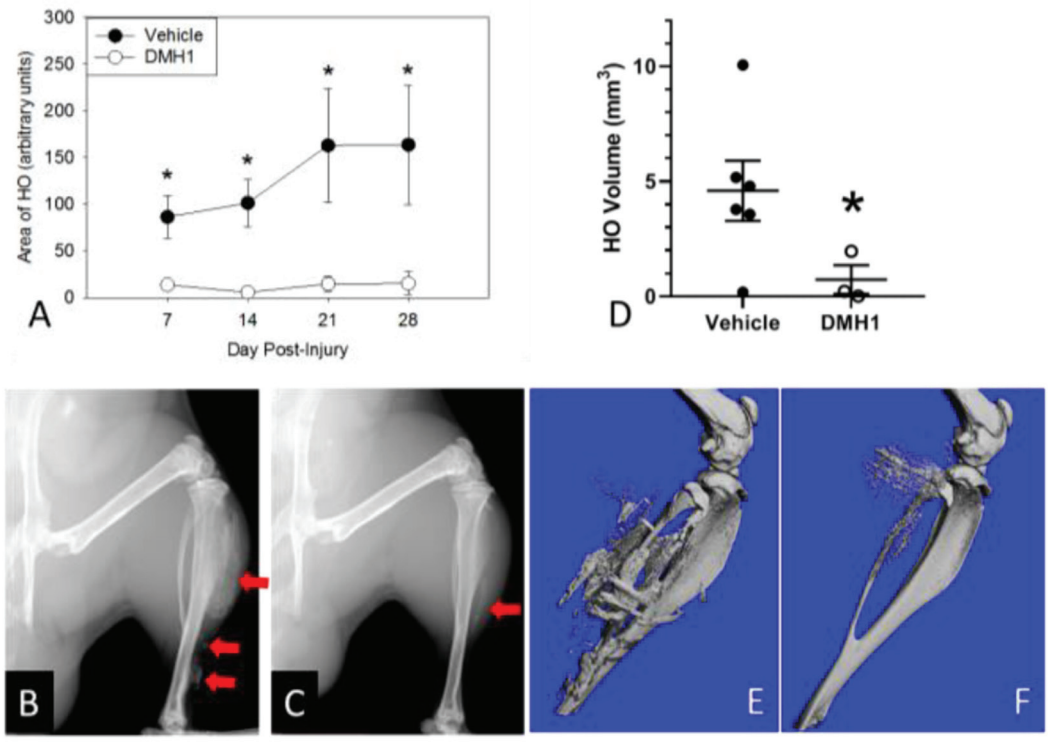


Figure 6. Small molecule ALK inhibition prevents HO in 28-day-old *caALK2*$^{fl/fl}$ mice. Intramuscular injections of 4.44x10^9 PFU Adex-cre and 1.5 μg CTX were administered at p27. (A and B) Vehicle-treated mice developed extensive HO that expanded until 21 days post-injury, then plateaued to day 28. Intraperitoneal injection of the ALK-inhibitor DMH1 (10 mg/kg bid from the time of muscle injury until sacrifice) almost completely prevented the formation of HO, measured in weekly *in vivo* radiographs (A and C) and *ex vivo* microCT (D and F). Representative radiographs (B and C) and microCT projections (E and F) of legs from Vehicle (B and E) and DMH1 (C and F) treated mice at day 28 post-injury are shown in B and C, respectively. N=8 per group; *p<0.01 vs DMH1 at the same time point by ANOVA and Tukey's post-hoc test in A or Student's t-test in D.

two was injected into the lower hindlimb muscles. Weekly radiographs revealed detectable HO in 2 of 7 mice at 7 days post-injury, which increased to 6 of 7 at 14 days, and 7 of 7 at 21 days post-injury (Figure 5). Although the formation of HO appears to be slower in the 21-day-old mice, the robustness and variance of HO in 21- and 28-day-old *caALK2*$^{fl/fl}$ mice using this protocol appeared similar to that in younger 7-9-day-old mice (Figures 3-4). However, the efficiency of HO formation was drastically reduced when muscle injury was performed in 35- and 41-day-old mice using the same regimen and *caALK2*$^{fl/fl}$ mice (not shown).

Finally, we tested the ability of the Type 1 BMP Receptor inhibitor, DMH1 to block HO formation using our preferred protocol in 28-day-old mice. Both lower hindlimbs of twenty-eight (28) day-old *caALK2*$^{fl/fl}$ mice were injured with 1.5 μg CTX and injected with 5x10^9 PFU Adex-crein consecutive 10 μl injections. The mice received 2-hydroxypropyl-betacyclodextran (Vehicle) or 10 mg/kg DMH-1 in Vehicle via intraperitoneal injection twice-a-day from the time of injury until sacrifice, 28-days after injury. Intramuscular HO was evident on radiographs of Vehicle treated mice as early as 7 days post injury and increased in area until day 21 before plateauing between days 21 and 28 (Figure 6A). The radiographic area of HO in DMH1 treated mice was significantly less than that of Vehicle treated mice at all time points (Figure 6A) and was most pronounced at days 21 and 28 (Figures 6A-C). These findings were confirmed by *ex vivo* microCT analysis of HO volume in the excised hindlimbs (Figure 6D-F).

Discussion

Research on intramuscular ossification was long impeded by the lack of reproducible animal models. Creation of the inducible caALK2 mouse model[7,8], accurate mouse models of FOP[17,19], and reproducible models of traumatic HO[36-46] has tremendously increased the pace of research and drug development in the field. However, the models still have limitations and can be challenging to use in a reproducible manner.

We observed a generally consistent pattern of HO in the

caALK2^{fl} *mice, regardless of the protocol. Following direct injection of Adex-cre and CTX in the gastrocnemius muscle, soft tissue mineralization can be detected as early as 7 days post-injury by planar digital radiography, although this was delayed in 21-28 day old mice. The earliest mineralization is generally localized to the lower hindlimb muscles, but mineralization is also found in the upper hindlimb muscles of most mice at post-injury day 14. In the more severe cases, ankylosis of the knee may occur between days 10 and 21. Therefore, it is important to monitor ambulation of the mice to ensure their ability to freely access food and water. Mice with bilateral ankylosis or those with unilateral ankylosis that severely impairs their ability to stand or walk should be humanely euthanized, regardless of the planned experimental endpoint.*

Our earliest experiments explored the effects of small variations in the dose of Adex-cre virus and/or the volume injected into the calf muscle of *caALK2*$^{fl/-}$ mice (Figure 3A-C). Small changes in the dose of intramuscular Adex-cre had little effect on HO in 9-day-old mice (Figure 3). However, larger increases (≥5.0 x 10^9 PFU) may enhance the formation rate and volume of HO and were required to reliably induce HO in 28-day-old mice (Figure 5). An age-related decline in the frequency or extent of HO forming flares has not been reported in FOP patients[14,32], suggesting this is a mouse- or method-specific limitation. The mechanisms responsible for the age-related changes in the caALK2 mouse are unclear, and multiple possibilities exist. This may be due to the larger muscle volume in older mice requiring a proportionate increase of virus in order to reach a theoretical minimum recombination rate that is required to initiate HO. Alternatively, aging could increase the fraction of recombined cells that is sufficient to induce HO or decrease the pool of susceptible HO progenitors. Indeed, the observation that consistent formation of HO in ALK2^{Q207D} mice required a dramatic increase in the PFUs of Ad-cre suggests this is related to the proportion of infected and recombined cells and/or the extent of injury relative to total muscle volume. In contrast, multiple publications have reported the consistent formation, though variable rate, of HO in conditional ALK2^{R206H} mice up to 6 months old[19,20]. These models circumvented the limitations of local Ad-cre injection by using globally expressed inducible cre transgenes to activate post-natal expression of ALK2^{R206H}, also suggesting the age-related phenotype in caALK2 mice may be dependent on the number of progenitor cells infected by Ad-cre. Unfortunately, the forced overexpression of ALK2^{Q207D} precludes global recombination due to adverse effects in other tissues[7]. Regardless, investigators using caALK2 mice must be careful to take this limitation into account.

Some had speculated that tissue expansion during bolus intramuscular injections could contribute to muscle injury and therefore enhance HO. However, that was not seen here (Figure 3). Instead, there was a non-significant trend toward less HO when injecting 40 µl vs 10 µl containing the same total PFUs of Adex-cre virus. This may suggest the concentration of Adex-cre is more important than volume or that the larger bolus washes the virus out of the muscle and into other compartments before infecting the local tissue. *The later possibility is supported by observations of rapid inflation and deflation of the of the lower hindlimb at the time of bolus injection and the appearance of GFP expression in the upper hindlimb muscles of mice receiving the smaller 10 µl injection (Figure 2)*.

A single injection of CTX also appears to create a larger volume and more consistent HO than multiple injections. Three injections of CTX on consecutive days immediately before injection of Adex-cre produced significantly less HO than a single injection of the same dose (1/3 of the cumulative dose) (Figure 3D-F). While three consecutive injections of CTX should cause more severe injury which typically correlates with HO formation, we speculate that the repeated CTX treatments may disrupt early inflammatory and repair processes required to trigger HO.

Although there are some reports of HO studies in mice injured at ≥9-days old[17,47,48], several laboratories have reported inconsistent or no HO when injuring older *caALK2*^{fl} mice (personal communications). Importantly, the methods presented here consistently induce HO in adolescent 21-28-day-old mice by increasing the doses of virus and CTX (Figures 5 and 6). The ability to induce HO in adolescent mice provides an opportunity to screen therapeutics that work by oral administration. This is an important requirement to establish a treatment for FOP since injections hold the potential to cause a flare and additional HO in FOP patients.

Our laboratories' extensive experiences with the *caALK2*^{fl} and conditional *ALK2*^{R206H} mice have revealed several additional variables that require attention for reproducible results. The exact location of the i.m. injections appears to be less important than the precision, which is critical for reproducible results. To this end, we recommend that all i.m. injections for a given study be performed by a single, well-trained and experienced person. Care should also be taken in sourcing and maintaining quality of Adex-cre and CTX. We have tested Adex-cre from multiple sources. In our experience, the activity Adex-cre from most commercial vendors was highly variable when used for i.m. injections. Hence, we recommend sourcing virus from a proven vendor and validating multiple lots in multiple labs, if possible. The activity and consistency of CTX is also critical. Although Sigma discontinued the product CTX product used in these studies, the CTX marketed by Latoxan produces comparable results (Supplemental Figure 1).

Early papers using caALK2 mice to study injury-induced intramuscular HO reported different sources of reagents, age at injury, genotype, and technique. The studies presented here exemplify the authors' most reproducible methods for injury-induced formation of HO in 9-28-day-old *caALK2*^{fl} mice. While these can serve as thoroughly evaluated starting points for other laboratories, it is apparent that multiple variations can be used to adjust the extent of HO in this model. Over 300,000 patients receive

hip arthroplasties each year and HO occurs in over 65% of hip replacement patients[5,49]. More than 1,500,000 people living with limb loss in the US alone also have a high risk to develop HO[50]. It is likely those patients develop HO without known causative mutations for FOP. A common downstream event among traumatic and genetically induced HO is the increased BMP-Smad signaling, thus *caALK2*^{fl} mice and other FOP-model mice will complement each other to further deepen our understanding of the mechanisms of HO. These methods described here may accelerate the establishment of this and related models of HO in additional laboratories, speeding research and the eventual creation of therapeutics.

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