HHS Public Access

Author manuscript

Biochem Biophys Res Commun. Author manuscript; available in PMC 2024 December 25.

Published in final edited form as:

Biochem Biophys Res Commun. 2023 December 25; 688: 149147. doi:10.1016/j.bbrc.2023.149147.

Enhanced BMP signaling in *Cathepsin K*-positive tendon progenitors induces heterotopic ossification

Hiroyuki Yamaguchi[1,#], **Margaret Li**[1,2,#], **Megumi Kitami**[3,4], **Sowmya Swaminathan**[1,5], **Yuji Mishina**[6], **Yoshihiro Komatsu**[1,7,*]

[1]Department of Pediatrics, McGovern Medical School, The University of Texas Health Science Center at Houston, Houston, TX 77030, USA

[2]Department of Kinesiology, Rice University Wiess School of Natural Science, Houston, TX 77005, USA

[3]Division of Dental Pharmacology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, 951-8514, Japan

[4]Center for Advanced Oral Science, Niigata University Graduate School of Medical and Dental Sciences, Niigata, 951-8514, Japan

[5]The College of Natural Sciences, The University of Texas at Austin, Austin, TX 78712, USA

[6]Department of Biologic and Materials Sciences & Prosthodontics, School of Dentistry, University of Michigan, Ann Arbor, MI 48109, USA

[7]Graduate Program in Genetics and Epigenetics, The University of Texas Graduate School of Biomedical Sciences at Houston, Houston, TX 77030, USA

Abstract

Heterotopic ossification (HO) is abnormal bone growth in soft tissues that results from injury, trauma, and rare genetic disorders. Bone morphogenetic proteins (BMPs) are critical osteogenic regulators which are involved in HO. However, it remains unclear how BMP signaling interacts with other extracellular stimuli to form HO. To address this question, using the Cre-loxP recombination system in mice, we conditionally expressed the constitutively activated BMP type I receptor ALK2 with a Q207D mutation (Ca-ALK2) in *Cathepsin K-Cre* labeled tendon progenitors (hereafter "*Ca-Alk2:Ctsk-Cre*"). *Ca-Alk2:Ctsk-Cre* mice were viable but they formed spontaneous HO in the Achilles tendon. Histological and molecular marker analysis revealed that HO is formed via endochondral ossification. Ectopic chondrogenesis coincided with enhanced GLI1 production, suggesting that elevated Hedgehog (Hh) signaling is involved in the pathogenesis of HO. Interestingly, focal adhesion kinase, a critical mediator for the mechanotransduction pathway, was also activated in *Ca-Alk2:Ctsk-Cre* mice. Our findings suggest

*Correspondence: Yoshihiro.Komatsu@uth.tmc.edu.

Author Contribution

Y.K. designed the study. H.Y., M.L., M.K., S.S., and Y.K. performed the experiments. H.Y., M.L., M.K., and Y.K. analyzed the data. H.Y., M.L., Y.M., and Y.K. wrote the manuscript.

#The first two authors contributed equally to this work

Declaration of Conflict of Interests

The authors declared no potential conflicts of interest for the research, authorship, and/or publication of this article.

that enhanced BMP signaling may elevate Hh and mechanotransduction pathways, thereby causing HO in the regions of the Achilles tendon.

Keywords

BMP signaling; Hedgehog signaling; Heterotopic ossification; Mechanotransduction; Mouse

Introduction

Heterotopic ossification (HO) is characterized by extraskeletal bone formation in soft tissues, where the bone tissues normally do not develop[1,2]. HO frequently results in patients with reduced mobility and severe pain, and thus severely affects their quality of life.

Following trauma and/or injuries, the destruction of connective tissue structure occurs and osteogenic growth factors including bone morphogenetic proteins (BMPs) are released and initiate the induction process of ectopic bone formation[3]. After the discovery of BMPs, accumulated studies have revealed their significant roles in skeletal development[4,5]. Upon the binding of BMP ligands, BMP type II receptors phosphorylate and activate associated BMP type I receptors, which in turn transduce intracellular signaling by phosphorylating Smad1/5/9, a canonical downstream of BMP signaling[6,7]. Previous studies have demonstrated that a point mutation in one of the BMP type I receptors ALK2 (R206H) leads to the alteration of ligand selectivity and causes fibrodysplasia ossificans progressiva (FOP), a severe form of HO[8,9]. Recent work has led to clinical trials to develop therapies for curing FOP[10-12]. While it is now clear that multiple growth factor signaling pathways such as Hedgehog (Hh) signaling are responsible for HO[13,14], recent studies highlight the importance of mechanical cues from the microenvironment during skeletal development[15,16]. For example, suppression of the mechanotransduction pathway by genetic inactivation and/or a chemical blocker approach inhibits burn and injury-induced HO in mice[17,18]. These studies demonstrate that dysregulated mechanotransduction pathway also plays a role in the etiology of HO.

In this study, we examined the etiology of HO in the Achilles tendon. Interestingly, Hh and mechanotransduction pathways were elevated during ectopic osteochondrogenesis, suggesting dysregulated molecular and mechanical stimuli may be associated with HO in *Ca-Alk2:Ctsk-Cre* mice.

Materials and Methods

Animals

To generate *Ca-Alk2:Ctsk-Cre* mice, we crossed *Ca-Alk2* transgenic mice with *Ctsk-Cre* knock-in mice[19,20]. To examine *Ctsk-Cre* activity, we crossed *Ctsk-Cre* mice with *Rosa26-GFP* reporter mice[21]. These mice were maintained in the animal facility of The University of Texas McGovern Medical School in Houston. The experimental protocol (AWC-21-0127) was reviewed and approved by the Animal Welfare Committee and the Institutional Animal Care and Use Committee of The University of Texas McGovern Medical School at Houston.

The sex of animals

In this study, we focused on analyzing male mice to avoid the confounding variable of the hormone cycle in females, which may indirectly affect the bone-related phenotypes in *Ca-Alk2:Ctsk-Cre* mice.

Micro-computed tomography analysis

Micro-computed tomography (μCT) images were scanned with a CT system at 90 kV energy and 88 μA intensity (CosmoScanGXIII; Rigaku Corporation, Tokyo, Japan). The slices were reconstructed to produce 2D and 3D images, and bone volume was measured using Analyze12.0 (AnalyzeDirect Inc., Overland Park, KS).

Histological Analysis

Hematoxylin and eosin, Safranin O, Picrosirius red staining, and immunostaining analysis were performed as described previously[22-25]. Primary antibodies used in immunostaining were as follows: p-SMAD1/5/9 (Cell Signaling; 9511, 1:50,), Ki-67 (BD Biosciences; 550609, 1:250), SOX9 (Santa Cruz; sc-20095, 1:50), RUNX2 (Cell Signaling; 12556, 1:250), GLI1 (R&D System; AF3455, 1:100), and p-FAK (Invitrogen; 44-626G, 1:200). Stained slides were examined with an Olympus FluoView FV1000 laser scanning confocal microscope using the software FV10-ASW Viewer (version 4.2). At least three images from each slide were obtained and quantified for histological analysis.

Statistical analysis

A two-tailed Student's *t-test* was used for comparisons between the two groups (GraphPad Prism 9 Software). A p-value of less than 0.05 was considered statistically significant.

Results and Discussion

Enhanced BMP signaling in *Cathepsin K*-labeled tendon progenitors leads to HO in mice.

The previous report demonstrates that *Scleraxis* (*Scx*) lineage cells in muscle and tendon contribute to form HO[26]. A recent study demonstrates that *Cathepsin K (Ctsk)-Cre* labels a subpopulation of tendon-derived progenitors marked by *Scx* in mice[27]. To investigate whether enhanced BMP signaling in *Ctsk-Cre* labeled tendon progenitors effectively induces HO, we crossed *Ca-Alk2* mice with *Ctsk-Cre* mice (hereafter "*Ca-Alk2:Ctsk-Cre*"). At 5 months, the *Ca-Alk2:Ctsk-Cre* mice began to show mobility problems with loss of body weight (Fig. 1A). By crossing with *Ctsk-Cre* mice and *Rosa26-GFP* reporter mice, we confirmed that the Achilles tendon was robustly labeled by *Ctsk-Cre* (Fig. 1B). Micro-computed tomography (μCT) analysis showed spontaneous and progressive tendon ossification in the Achilles tendon with a 100% phenotypic penetrance in *Ca-Alk2:Ctsk-Cre* mice (Fig. 1C, D). These data suggest that *Ctsk-Cre* labeled tendon-derived progenitors are critical to forming HO in *Ca-Alk2:Ctsk-Cre* mice.

Enhanced BMP signaling stimulates ectopic osteochondrogenesis in the Achilles tendons.

To reveal the mechanisms of how enhanced BMP signaling via ALK2 in tendon-derived progenitors induces HO, we first examined the levels of phosphorylated SMAD1/5/9 using

2-month-old mice when ectopic ossification is still not evident in *Ca-Alk2:Ctsk-Cre* mice. Achilles tendons of *Ca-Alk2:Ctsk-Cre* mice exhibited higher levels of phosphorylated SMAD1/5/9 compared with controls (Fig. 2A). Next, we examined the cell proliferation activity in *Ca-Alk2:Ctsk-Cre* mice. Immunohistological analysis using the Ki-67 antibody showed that the cell proliferation activity is intact in *Ca-Alk2:Ctsk-Cre* mice (Fig. 2B). However, expression analysis using SOX9 (chondrogenic marker), and RUNX2 (osteogenic marker) antibodies revealed that osteochondrogenic differentiation is ectopically enhanced in the Achilles tendons of *Ca-Alk2:Ctsk-Cre* mice (Fig. 2C). Histological analysis by Hematoxylin and eosin staining (revealing cell/tissue morphology) (Fig. 2D), Safranin O staining (detecting cartilage) (Fig. 2E) and Picrosirius red staining (detecting collagen fibers) (Fig. 2F, G) further confirmed that HO is formed via endochondral ossification. These data suggest that the formation of HO was not caused by an increase in the proliferation of tendon-derived progenitors but by enhanced osteochondrogenic differentiation in the Achilles tendons.

Altered Hh and mechanotransduction pathways are associated with HO in *Ca-Alk2:Ctsk-Cre* mice.

Previous studies have shown that connective tissue abnormalities due to either developmental defects or injury can cause HO accompanied by the elevation of Hh signaling[28,29]. In addition, activation of Hh signaling in the tendon induces ectopic osteochondrogenesis[27]. Therefore, to reveal whether Hh signaling is dysregulated in *Ca-Alk2:Ctsk-Cre* mice, we examined GLI1 production. Compared with control mice, high levels of GLI1 were detected in the *Ca-Alk2:Ctsk*-Cre's tendon (Fig. 3A). Because the pattern of GLI1 production overlapped with the ectopic SOX9/RUNX2 production in *Ca-Alk2:Ctsk-Cre* mice (Fig. 2C), these results suggest that enhanced BMP signaling may lead to the dysregulation of Hh signaling.

Because the Achilles tendon is the largest tendinous structure which bears the highest mechanical stress in the body[30], and active mechanotransduction in mesenchymal progenitors impacts the osteochondrogenic cell fate in HO[17,18], we hypothesized that in addition to the elevation of Hh signaling, significant amount of mechanical stress in the Achilles tendon may participate in triggering ectopic osteochondrogenesis. To test our hypothesis, levels of phosphorylated focal adhesion kinase (p-FAK), a critical mediator for the mechanotransduction pathway, were examined by immunohistochemistry. Interestingly, higher levels of p-FAK were detected in the Achilles tendon of *Ca-Alk2:Ctsk-Cre* mice compared with controls (Fig. 3B), suggesting that an altered mechanotransduction pathway may also be involved in the pathogenesis of HO in the Achilles tendons.

To date, numerous studies demonstrate that activation of BMP signaling drives the cell fate of mesenchymal progenitors toward osteochondrogenesis[3,5]. However, it remains unclear how BMP signaling regulates skeletogenic cell fate in the context of HO. Recently, it has been reported that a self-amplifying loop of Shh and Yap drives the formation of HO[31]. In addition, mechanotransductive signaling through Yap/Taz/FAK is increased during the formation of trauma-induced HO[17]. These studies highlight the importance of Hh and mechanotransduction pathways in HO. However, the precise mechanism by which

Hh and mechanotransduction pathways in *Ca-Alk2:Ctsk-Cre* mice remains unclear. For example, what are the Hh ligand(s) to stimulate Hh signaling? Why tendon progenitors in *Ca-Alk2:Ctsk-Cre* mice are sensitive to mechanical stimuli (Fig. 3C)? Further studies will be required to clarify the mechanisms of how aberrant BMP signaling is associated with dysregulated Hh and mechanotransduction pathways to induce ectopic bone in multiple forms of HO such as injured and traumatic HO, and genetic HO.

Acknowledgments

We thank Dr. Brendan Lee for acquiring the *Ctsk-Cre* mice. This study was supported by NIDCR/NIH R01DE025897 (Y.K.), and the Bone Disease Program of Texas Rolanette and Berdon Lawrence Research Award (H.Y.).

References

1. Kaplan FS, Pignolo RJ & Shore EM Heterotopic Ossification: The Keys to the Kingdom. Bone 109, 1–2, doi:10.1016/j.bone.2018.03.001 (2018). [PubMed: 29567209]
2. Agarwal S, Sorkin M & Levi B Heterotopic Ossification and Hypertrophic Scars. Clin Plast Surg 44, 749–755, doi:10.1016/j.cps.2017.05.006 (2017). [PubMed: 28888300]
3. Salazar VS, Gamer LW & Rosen V BMP signalling in skeletal development, disease and repair. Nature reviews. Endocrinology 12, 203–221, doi:10.1038/nrendo.2016.12 (2016).
4. Urist MR Bone: formation by autoinduction. Science 150, 893–899, doi:10.1126/science.150.3698.893 (1965). [PubMed: 5319761]
5. Lyons KM & Rosen V BMPs, TGFbeta, and border security at the interzone. Current topics in developmental biology 133, 153–170, doi:10.1016/bs.ctdb.2019.02.001 (2019). [PubMed: 30902251]
6. Sanchez-Duffhues G, Williams E, Goumans MJ, Heldin CH & Ten Dijke P Bone morphogenetic protein receptors: Structure, function and targeting by selective small molecule kinase inhibitors. Bone 138, 115472, doi:10.1016/j.bone.2020.115472 (2020). [PubMed: 32522605]
7. Miyazawa K & Miyazono K Regulation of TGF-beta Family Signaling by Inhibitory Smads. Cold Spring Harbor perspectives in biology 9, doi:10.1101/cshperspect.a022095 (2017).
8. Kaplan FS, Al Mukaddam M, Stanley A, Towler OW & Shore EM Fibrodysplasia ossificans progressiva (FOP): A disorder of osteochondrogenesis. Bone 140, 115539, doi:10.1016/j.bone.2020.115539 (2020). [PubMed: 32730934]
9. Shore EM et al. A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. Nature genetics 38, 525–527, doi:10.1038/ng1783 (2006). [PubMed: 16642017]
10. Hatsell SJ et al. ACVR1R206H receptor mutation causes fibrodysplasia ossificans progressiva by imparting responsiveness to activin A. Sci Transl Med 7, 303ra137, doi:10.1126/scitranslmed.aac4358 (2015).
11. Shimono K. et al. Potent inhibition of heterotopic ossification by nuclear retinoic acid receptor-gamma agonists. Nature medicine 17, 454–460, doi:10.1038/nm.2334 (2011).
12. Lees-Shepard JB et al. Activin-dependent signaling in fibro/adipogenic progenitors causes fibrodysplasia ossificans progressiva. Nature communications 9, 471, doi:10.1038/s41467-018-02872-2 (2018).
13. Xu R, Hu J, Zhou X & Yang Y Heterotopic ossification: Mechanistic insights and clinical challenges. Bone 109, 134–142, doi:10.1016/j.bone.2017.08.025 (2018). [PubMed: 28855144]
14. Hwang CD et al. Contemporary perspectives on heterotopic ossification. JCI Insight 7, doi:10.1172/jci.insight.158996 (2022).
15. Zhou T. et al. Piezo1/2 mediate mechanotransduction essential for bone formation through concerted activation of NFAT-YAP1-ss-catenin. eLife 9, doi:10.7554/eLife.52779 (2020).
16. Wang L. et al. Mechanical sensing protein PIEZO1 regulates bone homeostasis via osteoblast-osteoclast crosstalk. Nature communications 11, 282, doi:10.1038/s41467-019-14146-6 (2020).

17. Huber AK et al. Immobilization after injury alters extracellular matrix and stem cell fate. The Journal of clinical investigation 130, 5444–5460, doi:10.1172/JCI136142 (2020). [PubMed: 32673290]
18. Pagani CA et al. Discoidin domain receptor 2 regulates aberrant mesenchymal lineage cell fate and matrix organization. Sci Adv 8, eabq6152, doi:10.1126/sciadv.abq6152 (2022). [PubMed: 36542719]
19. Fukuda T. et al. Generation of a mouse with conditionally activated signaling through the BMP receptor, ALK2. Genesis 44, 159–167, doi:10.1002/dvg.20201 (2006). [PubMed: 16604518]
20. Nakamura T. et al. Estrogen prevents bone loss via estrogen receptor alpha and induction of Fas ligand in osteoclasts. Cell 130, 811–823, doi:10.1016/j.cell.2007.07.025 (2007). [PubMed: 17803905]
21. Madisen L. et al. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat Neurosci 13, 133–140, doi:10.1038/nn.2467 (2010). [PubMed: 20023653]
22. Noda K, Kitami M, Kitami K, Kaku M & Komatsu Y Canonical and noncanonical intraflagellar transport regulates craniofacial skeletal development. Proceedings of the National Academy of Sciences of the United States of America 113, E2589–2597, doi:10.1073/pnas.1519458113 (2016). [PubMed: 27118846]
23. Yamaguchi H. et al. Temporospatial regulation of intraflagellar transport is required for the endochondral ossification in mice. Developmental biology 482, 91–100, doi:10.1016/j.ydbio.2021.12.004 (2022). [PubMed: 34929174]
24. Yamaguchi H, Meyer MD, He L & Komatsu Y Disruption of Trip11 in cranial neural crest cells is associated with increased ER and Golgi stress contributing to skull defects in mice. Developmental dynamics : an official publication of the American Association of Anatomists 251, 1209–1222, doi:10.1002/dvdy.461 (2022). [PubMed: 35147267]
25. Yamaguchi H. et al. The molecular complex of ciliary and golgin protein is crucial for skull development. Development 148, doi:10.1242/dev.199559 (2021).
26. Agarwal S. et al. Scleraxis-Lineage Cells Contribute to Ectopic Bone Formation in Muscle and Tendon. Stem Cells 35, 705–710, doi:10.1002/stem.2515 (2017). [PubMed: 27862618]
27. Feng H. et al. Tendon-derived cathepsin K-expressing progenitor cells activate Hedgehog signaling to drive heterotopic ossification. The Journal of clinical investigation 130, 6354–6365, doi:10.1172/JCI132518 (2020). [PubMed: 32853181]
28. Regard JB et al. Activation of Hedgehog signaling by loss of GNAS causes heterotopic ossification. Nature medicine 19, 1505–1512, doi:10.1038/nm.3314 (2013).
29. Liu H, Xu J & Jiang R Mkx-Deficient Mice Exhibit Hedgehog Signaling-Dependent Ectopic Ossification in the Achilles Tendons. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 34, 557–569, doi:10.1002/jbmr.3630 (2019). [PubMed: 30458056]
30. Freedman BR, Gordon JA & Soslowsky LJ The Achilles tendon: fundamental properties and mechanisms governing healing. Muscles Ligaments Tendons J 4, 245–255 (2014). [PubMed: 25332943]
31. Cong Q. et al. A self-amplifying loop of YAP and SHH drives formation and expansion of heterotopic ossification. Sci Transl Med 13, doi:10.1126/scitranslmed.abb2233 (2021).

Highlight

- *Cathepsin K-Cre*-driven enhanced BMP signaling induces heterotopic ossification (HO) in mice.
- Ectopic endochondral ossification is associated with the pathogenesis of HO in the tendon.
- Elevated hedgehog and mechanotransduction pathways are associated with HO in mice.

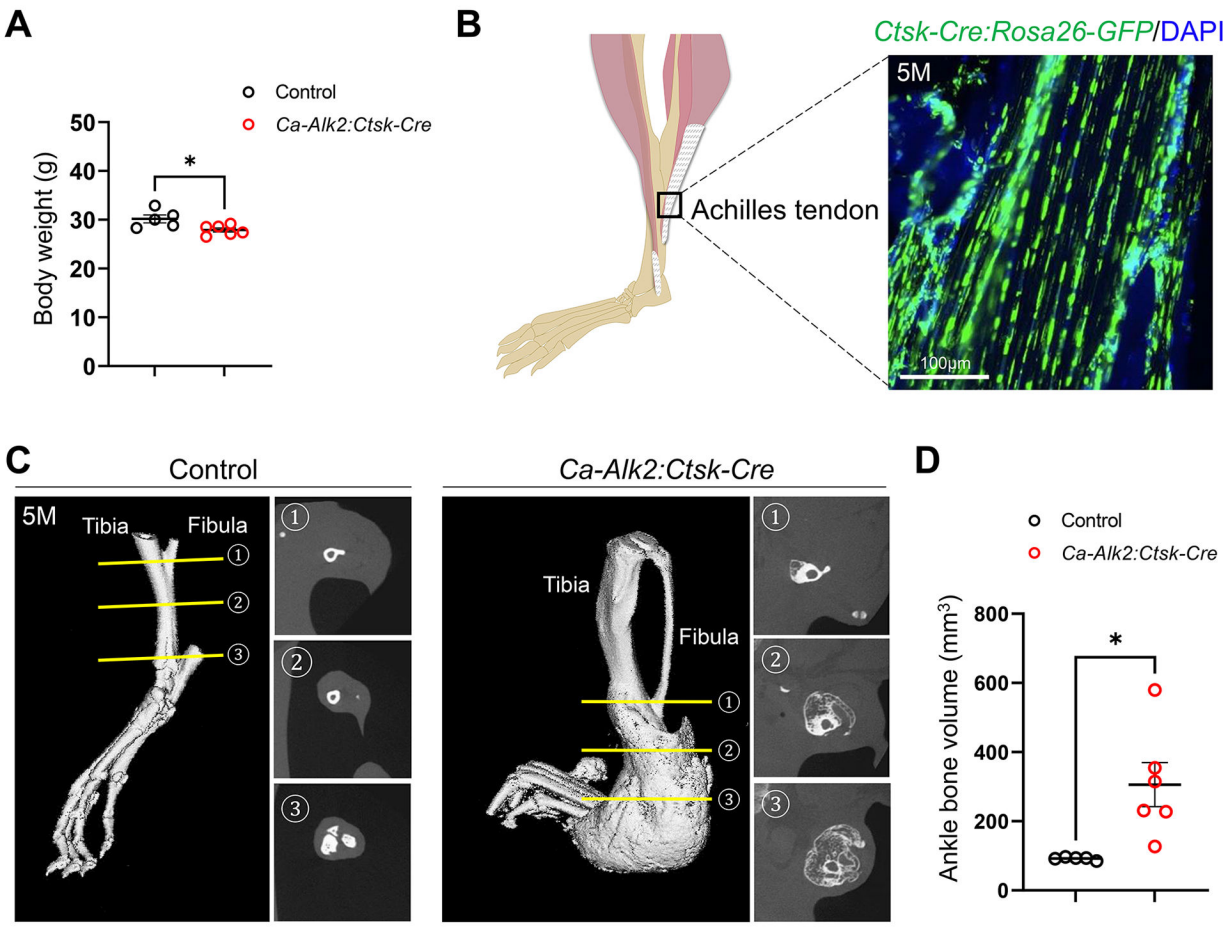

Fig. 1. Enhanced BMP signaling causes HO in the Achilles tendons.

(A) Quantification of the body weight of 5-month-old mice (5M). Data are as mean ± SD, n=5-6, Student's Ttest, *p<0.05. **(B)** The *Ctsk-Cre* activity was examined by crossing *Ctsk-Cre* mice and *Rosa26-GFP* reporter mice using 5-month-old mice (5M). After obtaining cryosection, tissues were counterstained with DAPI. **(C)** μCT images of the ankle. Yellow lines indicate the levels of the sectional images. **(D)** Quantification of the HO volume at 5M. Data are as mean ± SD, n=5-6, Student's T-test, *p<0.05.

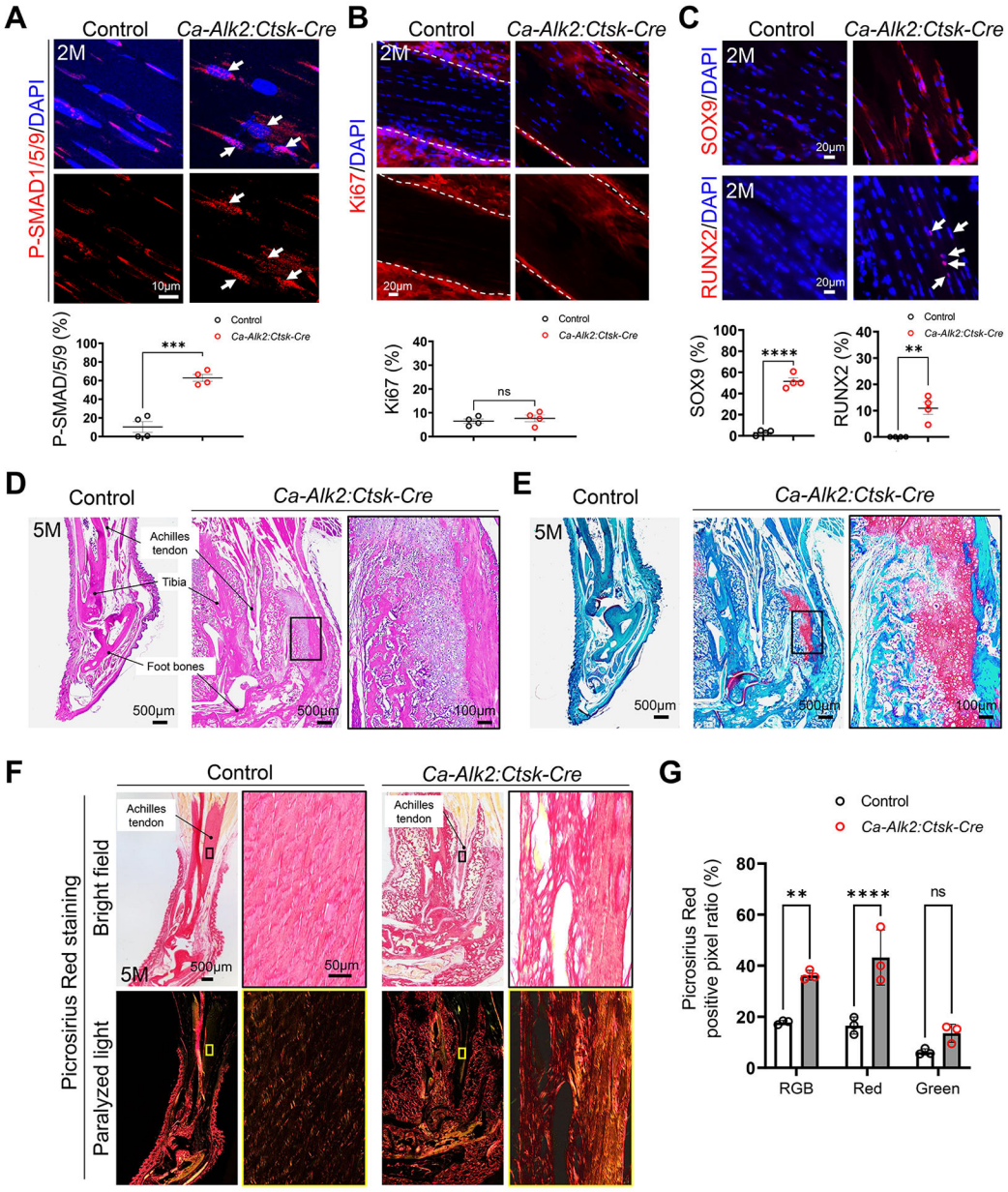

Fig. 2. Enhanced BMP signaling stimulates ectopic osteochondrogenesis in the Achilles tendons in *Ca-Alk2:Ctsk-Cre* mice.

(A, B, C) Immunohistochemistry of phospho (P)-SMAD1/5/9, Ki-67, SOX9, and RUNX2, and corresponding quantification of the Achilles tendons in 2-month-old mice (2M). Data are as mean ± SD, n = 4 individual samples in each group. Arrows indicate the nuclear localization of P-SMAD1/5/9 or RUNX2. **p<0.01, ***p<0.001, ****p<0.0001, ns, not significant. **(D)** Hematoxylin and eosin staining, **(E)** Safranin O staining, and **(F)** Picrosirius red staining at 5-month-old mice (5M). Boxes show high-magnification images of each staining. **(G)** In Picrosirius red staining, the signal intensity of type I collagen was measured and quantified. Data are as mean ± SD, n = 4 individual samples in each group. **p<0.01, ****p<0.0001, ns, not significant.

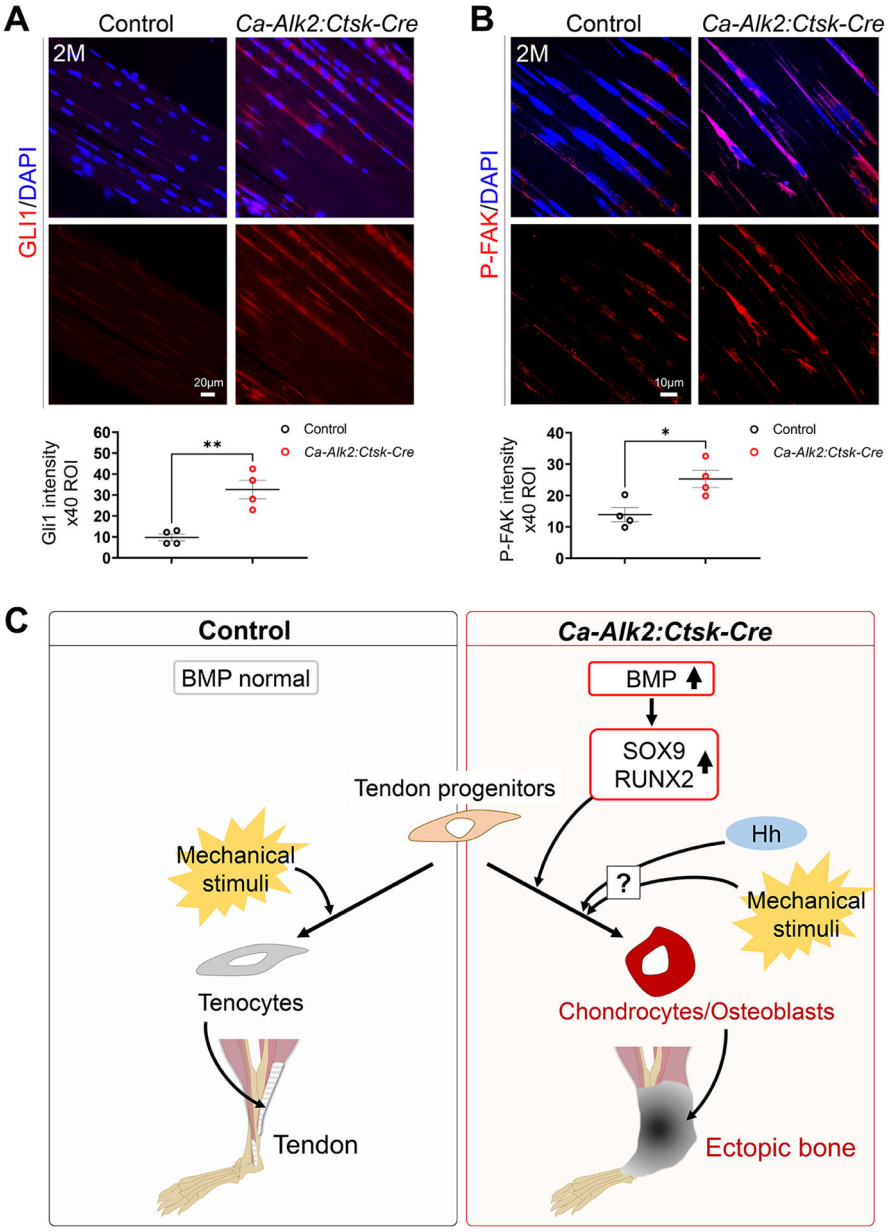

Fig. 3. Dysregulated Hh and mechanotransduction pathways are associated with HO in *Ca-Alk2:Ctsk-Cre* mice.

(A) Immunohistochemistry of GLI1 in the Achilles tendons at 2-month-old mice (2M). Data are as mean ± SD, n = 4 individual samples in each group. **p<0.01. **(B)** Immunohistochemistry of phospho (P)-FAK in the Achilles tendons at 2M. Data are as mean ± SD, n = 4 individual samples in each group. *p<0.05. **(C)** A schematic model of this study.