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Tendon Mineralization Is Progressive and Associated with Deterioration of Tendon Biomechanical Properties, and Requires BMP-Smad Signaling in the Mouse Achilles Tendon Injury Model

Kairui Zhang^{1,2}, Shuji Asai^{1,3}, Michael W. Hast⁴, Min Liu¹, Yu Usami¹, Masahiro Iwamoto¹, Louis J. Soslowsky⁴, and Motomi Enomoto-Iwamoto¹

¹Division of Orthopaedic Surgery, Children's Hospital of Philadelphia, Philadelphia, PA, United States

²Department of Orthopaedics and Traumatology, Nanfang Hospital, Southern Medical University, Guangzhou, China

³Department of Orthopaedic Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan

⁴McKay Orthopaedic Research Laboratory, Department of Orthopaedic Surgery, University of Pennsylvania, Philadelphia, PA, United States

Abstract

Ectopic tendon mineralization can develop following tendon rupture or trauma surgery. The pathogenesis of ectopic tendon mineralization and its clinical impact have not been fully elucidated yet. In this study, we utilized a mouse Achilles tendon injury model to determine whether ectopic tendon mineralization alters the biomechanical properties of the tendon and whether BMP signaling is involved in this condition. A complete transverse incision was made at the midpoint of the right Achilles tendon in 8-week-old CD1 mice and the gap was left open. Ectopic cartilaginous mass formation was found in the injured tendon by 4 weeks post-surgery and ectopic mineralization was detected at 8–10 weeks post-surgery. Ectopic mineralization grew over time and volume of the mineralized materials of 25-weeks samples was about 2.5 fold bigger than that of 10-weeks samples, indicating that injury-induced ectopic tendon mineralization is progressive. *In vitro* mechanical testing showed that max force, max stress and mid-substance modulus in the 25-weeks samples were significantly lower than the 10-weeks samples. We observed substantial increases in expression of bone morphogenetic protein family genes in injured tendons 1 week post-surgery. Immunohistochemical analysis showed that phosphorylation of both Smad1 and Smad3 were highly increased in injured tendons as early as 1 week post-injury and remained high in ectopic chondrogenic lesions 4 weeks post-injury. Treatment with the BMP receptor kinase inhibitor (LDN193189) significantly inhibited injury-induced tendon

Correspondence: Motomi Enomoto-Iwamoto PhD, DDS, The Children's Hospital of Philadelphia Division of Orthopaedic Surgery, 3615 Civic Center Boulevard, ARC 902, Philadelphia PA 19104, USA, Phone: 267-425-2071, Fax: 267-426-2215, ; Email: iwamotom1@email.chop.edu.

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mineralization. These findings indicate that injury-induced ectopic tendon mineralization is progressive, involves BMP signaling and associated with deterioration of tendon biomechanical properties.

Keywords

Achilles Tendon; Injury; Ectopic Mineralization; Biomechanics; Smad; BMP

1. Introduction

Ectopic tendon mineralization can develop following tendon rupture or tendon repair surgery and has been found in degenerative tendons that present clinical symptoms and pathological features of tendinopathy (O'Brien et al., 2012;Oliva et al., 2012;Richards et al., 2008;Oliva et al., 2011). Several studies have systematically analyzed incidence and clinical consequences of tendon mineralization in ruptured Achilles tendons after repair surgery. Kraus et al. (Kraus et al., 2004) reported that 10 of 36 patients (28%) had ectopic mineralization in their Achilles tendons after open repair surgery and that the development of mineralization was associated with clinical symptoms such as chronic swelling, pain, and limited range of motion. Ateschrang et al. (Ateschrang et al., 2008) performed a retrospective study for post-operative Achilles tendon mineralization and found that 14.4% patients (15 out of 104 patients) had ectopic mineralization at intratendinous or peritendinous locations after receiving an open-augmented repair using the Silfverskjöld technique. In this study, they did not find significant differences between the clinical outcome and the presence of post-operative mineralization.

Anecdotal experiences have suggested that mineralization may weaken the mechanical integrity of affected tendons, as mineralization has been found in ruptured Achilles and other tendons at a higher frequency compared to control tendons (Kannus et al., 1991). However, O'Brien *et al.* (O'Brien et al., 2013) demonstrated that ectopic tendon mineralization did not change stiffness, failure load or failure strain in murine Achilles tendons. Thus it is still unclear whether peri- and intratendinous mineralization affects the recovery of biomechanical properties and function of the ruptured tendon.

Ectopic mineralization can be caused by endochondral ossification. (O'Brien et al., 2012;Fenwick et al., 2002;Agabalyan et al., 2013;Hatori et al., 2002). It has been shown that transection of Achilles tendons (Tenosectomy) in rodents induces ectopic mineralization via endochondral ossification (Rooney et al., 1992;Lin et al., 2010;Peterson et al., 2015). Using a murine model, we recently demonstrated that tendon progenitor-like cells appear in injured tendons, have strong chondrogenic potential, and may contribute to ectopic mineralization (Asai et al., 2014). Furthermore, we have shown that injured tendons contain CD105-positive and negative progenitor cells represent different degrees of Smad1/5 signaling in response to TGFβ1 proteins. We have suggested that CD105, a co-receptor of TGFβ receptors may be involved in regulation of TGFβ/BMP signaling in ectopic chondrogenic differentiation of progenitors in injured tendons (Asai et al., 2014). Previous studies have reported that up-regulation of expression of various TGFβ/BMP superfamily proteins is

associated with ectopic tendon mineralization (Lin et al., 2010; Yee Lui et al., 2011; Rui et al., 2012), suggesting that TGF β /BMP signaling is involved in pathogenesis of ectopic tendon mineralization as reported in hereditary heterotopic ossification (HO) (Yu et al., 2008; Kaplan et al., 2012) and vascular calcification (Cai et al., 2012; Malhotra et al., 2015).

In this study, we sought to determine whether injury-induced tendon ectopic mineralization is progressive, whether it affects biomechanical properties, and whether BMP-Smad signaling contributes to this condition. We induced ectopic tendon mineralization in the mouse with the use of the Achilles transection model. We compared the volume of ectopic mineralization and biomechanical properties of ruptured tendons between 10 weeks and 25 weeks post-injury. We also examined spatiotemporal changes in BMP-Smad signaling during the healing process and further investigated whether the inhibitors of the BMP receptor kinase inhibit mineralization in injured tendons.

2. Results

2.1. Progressive ectopic mineralization in injured Achilles tendons

The Achilles tendons were harvested together with the bone 10 and 25 weeks post-injury. Ectopic mineralization was detected in the all Achilles tendons that had been subjected to injury surgery (Fig. 1A and B). The mineralized materials in the 25 week injured tendons were bigger in size and extended toward the midsubstance (Fig. 1B) compared to those in the 10 week injured tendons (Fig. 1A). The quantification analysis of the μ CT images revealed that volume of mineralized materials was significantly larger in the 25-week injured tendons compared to the 10-week injured tendons (Fig. 1C), indicating that ectopic tendon mineralization is progressive.

2.2. Deterioration of biomechanical properties in injured Achilles tendons

Biomechanical assays indicated deleterious effects associated with the progression of ectopic mineralization. Although, stiffness and maximum force of the injured tendons 10 weeks and 25 weeks post-injury were not significantly different from those of the control tendons (Fig. 2A and 2B), the maximum stress at 25 weeks and modulus at 10 weeks and 25 weeks were significantly lower than controls (Fig. 2C and 2D). These results are associated with the significant increase in cross-sectional area that was measured in the 25 weeks post-injury group compared to the control (Fig. 2E). Collagen fiber alignment was evaluated by measuring circular variance of collagen fibers during mechanical testing. The injured tendons yielded higher values compared to the control (Fig. 2F), indicating that the collagen fiber alignment in injured tendons were more disorganized than controls. The average of the values of the 10-week group were lower than those of the 25-week group ($p=0.053$).

2.3 No ectopic mineralization in the contralateral Achilles tendons

It has been reported that ectopic tendon mineralization was induced in the contralateral Achilles tendons in C57BL mice when the needle injury was made in the midpoint of the Achilles tendon (O'Brien et al., 2013). However, μ CT analysis did not detect ectopic mineralization in the any mice for both the 10 and 25 weeks post-injury groups (Fig. 3A and 3B). We examined biomechanical properties and collagen fiber alignment of the

contralateral Achilles tendons, and found no significant difference in circular variance of collagen alignment, stiffness, maximum force, maximum stress and modulus between 10- and 25-week groups (Fig. 3C–3G). The same injury surgery was performed in C57BL/6j mice, mineralization in the contralateral Achilles tendons was not detected in any of the mice (Supplemental figure 1).

2.4. TGF β BMP-Smad signaling in ectopic cartilaginous lesion

Previous studies have reported that expression of TGF β s and BMPs are up-regulated in incised Achilles tendons in rats (Lin et al., 2010). To obtain comprehensive information on gene expression of TGF β /BMP signaling-related molecules after tendon injury, total RNAs were prepared from uninjured Achilles tendons and injured Achilles tendons 1, 2 and 4 weeks after the transection surgery and were subjected to the qPCR (TGF β /BMP signaling PCR array) (Supplement File 1). We observed up-regulation of *Bmp3*, *Bmp7* and *Bmp12* (*Gdf7*) gene expression at 1, 2 and 4 weeks post-injury. In addition, expression of *Dlx2*, a target gene of the BMP pathway, was also strongly increased. Expressions of *Tgfb1-3* were moderately up-regulated at all time points.

To examine the spatiotemporal activity of BMP-Smad signaling in injured tendons, immunohistochemical staining for phospho-Smad 1 and phospho-Smad 3 was performed. The mid-substance and the ends of injured tendons were separately examined because these two regions represent distinct histology in later healing phases: Previous research has indicated that the mid-substance of the injured tendon is composed of longitudinally aligned cells (Fig. 4E) while the end of the injured tendons form ectopic cartilaginous tissue (Fig. 4F) leading to endochondral bone formation (Asai et al., 2014). In uninjured control tendons, the tendon cells showed limited positive signals for both phospho-Smad1 (Fig. 4H and I) and -Smad3 (Fig. 4N and O) staining. The mid-substance and ends of the neo-formed tendons showed high immuno-reactivity for phospho-Smad1 (Fig. 4J and K) and phospho-Smad 3 (Fig. 4P and Q) at 2 weeks post-injury, indicating that the entire tendon present high activities of TGF β /BMP-Smad signaling. The staining of phospho-Smad1 and phospho-Smad 3 became weaker 4 weeks post-injury (Fig. 4L, M, R and S). In particular, the mid-substance of the tendons contained fewer positive cells (Fig. 4L and R) compared to the tendons at 2 weeks post-injury (Fig. 4J and P). The ends of the injured tendons contained a large number of positive cells to phospho-Smad1 (Fig. 4M) and phospho-Smad 3 (Fig. 4S). These stained cells were polygonal in shape (Fig. 4G, N and T), which is a feature of chondrogenic phenotype, further indicating that the cells undergo chondrogenic differentiation retain high activity of TGF β /BMP-Smad signaling.

2.5. Inhibition of ectopic tendon mineralization by BMP kinase inhibitors

Lastly we tested whether BMP signaling is required for induction of ectopic endochondral ossification in injured tendons. LDN193189 is a small chemical compound that has been developed to inhibit Smad 1/5/8 phosphorylation by BMP type 1 receptors (Cuny et al., 2008). Administration of LDN193189 strongly inhibits heterotopic ossification (HO) induced by constitutive activation of the ALK2 receptor in mice (Yu et al., 2008). Mice were treated with LDN193189 after Achilles tendon injury surgery and subsequently examined whether this drug inhibits ectopic mineralization within the tendon. The drug was given to

the mice every day from 1 to 3 weeks post-surgery, and the tendons were harvested 12 weeks post-surgery. To assure the action of LDN193189, we harvested the tendons 2 days after the drug treatment and examined phosphorylation of Smad1/5 by immunostaining. The vehicle-treated tendons showed strong immunoreactivity at both ends and midsubstance of the injured tendons while the LDN193189-treated tendons showed much weaker staining (Supplement Figure 2), indicating that the LDN193189 indeed inhibited the Smad1/5 pathway. The μ CT analysis revealed that the LDN193189 treatment reduced the volume of the ectopic mineralized materials to about 40% of that of the control group (Fig. 5A and B). In addition, the LDN193189-treated tendons contained less cartilaginous lesion positive to alcian blue (Fig. 5C). The treatment with LDN193189 significantly inhibited gain of body weight and bone growth (Fig. 5D and E), but the body weight of the treated group caught up to the control group after cessation of the drug treatment (Fig. 5D, 8 weeks).

3. Discussion

3.1. Ectopic tendon mineralization and biomechanical properties

Mineralization has been reported as a pathological feature of tendinopathy in rotator cuff tendons, Achilles tendons, and other tendons and ligaments (Oliva et al., 2012;Oliva et al., 2011). Tendon mineralization has been also found in Achilles tendons after surgical restoration of ruptured tendons (O'Brien et al., 2012;Richards et al., 2008;Kraus et al., 2004;Ateschrang et al., 2008). Although tendon mineralization is anticipated to cause clinical symptoms such as pain and limitation of motion and to impair tendon function (Richards et al., 2008;Kraus et al., 2004;Blankstein et al., 2001), the clinical and basic studies on this condition are limited. Achilles tendon transection in rodents serves as a useful model to study tendon mineralization following tendon rupture and trauma surgery. Using the mouse Achilles tendon transection model, we have demonstrated that the volume of mineralized materials increased for a long term while the elastic modulus and maximum stress of the affected tendons decreased. Further, the cross-sectional area of the midsubstance was increased at the later time point. The mineralized materials were extended from the ends to the mid-substance in 25 weeks post-surgery. An increase in the cross-sectional area and decreases in the elastic modulus and maximum stress values may be not only due to a change in the strain of the regenerating tendon tissue in the midsubstance but also due to an increase in mineralized materials in the midsubstance. The biomechanical parameters of the contralateral tendons were similar between 10-week and 25-week groups, confirming the previous findings that the biomechanical properties in tendons do not change with age (Connizzo et al., 2013). These findings suggest that tendon mineralization following surgical trauma is progressive and might deteriorate biomechanical properties of the affected tendons for a long time period. Case studies have validated this model, as enlargement of ossification in Achilles tendons over monitoring periods of 1–2 years has been reported (Hatori et al., 2002;Hatori et al., 1994). Thus, monitoring of tendon mineralization after tendon restoration surgery may be clinically important, and if tendon mineralization is detected, inhibition of progression of mineralization may minimize the associated weakening of the tendon's biomechanical integrity.

Results contradict previous studies investigating ectopic mineralization due to punching a small hole in the mid-substance of the Achilles tendon in C57BL mice using a 23 G needle (O'Brien et al., 2013). They found that ectopic mineralization develops in injured tendons at 20 weeks post-injury, but did not detect significant differences in the tangent stiffness and failure load between control uninjured and injured tendons (O'Brien et al., 2013). They have also demonstrated that the contralateral tendons to the injured tendons have greater static and total creep strain than the injured site tendons and the control uninjured tendons (O'Brien et al., 2013). It is not clear if the elastic modulus of the injured tendons is similar or different from the uninjured control tendons or if the mineralization is intra- or peri-tendinous and involves endochondral ossification in this injury model. The needle injury generates a small size of defect while our model produces a big gap in the tendon and induces neo-formed tendon filling the gap. Thus the healing process and pathogenesis of mineralization may be different between these two models. Further histological and biomechanical examinations are required to define similar and distinct points between these two models. We did not detect mineralization in the contralateral tendons in CD1 and C57BL/6 mice. This is different from previous reports that used the needle injury model in C57BL/6 mice (O'Brien et al., 2013). The discrepancy may come from not only the injury surgery procedures but also external variables, such as differences in environment of mouse facilities or dietary components.

3.2. BMP-Smad signaling in tendon mineralization

BMP signaling has been recognized as a causative pathway for mineralization in pathological conditions including hereditary HO (Yu et al., 2008; Kaplan et al., 2012) and vascular calcification (Cai et al., 2012). The results in this study demonstrated that expression of several BMP family members, *Bmp3*, *Bmp7* and *Bmp12* (*Gdf7*) were strongly up-regulated in injured tendons and remained high at least until 4 weeks post-injury. Taken together with BMP inhibitors inhibited mineralization in injured tendons, it is very likely that BMP signaling is a causative pathway in ectopic tendon mineralization in this injury model as other pathological mineralization.

LDN198318 treatments were made only from 1 week to 3 weeks post-injury in our experiment, but we observed the inhibition of ectopic mineralization at 12 weeks post-injury, suggesting that an initial stage of BMP signaling is important for inhibition of mineralization. At this stage, the cells positive for phospho-Smad1 and phospho-Smad3 were widely distributed in injured sites; therefore, the inhibitors can target multiple types of cells. Previous studies have revealed that BMP signaling in HO targets various kinds of cells including inflammatory cells, satellite cells, endothelial cells, pericytes, neurons, circulating and local connective tissue progenitors (Kaplan et al., 2007; Lounev et al., 2009; Suda et al., 2009; Salisbury et al., 2011; Bi et al., 2007). We have recently reported that progenitors appear in injured tendons and that these cells have strong chondrogenic potential compared to the tendon progenitors from uninjured tendons and bone marrow stromal cells (Asai et al., 2014). When the injured tendon-derived progenitors were cultured at a high density, they increased expression of aggrecan and collagen 2 genes without exogenous chondrogenic factors treatment (Asai et al., 2014). Increases in these genes were inhibited by LDN198318 (data not shown). Further we showed that stimulation of phosphorylation of Smad1/5/8 is

more evident in the CD105-negative tendon progenitors than that in the CD105-positive tendon progenitors and the former cells had stronger chondrogenic potential than the latter cells (Asai et al., 2014). Thus, it is likely that the injured tendon-derived progenitor is one of the target cells for LDN198318 and is responsible for ectopic tendon mineralization involving Smad1/5/8 signaling in this model. Both BMP family members and TGF β s were increased in injured tendons. Interestingly, progenitors isolated from injured Achilles tendons increased phosphorylation of both Smad1/5 and Smad2/3 in response to BMP2 and TGF β 1 (Asai et al., 2014), suggesting that Smad1/5/8 may mediate both TGF β and BMP signaling in induction of ectopic endochondral ossification in injured tendons. The involvement of Activin-Activin receptor signaling should be investigated, as very recent studies have reported that Activin signaling antagonizes BMP-Smad1/5/8 signaling (Hatsell et al., 2015). Because we detected impairment of tibial growth and body weight gain by LDN198318, we could not perform longer treatment. Therefore, we have not clarified whether LDN198318 inhibits progression of ectopic mineralization at later stages, which is a limitation of this study.

Recently studies have demonstrated that nuclear retinoic acid receptor gamma (RAR γ) agonists strongly inhibited BMP-2 induced ectopic endochondral ossification (Shimono et al., 2011). It has also been shown that RAR γ agonists inhibited HO induced by constitutive activation of ALK2 (Shimono et al., 2011). Thus, the inhibitory action of RAR γ agonists on HO is likely mediated by inhibition of BMP-Smad1/5/8 signaling. Surprisingly and interestingly, RAR γ agonists did not inhibit mineralization in our Achilles tendon transection model. The reasons why RAR γ agonists did not inhibit ectopic tendon mineralization could be low expression of RAR γ or the presence of negative factors for RAR γ action in injured tendons.

In conclusion, our data indicates that injury-induced tendon mineralization is progressive for a long period of time and that an increase in tendon mineralization is associated with deterioration of the tendon's biomechanical integrity. Therefore, prevention of progressive tendon mineralization after injury or surgery, by the use of drugs such as BMP receptor kinase inhibitors (LDN193189) or other means, may be clinically important for recovery of tendon function.

4. Experimental Procedures

4.1. Mice

All mouse studies were conducted with approval by the Institutional Animal Care and Use Committee of the Children's Hospital of Philadelphia. The CD-1 and C57BL/6j mice were purchased from Charles River Laboratories International, Inc. (Wilmington, MA) and Jackson Laboratory (Bar Harbour, ME), respectively.

4.2. Mouse surgery and treatment

A complete transverse incision, without attempt at repair, was made at the midpoint of the right Achilles tendon in 6–8 week-old CD-1 or C57BL/6 mice (Asai et al., 2014). Animals were euthanized 1, 2, 4, 10, 12 or 25 weeks post-operatively (n=3–10/group) and tendon

tissues were carefully harvested to perform histological and immunohistochemical examination, gene expression analysis, μ CT scan, X ray analysis or biomechanical analysis. LDN 193189 (Axon Medchem LLC, Reston, VA) (3 mg/kg) was intraperitoneally given to the mice every day from 1 to 3 weeks post-surgery, and the tendons were harvested 2 days or 12 weeks post-surgery.

4.3. Histological and immunohistochemical analyses

The distal half hind limbs were dissected and fixed with 4% (v/v) paraformaldehyde, decalcified with Immunocal (Decal Chemical Corporation, Tallman, NY) or EDTA and embedded in paraffin. Longitudinal sections of the Achilles tendons were prepared and subjected to histological staining with hematoxylin/eosin or alcian blue (pH 1.0)/eosin. For detection of phospho-Smad1 or phospho-Smad3 proteins, the sections were treated with 0.25% Trypsin/EDTA for 10 min at room temperature, and blocked with 10% goat serum and 10% fetal bovine serum in PBS. Endogenous avidin/biotin was quenched with Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, CA) followed by overnight incubation with rabbit anti-phospho-Smad1 or anti-phospho-Smad3 antibody (1:250, Invitrogen, Carlsbad, CA) at 4°C. Following washes with PBS the sections were incubated with goat anti-rabbit biotinylated secondary antibody (1:200, Vector Laboratories) at room temperature for an hour, and incubated with ABC reagent (Vector Laboratories) for an hour followed by visualization of the antibody with ImmPACT NOVAREd (Vector Laboratories) and counterstaining with Fast Green. For immunofluorescence staining of pSmad1/5, the sections were incubated with 10 mM sodium citrate buffer (pH 6.0) at 95°C for 10 min and incubated with the rabbit monoclonal antibody (pSer463+465 antibody, ThermoFisher Scientific, Waltham, MA) followed by incubation with goat anti-rabbit biotinylated secondary antibody (1:200, Vector Laboratories) and then Texas Red-conjugated NeutrAvidin (1:200, ThermoFisher Scientific).

4.4. RNA isolation and gene expression assays

Total RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol and reverse-transcribed into cDNA. The resulting cDNA was subjected to a polymerase chain reaction (PCR). To profile changes in gene expression of TGF β /BMP signaling related molecules, we carried out a PCR array using RT2 Profiler PCR array for TGF β /BMP signaling pathway (Qiagen, Valencia, CA) following the manufacturer's protocol. Average threshold cycle value (Ct value) was calculated from 4-fold reactions and normalized to that of housekeeping gene GAPDH.

4.5. μ CT analyses

Injured tendons (n=10) were fixed with 4% paraformaldehyde or 10% neutralized formalin at 4°C and subjected to μ CT analysis using a CT40 scanner (Scanco USA, Inc., Wayne, PA) at 55 kV and 70 mA. Data were analyzed at threshold 244 for detection of ectopically mineralized components.

4.6. Mechanical testing and collagen alignment measurement

Achilles tendons with calcanei were fine dissected, so that all musculature and surrounding soft tissue was removed. Specimens were hydrated in phosphate buffered saline. Tendon cross-sectional area was calculated with a custom laser-based device. During mechanical testing, tendons were placed in a custom fixture that grips the calcaneus and tendon ends with a gauge length of 5mm. Specimens underwent a previously described protocol consisting of preconditioning, stress-relaxation, and ramp to tensile failure at a rate of 0.1%/s using an Instron 5542 test frame (Instron, Norwood, MA) (Connizzo et al., 2013). Images for analysis of fiber alignment were captured during mechanical testing with a cross-polarized light protocol, according to previously published methods (Lake et al., 2009).

4.7. Statistics

Results were analyzed using InStat 3 version 3.1a (GraphPad Software, Inc., La Jolla, CA). Student's t-tests or two-way factorial ANOVA followed by Bonferroni post-hoc multiple comparison tests were used to identify the differences.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Long-term changes in the biomechanical properties of the injured tendons were characterized in the mouse Achilles tendon injury model.
- The molecular mechanism underlying ectopic mineralization was studied in the injured Achilles tendons in mice.
- The pharmacological approach was effective to inhibit ectopic mineralization occurring in Achilles tendons after complete rupture.

Figure 1. Tendon mineralization is progressive in mouse Achilles transection model. A and B, The μ CT image of injured tendons 10 weeks (A) and 25 weeks (B) post-injury. Arrows indicate mineralized materials. C, Quantification of the volume of mineralized materials (n=10/group). *, $P<0.05$. Scale bar, 2 mm.

Figure 2.

Biomechanical properties, cross sectional area and collagen fiber alignment of uninjured and injured Achilles tendons. Stiffness (A), max force (B), max stress (C), modulus (D), cross sectional area (E) and circular variance of collagen alignment (F) of uninjured tendons (Control) or injured tendons 10 weeks and 25 weeks post-injury were measured (n=10/group).

Figure 3. Mineralization and biomechanical properties of the contralateral tendons to the injured Achilles tendons. μ CT image (A and B), circular variance of collagen alignment (C), stiffness (D), maximum force (E), maximum stress (F) and modulus (G) of the contralateral tendons to the injured site 10 weeks and 25 weeks post-injury (n=10/group). Scale bar, 2 mm.

Figure 4.

Distribution of phospho-Smad1 and -Smad3 in injured Achilles tendons. Images of hematoxylin-eosin (HE, A–G) staining and immunostaining with phospho-Smad1 (p-Smad1, H–N) and -Smad3 (p-Smad3, O–U) of the uninjured (Control, A,B,H,I,O,P) and injured Achilles tendons 2 weeks (2 weeks, C,D,J,K,Q,R) and 4 weeks (4 weeks, E–G, L–N, S–U) post-injury. Images of the mid-substance O–T; 12.5 mm for G, N and U. G, N and U are magnified images of the boxed areas of F, M and T, respectively.

Figure 5.

Inhibition of mineralization by LDN193189 in injured Achilles tendons. LDN193189 (3 mg/kg) or vehicle was daily administered by intraperitoneal injections from 1 to 3 weeks post-injury (n=10/group). The Achilles tendons were harvested 12 weeks post-injury and subjected to μ CT analysis (A) to determine the volume of mineralized materials (B). The sections of tendons were stained with alcian blue and eosin staining (C). The body weight

was measured at 2, 4 and 8 weeks post-injury (D) and the tibial length was measured by the X-ray analysis at 12 weeks post-injury (E). *, $p < 0.05$. Scale bars, 2 mm for A; 50 μm for C.