Promethazine downregulates Wnt/β-catenin signaling and increases biomechanical forces of injured Achilles tendon

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The authors declare that no competing interests exist.

Running title : Promethazine for tendon injury

Author contributions

T.S., B.O., and K.O. conceived the study and interpreted the results. T.S. and K.M. contributed to the rat experiments. T.S. and Y.K. contributed to the experiments using tendon-derived cells. S.I., and H.H. supervised the project. N.I. and K.O. provided financial support. T.S., B.O., and K.O. prepared the paper with assistance from the other authors.

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Abstract

Background: The tendon rarely regains its initial functionality with sufficient biological and biomechanical properties after injury. One of the major challenges in tendon repair is that the cellular mechanisms underlying incomplete tendon repair remain largely elusive.

Hypothesis/Purpose: It has previously been reported that Wnt/β-catenin signaling suppresses the differentiation of tenocytes. We hypothesized that the inhibition of the Wnt/β-catenin signaling would promote the tendon repair and improve the biomechanical properties of the healing tendons.

Study Design: Controlled laboratory study.

Methods: The right Achilles tendon was injured by a dermal punch in rats. The left Achilles tendon was sham-operated as a control. The tendons were histologically analyzed on postoperative weeks 0.5, 1, 2, 3, 4, and 8. Starting from the first postoperative day for 2 weeks, a Wnt/β-catenin inhibitor, IWR-1, was subcutaneously injected at the injured tendon, or a first-generation H1-antihistamine agent, promethazine (PH), was intramuscularly injected. Histological and biomechanical analyses were performed with the injured tendons. Tendon-derived cells (TDCs) were isolated to evaluate the effects of PH on the expression of the tendon marker genes by qRT-PCR.

Results: The amount of β-catenin protein in tendon cells was increased in the injured tendons from postoperative weeks 0.5 to 2. Histological analyses showed that the Bonar score representing the histological properties and the amount of β-catenin were lower in the IWR-1-treated injured tendons compared to those in the saline-treated injured tendons. IWR-1, however, compromised the biomechanical properties of the healing tendons. We first observed that PH increased the expressions of the tendon marker genes in TDCs. The amount of β-catenin in the PH-treated injured tendons was lower compared to that in the saline-treated injured tendons. In contrast to IWR-1, PH increased the peak force and stiffness of the healing tendons.

Conclusion: The inhibition of the Wnt/β-catenin signaling by IWR-1 improved histological repair of the Achilles tendons, but compromised the biomechanical properties. In contrast, PH reduced Wnt/β-catenin signaling and increased the biomechanical forces of the injured Achilles tendons.

Clinical Relevance: PH is a candidate repositioned drug that potentially increases the biomechanical forces of the healing tendons.

What is known about this subject: The tendon rarely regains its initial functionality with sufficient biological and biomechanical properties after injury. Wnt/β-catenin signaling suppresses the differentiation of cultured tenocytes.

What this study adds to existing knowledge: Injection of an inhibitor of the Wnt/β-catenin signaling, IWR-1, improved histological repair of the Achilles tendons, but compromised the biomechanical properties. In contrast, promethazine reduced Wnt/β-catenin signaling and increased the biomechanical forces of the injured Achilles tendons.

Key Terms: Promethazine; Achilles tendon; tendon healing; rat; drug repositioning; and biomechanics of the tendon

Introduction

Tendons are largely constituted of connective tissue that connects muscle and bone to transmit the biomechanical force for allowing for body movement.[5] Rupture of the Achilles tendon occurs with an incidence of 18.0 to 31.2 per 100,000 person-years, and its frequency is increasing.[6,28] Conservative and operative approaches are currently employed, but an injured tendon sometime heals with scar tissue and rarely achieves the functionality equivalent to that of the pre-injured state with appropriate biological and biomechanical properties.[10] Incomplete tendon healing may induce the recurrence of tendon rupture, which requires prolonged rehabilitation and compromises quality of life. The biomechanical properties of the tendon are dependent on the composition of the extracellular matrix proteins and fiber orientation.[27] In the last decades, many strategies to augment the biomechanical properties of injured tendons have been developed. Some growth factors, such as IGF-1[16], BMPs[26], and TGF-β[15], and their combinations[20] are promising therapeutic modalities in the healing of injured tendons. Biologically active adjuncts such as platelet-rich plasma are used in clinical practice.[13] Transplantation of embryonic stem cells, bone marrow-derived stromal cells, and endogenous tendon-derived cells (TDCs) enhances tendon repair in animal models.[19] Although cellular therapies are predicted to exert their effects via secreted proteins such as growth factors and extracellular matrix, the details need to be further dissected. A clinically applicable drug to augment the existing conservative and operative therapies is expected to be developed.

Tendon-related transcriptional factors, scleraxis (encoded by *Scx*) and mohawk (encoded by *Mkx*), are required for the differentiation of tenocytes, which secrete specific extracellular matrix including collagen types I and III. *Scx* is required for proper differentiation and is an important early marker in tendon development. Knockout of *Scx* results in the development of hypoplastic tendon tissues in the entire mouse body.[23] *Mkx* is required for tendon maturation by regulating type I collagen production in tendon cells. *Mkx*-/- mice show hypoplastic tendon tissues throughout the body.[11] An extracellular matrix protein, tenomodulin (encoded by *Tnmd*), is required for the maturation of collagen fibrils. *Tnmd* is highly expressed in mature tenocytes and is a late-phase marker of tendon development. The loss of *Tnmd* in tendons results in a low proliferative capacity of

tenocytes; hence, the collagen fiber bundles in tendons exhibit a non-uniform morphology.[24]

Similar to other growth factors, Wnt ligands and its downstream Wnt/β-catenin signaling play vital roles in the regulation of cellular functions and is involved in tissue healing and regeneration.[25,2] In the Wnt/β-catenin signaling, a Wnt ligand interacts with Frizzled (FZD) receptors on the cell surface. This induces the inhibition of the β-catenin destruction complex and then promotes the stabilization and nuclear translocation of β-catenin, which results in the activation of the target genes. It has previously been reported that the activation of Wnt/β-catenin signaling attenuates the differentiation of TDCs by suppressing gene expressions of *Scx*, *Mkx*, and *Tnmd*[14], as well as by partially suppressing TGF-β signaling. However, little is known about the spatiotemporal roles of Wnt/β-catenin signaling in injured tendons.

The purpose of this study was to identify the roles of Wnt/β-catenin signaling in injured tendons and to identify a potential drug that regulates the Wnt/β-catenin signaling to improve biomechanical properties of healing tendons. First, we detected a significant activation of Wnt/β-catenin signaling within 2 weeks after the tendon injury. When the activity of Wnt/β-catenin signaling was inhibited by IWR-1, abnormal production of mucopolysaccharides was reduced in the tendon tissue. In TDCs, promethazine (PH), a first-generation antihistamine agent that blocks the histamine H1 receptor, suppressed Wnt/β-catenin signaling and increased the expressions of *Mkx* and *Tnmd*. In a rat model of tendon injury, PH suppressed the activity of Wnt/β-catenin signaling and improved the mechanical forces of healing tendons.

Materials and Methods

A rat model of tendon injury

All animal studies were approved by the Animal Care and Use Committee of the [redacted] and followed the guideline of the Institusional Animal Care and Use Committee. Six-week-old male Sprague-Dawley rats (weighting 170–220 g, Japan SLC, Inc.) were subjected to isolation of TDCs, as well as to histological and biomechanical studies. The rats for histological and mechanical studies were anesthetized with 2.5% sevoflurane. Under aseptic

conditions, the right Achilles tendon was injured with a dermal punch (Seiken Torepan, KAI) at the midpoint between the calcaneus and the gastrocnemius muscle. Thereafter, the skin was sutured with a 5-0 nylon thread. The left Achilles tendon was exposed, but the tendon remained uninjured (sham-operated tendon). On postoperative weeks 0.5 (postoperative day 3), 1, 2, 3, 4 and 8, the rats were euthanized with carbon dioxide (CO_2), and the Achilles tendon complex (Achilles tendon with the calcaneus and the gastrocnemius muscle) was isolated and stained with hematoxylin-eosin (HE) and Alcian blue. Tendon repairs were graded according to the Bonar score[3] on the operated tendon tissues. The Bonar score is a sum of the following four parameters; appearance of tenocytes, grades 0 (elongated spindle shape) to 3 (large cell with round nucleus and abundant cytoplasm); ground substance, grade 0 (no stainable ground substance) to 3 (abundant mucin with inconspicuous collagen staining); collagen, grade 0 (tightly cohesive arrangement of fibers) to 3 (marked separation of fibers with loss of architecture); and vascularity, grades 0 (inconspicuous blood vessels) to 3 (more than two clusters of capillaries per tissue). A blinded observer evaluated the grades of tendon repair from 0 (healthy tendon) to 12 (severely injured tendon), and the average in each group of rats was calculated.

Immunostaining for β-catenin protein

For immunostaining, sections of the paraffin-embedded tendon were first deparaffinized and rehydrated. Serial sections were incubated with a rabbit antibody against β-catenin (BD Transduction Laboratories, 1:200 dilution) at 4˚C overnight and then incubated with a secondary donkey antibody against rabbit IgG (H+L) conjugated with Alexa Fluor 488 (Thermo Fisher #A21206, 1: 1,000 dilution) at room temperature for 1 h. The sections were mounted in Vectashield containing 2 ng/ml diamidino-2-phenylindole (DAPI, Vector Laboratories), as described previously.[14] The image was observed using a confocal laser scanning microscope system (TiE-A1R, Nikon). The total and nuclear signal intensities of β-catenin were automatically quantified in three tendons in each group using the MetaMorph software (Molecular Device). We analyzed two areas of the injured tendon, and one area of the sham-operated tendon. Each area was comprised of ~36,000 μm^2. Nucleus was

stained with DAPI. Total cellular and total nuclear signal intensities of β-catenin were normalized by those of the sham-operated tendons on postoperative week 0.5 or those of vehicle-treated tendons.

Drug administration

IWR-1 (Tocris #3532) was first dissolved in dimethyl sulfoxide (DMSO) to make a 50 mM stock solution. IWR-1 was then diluted in saline at 5 μM, and 100 μl of 5 μM IWR-1 or saline with DMSO was administered subcutaneously around the right Achilles tendon every day from the first postoperative day for 2 weeks. PH (20-30 mg, TCI P2029) was dissolved in saline, and 1 mg/kg/day of PH or saline was intramuscularly injected into the quadriceps muscle twice a day from the first postoperative day for 2 weeks.

Primary culture of tendon-derived cells (TDCs)

SD rats (6-week-old males, weighting 170–220 g) were euthanized with CO_2, and TDCs were isolated from the Achilles tendon, as previously reported.[14] After resecting the paratenon and muscle, the Achilles tendon was cut into ~1-mm pieces and placed in a 10-cm culture plate filled with Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (10,000 U/ml, Gibco). After incubating the cells in a humidified chamber with 5% CO_2 at 37˚C for 14 days, the cells were detached with trypsin-EDTA, and seeded in a new plate. After two passages, the cells were seeded in a six-well plate at a density of 3×10^5 cells/well and cultured for 2 days. Then, the cells were supplemented with 0, 0.5, 1, 2, or 5 μM PH (Wako, 165-24142), 2 μM ethopropazine (FCS, 10-1559), 2 μM hydroxyzine (LKT, H97171), 2 μM IWR-1, 2 μM BIO (Sigma, #B1686), 50 ng/ml human recombinant Wnt3a protein (R&D Systems, #5036-WN), 2 μM SD208 (Wako, 193–16331), and/or 1 μM LDN-193189 (Cayman, 11802) for 48 h.

Total RNA extraction and quantitative RT-PCR

Total RNA in TDCs was isolated using QuickGene RNA cultured cell kit (Kurabo) on

QuickGene-800 (Kurabo). The first strand cDNA was synthesized with ReverTra Ace (Toyobo). We quantified mRNA expressions for *Scx*, *Mkx*, and *Tnmd* as tenogenic genes, and for *Axin2* as an indicator of activated Wnt/β-catenin signaling using LightCycler 480 (Roche) and SYBR Green (Takara). The mRNA levels were normalized by *Gapdh*. Primer sequences are shown in Supplementary Table S1.

Biomechanical tests

Biomechanical tests were performed as described elsewhere.[1,4] Briefly, at postoperative week 2, rats were euthanized with CO_2. Bilateral Achilles tendons with the calcaneal bone and the gastrocnemius/soleus muscles were harvested. The sagittal and transverse diameters of the mid part of the Achilles tendons were measured by an electronic digital caliper. The cross sectional areas were calculated assuming an elliptic cylindrical shape as described previously.[9] The gastrocnemius and soleus muscles were scraped off from the tendon, and tendon fibers were fixed in a metal clamp with sandpaper. The calcaneal bone was fixed in a metal clamp at 30° dorsiflexion. The mechanical testing machine (ZTA-500N/EMX-1000N IMADA) pulled the fixed tendon at a constant speed (0.1 mm/s) until the tendon was ruptured. The data acquisition rate was set at every 0.03 sec. The peak force at failure (N) and stiffness (N/mm) were calculated by the testing machine. We quantified the stiffness from the linear part of the elastic phase in the force extension curve. The elastic modulus and peak stress were estimated by the cross-sectional areas.

Statistical analysis

All data were presented as the mean ± standard error of the mean (SEM). Statistical significance was evaluated by either Student's *t*-test, one-way ANOVA with Tukey-Kramer post-hoc test, or two-way repeated measures ANOVA with Tukey-Kramer post-hoc test. The Jonckheere - Terpstra trend test was used to evaluate the dose dependence. The threshold for significance was all set to be $p < 0.05$. The statistical analyses were performed with SPSS statistics 23 (IBM).

Results

Tendon injury activates Wnt/β-catenin signaling and worsens the biomechanical properties

It has previously been reported that Wnt/β-catenin signaling attenuates the differentiation of TDCs by suppressing gene expressions of *Scx*, *Mkx*, and *Tnmd*, partially through suppressing TGF-β signaling.[14] To examine the changes of activity of Wnt/β-catenin signaling over time in tendon cells following tendon injury, we analyzed the accumulation of β-catenin during the healing process of the injured tendons. The rat Achilles tendon was injured by a dermal punch on day 0, and its sagittal section was stained with HE or Alcian blue, and immunostained for β-catenin on postoperative weeks 0.5, 1, 2, 3, 4 and 8 (Fig. 1A, B). HE staining showed that collagen fibers at the injured sites were first replaced by small and round inflammatory cells on postoperative week 0.5. The inflammatory cells were gradually replaced by flattened and elongated tendon cells on postoperative weeks 1 to 4, and finally changed to scar tissue on postoperative week 8 (Fig. 1A). Alcian blue staining on postoperative weeks 1 and 2 showed an abnormal accumulation of mucopolysaccharides at the injured site, which probably represented chondroid tissue (Fig. 1A). In the tendon cells at the injured site, the accumulation of total cellular and total nuclear β-catenin protein peaked at postoperative week 1 and gradually decreased until postoperative week 8 (Fig. 1B-D). We then measured biomechanical properties, and found that peak force, stiffness, peak stress, and elastic modulus were lower in the injured Achilles tendons, although statistical significance was observed only in elastic modulus on postoperative weeks 2 (Fig. 1E). We also found that tendon injury increased the cross-sectional area of the tendon. Thus, tendon injury activated Wnt/β-catenin signaling in tendon cells at the injured site, and compromised biomechanical properties.

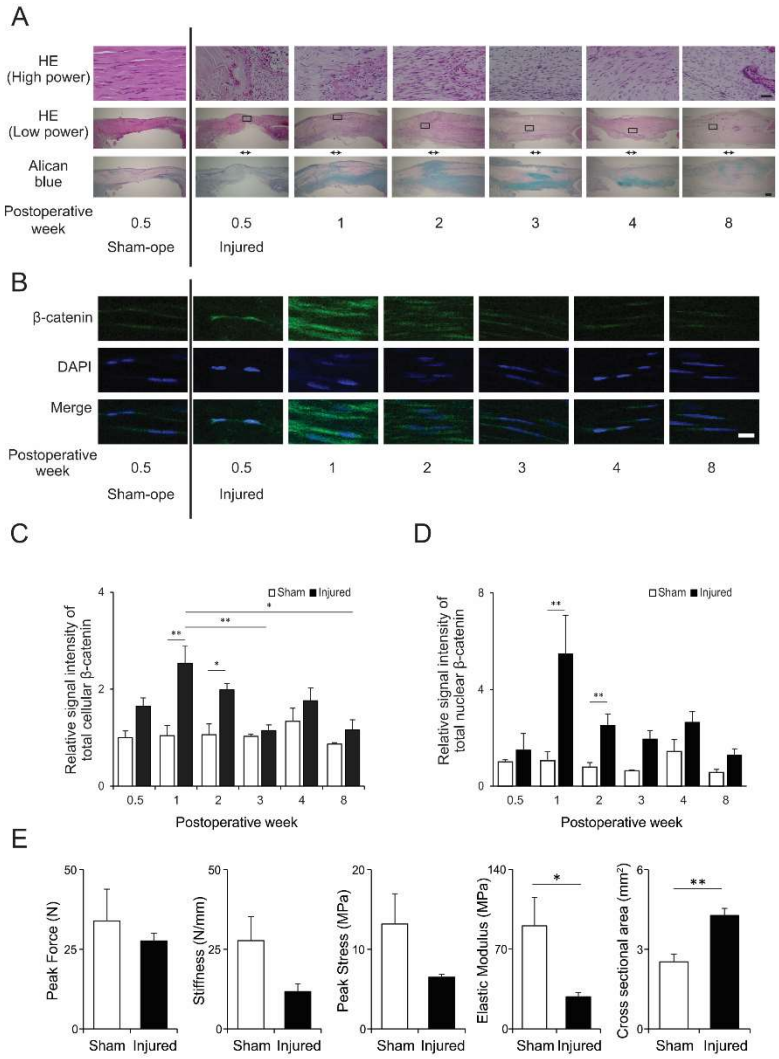


Fig 1. Time course of tendon histology and β-catenin protein in injured rat Achilles tendon. **(A)** High- and low-power field images of hematoxylin-eosin (HE) staining, and low-power field images of Alcian blue staining of sagittal sections of the injured tendon on postoperative weeks 0.5 to 8 and sham-operated tendons on postoperative week 0.5. Positions of high-power field images are indicated by squares. Positions of the injured sites are indicated by a double-headed arrow. Scale bar = 100 μm (higher power field) or 500 μm (lower power field). **(B)** Immunostaining for β-catenin (green) with DAPI (blue). Scale bar = 10 μm. **(C, D)** Mean and SEM (n = 3 rats) of signal intensities of total cellular and total nuclear β-catenin of the tendon cells. Each intensity is normalized by the intensity of sham-operated tendon on postoperative week 0.5. *$p < 0.05$ and **$p < 0.01$ by two-way repeated measures ANOVA followed by Tukey-Kramer post-hoc test. **(E)** Mean and SEM (n = 3

rats) of peak force, stiffness, peak stress, elastic modulus, and cross sectional area of injured and sham-operated Achilles tendons on postoperative weeks 2. *$p < 0.05$ and **$p < 0.01$ by Student's *t*-test.

Suppression of Wnt/β-catenin signaling by IWR-1 reduces the formation of scar tissue, but compromises the biomechanical properties of the injured tendon

To examine the role of Wnt/β-catenin signaling in the early phase of the healing process for the tendon tissue, IWR-1 was injected to the subcutaneous space around the tendon once a day from the first postoperative day for 2 weeks. IWR-1 is a specific inhibitor for Wnt/β-catenin signaling via the stabilization of β-catenin destruction complex. Compared to vehicle-injected tendons, abnormal accumulation of mucopolysaccharides was decreased in the middle part of the IWR-1-treated tendon, while no remarkable difference in the cell shapes of tendon cells was observed in both vehicle- and IWR-1-treated tendons by HE staining on postoperative weeks 2 (Fig. 2A). The histological Bonar scores for the IWR-1-treated tendons were low (Fig. 2B). We assessed immunostaining for β-catenin of the injured tendon, and showed that the signal intensities of total cellular and total nuclear β-catenin were both significantly lower in the IWR-1-treated group (Fig. 2C, D). We then measured the effects of IWR-1 on biomechanical properties of the injured tendons, and found that IWR-1 treatment tended to compromise biomechanical properties including peak force, stiffness, peak stress, and elastic modulus. We also examined the sham-operated tendon, and found that IWR-1 injected on the injured side abnormally lowered the Bonar score and impaired biomechanical properties (Supplementary Fig. S1A, B). These results suggest that IWR-1 suppressed Wnt/β-catenin signaling, reduced accumulation of β-catenin in the nucleus and the cell, and compromised biomechanical features of the healing tendon.

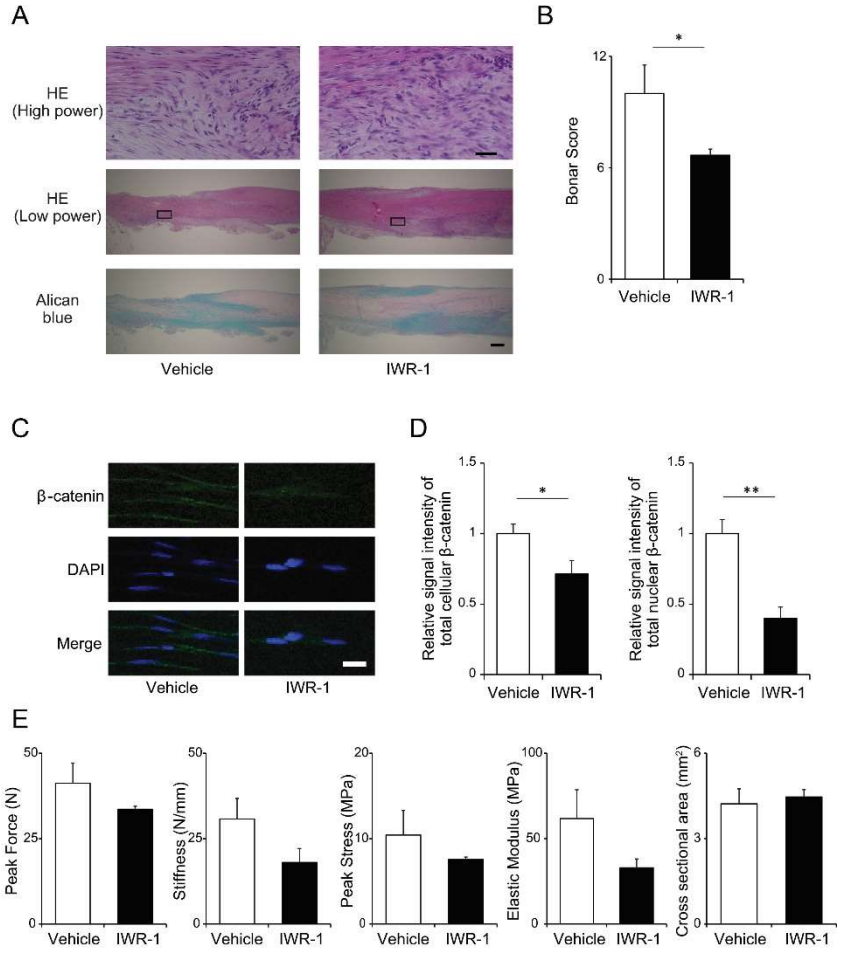


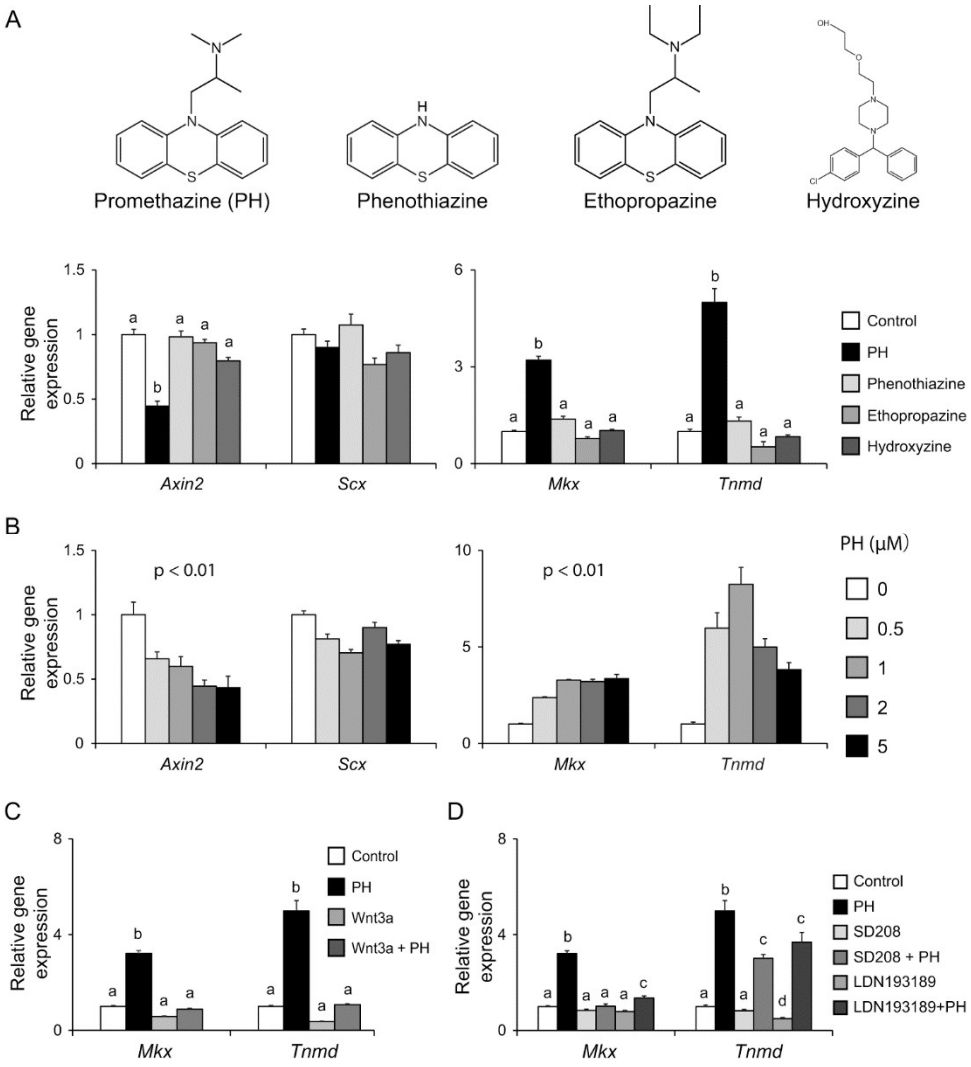
Fig 2. IWR-1 suppressed β-catenin signal, and compromised biomechanical properties in the injured rat Achilles tendon.

(A) Hematoxylin-eosin (HE) and Alcian blue staining of sagittal sections of injured tendons on postoperative weeks 2 with/without subcutaneous IWR-1 administration. Scale bar = 500 μm. **(B)** The average of the Bonar scores of the vehicle- and IWR-1-treated groups is indicated by mean and SEM (n = 3 rats each). *$p < 0.05$ by Student's test. **(C)** Immunostaining of vehicle- and IWR-1-treated groups for β-catenin (green) with DAPI (blue). Scale bar = 10 μm. **(D)** Mean and SEM (n = 3 rats) of signal intensities of total cellular and total nuclear β-catenin of the tendon cells. Signal intensities of β-catenin in IWR-1-treated tendon are normalized by those in vehicle-treated tendons. *$p < 0.05$ and **$p < 0.01$ by Student's test. **(E)** Mean and SEM (n = 3 rats) of peak force, stiffness, peak stress, elastic modulus, and cross sectional area of injured Achilles tendons of vehicle- and IWR-1-treated groups. No statistical significance by Student's t-test.

Promethazine (PH) suppresses Wnt/β-catenin signaling and increases *Mkx* and *Tnmd* expressions in TDCs

It has previously been reported reported that 5-10 μM of IWR-1 increased the gene expressions of *Scx*, *Mkx*, and *Tnmd* ~1.2- to ~1.8-folds in TDCs.[14] However, Wnt/β-catenin signaling suppresses apoptosis of cultured TDCs caused by anti-inflammatory drugs[30], suggesting that suppression of a basal level of Wnt/β-catenin signaling may have an adverse effect on cell survival in the tendon. Indeed, the IWR-1 injection attenuated the biomechanical properties of both injured and sham-operated tendons (Fig. 2E and Supplementary Fig. S1B). We accordingly looked for a clinically approved drug that weakly suppresses Wnt/β-catenin signaling. It has previously been reported that antihistamine agents suppressed Wnt/β-catenin signaling in HCS-2/8 human chondrosarcoma cells.[22] We thus examined the effects of promethazine (PH), phenothiazine, ethopropazine, and hydroxyzine. PH is a first-generation antihistamine agent that is derived from phenothiazine. Ethopropazine is another derivative of phenothiazine. Hydroxyzine is another first-generation antihistamine agent that was not derived from phenothiazine.

We isolated TDCs from the rat Achilles tendon[14], and examined the expressions of tendon-specific genes of *Scx*, *Mkx*, and *Tnmd*, as well as Wnt/β-catenin signaling by examining the expression of its target gene, *Axin2*. We found that only PH downregulated the expression of *Axin2,* and upregulated the expressions of *Mkx* and *Tnmd* in TDCs in a dose-dependence manner (Fig. 3A, B). PH, however, had no effect on the expression of *Scx.* The other three compounds had no effects on the expression of these genes. Wnt3A reduced the expressions of *Mkx* and *Tnmd*, and PH rescued the reduction (Fig. 3C). Additionally, PH-mediated increase of *Mkx* and *Tnmd* expressions was attenuated by inhibitors of the TGF-β superfamily receptors (SD208 for ALK5 and LDN-193189 for ALK2/3). These results suggest that PH increased the expressions of *Mkx* and *Tnmd* in TDCs, likely by down-regulating Wnt/β-catenin signaling and up-regulating TGF-β signaling.



280

281 **Fig 3. Promethazine (PH) suppressed Wnt/β-catenin signaling and increased mRNA**

282 **expressions of *Mkx* and *Tnmd* in rat tendon-derived cell (TDCs).**

283 Relative expressions of *Axin2*, *Scx*, *Mkx*, and *Tnmd* in TDCs treated with 2 μM of PH,

284 phenothiazine, ethopropazine, hydroxyzine **(A)**, 0 to 5 μM of PH **(B)**, 2 μM of PH and/or 50 ng/ml

285 of Wnt3A **(C)**, 2 μM of PH with 2 μM of SD208 or 1 μM of LDN193189 **(D)** for 48 h. Each mRNA

286 expression is normalized by *Gapdh* mRNA, and then normalized with those for untreated cells.

287 Mean and SEM are indicated (n = 3 wells each). After one-way ANOVA, statistically similar items

288 were grouped together according to the Tukey-Kramer post-hoc test ($p < 0.05$), and each group was

289 labeled by an identical single lowercase letter **(A, C, D)**. P-values by the Jonckheere-Terpstra trend

290 test to evaluate dose dependency are indicated at the top of the graph **(B)**.

PH suppresses Wnt/β-catenin signaling and improves biomechanical properties in a rat model of tendon injury

PH is approved to be administered subcutaneously, intramuscularly, and intraorally.[8] To evaluate the effect of PH on injured tendons, PH was administered intramuscularly twice a day from the first postoperative day for 2 weeks in a rat model of tendon injury. PH treatment demonstrated no remarkable changes in HE and Alcian blue stainings as well as in the Bonar score (Fig. 4A, B). We, however, observed that immunofluorescent signals for total cellular and total nuclear β-catenin in tendon cells were significantly reduced by PH treatment (Fig. 4C, D). Additionally, the injured tendons treated with PH had higher biomechanical properties in peak force and stiffness, and higher cross section areas (Fig. 4E). In contrast to the adverse effects of IWR-1 on the sham-operated tendon (Supplementary Fig. 1A, B), PH had no effect on the biomechanical properties of sham-operated tendons (Supplementary Fig. S1C, D). These results suggest that intramuscular injection of PH suppressed Wnt/β-catenin signaling and improved the biomechanical features of the healing tendon in a rat model.

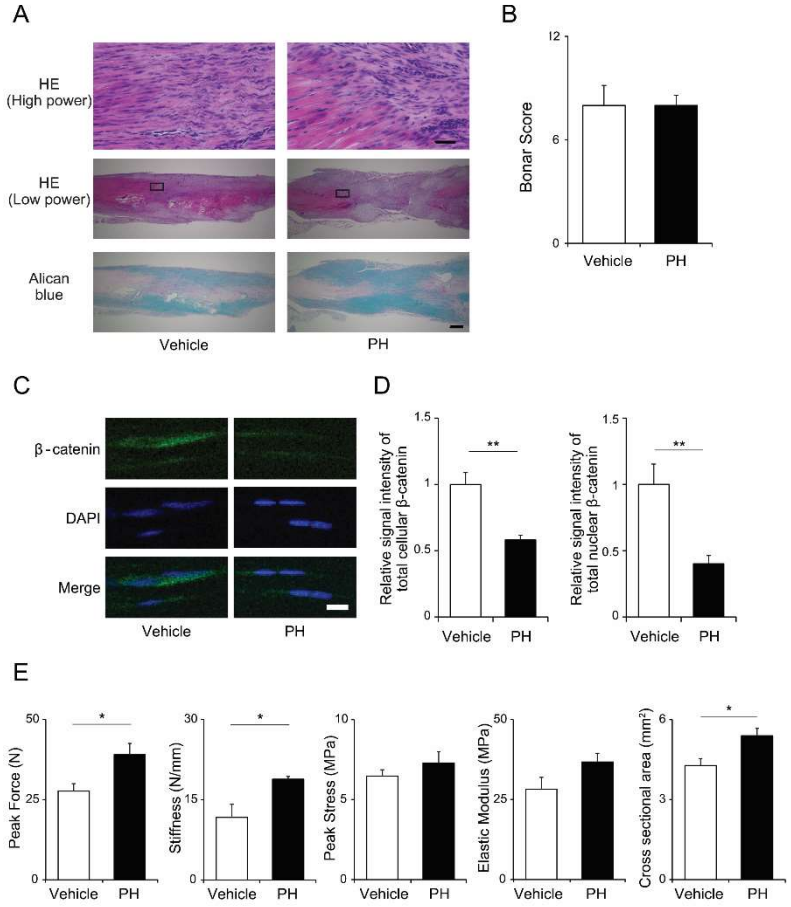


Fig 4. Suppression of β-catenin signal by promethazine (PH) improved biomechanical properties in the injured rat Achilles tendon.

(A) Hematoxylin-eosin (HE) and Alcian blue staining of sagittal sections of injured tendons on postoperative day 14 with/without PH administration. Scale bar = 500 μm. **(B)** The average of the Bonar scores of vehicle- and PH-treated groups are indicated by mean and SEM (n = 3 rats each). No statistical difference by Student's t-test. **(C)** Immunostaining of vehicle- and PH-treated groups for β-catenin (green) with DAPI (blue). Scale bar = 10 μm. **(D)** Mean and SEM (n = 3 rats each) of signal intensities of total cellular and total nuclear β-catenin of the tendon cells. Each intensity is normalized by the intensity of vehicle. *$p < 0.05$ and **$p < 0.01$ by Student's t-test. **(E)** Mean and SEM (n = 3 rats each) of peak force, stiffness, peak stress, elastic modulus, and cross sectional area of injured Achilles tendons of vehicle- and PH-treated groups. *$p < 0.05$ by Student's t-test.

Discussion

Wnt/β-catenin signaling plays a vital role in tissue healing and regeneration, as well as in tissue development.[21,17] However, little is known about the roles of Wnt/β-catenin signaling in tendon repair. In this study, we found that Wnt/β-catenin signaling was activated in tendon cells in 2 weeks after tendon injury (Fig. 1B), when the tissue had abnormal accumulation of mucopolysaccharides (Fig. 1A). We also found that the accumulation of mucopolysaccharides (Fig. 2A) and high Bonar scores (Fig. 2B) were ameliorated by local administration of IWR-1, an inhibitor for Wnt/β-catenin signaling on 2 weeks after tendon injury. In accordance with our observations, other authors reported that prolonged upregulation of Wnt signaling led to delayed tendon-to-bone healing.[29] Taken together, Wnt/β-catenin signaling is activated for 2 weeks after tendon injury and is likely to increase extracellular matrix proteins including mucopolysaccharides at the injured site.

As inhibition of Wnt/β-catenin signaling is a potential therapeutic target for injured tendons, we injected IWR-1 subcutaneously at the injured Achilles tendon to deliver a high concentration of IWR-1 to the tendon but minimally to the neighboring tissues. Although IWR-1 injection ameliorated abnormal accumulation of mucopolysaccharides and lowered the Bonar score, IWR-1 injection rather compromised the biomechanical properties of both injured and sham-operated tendons (Fig. 2E). As IWR-1 is a potent and extensive inhibitor of Wnt/β-catenin signaling and has not been developed for therapeutic purposes, we looked for a drug-repositioned compound with similar Wnt/β-catenin signaling-inhibiting effects.

Here we found that PH, a first-generation antihistamine agent, suppressed Wnt/β-catenin signaling in TDCs. Similar effects, however, were not observed in its parental compound, phenothiazine, or in the other antihistamine agents, ethopropazine or hydroxyzine (Fig. 3A, B). PH significantly upregulated the expressions of both *Mkx* and *Tnmd* in TDCs, probably through down-regulation of Wnt/β-catenin signaling and up-regulation of the TGF-β signaling (Fig.3C, D). In addition to the anthihistamine effect of PH, facilitation of tendon healing was reported in chicken in 1961,[18] and prevention of peritoneal adhesions was reported in rat in 1975.[7] We also reported that

PH suppressed the abnormal osteogenic differentiation of platelet-derived growth factor receptor α (PDGFRα)-positive mesenchymal progenitors in skeletal muscle without overt adverse effects in an animal model.[12] We showed in a rat model of tendon injury that intramuscular injection of PH significantly reduced Wnt/β-catenin signaling (Fig. 4C, D) and improved the biomechanical features of the healing Achilles tendon in 2 weeks after the injury (Fig. 4E).

In conclusion, we identified that PH suppressed Wnt/β-catenin signaling in tendon cells. Although additional non-clinical and clinical studies are required to optimize the dosage and the administration protocol, we propose that a pre-approved drug, PH, is a promising compound that can be potentially applied for the treatment of tendon injury in humans.

Author contributions

T.S., B.O., and K.O. conceived the study and interpreted the results. T.S. and K.M. contributed to the rat experiments. T.S. and Y.K. contributed to the experiments using tendon-derived cells. S.I., and H.H. supervised the project. N.I. and K.O. provided financial support. T.S., B.O., and K.O. prepared the paper with assistance from the other authors.

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