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

Competing Interests:
The authors declare that no competing interests exist.

Running title: Promethazine for tendon injury

Abstract

Background: Wnt/β-catenin signaling suppresses the differentiation of cultured tenocytes, but its roles in tendon repair remain mostly elusive. No chemical compounds are currently available to treat tendon injury.

Hypothesis/Purpose: We hypothesized that the inhibition of the Wnt/β-catenin signaling would accelerate the tendon healing.

Study Design: Controlled laboratory study.

Methods: Tendon-derived cells (TDCs) were isolated from the rat Achilles tendon. The right rat Achilles tendon was injured by a dermal punch, while the left tendon was sham-operated. A Wnt/β-catenin inhibitor, IWR-1, and an antihistamine agent, promethazine, were locally and intramuscularly injected, respectively, for two weeks after surgery. The healing tendons were histologically and biomechanically evaluated.

Results: The amount of β-catenin protein was increased in the injured tendons from postoperative weeks 0.5 to 2. Inhibition of Wnt/β-catenin signaling by IWR-1 in healing tendons improved the histological abnormalities and decreased β-catenin, but compromised the biomechanical properties. As we previously reported that antihistamine agents suppressed Wnt/β-catenin signaling in human chondrosarcoma cells, we examined the effects of antihistamines on TDCs. We found that a first-generation antihistamine agent, promethazine, increased the expressions of the tendon marker genes, *Mkx* and *Tnmd*, in TDCs. Intramuscular injection of promethazine did not improve histological abnormalities, but decreased β-catenin in healing tendons, and also increased the peak force and stiffness of the healing tendons on postoperative week 2. On postoperative week 8, however, the biomechanical properties of vehicle-treated tendons became similar to those of promethazine-treated tendons.

Conclusion: Both IWR-1 and promethazine suppressed Wnt/β-catenin signaling and improved the histological abnormalities of healing tendons. IWR-1, however, compromised the biomechanical properties of healing tendons, whereas promethazine improved them.

Clinical Relevance: Promethazine is a candidate repositioned drug that potentially accelerates tendon repair.

What is known about this subject: The tendon rarely regains its initial functionality with sufficient biological and biomechanical properties after injury. The roles of Wnt/β-catenin signaling in tendon repair remained largely unknown.

What this study adds to existing knowledge: Wnt/β-catenin signaling is activated in the injured Achilles tendons, and inhibition of Wnt/β-catenin signaling by an antihistamine agent, promethazine, accelerated tendon healing with improved biomechanical properties.

Key Terms: Promethazine; Achilles tendon; tendon healing; 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.[7] 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.[8,33] 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.[12] 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.[31] In the last decades, strategies to augment the biomechanical properties of injured tendons have been investigated. Transplantation of embryonic stem cells, bone marrow-derived stromal cells, and endogenous tendon-derived cells (TDCs) enhances tendon repair in animal models.[22] Although these cell therapies are predicted to exert their effects via secreted proteins such as growth factors and extracellular matrix, the details need to be further dissected. In addition, the effects of growth factors, such as IGF-1[18], BMPs[30], and TGF-β[17], and their combinations[24] on tendon healing have been reported in animal and cellular models. Clinical effects of platelet-rich plasma are likely to be partly accounted for by local enrichment of these growth factors.[15]

The tendon-related extracellular matrix such as collagen types I and III, are secreted by differentiated tenocytes, which is promoted by transcriptional factors, scleraxis (encoded by *Scx*) and mohawk (encoded by *Mkx*). *Scx* is required for proper differentiation of tendon cells and is an important early marker in tendon development. Knockout of *Scx* impairs the development of the force-transmitting tendons of the limbs and the tail, but not of the muscle-anchoring tendons.[27] *Mkx* is required for tendon maturation by regulating type I collagen production in tendon cells. *Mkx*-/- mice show hypoplastic tendon tissues throughout the body.[13] A regulator for extracellular matrix proteins, 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.[5]

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.[28,3] In 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. We previously reported that the suppression of activated Wnt/β-catenin signaling by a specific inhibitor of β-catenin stabilization, IWR-1, induces gene expressions of *Scx*, *Mkx*, and *Tnmd*, partially through the suppression of TGF-β signaling[16]. However, little is known about the spatiotemporal and physiological roles of Wnt/β-catenin signaling in injured tendon tissues.

Our previous report showed that antihistamine agents had a class effect on the inhibition of Wnt/β-catenin signaling in human chondrosarcoma-derived HCS-2/8 cells.[26] We also showed that antihistamine agents including promethazine (PH) suppressed abnormal adipogenic differentiation of platelet-derived growth factor receptor α (PDGFRα)-positive mesenchymal stem cells in skeletal muscle.[14] In addition, PH was reported to promote the healing of injured tendon of digital flexor in chicken in 1961.[21] PH is one of the first-generation antihistamine agents including ethopropazine and hydroxyzine. PH and ethopropazine are derivatives of phenothiazine. PH has antidopaminergic, antihistaminergic, and anticholinergic properties, and has been used to treat allergies, insomnia, and nausea without major adverse effects[32,35].

Here we analyzed the activation of Wnt/β-catenin signaling in injured tendons, and examined the effects of inhibition of Wnt/β-catenin signaling by IWR-1 and PH on tendon healing.

Materials and Methods

A rat model of tendon injury

All animal studies were approved by the Animal Care and Use Committee of the Nagoya University (No. 20277) and were conducted following relevant guidelines. Six-week-old male Sprague-Dawley rats (weighting 170–220 g, Japan SLC, Inc.) were subjected to isolation of TDCs (*n*

= 15 rats), as well as to histological (n = 30 rats) and biomechanical studies (n = 24 rats). 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 (Supplementary Fig. S1A). 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 (n = 6 rats at each time point). The samples were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned into 3 μm-thick slices (n = 3 rats at each time point). The remaining samples were subjected to RNA isolation for quantitative RT-PCR (n = 3 rats at each time point). The sections were stained with hematoxylin-eosin (HE) and Alcian blue. Tendon repairs were graded according to the Bonar score[4] 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 healing tendon, and gave the Bonar scores of 0 (healthy tendon) to 12 (severely injured tendon). The average Bonar score in each group of rats (n = 3 tendons for each group) was calculated.

Immunostaining for β-catenin protein

For immunostaining, 3 μm-thick sections of the paraffin-embedded tendon were first deparaffinized and rehydrated. Serial sections were incubated with a rabbit antibody against β-catenin (Cell Signaling Technology, 9587S, 1:200 dilution for single staining; and BD Transduction Laboratories, #610153, 1:200 dilution for double staining with Scx), Scx (abcam, ab58655, 1:200 dilution), and Tnmd (abcam, ab203676, 1:200 dilution) at 4˚C overnight, and then incubated with an

antibody against mouse IgG (H+L) conjugated with biotin (Vector Laboratories, BA-2000, 1:200 dilution) followed by incubation of Alexa Fluor 546 (Thermo Fisher Scientific, S11225, 1:200 dilution) with or without 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.[16] The image was observed using a confocal laser scanning microscope system (TiE-A1R, Nikon). The total and nuclear signal intensities of β-catenin, as well as the number of DAPI-positive cells, were automatically quantified in three tendons in each group using the MetaMorph software (Molecular Device). We analyzed two areas for the injured tendon, and one area for the sham-operated tendon. Each area was comprised of ~36,000 μm^2. Total cellular and total nuclear signal intensities of β-catenin were normalized for the average of three sham-operated or vehicle-treated tendons on postoperative week 0.5. The β-catenin-stained or/and Scx-stained cells were counted by a blind observer.

Drug administration

IWR-1 (Tocris #3532) was first dissolved in dimethyl sulfoxide (DMSO) to make a 50 mM stock solution. As ubiquitous inhibition of Wnt/β-catenin signaling by systemic administration of IWR-1 would be toxic to rats, we locally administered IWR-1 around the injured Achilles tendon. The size of rat Achilles tendon was ~40 mm^3 with ~10 mm in length, ~2 mm in thickness, and ~2 mm in width. As 1 μM IWR-1 attenuated Wnt/β-catenin signaling in TDCs (see Results), 10 μl of 5 μM IWR-1 in saline was subcutaneously administered around the healing Achilles tendon twice a day to locally attain ~1.25 μM IWR-1. The same amount of saline with 0.01% DMSO was similarly administered as a control. In contrast to IWR-1, intramuscular injection of PH has been approved in clinical settings. We previously showed that oral administration of 1 mg/Kg/day PH for mice ameliorated abnormal adipogenesis in the lower leg muscles, which was induced by resection of the Achilles tendon. [14] In addition, muscle injection of PH was reported to promote the healing of injured tendon of digital flexor in chicken in 1961.[21]We thus injected 1 mg/Kg/day PH [100 μl of 6.2 ~ 9.4 μM PH (TCI P2029) in

158 saline] in the quadriceps muscle twice a day from the first postoperative day for 2 weeks in a rat model
159 of Achilles tendon injury.
160
161 Primary culture of tendon-derived cells (TDCs)
162 Twelve SD rats (6-week-old males, weighting 170–220 g) were euthanized with CO_2, and TDCs
163 were isolated from the Achilles tendon, as previously reported.[16] After resecting the paratenon and
164 muscle, the Achilles tendon was cut into ~1-mm pieces and placed in a 10-cm culture plate filled with
165 Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies) supplemented with 10% fetal
166 bovine serum and 1% penicillin-streptomycin (10,000 U/ml, Gibco). After incubating the cells in a
167 humidified chamber with 5% CO_2 at 37˚C for 14 days, the cells were detached with trypsin-EDTA,
168 and seeded in a new plate. After two passages, the cells were seeded in a six-well plate at a density of
169 3×10^5 cells/well and cultured for 2 days. Then, the cells were supplemented with 0, 0.5, 1, 2, or 5
170 μM PH (Wako, 165-24142), 2 μM ethopropazine (FCS, 10-1559), 2 μM hydroxyzine (LKT, H97171),
171 2 μM IWR-1, 2 μM BIO (Sigma, #B1686), 50 ng/ml human recombinant Wnt3a protein (R&D
172 Systems, #5036-WN), 2 μM SD208 (Wako, 193–16331), and/or 1 μM LDN-193189 (Cayman, 11802)
173 for 48 h.
174
175 Total RNA extraction and quantitative RT-PCR
176 Total RNA in TDCs or in the Achilles tendon (n = 6 tendons each for sham-operated and injured
177 tendons treated with either vehicle, IWR-1, or PH) was isolated using QuickGene RNA cultured cell
178 kit (Kurabo) on QuickGene-800 (Kurabo). The first strand cDNA was synthesized with ReverTra Ace
179 (Toyobo). We quantified mRNA expressions using LightCycler 480 (Roche) and SYBR Green
180 (Takara). The mRNA levels were normalized for *Gapdh*. Primer sequences are shown in
181 Supplementary Table S1.
182
183 Biomechanical tests
184 Biomechanical tests were performed as described elsewhere.[1,6] Briefly, on postoperative weeks

2 and 8, rats (n = 6 tendons each for sham-operated and injured tendons treated with vehicle, IWR-1, or PH at each time point) 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.[11] 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 compromises the biomechanical properties

Our previous report showed 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.[16] To examine the change of activities of Wnt/β-catenin signaling over time 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 (Supplementary Fig. S1A). On

postoperative weeks 0.5, 1, 2, 3, 4 and 8, a sagittal section of the injured Achilles tendon was stained with HE or Alcian blue, and immunostained for β-catenin (Fig. 1A, B). HE staining showed that collagen fibers at the injured site 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, total cellular and total nuclear β-catenin levels were significantly high up to postoperative week 2 (Fig. 1B-D). In addition, the ratio of β-catenin-positive cells remained high at the injured site up to postoperative week 8 (Fig. 1E), although the signal intensities were gradually decreased (Fig. 1C, D). qRT-PCR of the whole tendon tissue showed that expression of *Tnmd,* but not of *Scx* or *Mhx*, was increased in the injured tendon tissue on postoperative week 2 (Supplementary Fig. S1B). Furthermore, immunostaining for β-catenin, Scx, and Tnmd in serial sections revealed that β-catenin- and Scx-positive cells were diffusely observed adjacent to the injured site, whereas Tnmd-positive cells were abundantly observed more than 10 μm away from the injured site (Supplementary Fig. S1C) on postoperative week 2. Double immunostaining of the injured site revealed that 48.6% of DAPI-positive cells expressed Scx protein and 92.6% of the Scx-positive cells expressed nuclear β-catenin protein on postoperative week 2 (Supplementary Fig. S1D). These results suggested that Wnt/β-catenin signaling was activated in the tendon precursor cells in the early stage of healing processes. We then measured biomechanical properties, and found that peak force, stiffness, peak stress, and elastic modulus were lower in the injured Achilles tendons compared to those in the sham-operated tendons (Fig. 1F). 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 the biomechanical properties.

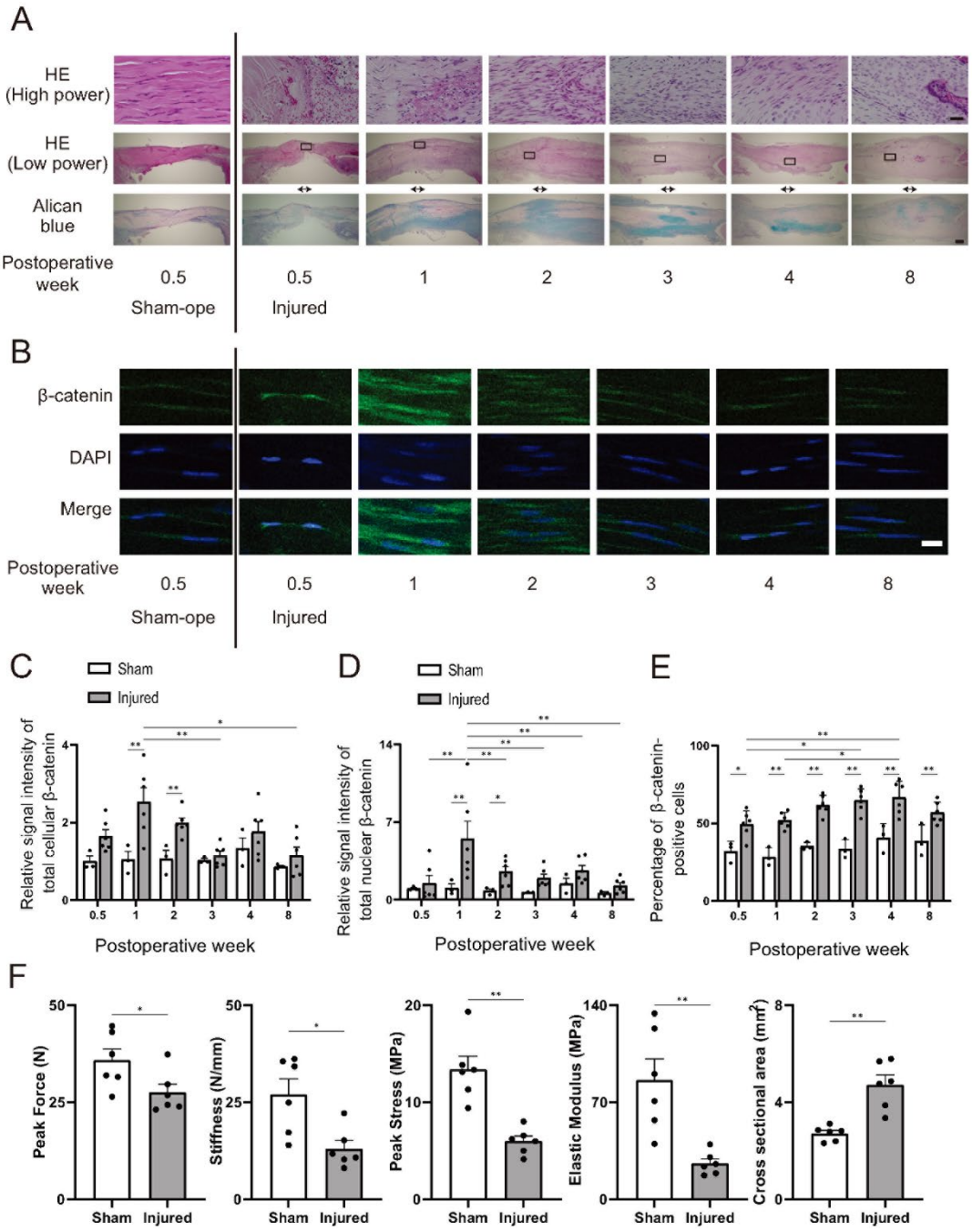


Fig 1. Time course of tendon histology and β-catenin protein in injured rat Achilles tendon.

(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 for 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 percentage of β-catenin-positive cells. *$p < 0.05$ and **$p < 0.01$ by two-way repeated measures ANOVA followed by Tukey-Kramer post-hoc test. **(F)** Mean and SEM (n = 6 rats) of peak force, stiffness, peak stress, elastic modulus, and cross-sectional area of injured and sham-operated Achilles tendons on postoperative week 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 of the tendon injury, 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 between vehicle- and IWR-1-treated tendons by HE staining on postoperative week 2 (Fig. 2A). The histological Bonar scores of the IWR-1-treated tendons were lower than those of vehicle-treated tendons (Fig. 2B). We assessed immunostaining for β-catenin of the injured tendon, and found 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 lowered the Bonar score of the sham-operated tendon (Supplementary Fig. S2A) and impaired biomechanical properties (Supplementary Fig. S2C). These results suggest that IWR-1 suppressed Wnt/β-catenin signaling, reduced accumulation of β-catenin in the nucleus and the cell,

and compromised the biomechanical features of the healing tendon.

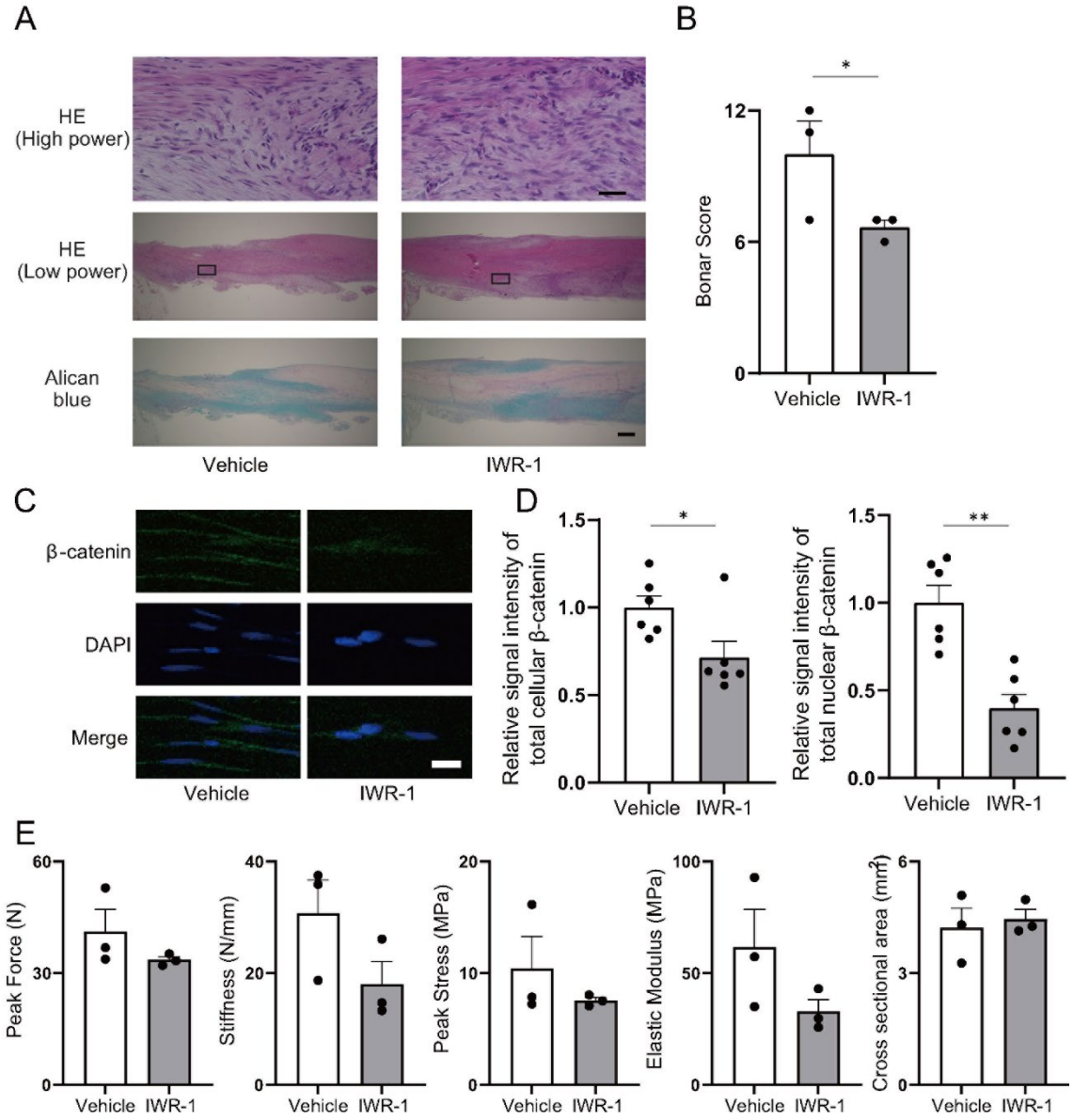


Fig 2. IWR-1 suppressed β-catenin signaling, and compromised the biomechanical properties of the injured rat Achilles tendon.

(A) Hematoxylin-eosin (HE) and Alcian blue staining of sagittal sections of injured tendons on postoperative week 2 with or without subcutaneous IWR-1 administration. Scale bar = 500 μm. **(B)** Bonar scores of the vehicle- and IWR-1-treated injured tendons are indicated by mean and SEM (n = 3 rats each). *$p < 0.05$ by Student's test. **(C)** Immunostaining of vehicle- and IWR-1-treated tendons 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 the biomechanical features (peak force, stiffness, peak stress, elastic modulus, and cross-sectional area) of vehicle- and IWR-1-treated injured Achilles tendons. No statistical significance by Student's *t*-test. The effects of IWR-1 on the Bonar score and the biomechanical features of the sham-operated tendon are shown in Supplementary Figs. S2A and S2C, respectively.

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

We previously showed that 5-10 μM of IWR-1 increased the gene expressions of *Scx*, *Mkx*, and *Tnmd* ~1.2- to ~1.8-folds in TDCs.[16] However, Wnt/β-catenin signaling suppresses apoptosis of cultured TDCs caused by anti-inflammatory drugs[38], 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. S2C). We accordingly looked for a clinically approved drug that appropriately suppresses Wnt/β-catenin signaling. We previously reported that antihistamine agents have a class effect on the suppression of Wnt/β-catenin signaling in HCS-2/8 human chondrosarcoma cells.[26] We thus examined the effects of antihistamine agents (PH, phenothiazine, ethopropazine, and hydroxyzine) on tendon healing. PH and ethopropazine are the first-generation antihistamine agents derived from phenothiazine. Hydroxyzine is another first-generation antihistamine agent that was not derived from phenothiazine.

We isolated TDCs from the rat Achilles tendon[16], 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 PH, but not the other three antihistamine agents, 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.* We found that Wnt3A reduced the expressions of *Mkx* and *Tnmd*, and PH did not rescue the reduction (Fig. 3C),

suggesting that PH should have worked on upstream of Wnt3a. Then, we analyzed the effects of PH on the gene expressions for Wnt ligands (*Wnt1*, *Wnt3*, *Wnt4*, and *Wnt7a*), a mediator for secretion of Wnt ligands (*Wls*), and an inhibitor for Wnt ligands (*Dkk1*). *Wnt1*, *Wnt3*, *Wnt4*, and *Wnt7a* encode proteins in the Wnt ligand family, and are expressed in the regions where Scx- and Mkx-positive-tendon precursors originate in E15.5 embryos (Allen Brain Atlas). *Wls* encodes a membrane protein and is required for the secretion of Wnt ligands for limb morphogenesis including tendon tissues.[40] *Dkk1* encodes an inhibitor for Wnt ligands, and induces the differentiation of adipocytes from tendon stem cells.[2] As 0 to 5 μM PH induced the expressions of *Mkx* and *Tnmd*, and suppressed the expression of *Axin2* in dose-dependent manners (Fig. 3B), we examined the effects of this range of PH on the expressions of *Wnt1, Wnt3, Wnt4, Wnt7a, Wls*, and *Dkk1*. Contrary to our expectations, however, PH rather induced *Wnt1* expression and tended to induce *Wnt3* expression in dose-dependent manners, although *Wnt4* tended to be suppressed in a dose-dependent manner (Supplementary Fig. S3). PH-mediated suppression of Wnt/β-catenin signaling was thus likely to be accounted for by unidentified or unanalyzed Wnt-related molecule(s).

We next examined the involvement of TGF-β signaling in PH-mediated tendon healing, and found that 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) (Fig. 3D). TGF-β signaling is required for the maintenance of differentiated tenocytes via phosphorylation of Smad2/3.[36] We found that PH affected neither Smad 2/3 phosphorylation nor the expression of *Id1*, one of direct target genes for TGF-β signaling,[20] in TDCs (Fig. 3E). Thus, TGF- β signaling was required for the effect of PH, but was not upregulated by PH. To summarize, PH increased the expressions of *Mkx* and *Tnmd* in TDCs, likely by down-regulating Wnt/β-catenin signaling but not by up-regulating TGF-β signaling.

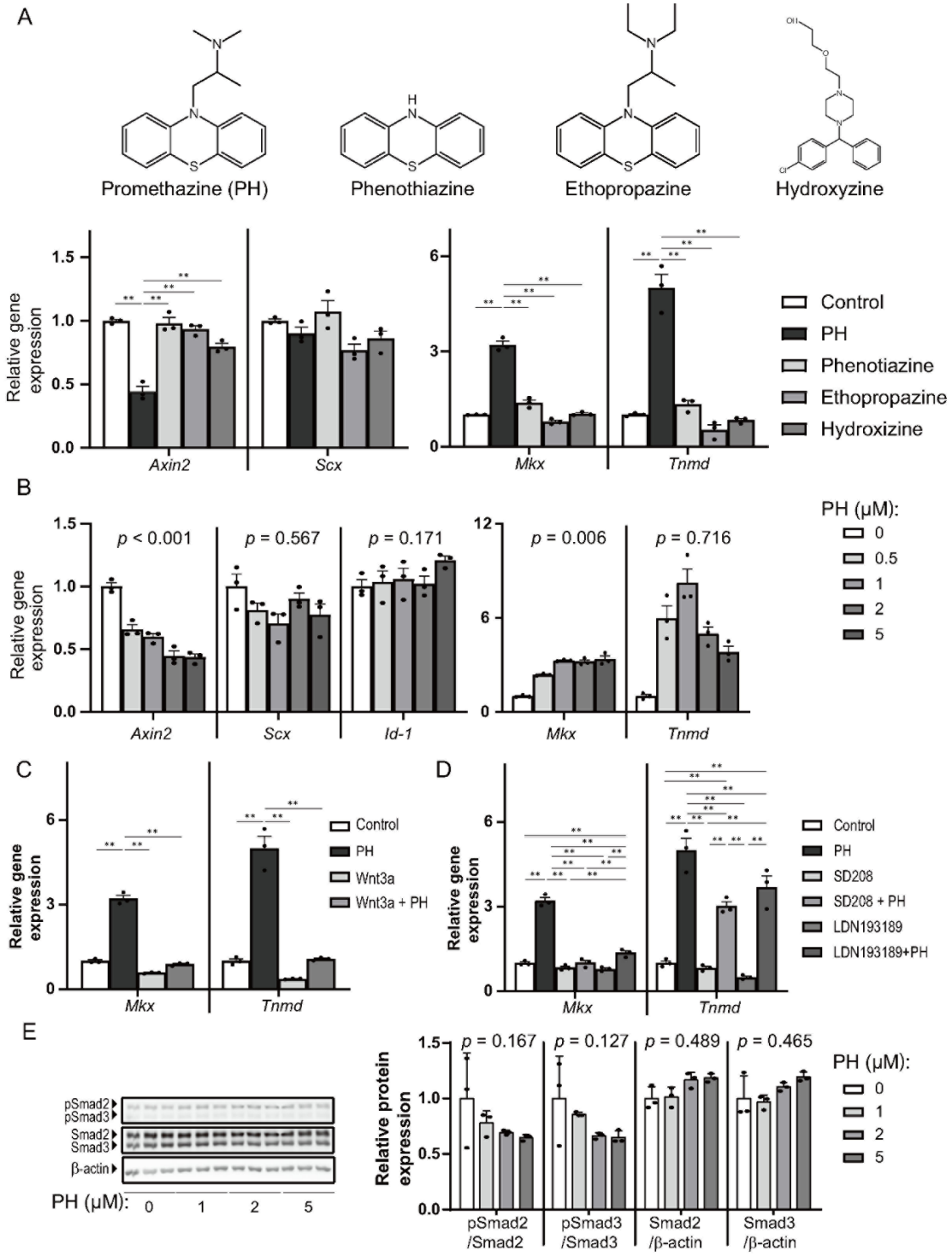


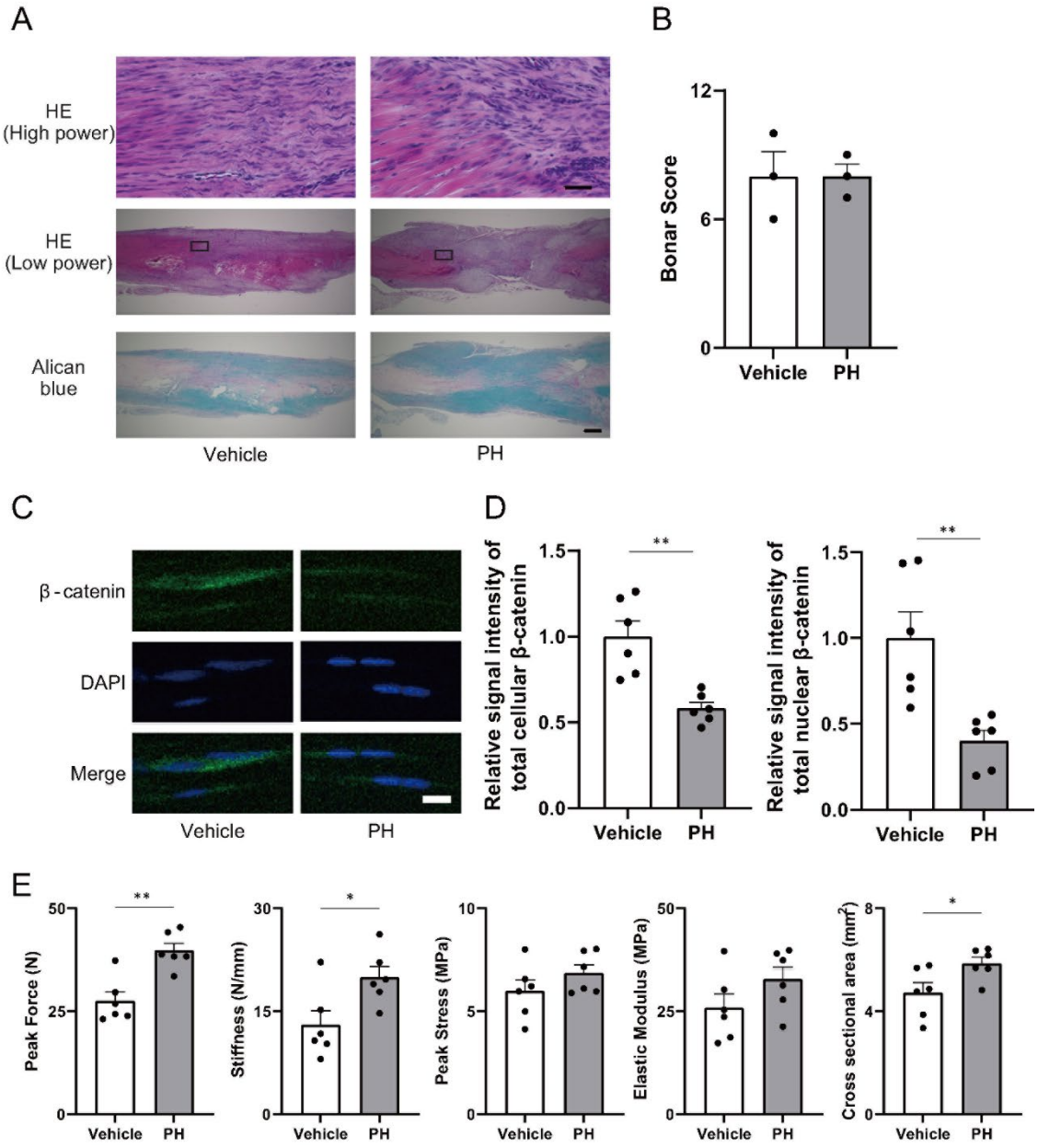
Fig 3. Promethazine (PH) suppressed Wnt/β-catenin signaling and increased mRNA expressions of *Mkx* and *Tnmd* in rat tendon-derived cell (TDCs).

Relative expressions of *Axin2*, *Scx*, *Mkx*, *Tnmd*, and *Id1* in TDCs treated with 2 μM of four antihistamine agents (PH, phenothiazine, ethopropazine, and hydroxyzine) **(A)**, 0 to 5 μM of PH **(B)**, 2 μM of PH and/or 50 ng/ml of Wnt3A **(C)**, and combinations of 2 μM of PH, 2 μM of SD208, and 1 μM of LDN193189 **(D)** for 48 h. Each mRNA expression was normalized for *Gapdh* mRNA, and then for the average of untreated cells. **(E)** Western blotting of phosphorylated Smad2 and Smad3 (pSmad2 and pSmad3, respectively), as well as total Smad2 and Smad3, in TDCs treated with 0, 1, 2, and 5 μM of PH for 48 h. For quantitative analysis, phosphorylated Smad2 and Smad3 were normalized for total Smad2 and Smad3, respectively, and then for the average of untreated cells. Total Smad2 and Smad3 were normalized for β-actin, and then for the average of untreated cells. Mean and SEM are indicated (n = 3 wells each). **(A, C, D)** One-way ANOVA followed by Tukey-Kramer post-hoc test. *$p < 0.05$ and **$p < 0.01$. **(B, E)** P-values by the Jonckheere-Terpstra trend test to evaluate dose dependency are indicated at the top of each graph.

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

PH is clinically approved to be administered subcutaneously, intramuscularly, and intraorally.[10] We first confirmed that, in contrast to the adverse effects of IWR-1 on the biomechanical properties of sham-operated tendon (Supplementary Fig. S2A, C), intramuscular injection of PH for 2 weeks had no effect on the biomechanical properties of sham-operated tendons (Supplementary Fig. S2B, D). 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. On postoperative week 2 when PH administration was terminated, we first confirmed that PH had no effect on the number of DAPI-positive cells at the injured site (Supplementary Fig. S4A). Then we analyzed the expressions of 18 genes at the injured site by quantitative RT-PCR, and found that PH treatment decreased the expressions of *Scx*, a gene for tendon precursors; *Il1b,* a gene for inflammation; and *Mmp2*, a gene for ECM degradation; and *Fn1*, a gene for fibrosis (Supplementary Fig. S4B-D). Reduced *Scx* may indicate accelerated maturation of tendon cells, because *Scx* is an early marker of tendon cell differentiation.[27] Reduced *Il1b*, *Mmp2*, and *Fn1* should be in favor of the healing process. Although

PH treatment failed to reduce the Bonar score (Fig. 4A, B), we observed that PH treatment significantly reduced immunofluorescent signals for total cellular and total nuclear β-catenin in tendon cells (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). On postoperative week 8, however, the biomechanical properties of the injured Achilles tendon became similar between the vehicle and PH treatment groups (Supplementary Fig. S2E), indicating that PH accelerated tendon healing, but the advantage disappeared in the long term. Taken together, intramuscular injection of PH suppressed Wnt/β-catenin signaling and accelerated improvement of biomechanical features of the Achilles tendon in the early stage of tendon healing in a rat model.



370 **Fig 4. Suppression of β-catenin signal by promethazine (PH) improved biomechanical properties**

371 **of the injured rat Achilles tendon.**

372 **(A)** Hematoxylin-eosin (HE) and Alcian blue staining of sagittal sections of injured tendons on

373 postoperative day 14 with or without PH administration. Scale bar = 500 μm. **(B)** Mean and SEM (*n*

374 = 3 rats each) of the Bonar scores of vehicle- and PH-treated tendons. No statistical difference by

375 Student's *t*-test. **(C)** Immunostaining of vehicle- and PH-treated groups for β-catenin (green) with

376 DAPI (blue). Scale bar = 10 μm. **(D)** Mean and SEM (*n* = 3 rats each) of signal intensities of total

377 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 ($n = 6$ 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. The effects of PH on the Bonar score and the biomechanical features of the sham-operated tendon are shown in Supplementary Figs. S2B and S2D, respectively.

Discussion

Wnt/β-catenin signaling plays a vital role in tissue healing and regeneration, as well as in tissue development.[25,19] Indeed, Wnt3a expression is temporally increased in the fibroblast-like cells and chondrocyte-like cells in a collagenase-injected rat model of tendon injury and in some clinical samples of tendinopathy.[23] Wnt/β-catenin signaling inhibits apoptosis of tendon stem cells.[39] 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-D), 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.[37] 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 healing tendon but not to the other 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.

Being prompted by our previous observation that antihistamine agents have a class effect on the inhibition of Wnt/β-catenin signaling in human chondrosarcoma-derived HCS-2/8 cells,[26] we screened for the suppressive effects of four antihistamine agents on Wnt/β-catenin signaling in TDCs. 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 through down-regulation of endogenous Wnt/β-catenin signaling probably at the level of Wnt ligands (Fig. 3C and Supplementary Fig. S3). We found that TGF-β signaling is required for the upregulation of *Mkx* and *Tnmd* (Fig. 3D), although PH did not enhance TGF-β signaling (Fig. 3E). Both Scx [34] and *Mkx*[29] upregulate the expression of *Tnmd*. As PH upregulated *Mkx* but not *Scx* in TDCs (Fig. 3B), PH might have promoted the expression of *Tnmd* through *Mkx*. Further studies are required to understand how PH inhibits Wnt/β-catenin signaling and how Wnt/β-catenin signaling regulates *Mkx* and *Tnmd* expressions. Interestingly, the effects of PH have been reported in multiple pathological states. Facilitation of tendon healing by PH was reported in chicken in 1961.[21] Prevention of peritoneal adhesions by PH was reported in rat in 1975.[9] We also reported that PH suppressed abnormal adipogenic differentiation of PDGFRα-positive mesenchymal progenitors in skeletal muscle without overt adverse effects in an animal model.[14] We currently showed in a rat model of tendon injury that intramuscular injection of PH significantly reduced Wnt/β-catenin signaling (Fig. 4C, D), suppressed inflammation and fibrogenesis (Supplementary Fig. S4B-E), and accelerated the improvement of biomechanical features of the healing Achilles tendon at 2 weeks (Fig. 4E) but not at 8 weeks (Supplementary Fig. S2E, F) after the injury. The accelerated tendon healing, however, did not make the healed tendon vulnerable in the late stage. Although PH is primarily an antihistamine agent, PH might have exerted its effects via its inhibition of dopamine D_2, and/or muscarinic cholinergic receptors.

In conclusion, we identified that PH suppressed Wnt/β-catenin signaling in tendon cells at the early stage of the healing processes of the injured tendon. Accelerated tendon healing would enable

range of motion (ROM) exercises in the early clinical stage. Here, 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. Further non-clinical and possibly clinical studies are required to determine the optimal dosage and the optimal administration protocol.

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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