Tendinopathy, the most common disorder affecting tendons, is characterized by chronic disorganization of the tendon matrix, which leads to tendon tear and rupture. The goal was to identify a rational molecular target whose blockade can serve as a potential therapeutic intervention for tendinopathy. We identified C1q/TNF-related protein-3 (CTRP3) as a markedly up-regulated cytokine in human and rodent tendinopathy. Overexpression of CTRP3 enhanced the progression of tendinopathy by accumulating cartilaginous proteoglycans and degenerating collagenous fibers in the mouse tendon, whereas CTRP3 knockdown suppressed the tendinopathy pathogenesis. Functional blockade of CTRP3 using a neutralizing antibody ameliorated overuse-induced tendinopathy of the Achilles and rotator cuff tendons. Mechanistically, CTRP3 elicited a transcriptomic pattern that stimulates abnormal differentiation of tendon stem/progenitor cells and ectopic chondrification as an effect linked to activation of Akt signaling. Collectively, we reveal an essential role for CTRP3 in tendinopathy and propose a potential therapeutic strategy for the treatment of tendinopathy.

INTRODUCTION

Tendons, which are primarily constructed of parallel arrangements of collagenous fibers, resist tensile stresses transmitted from the muscle to the bone (1). Tendon pathologies range from chronic tendon disorder, tendinopathy, to acute injury involving partial or complete tendon rupture (1). Tendon pathologies are frequently found in athletes and individuals subjected to excessive or repetitive musculoskeletal activities (1, 2). More than 30 million tendon-related procedures are performed annually worldwide, presenting a considerable socioeconomic burden (3).

Tendinopathy is the most common form of chronic tendon disorders, characterized by progressive disorganization and alteration of the tendon matrix that predisposes the tendons to eventual tears and ruptures (4). Despite relatively well-recognized risk factors for tendinopathy, such as mechanical stresses caused by tendon injury or overuse (1, 4), the pathogenesis of tendinopathy remains unclear. Acute tendon injury is characterized by a discontinuity of the tendon as a result of partial tear or complete rupture (1). Although acutely injured tendons attempt a healing process accompanying inflammation and extracellular matrix (ECM) remodeling, repaired tendons often contain fibrovascular scars with disorganized matrix composition, which results in inferior mechanical properties and impaired force-transmitting functions (5, 6).

Therapeutically, symptomatic or surgical treatments are performed in patients with tendon disorders (7, 8). However, surgical tendon repair is frequently associated with postoperative failure such as rerupture, as the repaired tendon often retains degenerative changes associated with tendon pathologies (7). Currently, effective disease-modifying treatments for degenerative tendon diseases are not available (9). Hence, new molecular targets and methods for therapeutic intervention are required.

The ECM of a tendon predominantly consists of collagen fibrils and a small fraction of proteoglycans (PGs) and glycoproteins (1, 2). Collagen fibrils consist of >95% type I collagen and low levels of other types of collagen such as type III (8). Disruption of the integrity of collagen fibrils and altered matrix composition are the primary concerns in tendon pathologies (10). Type I collagen fibrils are responsible for the overall tensile strength of tendon tissues (2). A diseased tendon typically exhibits an increased proportion of type III collagen relative to type I collagen (2). Accumulation of cartilage-like matrix consisting of ground substances is a hallmark of tendinopathy (11). This event is linked to impairment of tensile strength of the tendon matrix (12). Degenerative tendons frequently display chondroid metaplasia characterized by the deposition of hyaluronan and PGs (13). The pathophysiological mechanisms underlying this abnormal matrix remodeling in tendons remain unclear.

Biological therapies targeting cytokines hold great promise for the treatment of various diseases (14). Here, we sought to identify a therapeutic target for treating tendinopathy, with a goal to identify soluble factors that directly regulate tendon degeneration. C1q/tumor necrosis factor (TNF)–related protein-3 (CTRP3) is a highly hydrophilic, secreted protein that belongs to the C1q/TNF superfamily (15). It is highly conserved among vertebrates and consists of N-terminal collagenous repeats (Gly-X-Y) and a C-terminal C1q-like globular domain (15). Notably, a 95.9% amino acid sequence identity exists
between human and mouse CTRP3 proteins (16). CTRP3 plays important functions, such as regulation of metabolism (17), inflammation (16), and cell growth (15, 18). The functional aspects of CTRP3 are under active investigation, particularly in the context of metabolic syndrome (19) and inflammatory diseases (16), such as fatty liver (20) and rheumatoid arthritis (21). Among the mesenchymal lineage cells, CTRP3 is expressed in adipocytes and chondrocytes (16). CTRP3 expression is up-regulated during chondrogenic differentiation of progenitor cells (15), suggesting its possible roles in cartilage and bone development. CTRP3 is essential to support the organized architecture of cartilage in the fetal mouse mandible (22). However, a pathological association between dysregulation of CTRP3 and musculoskeletal disorders has yet to be explored. In this study, we elucidated an essential role of CTRP3 in promoting tendon pathologies and developed a potential disease-modifying, biological therapy for the treatment of tendinopathy.

RESULTS

CTRP3 expression is up-regulated in human and mouse tendinopathy

To identify a mediator of tendon pathologies whose expression is specifically increased in the damaged condition, we first analyzed the transcriptome dataset of human tendinopathy (fig. S1A) (23). Three hundred and thirty-six genes were identified to be differentially up-regulated in damaged tendons ($n = 23$) in comparison with normal tendons ($n = 23$) with $P < 0.05$ and fold change $\geq 1.5$. We further focused on candidates classified as “cytokines” using the databases from the Ingenuity Pathway Analysis (IPA) (1740 genes) and UniProt (1662 genes), considering their potential advantages in terms of druggability by antibody-based therapy (fig. S1B and table S1). Nine cytokine genes were differentially up-regulated in the damaged tendons of humans in comparison with their normal counterparts (table S2). We then conducted RNA sequencing using samples from sham-operated and partially transected mouse Achilles tendons (fig. S2, A to D, and data file S1). A total of 44 cytokine genes were differentially up-regulated in the acutely injured tendons with $P < 0.05$ and fold change $\geq 1.5$. We identified CTRP3, SPP1, IL11, SPARC, and VEGFC as the commonly up-regulated cytokine genes in damaged tendons of human and mouse (Fig. 1A, fig. S3A, and table S3). Among these cytokines, CTRP3 has remained unexplored in the context of tendinopathy pathogenesis (table S2) and was therefore investigated further.

In an independent patient cohort of rotator cuff tendinopathy, CTRP3 expression was correlated with the severity of tendinopathy marked by collagen disorganization, ground substance accumulation, and catabolic enzyme expression (Fig. 1B, fig. S3B, and table S4). Tendon and ligament are closely related connective tissues and exhibit densely arranged collagenous fibers that resist tensile stresses. Upon injury or overuse, ligaments similarly undergo degenerative changes resembling tendinopathy (6). Disorganization of collagen fiber arrangement and accumulation of ground substances were observed in ligaments with high-grade anterior cruciate ligament injury. CTRP3 was markedly up-regulated in these high-grade ligament injury specimens (fig. S3C).

CTRP3 was the most significantly up-regulated cytokine in acutely injured mouse tendons following 3 weeks of their partial transection (fold change $= 57.302$; $P = 7.30 \times 10^{-95}$) (Fig. 1C), the time point at which tendinopathy-associated molecular signatures were clearly observed (fig. S4, A and B). In this condition, Ctrp3 became one of the most abundantly expressed genes among the whole transcriptome (fig. S5A) and the cytokine transcripts (fig. S5B) when ordered according to their percentile rank of transcripts per million mapped reads. Among the tendinopathy-associated inflammatory cytokines including interferons (IFNs), interleukin-1β, and TNF-α (7, 24), IFN-α significantly promoted Ctrp3 expression in primary cultured tenocytes (fig. S6, A to F). In time course observations of partially transected Achilles tendons, the manifestations of tendon injury, including abnormal accumulation of PGs between fibers and hypercellularity at the injury sites, were clearly observed starting at 2 weeks after the partial transection and persisted even after 5 weeks of the injury (fig. S7, A and B). Moreover, upon long-term follow-up using micro–computed tomography, mineralized lesions were clearly detected in the injured Achilles tendons, but not in sham controls, indicating the incomplete repair and chronic pathological status (fig. S7C). CTRP3 expression was substantially elevated from 1 week after the injury, preceding the onset of histopathological changes. CTRP3 up-regulation was maintained until 3 weeks after the injury, and its expression gradually decreased afterward.

Next, we used intensive treadmill running (ITR) with varying durations as a rodent model of overuse tendinopathy (25–27). Three weeks of ITR caused mild accumulation of PG-positive ground substances, as detected by Alcian blue staining, and led to a modest increase in the Bonar score (Fig. 1, D and E, and fig. S8, A and B). However, these histological changes reverted to normalcy after 3 weeks of resting, suggestive of adaptive remodeling of tendon matrix in response to mechanical overloading. Similarly, although 3 weeks of ITR elicited a mild up-regulation of CTRP3 at both mRNA and protein levels, CTRP3 expression returned to basal levels after resting (Fig. 1, D and E, and fig. S8A). In contrast, PG accumulation and tendon matrix disorganization were markedly increased following 6 weeks of ITR (Fig. 1D and fig. S8A). These pathological changes persisted even after 3 weeks of resting. Consistently, no significant difference in the Bonar score was observed before and after resting (Fig. 1E and fig. S8B). Under this overload condition, CTRP3 was markedly up-regulated at both mRNA and protein levels (Fig. 1, D and E, and fig. S8A). CTRP3 expression decreased and remained at a moderate level after resting.

To further explore molecular events occurring with the onset of chronic tendon injury, we conducted immunofluorescence studies using antibodies against type I collagen cleavage site, which detects permanent collagen damage caused by catabolic proteases (Fig. 1G and S8C). Achilles tendons exhibited markedly higher levels of type I collagen cleavage epitopes after 6 weeks of ITR than after 3 weeks of ITR or naïve control. Notably, the region with increased collagen cleavage overlapped with the sites of extensive PG accumulation. This chronic tendinopathic area was also characterized by marked up-regulation of SOX9 (Fig. 1G and S8C), known as the chondrogenic master regulator (28) and CTRP3 (fig. S8, D and E). Together, CTRP3 expression is up-regulated in human and mouse tendinopathy and correlates with the onset of damage irreversibility in tendons.

Overexpression of CTRP3 augments tendinopathy progression in mice

Increased expression of CTRP3 in chronically damaged tendons suggests its possible involvement in the pathogenesis of tendinopathy. The role of CTRP3 in tendons was examined by overexpressing this
**Fig. 1.** CTRP3 expression is up-regulated in human and mouse tendinopathy. (A) Venn diagram of differentially up-regulated cytokine genes in the transcriptomes of human tendinopathic tendons (GSE26051) and partially transected mouse Achilles tendons. (B) Histological and immunohistochemical staining of human normal and tendinopathic tendons (n ≥ 6). Alcian blue/Fast Red, hematoxylin and eosin (H&E), and Picrosirius red (PSR) staining and immunohistochemistry (IHC) for matrix metalloproteinase 13 (MMP13) and CTRP3 are shown. Images for PSR staining were acquired using polarized light microscopy. (C) Volcano plot of gene expression changes in injured mouse Achilles tendons (3 weeks after partial transection) compared to that in sham-operated tendons (n = 4). (D) Histological and IHC staining of naïve and overused mouse Achilles tendons with or without the 3-week rest period. (E) Assessment of tendinopathy using the total Bonar score of the tendons from (F) (n = 5). ITR, intensive treadmill running. (F) Relative Ctrp3 mRNA level assessed by quantitative reverse transcription polymerase chain reaction (qRT-PCR; n = 4). (G) Histological and immunofluorescence (IF) staining against type I collagen cleavage site and SOX9. The relative fluorescence intensity or the percentage of immunopositive cells is indicated. Scale bars, 25 μm. Data represent means ± SD. P values were determined by the Kruskal-Wallis test (E) and analysis of variance (ANOVA) (F). DAPI, 4′,6-diamidino-2-phenylindole.
protein in mouse Achilles tendon. We first verified that exogenously expressed CTRP3 is secreted into the culture medium by primary cultured mouse tenocytes (Fig. S9A). Intratendinous injection of a Ctrp3 expression vector followed by electroporation resulted in robust overexpression of CTRP3 in the Achilles tendon (Fig. 2A and fig. S9, B and C). CTRP3 overexpression increased PG deposition between fibers in the Achilles tendon (Fig. 2A and fig. S9D), causing an increase in the grade of tendinopathy, as evaluated by the Bonar scoring system (Fig. 2B and fig. S9E). Furthermore, behavioral assessment by treadmill running experiments showed that CTRP3 overexpression impaired exercise capacity in mice (Fig. 2C). CTRP3 overexpression increased the expression of chondrogenic markers such as Col2a1, Acan, Fn1, and Sox9, as well as that of tendinopathy-associated collagen type, Col3a1 (Fig. 2D). Although CTRP3 overexpression caused modest up-regulation of Mmp13 and Mmp14, which are capable of degrading the tendon matrix (2), irreversible collagen damage was not clearly detected by immunofluorescence using antibodies against type I collagen cleavage site (fig. S9F). The gene delivery of CTRP3 using the adenoviral vector similarly elicited tendinopathy-associated phenotypes in terms of histopathology and behavior in mice (fig. S10, A to D).

Next, we further explored how CTRP3 overexpression affects the appearance of a chronically damaged tendon under mechanical stress. We examined the progression of tendinopathy in control and CTRP3 overexpression groups following 3 weeks of ITR, which caused adaptive remodeling of the tendon matrix in wild-type mice (Fig. 1, D to G). Under this condition, the Achilles tendons overexpressing CTRP3 exhibited marked accumulation of PGs and collagen disorganization (Fig. 2E and fig. S11A). Notably higher levels of type I collagen cleavage sites and SOX9 was observed, indicative of chronic tendon damages (Fig. 2F). CTRP3 overexpression markedly increased the severity of tendinopathy evaluated by the Bonar scoring (Fig. 2G and fig. S11B) and impaired exercise capacity assessed by treadmill running experiments (Fig. 2H). Together, these results suggest that CTRP3 overexpression increases susceptibility to tendon damage and substantially accelerates the development of chronic tendinopathy in mice.

CTRP3 knockdown ameliorates overuse-induced tendinopathy in mice

We next investigated the effect of CTRP3 knockdown on the progression of tendinopathy induced by mechanical overload. The delivery of Ctrp3-targeting short hairpin RNA (shRNA) effectively reduced the expression of Ctrp3 in primary cultured mouse tenocytes (Fig. 3A and fig. S12A). Intratendinous injection of the Ctrp3 shRNA vector followed by electroporation effectively abolished Ctrp3 expression induced by tendon overuse (Fig. 3B). After 6 weeks of ITR, the control shRNA-delivered Achilles tendons developed severe tendinopathic phenotypes, such as abnormal accumulation of PGs and disorganization of tendon fibers (Fig. 3C and fig. S12B). CTRP3 knockdown effectively mitigated the appearance of these phenotypes, resulting in a decrease in the grade of tendinopathy, as evaluated by the Bonar scoring system (Fig. 3D and fig. S12C). Furthermore, behavioral assessment by treadmill running experiments showed that CTRP3 knockdown markedly attenuated the development of tendinopathy-associated dysfunctions (Fig. 3E). CTRP3 knockdown suppressed overuse-induced expression of chondrogenic genes (Col2a1, Acan, Fn1, and Sox9), Col3a1, and matrix metalloproteinase (Mmp) genes in the Achilles tendons. In contrast, the expression of Mkx, a tendon-specific transcription factor, was rescued by CTRP3 knockdown in tendons (Fig. 3F).

Blockade of CTRP3 prevents the development of experimental tendinopathy in mice

Our results suggest that CTRP3 exacerbates the tendinopathy pathogenesis. Moreover, a genetic approach to suppress CTRP3 expression can effectively prevent disease progression. We, therefore, tested the therapeutic utility of a function-blocking antibody against CTRP3 in tendinopathy development. The selected anti-CTRP3 antibody effectively bound a native form of secreted CTRP3, supporting its capacity to neutralize CTRP3 function (fig. S13A). The antibody therapy was first tested in an Achilles tendinopathy mouse model subjected to 6 weeks of ITR treatment (Fig. 4A). Compared to control immunoglobulin G (IgG) treatment, anti-CTRP3 antibody treatment markedly reduced the major manifestations of overuse-induced tendinopathy, including accumulation of PGs and disorganization of collagen fibers (Fig. 4B and fig. S13B). At the molecular level, the levels of type I collagen cleavage sites and SOX9 was decreased upon anti-CTRP3 antibody treatment, thereby preventing the onset of irreversible damage in tendons (Fig. 4C). Consistently, CTRP3 blockade not only resulted in a substantial decrease in tendinopathy severity, as evaluated by the Bonar scoring system (Fig. 4D and fig. S13C), but also restored the exercise capacity of the tendinopathy mouse model (Fig. 4E). Overuse-exposed mouse Achilles tendons treated with the anti-CTRP3 antibody withstood markedly higher mechanical loads compared with those treated with control IgG, indicating enhanced biomechanical integrity (fig. S13, D and E). Last, overuse-induced expression of tendinopathy-associated genes in tendons was also decreased upon treatment with the anti-CTRP3 antibody (Fig. 4F).

As an alternative mouse model of tendinopathy, we used a collagenase injection model that causes irreversible collagen damage (29). Collagenase injection triggered an increase in cellularity with a mild deposition of ground substance in the Achilles tendon (Fig. 4G and fig. S13F). After 3 weeks of ITR treatment, these mice accumulated substantial amounts of ground substances in their tendons, along with concomitant up-regulation of SOX9 and CTRP3 (Fig. 4, G and H, and fig. S13F). The treatment of collagenase-injected tendons with an anti-CTRP3 antibody decreased tendinopathy-associated histological changes, suppressed SOX9 and CTRP3 expression upon mechanical overload, and eventually decreased tendinopathy severity, as evaluated by Bonar scoring (Fig. 4, G to I, and fig. S13, F and G). Collectively, our results highlighted the therapeutic effects of CTRP3 neutralization in mouse tendinopathy.

CTRP3 activates cartilage gene program in tendons

To gain insights into the role of CTRP3 in tendinopathy development, we performed transcriptomic analysis using tendons isolated from various mouse tendinopathy models. The transcriptomes of tendons transfected with Ctrp3-overexpression vector were compared with those of control vector-transfected tendons and naive tendons (data file S2). Unsupervised hierarchical clustering analysis grouped differentially expressed genes into three clusters (Fig. 5A). Among them, “cluster 2” represents the gene set that was specifically activated upon CTRP3 overexpression (table S5). Transcriptome analysis was conducted to examine naive tendons and overuse-induced tendinopathy tendons treated with control IgG or anti-CTRP3 antibody, respectively (data file S3). Among the
Fig. 2. Ectopic overexpression of CTRP3 promotes tendinopathy in mouse Achilles tendons. (A) Histological and IHC staining of mouse Achilles tendons transfected with an empty vector, Ctrp3-3xflag vector, or vehicle. The vectors were transfected via electroporation (EP), while vehicle-treated mice were not subjected to EP. (B) Assessment of tendinopathy by the total Bonar score of tendons from (A) (n ≥ 3). (C) Resting behavior during treadmill running. Mice prepared for histological analysis in (A) or RNA extraction in (D) were used for this evaluation (n ≥ 9). (D) Quantification of indicated mRNA levels by qRT-PCR in mouse Achilles tendons transfected with an empty vector or Ctrp3-3xflag vector (n ≥ 3). (E and F) Histological (E) and IF staining (F) of Achilles tendons from naïve and overuse-subjected mice transfected with empty or Ctrp3-3xflag vectors or treated with vehicle. (G) Assessment of tendinopathy by the total Bonar score of tendons from (E) (n = 5). (H) Resting behavior of mice from (E) during treadmill running (n = 5). Scale bars, 25 μm. Data represent means ± SD. P values were determined by the Mann-Whitney U test (B and C), the Student’s t test (D), and the Kruskal-Wallis test (G and H).
two clusters identified, “cluster 4” represents the gene set that was activated by tendon overuse but alleviated by blocking of CTRP3 function (Fig. 5B and table S6). Cluster 4 highly overlapped with cluster 2 (hypergeometric $P = 1.55 \times 10^{-34}$) (Fig. 5C).

To assess the role of CTRP3 in causing tendinopathy, we analyzed the biological processes performed by cluster 2 genes using the Biological Networks Gene Ontology (BiNGO) tool (30). Hierarchically structured annotations related to cartilage development and PG metabolic processes were highly enriched in cluster 2 (Fig. 5D, fig. S14A, and table S7). Moreover, gene set enrichment analysis (GSEA) revealed that the “cartilage development” and “synthesis of proteoglycan” gene sets (tables S8 and S9) from IPA were positively enriched in the whole transcriptome of CTRP3-overexpressing tendons (Fig. 5E and fig. S14B). Similarly, the genes listed in the two annotations were up-regulated in the tendons of the overuse-induced tendinopathy mouse model than in those of naïve mice. Furthermore, CTRP3 antibody therapy effectively suppressed these gene sets in mice with tendinopathy (Fig. 5E and fig. S14B). Last, we examined how the gene cluster activated upon CTRP3 overexpression is regulated in human tendinopathy. The cluster 2 gene set was positively enriched in tendinopathy-affected tendons than in their nonlesional counterparts (Fig. 5F). Together, CTRP3 activates the cartilaginous transcriptional program in tendons, which accounts for key molecular and morphological changes associated with tendinopathy.

**CTRTP3 enhances chondrogenesis of tendon stem/progenitor cells**

In tendons with tendinopathy, chondroid metaplasia is commonly observed in lesional regions along with concomitant accumulation of PGs (13). There has been a notion that tendinopathy features observed in rodent models are attributable to aberrant differentiation of tendon stem/progenitor cells (TSPCs) into nontenogenic cell
Fig. 4. Blockade of CTRP3 ameliorates experimental tendinopathy in mice. (A) Schematic illustration of experimental antibody therapy targeting CTRP3 in the treatment of tendinopathy in mice. (B and C) Histology (B) and IF staining (C) of Achilles tendons from naïve and overuse-induced tendinopathy mice treated with control IgG or anti-CTRP3 antibody (peritendinous injection at 0.063 mg/kg; twice a week for 3 weeks). (D) Assessment of tendinopathy by the total Bonar score of the tendons from (B) (n = 6). (E) Resting behavior of mice from (B) during treadmill running (n = 6). (F) Quantification of indicated mRNA levels by qRT-PCR (n = 3). (G and H) Mice were subjected to intratendinous injection of collagenase and then subjected to 3 weeks of ITR or left in their cage (naïve). The Achilles tendons of these mice were peritendinously treated with control IgG or anti-CTRP3 antibody. Histology (G) and SOX9 IF staining (H) of indicated Achilles tendons. (I) Assessment of tendinopathy by total Bonar score of the tendons from (G) (n = 5). Scale bars, 25 μm. Data represent means ± SD. P values were determined by the Kruskal-Wallis test (D, E, and I) or ANOVA (F).
types such as chondrocytes (25). We observed that nestin-positive cells, previously described as TSPCs found in mouse tendons (31), were increased and recruited to the injury site of Achilles tendons in mice (Fig. 6, A and B). Because our results indicate a role for CTRP3 in activating a cartilage-associated transcriptional program, we examined how CTRP3 influences the fate determination of TSPCs into chondrogenesis. After the isolation of mouse TSPCs, the unique expression profiles of TSPC markers, including nestin, CD90, and SSEA4 (Stage-specific embryonic antigen 4) (31–33), were confirmed (Fig. 6C and fig. S15), and their self-renewal capacity and multipotencies were fully verified (Fig. 6, D to F) (31–33).

CTRP3 overexpression effectively promoted chondrogenesis of TSPCs grown in pellet culture, as evidenced by Alcian blue staining and the pellet size (Fig. 6, G and H). Similarly, CTRP3 overexpression enhanced chondrogenic differentiation of human umbilical cord–derived mesenchymal stem cells (UC-MSCs) grown in a pellet culture (Fig. 6I and fig. S16A). At the molecular level, a significant increase in the expression of Col2a1, Acan, and Sox9 following CTRP3 overexpression in pellet-cultured TSPCs was observed. In contrast, an overall decrease in the expression of nonchondrogenic collagens such as Col1a1 and Col3a1 and tenogenic markers such as Scx, Mkx, and Tnmd was observed (Fig. 6J). We further confirmed increased protein levels of chondrogenic markers, type II collagen, aggrecan (ACAN), and SOX9 upon CTRP3 overexpression (Fig. 6K and fig. S16B). Collectively, these results support the prochondrogenic roles of CTRP3 on tendon progenitor cells.

**CTRP3-mediated signaling disrupts tenogenesis**

We next examined the effect of CTRP3 on tenogenic differentiation of tendon progenitor cells. CTRP3 overexpression substantially suppressed neotendon formation of TSPCs and UC-MSCs grown in anchored fibrin gels and markedly decreased the thickness of neotendon constructs (Fig. 7, A to C, and fig. S17A). Upon CTRP3 overexpression in fibrin gel–cultured TSPCs, there was an overall decrease in the expression of tendon-specific transcription factors such as Scx and Mkx and tendon-related markers such as Col1a1, Col3a1, and Tnmd. Instead, an overall up-regulation of chondrogenic markers was observed (Fig. 7D). Irrespective of the inhibitory effect of CTRP3 on tenogenesis, CTRP3 knockdown did not further affect neotendon formation of TSPCs, presumably because of the low CTRP3 expression in TSPCs (fig. S17, B to D). We then examined how CTRP3 overexpression affects the expression of
CTRP3 promotes chondrogenic differentiation of TSPCs. (A) Micrographs of the gastrocnemius muscle (GAS) and Achilles tendon before and after tissue clearing using the CUBIC method. (B) Three-dimensional (3D)–reconstructed IF of nestin in the Achilles tendon after indicated surgical procedures. (C) IF staining against molecular markers for TSPCs. The percentage of immunopositive cells is indicated. (D) Sphere formation assay was conducted with mouse tenocytes and TSPCs. (E) Number of spheres that are bigger than 50 μm in diameter (n = 6). (F) Alizarin Red S and Oil Red O staining of mouse TSPCs. (G) Alcian blue/Fast Red staining and the gross appearance of pellet-cultured mouse TSPCs transduced with Ad-eGFP or Ad-Ctrp3. (H) Quantification of relative Alcian blue–stained area and the mean diameter of pellet-cultured mouse TSPCs from (F) (n = 5). (I) Quantification of relative Alcian blue–stained areas in pellet-cultured human UC-MSCs transfected with empty or Ctrp3-3xflag vectors (n = 6). (J and K) qRT-PCR analysis (J) and immunoblots (K) using pellet-cultured mouse TSPCs transduced with Ad-eGFP or Ad-Ctrp3 (n = 4). Scale bars, 3 mm (A), 25 μm (B), 50 μm (C, D, and F), and 300 μm (G). Data represent means ± SD. P values were determined by the Mann-Whitney U test (E, H, and I) and Student’s t test (J).

Tendinopathy-associated catabolic mediators (8) and metabolic genes (17, 20) in tenocytes. The overexpression of CTRP3 caused up-regulation of Mmp14 and Adams3 and down-regulation of Ptgss2 (fig. S17, E and F). Among the triglyceride synthesis genes, only Gpat3 expression was significantly decreased by CTRP3 overexpression. The transcripts of gluconeogenesis-related genes, such as G6pase and Pepck, were not detected in tenocytes (fig. S17G).

Next, we examined signaling pathways affected by CTRP3. Depending on the cell type and cellular context, CTRP3 elicits signaling through various subtypes of mitogen-activated protein kinase (MAPK) cascades (18) and the phosphatidylinositol-3-kinase (PI3K)–Akt pathway (17, 18). In TSPCs, UC-MSCs and tenocytes, CTRP3-conditioned media commonly activated PI3K–Akt signaling but not MAPK pathways mediated by extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 protein kinases (Fig. 7, E and F, and fig. S17H). Notably, the inhibitory effect of CTRP3 in forming neotendons from TSPCs and UC-MSCs was alleviated by inhibiting PI3K–Akt signaling (Fig. 7, G and H, and fig. S17, I and J). Meanwhile, the inhibition of PI3K–Akt signaling abolished CTRP3-mediated enhancement of chondrogenesis of TSPCs (fig. S17, K and L).

CTRP3 antibody treatment ameliorates rotator cuff tendinopathy in rats

Rotator cuff tendinopathy is the primary cause of shoulder disability (34). Rotator cuff tendinopathy occurs when the rotator cuff tendon is exposed to overload or repeated stresses with inadequate recovery periods (34). Hence, we induced rotator cuff tendinopathy in rats...
Fig. 7. CTRP3 suppresses tenogenic potentials as an effect linked to Akt signaling. (A) H&E staining of in vitro neotendon constructs from 3D fibrin gel–cultured mouse TSPCs transfected with empty or Ctrp3-3xflag vectors. Yellow arrowheads indicate neotendon constructs formed between sutures. (B and C) Quantification of the mean construct diameter of neotendon constructs from mouse TSPCs (B) \( (n = 6) \) and human UC-MSCs (C) \( (n = 6) \). Data are displayed as a box plot with minimum to maximum whiskers; dots indicate individual samples. (D) Quantification of indicated mRNA levels by qRT-PCR in neotendons constructed from 3D fibrin gel–cultured mouse TSPCs transfected with empty or Ctrp3-3xflag vectors \( (n = 4) \). (E and F) Immunoblots of indicated proteins in mouse TSPCs (E) and human UC-MSCs (F) treated with control conditioned media (CM) or CTRP3-CM in the absence or presence of the anti-CTRP3 antibody \( (n = 4) \). (G) H&E staining of neotendons constructed from mouse TSPCs transduced with Ad-eGFP or Ad-Ctrp3 in the presence of dimethyl sulfoxide (DMSO) or 10 \( \mu \)M LY294002. Yellow arrowheads indicate neotendon constructs formed between sutures. (H) Quantification of the mean construct diameter of neotendon constructs from (G) \( (n = 5) \). Scale bars, 100 \( \mu \)m. Data represent means ± SD. \( P \) values were determined by the Mann-Whitney \( U \) test (B and C), the Student’s \( t \) test (D), and the Kruskal-Wallis test (E, F, and H).
by using long-term downhill ITR (35) and tested the effect of antibody therapy targeting CTRP3 (Fig. 8A and fig. S13A). Peritendinous injection of the CTRP3-neutralizing antibody effectively suppressed abnormal ground substance deposition and collagen fiber disorganization induced by tendon overuse (Fig. 8B and fig. S18A). The level of type I collagen cleavage sites and SOX9 was reduced upon anti-CTRP3 antibody treatment, indicating effective prevention of permanent damages in the tendons (Fig. 8C). Antibody therapy ameliorated tendinopathy severity, as evaluated by the Bonar scoring system, in the supraspinatus rotator cuff tendons (Fig. 8D and fig. S18B). Consistently, forelimb grip strength jeopardized by tendinopathy was restored by CTRP3 antibody treatment (Fig. 8E). The development of overuse-induced tendinopathy significantly impaired the mechanical properties of rat supraspinatus tendons, causing a 51.3 and 57.2% reduction in the failure force and elastic modulus, respectively, compared with those of the naïve condition. The treatment with anti-CTRP3 antibody restored the mechanical properties to the levels of native tendons and significantly higher than those of tendinopathic tendons treated with control IgG (Fig. 8, F and G). At the molecular level, tendinopathy-associated biomarkers such as MMP13, fibronectin, and type III collagen were detected at minimal levels in rotator cuff tendons of CTRP3 antibody-treated rats (Fig. 8H). These results suggest that the blockade of CTRP3 alleviates the overall pathogenesis of rotator cuff tendinopathy in rats.

**DISCUSSION**

Tendinopathy is a chronic disease condition characterized by degenerative changes in the tendon matrix (4, 10). In this study, we identified CTRP3 as an essential regulator that triggers pathological matrix remodeling, resulting in tendinopathy. CTRP3 activated transcriptional programs that intensify ectopic chondrification and PG synthesis in tendons; neutralizing CTRP3 function effectively abrogated these transcriptome changes induced by tendon overuse. CTRP3 overexpression not only led to the accumulation of ground substance in mouse tendons but also promoted other major tendinopathy manifestations such as disorganization of collagen fibers and deposition of noncollagenous ECM. In contrast, specific blockade of CTRP3 suppressed overall tendinopathy phenotypes in the Achilles and rotator cuff tendons of rodent models. Therefore, CTRP3-driven chondrification of the tendon drives a series of degenerative events during the tendinopathy pathogenesis. This finding is corroborated by a recent finding in which a biocompound stimulating chondrogenic differentiation was shown to recapitulate the features of an overuse tendinopathy model (36).

CTRP3 is known to be expressed in proliferating chondrocytes in the growth plate and expands the population of chondrogenic precursors, regulating cartilage development and subsequent ossification (22). Moreover, CTRP3 has been documented to have opposing effects on inflammation. A negative correlation of the serum levels of CTRP3 with chronic inflammation in the diabetic milieu suggests a possible role of CTRP3 in enhancing insulin sensitivity. CTRP3 plays a protective role in hepatic steatosis by enhancing liver lipid metabolism (17) and promotes recovery of damage from heart attack (16). Despite the beneficial roles of CTRP3 in suppressing inflammation and improving bone and cartilage development, our results indicate that CTRP3 has detrimental effects in maintaining tendon homeostasis. Accumulation of cartilage-like matrix consisting of ground substances is a hallmark of tendinopathy (11). While cartilage matrix is optimized to absorb the compressive stresses imposed on the joint, the tendon mainly withstands tensile stresses transmitted from the muscle. Therefore, CTRP3 appears to exacerbate tendinopathy via abnormal deposition of cartilaginous matrix components and the consequent impairment of tendon matrix integrity and its mechanical properties.

The discovery of TSPCs provided new insights into the pathogenesis of tendinopathy (37). TSPCs maintain the population of tenocytes by modulating their own proliferation and differentiation into tenocytes in normal tendons (32). However, aberrant differentiation of TSPCs into nontenogenic lineages has been observed under external stimuli associated with tendinopathy development, such as mechanical overload (37). We showed that CTRP3 suppresses the tenogenic potential of TSPCs in a three-dimensional (3D) culture context under uniaxial tension. In contrast, CTRP3 stimulated chondrogenic differentiation of TSPCs grown in 3D pellet culture systems that mimic mesenchymal condensation. This finding is consistent with the earlier reports illustrating the role of CTRP3 in regulating the proliferation of chondrocytes and chondrogenic precursor cells (15, 18). Moreover, CTRP3 is highly expressed in the cartilage during embryonic development, suggesting its potential role in chondrogenesis (15). Therefore, CTRP3 might disrupt tendon homeostasis during tendinopathy by limiting the tenogenic potential and enhancing chondrogenesis of TSPCs.

Despite multiple efforts to develop a disease-modifying therapy for tendinopathy, the successful functional repair of a tendon is a clinically challenging task (6, 9). Surgical strategies for tendinopathy are considered the last treatment option, typically when a tendon tear or rupture is evident (38). However, degenerative processes often persist in surgically sutured tendons, resulting in a high postoperative failure rate (7). Therefore, numerous studies have been conducted to develop an effective therapeutic strategy to enable the biological repair of a damaged tendon (38). Platelet-rich plasma (PRP), known to enhance wound healing activity (8, 38), has been increasingly used in the clinical setting to treat tendinopathy (39). However, the efficacy of PRP treatment for tendinopathy remains controversial because of the inability of randomized controlled trials to demonstrate clinically substantial improvements upon PRP injection in chronic Achilles tendinopathy (39). Similarly, anti-inflammatory agents exhibit minimal efficacy in enhancing the healing process of a tendon (39). In this study, we focused on a biological therapy that neutralizes the function of a disease-causing target to oppose tendon degeneration and enable intrinsic biological repair of a damaged tendon. Previously, the blockade of TNF-α (40) and a subset of interleukin family members (41) showed promise in improving tendon healing in animal models, suggesting cytokines as a potential target for treating tendinopathy through biological therapies (40). We demonstrated that CTRP3, originally identified as a soluble adiponectin paralogous protein (15), is a rational cytokine target whose blockade directly opposes cellular events that cause tendinopathy and assists tendon repair in rodent models.

The present study has several limitations that will need to be addressed in future research. Our experiments were conducted primarily in rodents with overuse-induced tendinopathy. However, rodent models of tendinopathy do not fully recapitulate the human condition of the disease in terms of anatomy, movement kinematics, or pathology (29). Preclinical trials that involve large animals, preferentially those with orthograde posture, should be conducted
Fig. 8. Experimental antibody therapy targeting CTRP3 attenuates rotator cuff tendinopathy pathogenesis in rats. (A) Schematic illustration of experimental antibody therapy targeting CTRP3 in the treatment of rotator cuff tendinopathy in rats. (B and C) Histological, IHC (B), and IF (C) staining of supraspinatus tendons from rotator cuff of naïve and overuse-induced tendinopathy rats treated with control IgG or anti-CTRP3 antibody (peritendinous injection at 4.8 μg/kg; twice a week for 2 weeks). (D) Total Bonar scores of the rotator cuff tendons from (B) (n ≥ 4). (E) Assessment of forelimb grip strength of rats from (B) for evaluation of physical performance (n ≥ 4). (F and G) Quantification of failure force (F) and Young’s modulus (G) of rat supraspinatus tendons from naïve (n = 8) and overuse-induced tendinopathy rats treated with control IgG (n = 7) or anti-CTRP3 antibody (n = 7). (H) IF staining of rotator cuff tendons of overuse-induced tendinopathy rats treated with control IgG or anti-CTRP3 antibody to detect tendinopathy-associated proteins, MMP13, fibronectin (FN), and type III collagen. Scale bars, 25 μm. Data represent means ± SD. P values were determined by the Kruskal-Wallis test (D to G).
before human studies are undertaken. Moreover, note that patient tendon biopsies have been far more chronically exposed to pathological and inflammatory environments compared with the experimental conditions explored in murine models. Last, most therapeutic activities reported in this study were achieved by targeting mouse CTRP3; however, the precise molecular physiology of CTRP3 may differ between mice and humans (17). Therefore, careful consideration should be given to extrapolating the current data from mice to humans.

Tendinopathy involves a complex pathogenesis that is accompanied by multiple processes, including collagen degradation, PG accumulation, and ectopic calcification (11). Therefore, a single molecular factor such as CTRP3 may not solely account for all features of tendinopathy. Although CTRP3 overexpression alone elicited some of the major tendinopathic features, such as the accumulation of ground substances and collagen fibril disorganization, it was not sufficient to fully produce irreversible collagen cleavage, which represents a form of chronic damage frequently observed in tendinopathic tendons. Therefore, we speculate that a few other disease-associated factors that are up-regulated with tendon overuse may synergistically cooperate with CTRP3 to drive the full development of tendinopathy phenotypes. Moreover, although CTRP3 blocking may work prophylactically to delay further progression of tendinopathy, it remains unclear whether this approach is therapeutically effective in patients already at the advanced stage of tendinopathy. Nonetheless, we highlight that blockade of CTRP3 function by either its genetic knockdown or antibody-mediated neutralization mitigated the overall manifestations of tendinopathy, including irreversible collagen degradation. Therefore, although CTRP3 expression is not sufficient to solely induce tendinopathy, it is evidently a necessary factor for the development of tendinopathy.

CTRP3 is highly conserved among vertebrates. In particular, after cleavage of the signal peptide, the secreted forms of human and mouse CTRP3 proteins share 98.2% identity. Recent studies have indicated that successful drug targets exhibit high evolutionary conservation, providing insights into the selection of a rational target for biological therapies (42). Furthermore, we confirmed that CTRP3 is commonly up-regulated in tendinopathy disorders in both humans and mice. Differentially regulated genes by CTRP3 in mice were similarly enriched in human tendinopathy. Further investigations are warranted to determine the correlation between the experimental potential and clinical outcomes in human patients.

Here, we found that CTRP3 is an essential regulator that promotes the progression of tendinopathy. Biological therapy nullifying CTRP3 function effectively ameliorates the pathogenesis of tendinopathy in rodents, preserving the structural integrity, biomechanical property, and functionality of the Achilles and rotator cuff tendons. Our findings provide insights into the development of therapeutic strategies for the treatment of tendinopathy.

**MATERIALS AND METHODS**

**Study design**
The objectives of this study were to understand the molecular pathogenesis of tendinopathy and identify a key molecular target whose blockade effectively suppresses tendinopathy development and progression. We performed histological and immunohistochemical analyses on biopsy samples from human rotator cuff tendinopathy patients and parallel in silico analyses of publicly available transcriptome datasets from human tendinopathy patients. In all in vivo experiments testing the effects of CTRP3, animals were randomly assigned to each group with approximately equivalent numbers in each group, and all samples were analyzed in a blinded manner. The sample sizes for animal studies were determined by referencing previous studies involving the use of rodent tendinopathy models (25, 27). For the treadmill running experiments, animals that completely refused to run after several repeated trials were excluded from the exercise procedure. No other exclusion criteria were applied. To evaluate the pathogenesis of tendinopathy, histological, molecular, biomechanical, and behavioral studies were used. For each experiment, the sample size reflected the number of independent biological replicates and is indicated in the figure legend. All studies involving human samples and animals were approved by the appropriate ethics review boards. Informed consent was obtained from all participants before the operative procedure. Individual subject-level data are provided in data file S4.

**Collection of human tissue samples**
Human specimens were collected from Seoul Metropolitan Government–Seoul National University (SMG-SNU) Boramae Medical Center. Nineteen supraspinatus tendon samples were collected from patients with rotator cuff tears undergoing shoulder surgery. Human anterior and posterior cruciate ligament specimens were sourced from patients with osteoarthritis undergoing total knee arthroplasty. Tendon and ligament biopsies were acquired with a sufficient thickness and margin such that they did not deform extensively once they were fixed overnight in 4°C. Dehydration and embedding processes were performed without tissue shrinkage. An independent control group was obtained from OriGene Technologies Inc. (catalog nos. CB617745, CB621341, CB626826, CB627612, CB637060, and CB638844). Six samples of normal tendon were collected from patients with diseases unrelated to tendon disease. Our institutional review board approved this study, and written informed consent was obtained from all participants. The histological grade of the tendon specimens was analyzed according to the modified Bonar scoring system, which provides improved clarity and reproducibility as compared with the original Bonar scoring system when assessing the extent of tendon degenerative change (43). All histological or immunochemical images were independently evaluated by two pathologists. All specimens were analyzed in a blinded manner.

**Experimental tendinopathy rodent models**
Rodent models of overuse-induced Achilles and rotator cuff tendinopathy were constructed on the basis of the previously described ITR method with minor modifications (25–27). For the overuse-induced Achilles tendinopathy experiments, 12-week-old C57BL/6J male mice were subjected to 3 or 6 weeks of uphill treadmill activity. Before protocol initiation, mice were subjected to a training week of short uphill treadmill activity (15 m/min, 15 min/day, 7 days/week, 10° incline). After this training period, mice were subjected to treadmill activity at the same conditions (15 m/min, 7 days/week, 10° incline) for 1 and 2 hours/day in the first and second to sixth weeks, respectively. For the overuse-induced rotator cuff tendinopathy experiments, 12-week-old male Sprague-Dawley rats were subjected to 5 weeks of downhill treadmill activity. Before protocol initiation, rats were subjected to a training week of short downhill treadmill activity (15 m/min, 30 min/day, 6 days/week, 10° decline). Following this training period, rats were subjected to treadmill activity at the modified conditions (20 m/min, six days/week, 10° decline) for...
1 hour/day in the first to fifth weeks. A treadmill equipped with an air puff and electric shock grid was used to stimulate animals. Resting behavior was quantified as the number of shock events delivered to each animal. Animals were continuously monitored and motivated by pushing them with the end of stainless-steel forceps whenever they refused to run. With these efforts, most animals maintained the running states during the exercise period. Animals that persisted in their refusal to run were removed from the treadmill and reintroduced at a later time to complete the designated running time. A few mice completely refused to run even after several repeated trials. These mice were excluded from the exercise procedure. A collagenase injection tendinopathy model was induced using previously described methods with minor modifications (44). Briefly, 12-week-old C57BL/6J male mice were anesthetized, and the hindlimb was shaved. Collagenase type I (125 U/20 μl; Sigma-Aldrich) was introduced via intratendinous injection into the Achilles tendon using a 31-gauge insulin syringe. Animals were housed strictly in individual cages. A positive association of sex hormones such as estrogen with tendon healing has been reported (45). Because of the varying concentration profiles of sex hormones with the menstrual cycle in females, male animals were used to reduce individual differences between animals.

**In vivo transfection and transduction in mice**

For in vivo transfections, mice were anesthetized and the right hindlimb was shaved. Forty micrograms of plasmid DNA (in 30 μl of sterile saline) was introduced using intratendinous injection of the musculotendinous junction between the Achilles tendon and the gastrocnemius muscle using a 31-gauge insulin syringe. Conductivity gel was applied to the shaved skin, with the Achilles tendon and gastrocnemius muscle located between the caliper electrodes with a 5-mm gap. Electroporation was done with a BTX Gemini X2 (Harvard Bioscience Inc.) using 225 V/cm for 25 ms five times. After electroporation, the mice did not exhibit visible signs of tissue damage and appeared to show normal behavior during housing and exercise. For in vivo transduction, Ad-eGFP or Ad-CtRP3 (1 × 10⁹ plaque-forming units in 30 μl of sterile saline) was delivered to Achilles tendons of both hindlimbs, once a week for 3 weeks. Twelve-week-old C57BL/6J male mice were used for electroporation and transduction experiments.

**Partial transection Achilles tendon injury**

Eight-week-old C57BL/6J male mice were given an Achilles tendon injury. Before the surgery, the mice were anesthetized, and the surgical area was shaved and swabbed with alcohol. The skin and subcutaneous tissue on the right ankle were incised using a scalpel blade. The Achilles tendon was then separated from the soleus muscle. A 1.5-mm-diameter ear punch (NAPOX, catalog no. KN-291-1) was placed on the lateral side of the Achilles tendon to fit only about half the diameter. The Achilles tendon was then transected with the punch, leaving a 0.75-mm semicircular defect on the lateral side. Because the plantaris tendon is located on the medial side, we were able to generate the defect in the Achilles tendon without damaging the plantaris tendon. Therefore, the plantaris tendon was left in an essentially intact state. Last, the skin was closed with Mersilk sutures. After the surgery, mice were stabulated for 3 weeks.

**Behavioral experiments**

Behavioral experiments associated with tendinopathy were performed using rodents with experimentally induced tendinopathy. To assess the exercise performance of mice with Achilles tendinopathy, the mice were subjected to uphill treadmill activity (15 m/min, 30 min, 10° incline). An electric foot shock was used to stimulate mice to run. The number of electric foot shocks at the back of the treadmill was counted to measure the resting behavior of the mice. To assess the physical performance of rats with rotator cuff tendinopathy, the forelimbs of the animals were subjected to a grip strength test meter (Bioseb) according to the manufacturer’s guidelines.

**Neutralizing antibody therapy**

Before therapeutic antibody injection, animals were anesthetized. For treatment with the neutralizing antibody for mice, a peritendinous injection was given around the Achilles tendon with anti-CtRP3 antibody (0.063 mg/kg; Abcam). Antibody injections were carried out twice a week for 3 weeks throughout the course of the treadmill running experiments. For rats, a peritendinous injection of anti-CtRP3 antibody (4.8 μg/kg) was administered around the rotator cuff tendons. Antibody injections were carried out twice a week for 2 weeks over the course of the treadmill running experiments.

**Histology and immunohistochemistry**

Human rotator cuff tendons, human cruciate ligaments, mouse Achilles tendons, and rat rotator cuff tendons were washed in ice-cold phosphate-buffered saline (PBS) and fixed in prechilled 4% paraformaldehyde (PFA) for 7, 3, or 3 days, respectively. For the mouse Achilles tendons and rat supraspinatus tendon, the whole muscles and bones connected with tendons were preserved and harvested to prevent tissue shrinkage over the course of tissue processing. Tissues were processed by dehydration in increasing concentrations of ethanol, incubation in xylene, and infiltration with paraffin. After tissue processing, tendons were embedded in paraffin blocks. The paraffin-embedded samples were sectioned at 7-μm thickness, with 90 to 120 sections obtained per human tendon and ligament sample, 75 to 90 sections obtained per mouse Achilles tendon sample, and 60 to 75 sections obtained per rat supraspinatus tendon sample. The sections covered the full range of tendon tissues obtained. Four to five slides from the mid-depth of tendons were selected for each type of histological staining, which resulted in at least 16 histological sections. Tendinopathic phenotypes were identified by Alcian blue (pH 1.0) counterstained with Nuclear Fast Red and hematoxylin and eosin (H&E). At least 16 sections were stained and imaged for histological analysis. Images with coded labeling were assigned to pathologists, and histological or immunohistochemical images were independently evaluated by two pathologists, yielding similar results for the score and quantitation (overall, at least 90% match between the two pathologists; table S10). After completing the evaluation, histological or immunohistochemical scores were decoded and assigned to the respective experimental groups. Histological grade of tissue samples was analyzed using the modified Bonar scoring system (0 to 12), which evaluates the severity of tendinopathy based on cellularity, cell morphology, collagen organization, and ground substance categories (43). For immunohistochemistry and immunofluorescence, the sections were subjected to antigen retrieval with citrate buffer (pH 6.0) at 70°C for 1 hour or 0.05% trypsin at 37°C for 30 min and blocked with 1% bovine serum albumin (BSA) in PBS. The tissues were incubated with the indicated primary antibodies overnight at 4°C and with secondary antibodies at room temperature for 1 hour. Histological and immunohistochemistry staining images were acquired using a...
The CUBIC clearing and microscopy image analysis Full-size immunoblot images are provided in fig. S19. GOAT ANTI-RABBIT IgG + IgM (H&L) CONJUGATED WITH Dylight 594 NESTIN (MAB353) WERE PURCHASED FROM MILLIPORE. RABBIT ANTI-MOUSE (AB1031), ANTI–TYPE II COLLAGEN ANTIBODY (MAB8887), AND ANTI–P-phospho-AKT SER 473 (#9271), ANTI-AKT (PAN) (AB1031), ANTI–TYPE I COLLAGEN CLEAVAGE SITE ANTIBODY (#0217-050) WAS PURCHASED FROM SIGMA-ALDRICH. NORMAL MOUSE IgG (SC-2025), NORMAL RABBIT IgG (SC-2027), ANTI–Phospho-JNK THr 183/Tyr 185 antibody (sc-6254), ANTI–PHOSPHO–P38 Tyr 182 antibody (sc-16882), ANTI–COL3A1 antibody (sc-271249), ANTI–ACTIN antibody (sc-1615), ANTI–SOX9 antibody (sc-166505), AND ANTI–SSEA4 (SC-21704) WERE PURCHASED FROM SANTA CRUZ BIOTECHNOLOGY. ANTI–FIBRONECTIN antibody (A0245) WAS PURCHASED FROM DAKO. ANTI–CTRP3 antibody (ab36870), ANTI–MMP13 (ab51072), ANTI–CD146 (AB75769), AND ANTI–CHONDROITIN Sulfate (AB11570) WERE PURCHASED FROM ABcam. ANTI–PHOSPHO–AKT SER 473 (#9271), ANTI–AKT (PAN) (#4685), AND ANTI–PHOSPHO–ERK1/2 THr 202/Tyr 204 (#4377) WERE PURCHASED FROM CELL SIGNALING TECHNOLOGY. ANTI–ACAN antibody (AB1031), ANTI–TYPE II COLLAGEN antibody (MAB8887), AND ANTI–NASTIN (MAB353) WERE PURCHASED FROM MILLIPORE. RABBIT ANTI–MOUSE IgG + IgM (H&L) CONJUGATED WITH Dylight 488 (Jackson ImmunoResearch) OR GOAT ANTI–RABBIT IgG + IgM (H&L) CONJUGATED WITH Dylight 594 (Jackson ImmunoResearch). DAPI (4′,6-DIAMIDINO-2-PHENYLINDOLE) WAS USED AS A NUCLEAR STAINING (1 µg/ml). MICROSCOPIC IMAGES WERE ACQUIRED USING THE EVOS FL Cell Imaging System (Thermo Fisher Scientific). ImageJ software (National Institutes of Health) WAS USED FOR THE QUANTIFICATION OF IMMUNOFLOURESCENCE IMAGES. IMMUNOFLOURESCENCE-POSITIVE REGIONS WERE DEFINED ON THE BASIS OF THE CHosen THRESHOLD. FOR ECM PROTEINS, THE RELATIVE FLUORESCENCE INTENSITY WAS CALCULATED. FOR THE OTHER PROTEINS, THE PERCENTAGE OF IMMUNOPosITIVE CELLS WAS CALCULATED.

Antibodies
Anti–Type I Collagen Cleavage Site Antibody (#0217-050) Was PurCHASED FROM ImmunoGlobe. Anti–FLAG Antibody (F3165) AND ANTI–Vinculin Antibody (V9131) WERE PURCHASED FROM Sigma–Aldrich. Normal Mouse IgG (SC–2025), Normal Rabbit IgG (SC–2027), Anti–Phospho–JNK Thr 183/Tyr 185 Antibody (SC–6254), Anti–Phospho–P38 Tyr 182 Antibody (SC–16882), Anti–COL3A1 Antibody (SC–271249), Anti–Actin Antibody (SC–1615), Anti–Sox9 Antibody (SC–166505), AND Anti–SSEA4 (SC–21704) WERE PURCHASED FROM SANTA CRUZ BIOTECHNOLOGY. Anti–Fibronectin Antibody (A0245) WAS PURCHASED FROM DAKO. Anti–CTRP3 Antibody (Ab36870), Anti–MMP13 (Ab51072), Anti–CD146 (Ab75769), AND Anti–Chondroitin Sulfate (Ab11570) WERE PURCHASED FROM AbCAm. Anti–Phospho–Akt Ser 473 (#9271), Anti–Akt (Pan) (#4685), AND Anti–Phospho–ErK1/2 Thr 202/Tyr 204 (#4377) WERE PURCHASED FROM Cell Signaling Technology. Anti–AcAN Antibody (Ab1031), Anti–Type II Collagen Antibody (Mab8887), AND Anti–Nastin (Mab353) WERE PURCHASED FROM Millipore. Rabbit Anti–Mouse IgG + IgM (H&L) Conjugated With Dylight 488 (711-065-151) AND Goat Anti–Rabbit IgG + IgM (H&L) Conjugated With Dylight 594 (111-585-003) WERE PURCHASED FROM Jackson ImmunoResearch. Full–Size Immunoblots Images Are Provided In Fig. S19.

The CUBIC Clearing And Microscopy Image Analysis
Two CUBIC Reagents Were Prepared As Previously Reported (46). For Preparation Of CUBIC–Treated Samples, Mouse Achilles Tendons Were Fixed By Prechilled 4% PFA For 2 Days. The Samples Were Washed With PBS. These Samples Were Immersed Into 10 ml of 50% (V/V) ScaleCUBIC–1 Reagent (CUBIC–1) (1: 1 mixture of PBS:CUBIC–1) For 1 Day And Further Immersed In 10 ml of CUBIC–1 Reagent At 37°C With Gentle Shaking For 8 To 10 Days. Every 2 Days, The CUBIC–1 Solution Was Changed. For Counterstaining, DAPI (0.25 µg/ml) Was Added Into CUBIC–1 Reagent. Subsequently, The Samples Were Washed With PBS At Room Temperature With Gentle Shaking For 2 Days. These Samples Were Subjected To Immunostaining With The Primary Antibodies In 750 µl Of Antibody Working Solution For 3 Days At 37°C With Shaking. The Stained Samples Were Then Washed With 10 ml Of 0.1% (V/V) Triton X–100 In PBS Several Times At Room Temperature With Shaking And Then Stained With The Secondary Antibodies In 750 µl Of Antibody Working Solution. The Antibody Working Solution Was Prepared With 0.1% (V/V) Triton X–100, 0.5% (W/V) BSA, And 0.01% (W/V) Sodium Azide In PBS. These Samples Were Washed With PBS And Immersed Into 8 To 10 Ml Of 50% (V/V) ScaleCUBIC–2 Reagent (CUBIC–2) (1:1 mixture of PBS:CUBIC–2) For 1 Day And Further Immerged In 8 To 10 Ml Of CUBIC–2 Reagent For 3 Days. Mouse Achilles Tendon Fluorescence Images Were Acquired With Confocal Microscopy (Anti–Nestin; Leica, TCS SP8), Combined With 408- And 488-nm Lasers. Mouse Achilles Tendon Samples Were Immersed In CUBIC–2 Reagent During Image Acquisition. After 3D Images Were Acquired, Analysis Was Performed With Imaris Software (Bitplane).

Mechanical Testing
The biomechanical testing of mouse Achilles tendon and rat rotator cuff tendons were performed as described previously using the dynanometer device (Tinius Olsen) (47). Briefly, the sample was fixed to custom–made clamps, and the preconditioning of 5 cycles (1.0% strain, 5 mm/min) was performed. The cross–sectional area was measured at the preload. The sample then underwent a load to failure test at an elongation rate of 5 mm/min, and the failure force (N) was recorded. The strain was determined as a grip–to–grip displacement relative to the initial gauge length, and Young’s modulus (MPa) was calculated from the slope of stress–strain curves.

Primary Culture Of Human Mesenchymal Stem Cells
Human UC–MSCs Were Obtained From The SMG–SNU Boramae Medical Center. UC–MSCs Were Isolated From The Postpartum Umbilical Cords Of Healthy Adults, As Previously Described (48). Cells Were Cultured In Low–Glucose Dulbecco’s Modified Eagle’s Medium (DMEM; Welgene Inc.) Containing 10% Fetal Bovine Serum (FBS; Thermo Fisher Scientific) And 1% Antibiotics–Antimycotics [penicillin (100 U/ml), streptomycin (100 µg/ml), And Amphotericin B (250 ng/ml)] And Maintained At 37°C And 5% CO2. Cells Were Subcultured Every 6 Days Once They Have Reached Approximately 90% Confluence. Cells At Passages 5 To 7 Were Used For The Experiments.

Primary Culture Of Mouse TSPCs
Mouse TSPCs Were Isolated From The Tendon Tissues Of 7–Day–old Icr (Institute Of Cancer Research) Mice Using The Method Of Bi et al. (32), Zhang And Wang (33), And Yin et al. (31). Icr Mouse Tails Were Removed Using Scissors And Washed Three Times With Ice–Cold PBS Containing 1% Antibiotics–Antimycotics. Tendon Tissues Were Collected From These Tails With Sterilized Forceps And Scissors. During The Extraction Of Tail Tendon Sample, The Samples Were Kept In Ice–Cold PBS Containing 1% Antibiotics–Antimycotics. Tendon Tissues Were Washed With PBS And Centrifuged At 200g For 5 min; Then, The Supernatant Was Aspirated, And The Wash Was Repeated. After The Washes, Tendon Tissues Were Treated With 200 µl Of 0.25% Trypsin/EdtA, 400 µl Of Collagenase (10 mg/ml; Sigma–Aldrich), And 1.4 Ml Of DmEM Containing 1% Antibiotics–Antimycotics. Tendon Tissues Were Incubated At 37°C And 5% CO2 For 40 min And Pipetted Every 5 min During The Incubation. After The Incubation, 2 Ml Of DmEM Containing 10% Fbs And 1% Antibiotics–Antimycotics Were Added. The Cell Mixture Was Filtered Through A 70–µm Cell Strainer And Centrifuged At 200g For 5 min. A Single–Cell Suspension Was Obtained By Diluting The Suspension To 1 Cell/µl In DmEM Containing 10% Fbs.
and 1% antibiotics-antimycotics and then cultured in 100-mm dishes. TSPCs formed colonies on the surface of culture dishes. They were collected and expanded for the subsequent use in their tenogenesis and chondrogenesis experiments. In experiments involving adenoviral infection or electroporation of vectors, TSPCs were used at passage 2. The remaining experiments were performed with TSPCs at passage 1.

**Primary culture of mouse tenocytes**

For the primary culture of mouse tenocytes, 7-day-old ICR mice were sacrificed under general anesthesia. Tendon tissues were collected from tails using sterilized forceps and scissors. Collected tendon tissues were washed with PBS and centrifuged at 200g for 5 min, and the supernatant was aspirated. Tendon tissues were then treated with 200 μl of 0.25% trypsin/EDTA, 400 μl of collagenase (10 mg/ml; Sigma-Aldrich), and 1.4 ml of DMEM containing 1% antibiotics-antimycotics. Tendon tissues were incubated at 37°C and 5% CO₂ for 40 min. After the incubation, 2 ml of DMEM containing 10% FBS and 1% antibiotics-antimycotics were added. The cell mixture was filtered through a 70-μm cell strainer and centrifuged at 200g for 5 min. Tenocytes were resuspended with DMEM containing 10% FBS and 1% antibiotics-antimycotics and seeded at a concentration of 8 × 10⁵ cells per 35-mm dish. In experiments involving adenoviral infection or electroporation, tenocytes were used at passage 0. Cells were transduced with adenovirus at an 800–multiplicity of infection (MOI) Ad-eGFP or Ad-Ctpr3 and incubated at 37°C and 5% CO₂ for 24 hours.

**Primary culture of mouse chondrocytes**

Mouse chondrocytes were isolated from femoral condyles and tibial plateaus of 4- and 5-day-old ICR mice, as previously described (49). Cells were cultured in high-glucose DMEM (Welgene Inc.) containing 10% FBS and 1% antibiotics-antimycotics [penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (250 ng/ml)] and maintained at 37°C and 5% CO₂.

**In vitro chondrogenesis**

Before inducing chondrogenic differentiation, cells were transduced with adenovirus at a 100-MOI Ad-eGFP or Ad-Ctpr3 and incubated at 37°C and 5% CO₂ for 2 hours. During the incubation, dishes were gently shaken every 30 min to increase the efficiency of the infection. Culture medium was then removed and replaced. For electroporation, cells were resuspended in Opti-MEM (Thermo Fisher Scientific) at a concentration of 2.5 × 10⁵ cells per 200 μl. Two hundred microliters of this solution was then mixed with 30 μg of plasmid DNA (pCMV14, pCMV14-Ctpr3-3xflag, pLKO.1-Ctrl, and pLKO.1-shCtpr3), transferred to a sterile 2-mm gap cuvette, and electroporated once using a BTX Gemini X2 at 500 V for 0.1 ms. After electroporation, cells were seeded in 60-mm dishes. For adenoviral transduction cells were infected 100-MOI Ad-eGFP or Ad-Ctpr3 and incubated at 37°C and 5% CO₂ for 2 hours. After 3 days, 4.0 × 10⁴ mouse TSPCs or 2.0 × 10⁴ human UC-MSCs were seeded with 2 μg of fibrinogen (Sigma-Aldrich) and 1 U of thrombin (Sigma-Aldrich) to induce tenogenesis. Two hours later, culture medium was added to each well. Culture medium supplemented with 100 μM l-ascorbic acid 2-phosphate was changed every 2 days for 2 weeks. Tendon constructs were fixed in 4% PFA, embedded in a paraffin block, sectioned at 7 μm, and stained with H&E. The mean diameter of tendon constructs was analyzed using Image-Pro premier software. RNA was extracted using TRI Reagent (Molecular Research Center Inc.).

**In vitro neotendon construction**

Neotendon construction from mouse TSPCs and human UC-MSCs was performed as described previously (50). Culture dishes were pre-prepared as follows: Each well of a six-well plate was coated with SYLGARD (Dow Corning) and incubated at 55°C overnight. Two silk sutures were pinned in each well with 1 cm between sutures. The plates were sterilized with 70% ethanol and exposed to ultraviolet irradiation. Before inducing tenogenesis, cells were subjected to electroporation or transduction with an adenovirus. For electroporation, cells were resuspended in Opti-MEM at a concentration of 2.0 × 10⁷ cells per 200 μl. Two hundred microliters of this solution was mixed with 30 μg of plasmid DNA (pCMV14, pCMV14-Ctpr3-3xflag, pLKO.1-Ctrl, and pLKO.1-shCtpr3), transferred to a sterile 2-mm gap cuvette, and electroporated once using a BTX Gemini X2 at 500 V for 0.1 ms. After electroporation, cells were seeded in 60-mm dishes. For adenoviral transduction cells were infected 100-MOI Ad-eGFP or Ad-Ctpr3 and incubated at 37°C and 5% CO₂ for 2 hours. After 3 days, 4.0 × 10⁴ mouse TSPCs or 2.0 × 10⁴ human UC-MSCs were seeded with 2 μg of fibrinogen (Sigma-Aldrich) and 1 U of thrombin (Sigma-Aldrich) to induce tenogenesis. Two hours later, culture medium was added to each well. Culture medium supplemented with 100 μM l-ascorbic acid 2-phosphate was changed every 2 days for 2 weeks. Tendon constructs were fixed in 4% PFA, embedded in a paraffin block, sectioned at 7 μm, and stained with H&E. The mean diameter of tendon constructs was analyzed using Image-Pro premier software. RNA was extracted using TRI Reagent (Molecular Research Center Inc.).

**In vitro adipogenesis and osteogenesis**

TSPCs were seeded at 2.0 × 10⁵ cells per well in 12-well plate and then induced to differentiate toward an adipogenic lineage using StemPro Adipogenic Differentiation Kit (Thermo Fisher Scientific). After 23 days, cells were rinsed with PBS, fixed in prechilled 4% PFA at room temperature for 1 hour, and stained with Oil Red O (Sigma-Aldrich) for 30 min. To induce osteogenic differentiation, TSPCs were seeded at 1.75 × 10⁶ cells per well in 12-well plate and cultivated using StemPro Osteogenic Differentiation Kit (Thermo Fisher Scientific, A1007201). After 21 days, Alizarin Red S (Sigma-Aldrich) staining was performed. Cells were rinsed with PBS and fixed in prechilled 4% PFA at room temperature for 1 hour and then stained with Alizarin Red S for 30 min.

**Sphere formation assay**

Mouse tenocytes and TSPCs were suspended in DMEM-F12 medium supplemented with 2% of B-27 supplement (Thermo Fisher Scientific), epithelial growth factor (20 ng/ml; Invitrogen), and basic fibroblast growth factor (20 ng/ml; PeproTech). Cells were then seeded at a density of 1 × 10⁴ cells per well in a 24-well ultralow attachment plate (Corning). After 4 days, fresh medium was added to each well. Seven days later, the spheres were analyzed by sphere diameter, and the number of spheres larger than 50 μm was counted. Three representative fields per well were imaged (Zeiss, Axio Observer Z1), and
the diameters and the number of spheres were measured using the ImageJ program.

Adenovirus
Ad-Ctrp3 (ADV-254235), adenovirus expressing mouse Ctrp3, was purchased from Vector Biolabs. Ad-eGFP was a gift from J. B. Kim (Department of Biological Sciences, Seoul National University, South Korea). Adenovirus titers were determined using the Adenovector Rapid Titer Kit (Applied Biological Materials Inc.) according to the manufacturer’s protocol.

Production of conditioned media
Human embryonic kidney 293 T cells were used for the production of control or mouse CTRP3-enriched conditioned media. Cells were cultured in DMEM containing 10% FBS and 1% antibiotics-antimycotics. Cells were transfected with pCMV14-empty or pCMV14-Ctrp3-3xflag vectors using Opti-MEM and polyethylenimine transfection reagent (Sigma-Aldrich). After 24 hours, culture medium was changed to serum-free DMEM containing 1% antibiotics-antimycotics. After another 24 hours, culture medium was collected and centrifuged at 425g for 20 min at 4°C. The supernatant medium was harvested and filtered through a 0.22-μm filter to remove cell debris. Abundant secretion of CTRP3 into CTRP3-conditioned media was confirmed by immunoblotting.

Reverse transcription polymerase chain reaction and quantitative reverse transcription polymerase chain reaction
Human specimens and mouse tissue samples were homogenized by a tissue homogenizer (Automill) according to the manufacturer’s protocol. Total RNA was extracted using the TRI Reagent and reverse-transcribed using EasyScript Reverse Transcriptase (TransGen Biotech). Reverse transcription polymerase chain reaction (RT-PCR) was used in amplifying cDNA using SimpliAmp Thermal Cycler (Applied Biosystems). PCR primers used in the experiments are listed in table S11. For the quantitative analysis of mRNA transcript levels, quantitative RT-PCR (qRT-PCR) was performed using SYBR TOPrreal qRT-PCR 2x Premix (Enzymonics) on a StepOnePlus Real-Time PCR System (Applied Biosystems). Gene expression was normalized to Gapdh or GAPDH.

Plasmid
The Ctrp3 mRNA obtained from the Achilles tendon of C57BL/6J mice was converted into cDNA by RT as described above, and the mouse Ctrp3 cDNA was cloned into p3xFlag-CMV14 (pCMV14) mammalian expression vector. The shRNA sequence of mouse Ctrp3 gene obtained from MISSION shRNA database (Sigma-Aldrich) and the shCtrl, shCtrp3#1 (shCtrp3), shCtrp3#2, and shCtrp3#3 were cloned into pLKO.1-puro (pLKO.1) mammalian expression vector. The primers used for cloning are listed in table S12, and the construct was confirmed by sequencing.

RNA sequencing
Mouse Achilles tendon was injured by partial transection. A sham surgery was performed as the control. Tendon samples were harvested 3 weeks after surgery. Four biological replicates were used for each experimental group. For RNA extraction, each tendon homogenate was prepared from two mice from the same condition. RNA sequencing libraries were constructed with 1 μg of high-quality RNA (RNA integrity number, >7.0) using the TruSeq Stranded mRNA Library Prep Kit (Illumina). Libraries were validated using the Agilent 2100 Bioanalyzer. RNA sequencing was performed using the Illumina HiSeq 2500 sequencer at Macrogen Inc. The reads were trimmed with Trimmomatic (v0.39) and aligned to the mouse reference genome (mm10) using STAR aligner (v2.7.6a). Gene counts were subsequently estimated using StringTie (v1.3.5). Differential expression analysis was conducted by using the edgeR R package (v3.12). Genes with transcripts per million of >1 are plotted.

Microarray
Eight-week-old C57BL/6J male mice purchased from Daehan-Biolinek Co. were subjected to in vivo transfection and overuse-induced tendinopathy experiments, as described above. Mouse tissue samples were homogenized using a tissue homogenizer, and total RNA was extracted using TRI Reagent and the manufacturer’s protocol. Two biological replicates were used for each experimental group. Microarray services were provided by Macrogen Inc. and performed using the GeneChip Mouse Gene 2.0 ST Array (Affymetrix) as previously described (51). Differentially expressed gene probes were identified using comparison of the samples and the local-pooled-error test.

Bioinformatics analysis
Cytokine gene set was annotated using UniProt with “cytokine” term and IPA (52) with “cellular location of extracellular space” term. Among 1662 UniProt cytokine term genes and 1740 IPA cellular location of extracellular space term genes, 308 common genes were listed. Last, 126 genes with a transcripts per million value over 1 were used for data analysis. Hierarchical clustering of microarray data was performed with differential expressed genes according to their coexpression patterns based on Pearson’s correlation, Ward criterion, and Euclidean distance using R package version 3.3.2. Hypergeometric P value and representation factor between indicated cluster pairs were performed using R package version 3.3.2. The representation factor is the number of overlapping genes divided by the expected number of overlapping genes drawn from two independent gene groups. Gene ontology analysis of cluster 2 was performed using the BiNGO plugin of Cytoscape (30). GSEA was performed using GSEA version 2.2.3, as described previously (53). A prерanked gene list was used for analysis with default parameters. “Increases and affects cartilage development” gene set and “increases and affects synthesis of proteoglycan” gene set were annotated using IPA.

Study approval
Regarding human sample collection, the study was approved by the Institutional Review Board (IRB) of SMG-SNU Boramae Medical Center (IRB nos. 20150812/16-2015-115/091, 26-2016-143, E1803/003-009, and E2012/002-002). Regarding animal experiments, the study was approved by the Seoul National University Institutional Animal Care and Use committees (IACUC nos. SNU-170821-4-1 and SNU-170623-4). For animal experiments, we followed the Animal Research: Reporting of In Vivo Experiments guidelines (www.nc3rs.org.uk/arrive-guidelines).

Statistical analysis
All experiments were conducted independently for at least three times. Data are presented as means ± SD. Statistical significance was assessed by Mann-Whitney U test, the Wilcoxon matched-pairs signed-rank test, and the Kruskal-Wallis test, followed by post hoc
Mann-Whitney U test for pair-wise, paired, and multiple comparisons, respectively. For the analysis of real-time PCR results, a log-normal distribution of relative mRNA expression was confirmed (54), followed by parametric tests, Student’s t test, and analysis of variance (ANOVA) with Fisher’s least significant difference post hoc test. Statistical significance was accepted at P < 0.05. Statistical analysis was performed using IBM SPSS Statistics 24 and GraphPad Prism 7 software.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.abyg609

View/request a protocol for this paper from Bio-protocol

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