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RESEARCH ARTICLE

Suppression of TNF-α activity by immobilization rescues *Mkx* expression and attenuates tendon ossification in a mouse Achilles tenotomy model

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Abstract

Traumatic heterotopic ossification is a condition in which extraskeletal bone formation occurs in soft tissues after injury. It most commonly occurs in patients who had major orthopedic surgery and in those with severe extremity injuries. The lesion causes local pain and can impair motor function of the affected limb, but there is currently no established prophylaxis or treatment for this condition. In this study, we show that immobilization at an early stage of the inflammatory response after injury can attenuate ossification formation in a murine Achilles tenotomy model. Gene expression analysis revealed a decrease in the expression of *Tnf* and an increase in the expression of *Mkx*, which encodes one of the master regulators of tendon differentiation, Mohawk. Notably, we found that TNF-α suppressed the expression of *Mkx* transcripts and accelerated the osteogenic differentiation of tendon-derived mesenchymal stem cells (MSCs), suggesting that TNF-α acts as a negative regulator of *Mkx* transcription. Consistent with these findings, pharmaceutical inhibition of TNF-α increased the expression of *Mkx* transcripts and suppressed bone formation in this mouse model. These findings reveal the previously unrecognized involvement of TNF-α in regulating tendon MSC fate through suppression of *Mkx* expression and suggest that TNF-α is a potential target for preventing traumatic heterotopic ossification.

KEYWORDS
heterotopic ossification, mesenchymal stem cell, *Mkx*, tendon ossification, TNF-α

1 | INTRODUCTION

Posttraumatic heterotopic ossification is defined as the extraskeletal formation of bone in soft tissues such as muscle, tendon, and adipose tissue after injury.[1,2] It is most commonly associated with major orthopedic surgery, high-energy extremity injuries, and burns. This condition occurs in up to 30%–40% of patients with total hip replacement, fracture, or dislocation and in approximately 90% of patients with traumatic amputation.[1,3] Heterotopic ossification not only compromises muscle and tendon function but can also lead to chronic localized pain that can impede daily activities and rehabilitation. Heterotopic ossification is considered an irreversible event, and there is currently no established prophylaxis or treatment other than surgical removal of the lesion or administration of anti-inflammatory drugs to alleviate the pain. Therefore, it is clinically imperative to understand the mechanism underlying this condition and to develop a treatment modality to inhibit the bone formation process before it occurs.

Previous studies have shown that various factors, including inflammation, hypoxia, and neurogenic disorders, are associated with the etiology of this disease.[1,2] The nature of the cells that give rise to heterotopic ossification has not been fully defined; however, emerging data suggest that multipotent stem cells residing in soft tissues are responsible for this condition.[2,4–6] These tissue-resident multipotent stem cells, also known as mesenchymal stem cells (MSCs), are a heterogeneous subset of stromal cells, and their biological properties vary depending on the tissue in which they reside.[7,8] By definition, these cells can differentiate into several different cell lineages, including osteoblasts, chondroblasts, and adipocytes.[9] Studies in the last decade have shown that MSCs have diverse functions and are essential for tissue homeostasis and repair. However, under pathological conditions, the multipotency of MSCs is dysregulated and may contribute to the development of tissue degeneration.

In the present study, we show that post-injury immobilization suppresses the inflammatory response and prevents the development of heterotopic ossification in a mouse Achilles tenotomy model.[10,11] We found that the expression of the tendon-specific transcription factor Mohawk (encoded by the *Mkx* gene), which is critically involved in both tendon formation and homeostasis, is suppressed after tenotomy.[12–14] Furthermore, we found that TNF-α, one of the most crucial cytokines in the inflammatory and immune response, negatively regulates the expression of Mohawk transcription factor in tendon-derived MSCs and thereby drives the osteogenic differentiation of MSCs after tenotomy. These results reveal a novel role of TNF-α in negatively regulating Mohawk expression and may suggest one of the potential molecular mechanisms underlying the dysregulation of MSC differentiation under pathological conditions.

2 | METHODS

2.1 | Mice

Six-week-old C57BL/6J male mice were purchased from Japan SLC and maintained under specific pathogen-free conditions with constant temperature and humidity. All animal experiments were approved by the Animal Care Committee of the National Defense Medical College (approval number: 20024).

2.2 | The Achilles tenotomy model

Mice were subjected to Achilles tenotomy to induce bone formation in the tendon as previously described.[10,11] In this model, ossified lesions form in the stumps of the Achilles tendon 5–10 weeks after tenotomy without additional intervention. All procedures were performed under anesthesia induced by intraperitoneal injection of medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg). A 10 mm longitudinal incision was made in the posterior aspect of the right ankle to expose the Achilles tendon. The Achilles tendon was transected with a scalpel, and the incision closed with nonabsorbable sutures. In some experiments, the right hindlimb was immobilized with a rubber-coated steel wire in the knee extended and ankle flexed position after tenotomy as previously described (refer to Onda et al.[15] for a detailed method). To suppress the local inflammatory response, the JAK inhibitor peficitinib (Astellas Pharma) dissolved in 0.5% methylcellulose (24 mg/kg; Fujifilm Wako) was administered orally for 7 consecutive days after tenotomy.

2.3 | Isolation and culture of tendon-derived MSCs

Tendon-derived MSCs were collected from mouse tendon tissue as previously described with some modifications.[16,17] Tendon tissues from 6-week-old C57BL/6 J male mice were cut into pieces and digested with 4 mg/mL collagenase type I (Sigma–Aldrich, St. Louis, MO) and 1000 U/ml dispase I (Godo Shusei, Chiba, Japan) for 3 h at 37°C. After filtration through a 70 μm cell strainer, cells were cultured in complete Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum and penicillin–streptomycin (100 U/mL) at 37°C under 5% CO_2 and passaged at least two times to ensure a nearly homogeneous population before use. These cells had the potential to differentiate into osteogenic, chondrogenic, and adipogenic lineages (Supporting Information S1: Supplementary Methods and Supplementary Figure S1) and were considered to be predominantly composed of MSCs.[9] To induce osteogenic differentiation, the cells were incubated in osteogenic differentiation medium containing 10 mM β-glycerophosphate (Sigma–Aldrich), 50 μg/ml L-ascorbic acid (Sigma–Aldrich), and 100 ng/μL recombinant human BMP2 (PeproTech, Rocky Hill, NJ). Alizarin red staining was performed after 4 weeks of incubation to evaluate mineral deposition. For gene expression analysis, cells were collected after 2 weeks of incubation. In some experiments, MSCs were cultured in the presence of recombinant human TNF-α (PeproTech) with or without an anti-human TNF-α monoclonal antibody (infliximab; Nipponkayaku, Tokyo, Japan) to evaluate the effect of TNF-α on MSCs. Recombinant TNF-α was dissolved in distilled water and stored at −30°C before use.

2.4 | Radiological evaluation

Radiographs of the hindlimbs (TIFF-formatted grayscale images) were taken with an IVIS Lumina XR Series III (PerkinElmer). The density of ossified lesions in each image was evaluated using ImageJ software (National Institutes of Health). In short, the contour of the ossified lesion in each image was outlined using the "freehand selections" tool and a Wacom Intuos pen tablet (Saitama), and the integrated density value (RawIntDen) of the lesion, which is the sum of the values of the

pixels of the selected area, was determined. The RawIntDen value of ossified lesions in the tenotomy groups was normalized to 1.

2.5 | Histology

Mice were euthanized by cervical dislocation under isoflurane anesthesia. The hindlimbs were removed, fixed in phosphate-buffered saline containing 4% paraformaldehyde, and decalcified in 10% EDTA. Tissues were embedded in paraffin, sectioned at 4 μm thickness and stained with hematoxylin and eosin. Photomicrographs were taken with an IX71 microscope (Olympus).

2.6 | Quantitative polymerase chain reaction (PCR)

Total RNA from Achilles tendon tissue and MSCs was isolated using the RNeasy Plus Mini Kit (Qiagen) or ISOGEN II (Nippon Gene) and reverse-transcribed using the PrimeScript RT Reagent Kit (Takara Bio). Quantitative PCR was performed using TB Green Premix Ex Taq II (Takara Bio) on a Fast 7900HT Real-Time PCR System (Applied Biosystems). We normalized the expression level by calculating the ratio of the Ct value of a given gene to that of *Gapdh*. The expression levels of the transcripts of each gene in the untreated mouse group or the tenotomy group were set to 1. The data were processed using SDS2.4 software (Applied Biosystems). All analyzes were performed in triplicate. The nucleotide sequences of the oligos used in this study are listed in Table 1.

2.7 | Statistical analysis

Statistical analyzes were performed by one-way analysis of variance followed by Dunnett's or Tukey–Kramer's post hoc test for multiple comparisons or an unpaired *t* test for comparisons of two groups using GraphPad Prism 8 (GraphPad Software). The data are expressed as the mean ± standard error of the mean. $p < 0.05$ were considered to indicate statistical significance.

3 | RESULTS

3.1 | Immobilization in the early stages of inflammation is sufficient to suppress tendon ossification

To induce heterotopic ossification, we used a mouse Achilles tenotomy model as previously described.[10,11] In this model, tendon ossification, a form of heterotopic ossification that occurs in the tendon, can be reproducibly induced 5–10 weeks after Achilles tendon transection. As expected, ossification was observed at the cut end of the Achilles tendon 5 weeks after tenotomy (Figure 1A–D). However, when the surgically treated limb was immobilized after tenotomy, tendon ossification was completely suppressed, as evidenced by the absence of calcification on radiographs and bone tissue on histology (Figure 1B–D).

Considering that heterotopic ossification is often associated with inflammation, we next evaluated the time course changes in the expression of *Tnf* and *Il6* transcripts after tenotomy. As shown in Figure 1E, the expression of both the *Tnf* and *Il6* transcripts increased rapidly, peaked at approximately 1 week after tenotomy, and decreased to near basal levels by 3 weeks. Since, in general, immobilization attenuates local inflammation induced by injury, we hypothesized that tendon ossification could be suppressed by attenuating the inflammatory response at an early stage (i.e., the first week after tenotomy in this model) through immobilization. To test this hypothesis, we divided the mice into four groups after tenotomy and immobilized the surgically treated hindlimb for different periods after tenotomy (0, 1, 3, and 5 weeks; Figure 1F). As shown in Figure 1G,H, we found that 3 weeks of immobilization was sufficient to completely suppress tendon ossification and, more importantly, that even a brief immobilization period of 1 week

TABLE 1 Nucleotide sequences of the oligos.

Gene	Forward	Reverse
Gapdh	AACAGCAACTCCCACTCTTC	CTGTTGCTGTAGCCGTATT
Tnf	CCCACGTCGTAGCAAACCAC	AGGTACAACCCATCGGCTGGC
Il6	TCGGAGGCTTAATTACACATGTT	TGCCATTGCACAACTCTTTTCT
Mkx	CGGACGTTCAGTGGTTTCCTG	GCTTATGCCTTACCTTCCCTCC
Tnmd	TGTACTGGATCAATC CCACTCT	GCTCATTCTGGTC AATCCCCT
Runx2	CGACAGTCCCAACTTCCTGT	CGGTAACCACAGTCCCATCT
Alpl	GGAATACGAACTGGATGAGAAGG	GGAATACGAACTGGATGAGAAGG
Bglap	CTGACAAAGCCTTCATGTCCAC	GCGCCGGAGTCTGTTCACTA
Sox9	TGAAGAACGGACAAGCGGAG	AGATTGCCCAGAGTGCTCG
Acan	GCATGAGAGAGGCGAATGGA	TCTTCTGCCCGAGGGTTCTA

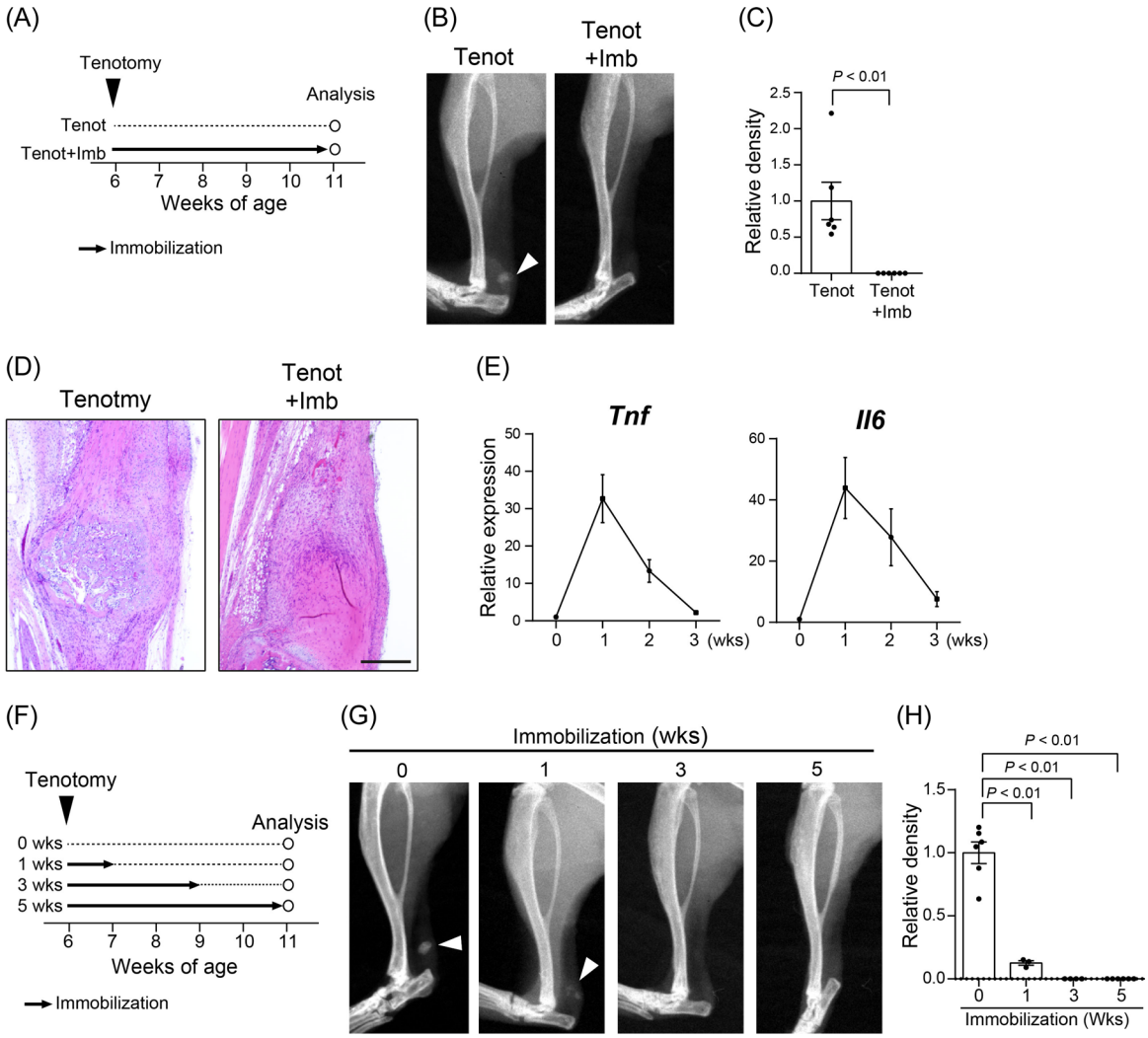

FIGURE 1 Early immobilization during the inflammatory response suppresses tendon ossification. (A) A diagram showing the schedule of immobilization after tenotomy. The hindlimb was immobilized for 5 weeks (Tenot+Imb) or left unimmobilized (Tenot) after tenotomy. (B) Representative radiographs of hindlimbs from mice in the Tenot group and Tenot+Imb groups. The arrowhead indicates tendon ossification. (C) Quantification of the tendon ossification area. The average ossified area in the Tenot group was set to 1. $n = 6$ for each group. (D) Representative hematoxylin and eosin-stained sections of the cut end of the Achilles tendon from mice in the Tenot and Tenot+Imb groups. Bar, 500 µm. (E) Time course changes in the expression of *Tnf* and *Il6* transcripts in Achilles tendon tissue after tenotomy. (F) Outline of the schedule for immobilization after tenotomy in each group. Black arrows indicate the duration of immobilization (0, 1, 3, and 5 weeks). (G) Representative radiographs of hindlimbs from one mouse in each group. Arrowheads indicate tendon ossification. (H) Quantification of the tendon ossification area. The average ossified area in the Tenot group was set to 1. $n = 3$–6 mice per group. wks, weeks.

markedly alleviated ossification in this model. These observations suggest that immobilization during the early phase of the inflammatory response is sufficient to attenuate the ossification that occurs after tenotomy.

3.2 | Tendon ossification is associated with increased *Tnf* transcript expression and reduced *Mkx* transcript expression

To elucidate the mechanism by which immobilization mitigates tendon ossification, we performed gene expression analysis of Achilles tendon tissues collected from mice that underwent tenotomy (Tenot group) and those that underwent immobilization for 1 week after tenotomy (Tenot+Imb group). As expected, there was a significant increase in the expression of *Tnf* and *Il6* transcripts in the Tenot group compared with the untreated control group (Ctrl) (Figure 2). Immobilization significantly suppressed the expression of *Tnf* transcripts after tenotomy; however, the expression of *Il6* transcripts was not affected by immobilization. Consistent with the decrease in ossification after immobilization, the expression of *Runx2* (a transcription factor essential for osteoblast differentiation), *Alpl* (encoding alkaline phosphatase, an early-stage marker of osteogenic differentiation), *Sox9* (a transcription factor essential for chondroblast differentiation), and

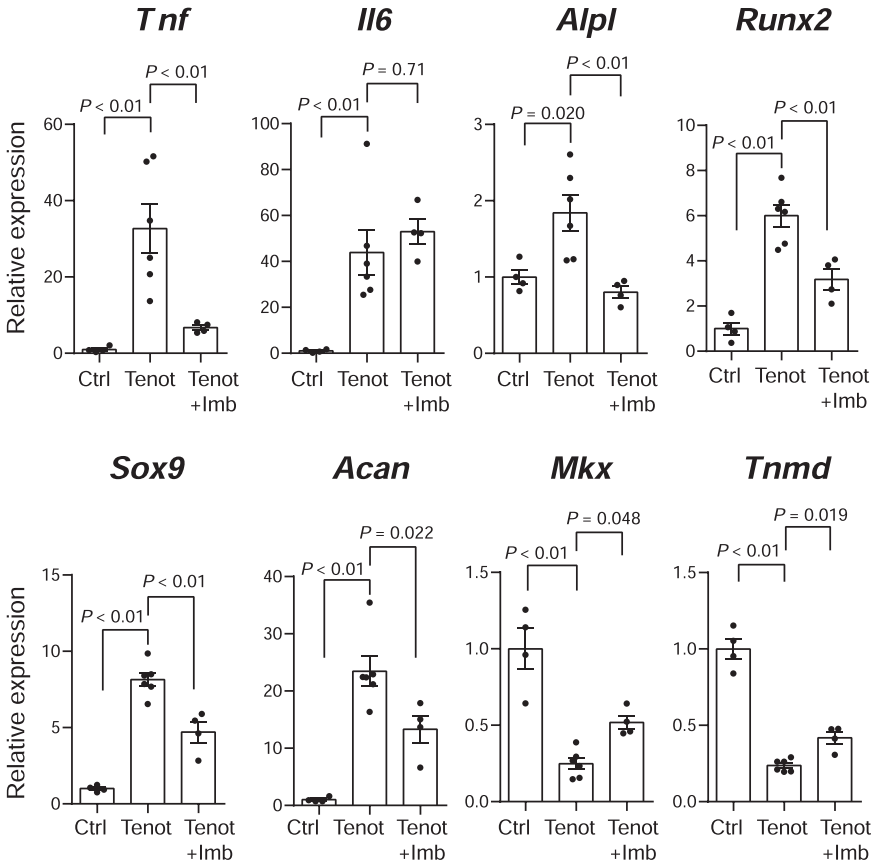

FIGURE 2 Gene expression analysis of the Achilles tendon tissues. Tendon tissues were collected from untreated (Ctrl) mice, mice that underwent tenotomy (Tenot), and mice that underwent tenotomy and hindlimb immobilization (Tenot+Imb) for 1 week. 4–6 mice per group. The expression levels of each gene in the untreated control mouse group (Ctrl) were set to 1.

Acan (encoding aggrecan, a cartilage-specific proteoglycan) transcripts was significantly lower in the Tenot+Imb group than in the Tenot group. Notably, we found that the expression of *Mkx*, which encodes the tenocyte-specific transcription factor Mohawk, was reduced in the Tenot group and partially rescued by immobilization (Tenot+Imb group). Nearly identical results were observed for *Tnmd* transcripts, which encode the tenocyte-specific cell-surface protein Tenomodulin. These observations suggest that ossification is associated with increased expression of *Tnf* transcripts and decreased expression of *Mkx* transcripts and that these changes in *Tnf* and *Mkx* transcript expression after tenotomy can be partially reversed by immobilization.

3.3 | TNF-α suppresses *Mkx* expression and promotes osteogenic differentiation of tendon-derived MSCs

To explore the potential causal relationship between the increased expression of *Tnf* transcripts and ossification, we performed osteogenic differentiation experiments using MSCs isolated from tendon tissue. MSCs were incubated with osteogenic differentiation medium in the presence or absence of recombinant TNF-α. As shown in Figure 3A, TNF-α significantly reduced the basal expression of *Mkx* transcripts. The expression of *Mkx* transcripts was partially restored when infliximab was added simultaneously with recombinant TNF-α (Figure 3B). Furthermore, Alizarin Red S staining of the cultured cells showed a marked increase in the number of calcified nodules in the cells cultured with TNF-α compared with that in the vehicle-treated cells (Figure 3C). Accordingly, there was a significant increase in the expression of the osteoblast differentiation markers *Alpl*, *Runx2*, and *Bglap* (encoding the osteoblast-specific extracellular protein osteocalcin) in TNF-α-treated cells compared with vehicle-treated cells (Figure 3D). Given that the Mohawk transcription factor is essential for maintaining the undifferentiated state of MSCs in a tendon,[12,13] these results suggest that TNF-α accelerates osteoblast differentiation, at least in part, by suppressing the transcription of *Mkx*.

3.4 | Janus kinase (JAK) inhibition attenuates tendon ossification

To further validate that TNF-α is causally associated with the development of tendon ossification, we investigated whether tendon ossification could be suppressed by reducing the activity of TNF-α in mice. Since biopharmaceuticals currently used in humans against TNF-α are not active or have limited activity in mice,[18–20] we used peficitinib, a small-molecule JAK inhibitor.[21] JAKs are critical regulators of the inflammatory response, and inhibition of JAK activity results in reduced production of pro-inflammatory cytokines, including IL-6 and TNF-α. We administered peficitinib to the mice for 1 week after tenotomy and examined the degree of tendon ossification (Figure 4A). As shown in Figure 4B and C, the administration of peficitinib significantly suppressed tendon ossification induced by tenotomy. Gene expression analysis of

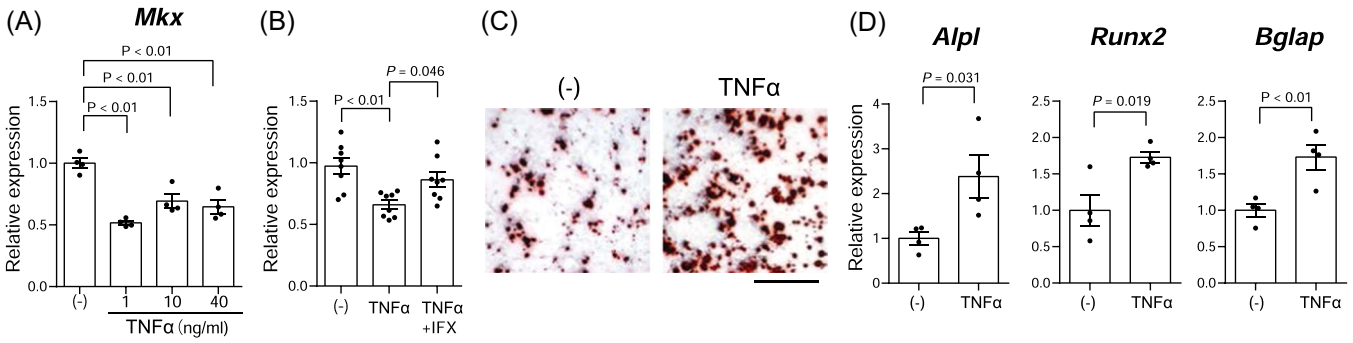

FIGURE 3 TNF-α suppresses the expression of *Mkx* transcripts and accelerates the osteogenic differentiation of MSCs. (A) Relative expression levels of *Mkx* transcripts in MSCs cultured with (1, 10, or 40 ng/mL) or without (−) TNF-α for 8 h. $n = 4$ independent experiments. (B) Relative expression levels of *Mkx* transcripts in MSCs cultured with TNF-α (10 ng/mL) in the presence or absence of infliximab (IFX, 10 μg/mL). $n = 8$ independent experiments. Representative microscopy images of MSCs cultured in osteogenic differentiation medium in the presence or absence (−) of TNF-α (1 ng/ml) for 3 weeks and stained with Alizarin red S. Bar, 1 mm. (D) Relative expression levels of osteogenic differentiation markers in MSCs cultured in osteogenic differentiation medium in the presence or absence of TNF-α (1 ng/ml) for 3 weeks. $n = 4$ independent experiments. The expression levels of each gene in the untreated cells (−) were set to 1.

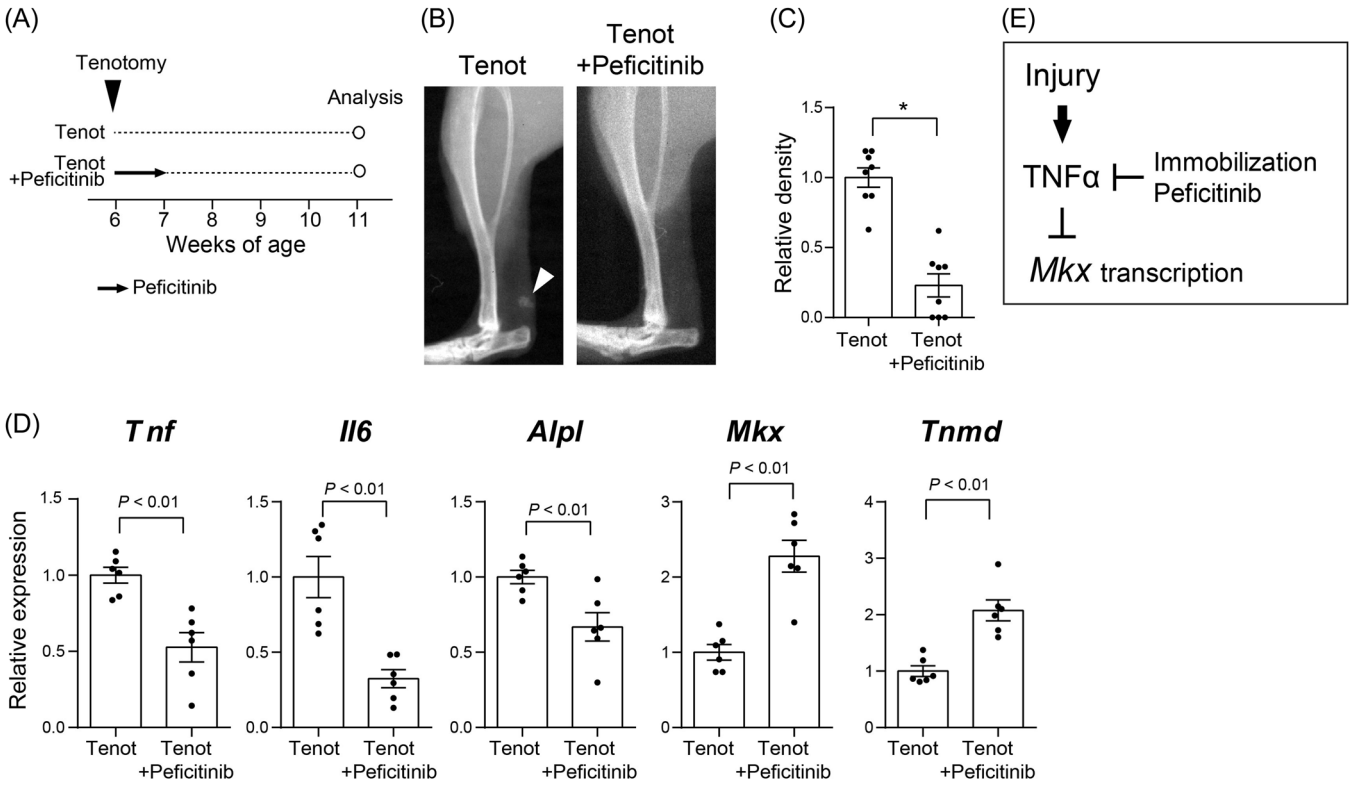

FIGURE 4 Administration of the JAK inhibitor peficitinib suppresses tendon ossification after tenotomy. (A) A diagram showing the schedule of peficitinib administration following tenotomy. (B) Representative radiographs of hindlimbs from mice that underwent tenotomy (Tenot) or tenotomy and treatment with peficitinib (Tenot+peficitinib) for 1 weeks. (C) Quantification of the tendon ossification area. The average ossified area in the Tenot group was set to 1. $n = 8$ for each group. (D) Gene expression analysis of inflammatory cytokines and differentiation markers in tendon tissues collected from the Tenot and Tenot+Peficitinib groups. (E) A diagram summarizing the results of the present study.

Achilles tendon tissue confirmed the decreased expression of *Tnf*, *Il6*, and *Alpl* transcripts and increased expression of *Mkx* and *Tnmd* transcripts in the peficitinib-treated mice (Figure 4D). These observations are consistent with the idea that tendon ossification after tenotomy can be impeded by suppressing TNF-α activity.

4 | DISCUSSION

It is a common practice in emergency medicine to apply a cast to the injured limb in patients with extremity trauma to reduce pain and protect the injury site from further damage. However, the mechanisms

underlying the potential benefits of limb immobilization are not fully understood. In this study, we found that immobilization attenuated the expression of TNF-α transcripts and suppressed tendon ossification in a mouse Achilles tenotomy model. Notably, we found that the expression of *Tnf* transcripts was induced after tenotomy but suppressed by immobilization. Furthermore, we found that the expression of *Mkx* transcripts was inversely correlated with that of *Tnf* in this model and that TNF-α suppressed the expression of *Mkx* transcripts in tendon-derived MSCs, thereby enhancing their osteogenic differentiation in vitro. Accordingly, the administration of peficitinib rescued the expression of *Mkx* transcripts and significantly suppressed tendon ossification after tenotomy. Taken together, our data reveal a previously unknown link between *Tnf* and *Mkx* and one of the potential mechanisms underlying the beneficial effects of limb immobilization after injury in preventing heterotopic ossification (Figure 4E).

Previous studies have shown that inflammation is one of the drivers of traumatic heterotopic ossification and have suggested that inflammation is a potential target for blocking the formation of heterotopic ossification.[22,23] However, only a few studies have investigated the time course changes in inflammatory cytokine expression after injury and the impact of interventions (e.g., immobilization, unloading, medication, etc.) on these changes.[24,25] In our mouse model, we found that the expression of inflammatory cytokines (*Tnf* and *Il6*) peaked after 1 week and gradually returned to basal levels by 3 weeks after tenotomy. Importantly, our data showed that 1 week of immobilization after tenotomy was sufficient to reduce the formation of tendon ossification, suggesting that the early-stage inflammatory response plays a crucial role in determining the fate of MSCs residing in tendons after injury. Given that the expression of *Tnf* transcripts was significantly reduced by 1 week of immobilization, we assumed that TNF-α is potentially one of the factors involved in the dysregulation of MSC differentiation. On the other hand, the finding that the expression of *Il6* transcripts was not affected by immobilization was unexpected. IL-6 is as crucial as TNF-α for eliciting inflammation and immune responses and is a valid molecular target for treating various autoimmune diseases in humans.[26,27] On the other hand, past studies have shown that IL-6 also functions as a myokine and is involved in the pathogenesis of disuse muscle atrophy and sarcopenia.[28–30] Considering that Achilles tenotomy results in muscle atrophy and atrophy of the tendon itself, it is tempting to speculate that the increased expression of *Il6* transcripts was due not only to the inflammatory response but also to atrophy of the tendon tissue after tenotomy.

Although it is evident that TNF-α is critically involved in the regulation of the fate and function of MSCs, the effects of TNF-α on MSCs are complex and appear to be highly context dependent.[31] Previous studies have suggested that the effect of TNF-α on the osteogenic differentiation of MSCs is dependent on the concentration of TNF-α, which promotes the osteogenic differentiation of MSCs at low concentrations and suppresses it at high concentrations.[2,32–34] As discussed in previous studies,[31,35] this may indicate that immediately after bone injury, TNF-α is released at low levels and thereby promotes fracture healing, whereas in chronic and overt inflammation, it is overproduced and inadvertently contributes to bone loss. Since the mouse model we used in the present study does not appear to induce an overt inflammatory response, it is likely that local levels of TNF-α were within the physical range observed under mild injury. Consistent with this assumption, we found that the inhibition of TNF-α alleviated the formation of tendon ossification in our mouse model and that TNF-α promoted the osteogenic differentiation of isolated MSCs in vitro at a low dose (1 ng/ml). Accordingly, a recent study showed a significant reduction in tendon ossification after tenotomy in mice lacking TNF-α.[36] Furthermore, we found that TNF-α increased the expression of *Alpl*, *Runx2*, and *Bglap* transcripts, as expected from the increase in mineral deposition observed in MSCs cultured in the presence of TNF-α; moreover, TNF-α suppressed the expression of *Mkx* transcripts in MSCs.

Mkx encodes the transcription factor Mohawk, which is specifically expressed in tendons during embryogenesis.[37] It plays an essential role in tendon differentiation, as evidenced by the severe hypoplasia of tendons in mice lacking *Mkx*.[14,38] Furthermore, abrogation of *Mkx* leads to tendon ossification even without injury in rats and mice, and *Mkx* deficiency accelerates the osteogenic and chondrogenic differentiation of tendon-derived cells.[12,15] These studies showed that *Mkx* is an indispensable regulator of tendon differentiation and is essential for the homeostasis of MSCs in tendons. A previous study showed that IL-1β suppresses the expression of *MKX* transcripts in ligament-derived cells in humans;[39] otherwise, the transcriptional regulation of *Mkx* transcripts remains poorly understood. Our data showed that the expression levels of *Mkx* transcripts inversely correlate with those of *Tnf* in our mouse model and that TNF-α suppresses the expression of *Mkx* in MSCs. Accordingly, the decrease in the expression of *Tnf* transcripts induced by the administration of peficitinib coincided with the increase in the expression of *Mkx* transcripts and the reduction in the formation of tendon ossification. These results support the idea that TNF-α promotes tendon officiation by suppressing *Mkx* transcription and is a previously unrecognized regulator of *Mkx*.

This study has several limitations. First, the mechanism underlying the suppressive effect of TNF-α on *Mkx* transcription in MSCs has not been elucidated. Considering that TNF-α suppresses the expression of *Mkx* transcripts in MSCs in a relatively short time (8 h), this effect is likely not mediated by newly synthesized proteins but is directly regulated downstream of TNF receptor signaling. In addition, understanding how *Mkx* regulates the maintenance of tendon cells and prevents osteogenic differentiation under normal conditions is important. Second, we used a JAK inhibitor rather than a specific inhibitor to study the effect of TNF-α in vivo. Because the JAK pathway has multiple functions, it is possible that the suppressed ossification we observed was not solely the result of TNF-α inhibition. Nevertheless, even with a specific inhibitor against TNF-α or by gene silencing or gene targeting, it would be difficult to distinguish direct from indirect effects of TNF-α on MSCs because inhibition of TNF-α activity could significantly alter the inflammatory response in vivo. Third, we only used the Achilles tenotomy model and tendon-derived MSCs in the present study, and it is unclear to

what extent the results of the present study are relevant to heterotopic ossification in other soft tissues and can be translated into the pathology of posttraumatic heterotopic ossification in humans. Finally, in this study, we focused on the effect of the inflammatory response after injury; however, it is highly likely that many other cell autonomous and environmental factors are involved in the progression of this condition. In fact, a recent study has shown that immobilization after limb injury leads to a reduction in mechanotransduction signaling in MSCs by altering extracellular matrix alignment and changes in genomic architecture that ultimately result in suppression of ossification.[40] Therefore, the results of the present study cannot be overly generalized and must be interpreted with caution until further data are accumulated.

In conclusion, the present study reveals one of the potential mechanisms underlying the beneficial effects of limb immobilization in suppressing ectopic tendon ossification. Our data may also be the first to show that TNF-α functions as a negative regulator of *Mkx* transcription and further underline the importance of alleviating the overt inflammatory response after injury to suppress heterotopic ossification. Despite some limitations, our data indicate that the osteogenic differentiation of MSCs, and therefore heterotopic ossification, can be controlled by targeting TNF receptor signaling in MSCs. Although unresolved issues remain to be addressed, the results of the present study may serve as a basis for further exploring the involvement of the inflammatory response in the pathogenesis of heterotopic ossification and the efficacy of anti-inflammatory intervention against this undesired condition with no effective treatment.

AUTHOR CONTRIBUTIONS

Masashi Isaji designed the study, performed most of the experiments, and wrote the paper. Keisuke Horiuchi designed and supervised the study, and wrote the paper. Shinya Kondo, Takahiro Nakagawa, and Takahiro Ishizaka contributed to the experimental work. Masatoshi Amako and Kazuhiro Chiba supervised the study. All authors have read and approved the final submitted manuscript.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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