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TIMP2 deficient mice develop accelerated osteoarthritis via promotion of angiogenesis upon destabilization of the medial meniscus

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ABSTRACT

Vascular invasion into the normally avascular articular surface is a hallmark of advanced osteoarthritis (OA). In this study, we demonstrated that the expression of tissue inhibitor of metalloproteinases-2 (TIMP2), an anti-angiogenic factor, was present at high levels in normal articular chondrocytes, and was drastically decreased shortly after destabilization of the medial meniscus (DMM). We also investigated the anti-angiogenic properties of TIMP2 via knockout. We hypothesized that the loss of TIMP2 could accelerate osteoarthritis development via promotion of angiogenesis. Loss of TIMP2 led to increased periarticular vascular formation 1 month post DMM, compared to wild-type mice, and did so without altering the expression pattern of matrix metalloproteinases and vascular endothelial growth factors. The increased vascularization eventually resulted in a severe degeneration of the articular surface by 4 months post DMM. Our findings suggest that reduction of TIMP2 levels and increased angiogenesis are possible primary events in OA progression. Inhibiting or delaying angiogenesis by TIMP2 expression or other anti-angiogenic therapies could improve OA prevention and treatment.

1. Introduction

Osteoarthritis (OA) is one of the most prevalent aging-associated diseases, and affects up to 15% of the population in the US [1]. The pathogenesis of OA is characterized by progressive degeneration of articular cartilage, vascular invasion of the articular surface, and osteophyte formation. Ultimately, these changes result in complete loss of cartilage and bone-on-bone articulation in the end stage of the disease. Under normal conditions a multitude of factors coordinate to maintain the structure and function of the joints. An imbalance in these factors may lead to joint degeneration. With a better understanding of OA, attempts have been made to prevent its genesis; however, current therapeutic approaches are still limited to symptom management and surgical replacement of the affected joints at later stages of the disease.

Although not widely discussed in terms of OA development, angiogenesis is acknowledged as a prominent contributor to synovial hyperplasia and cartilage vascularization in inflammatory joint diseases such as rheumatoid arthritis (RA) [2–4]. Numerous pro-angiogenic factors, such as vascular endothelial growth factors (VEGFs), angiopoietins, and hypoxia-inducible factors (HIFs) have been detected in RA synovial tissues [5,6]. Indeed, it has been demonstrated that synovial fluid from RA patients can stimulate endothelial cell proliferation and formation of capillary structures *in vitro*. Distinctive clinical presentation and histopathological studies have suggested that OA joints are also afflicted by increases in osteochondral angiogenesis and vascular invasion into the articular cartilage [7]. Human cartilage explants and animal injury models suggest that normal articular cartilage resists vascular invasion by expression of anti-angiogenic proteins, which are reduced or even lost in OA cartilage [8,9]. However, as this was traditionally thought to be a secondary event following cartilage degradation, the role of anti-angiogenic factors in suppressing OA progression has not been well investigated.

Tissue inhibitors of metalloproteinases (TIMPs) have received considerable attention in OA studies because they can inhibit the

Abbreviations: TIMPs, tissue inhibitor of metalloproteinases; OA, osteoarthritis; DMM, destabilization of the medial meniscus; MMPs, matrix metalloproteinases; VEGFs, vascular endothelial growth factors; HIF, hypoxia-inducible factors.

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catalytic ability of matrix metalloproteinases (MMPs). TIMPs have low specificity to their MMP substrates with overlapping targets; for example, active MMP-13 degrades collagen II and is inhibited by all TIMPs [10,11]. TIMP2, a member of the TIMP family, has the unique feature of biphasic regulation of MMPs. Low levels of TIMP2 are required for pro-MMP2 activation by MT1-MMP, while at higher levels TIMP2 binds to MMP2 in a ratio of 1:1, blocking its enzyme activity [12,13]. TIMP2 also regulates endothelial cell proliferation. Its N-terminal domain binds to alpha-3 beta-1 domain suppressing endothelial growth *in vitro* and TIMP2 has therefore been a candidate therapeutic agent for angiogenesis-related diseases [14–18].

In this study, we investigated the specific role of endogenous TIMP2 expression and its involvement in the pathological progression of OA using *Timp2* knockout mice. We utilized a model of destabilization of medial meniscus (DMM) which resected selected areas of medial meniscus in knee joints to create a uniform OA-like phenotype. Expeditiously, this model closely approximates OA progression resultant of either mechanical injury or aging-related degeneration [19]. Additionally, we investigated loss of TIMP2 as a possible mechanism behind cartilage vascularization and precipitation of OA-like changes in response to DMM.

2. Materials and methods

2.1. Experimental animals

Original *Timp2* knockout (*Timp2*−/−) mice (Strain Name: B6.129S4-TIMP2tm1Pds/J) and C57BL/6J wild-type (WT) were purchased from Jackson's lab. Mouse genotyping was determined by PCR using DNA extracted from tail biopsy tissues 21 days after birth. All procedures conducted in this study were approved by the University Committee of Animal Care of University of Rochester Medical Center and were in accordance with all ethics policy.

2.2. OA model

DMM was performed with micro-surgical techniques. 3-month-old mice were anesthetized with Ketamine (60 mg/kg) by intraperitoneal injection. Surgeries were then administered under aseptic conditions. Right knee joints of the mice were destabilized by partial resection of the medial meniscus. The skin and joint capsule were incised and immediately sutured on the left knees as sham surgery controls. Buprenorphine was administered in drinking water for pain relief for the first three days after operation.

2.3. Vascular perfusion and micro-CT analysis

Mice were sacrificed and given serial intracardiac injections of heparinized saline, 10% normal buffered formaldehyde and lead chromate microfil perfusion reagent (Flow Tech, Carver, MA). To eliminate noise from surrounding tissue and quantify the vascular signals accurately, perfused tissues were decalcified in 14% EDTA for two weeks before visualization and quantification of vascular volume (mm^3) and connectivity density ($1/mm^3$) by micro-CT ($n = 10$ for each group, Scanco VivaCT 40, Scanco Medical AG, Switzerland).

2.4. Histology and immunofluorescence

Knee sections were cut longitudinally by microtome at a thickness of 3-μm and prepared for Alcian blue/H&E and immunofluorescence staining. After deparaffinization, antigen epitopes were retrieved by boiling in 10 mM citrate buffer (pH 6.0) for 10 min followed by cooling in room temperature for 20 min. Any endogenous peroxidase activity was blocked by incubating the slides in 3% H_2O_2 for 5 min. The sections were then stained with a rabbit polyclonal anti-TIMP2 antibody at a concentration of 1:100 at 4 °C overnight. The antibody was then visualized using the appropriate biotinylated secondary antibody, followed by treatments with Texas Red conjugated streptavidin. The slides were visualized with a Zeiss Universal light and fluorescence microscope for image capture and analysis.

2.5. Metatarsal angiogenesis assay

The middle three metatarsals of E14.5 mice were dissected from each hind limb and cultured in 24-well plates with α-MEM containing 10% fetal calf serum with the indicated treatments for 15 days. At least 10 bones were included in each group. Explants were stained for CD31 and visualized with HRP substrate. Images were acquired with an Olympus multimode dissecting microscope. The expression levels of VEGFs in medium or whole metatarsal bone tissues were evaluated by ELISA or real-time PCR analysis *in vitro*.

2.6. OARSI scoring of mouse cartilage

A semi-quantitative histopathological grading was performed using a derivative of the Chambers scoring system. Methods were further described in OARSI histopathology [20].

2.7. Quantitative gene expression analysis

Total RNA was extracted from the cartilaginous knee tissue of the mice and cDNA was synthesized from 1 μg of RNA using the iScript cDNA Synthesis Kit (Bio-Rad, CA). All PCR conditions and specific primer sequences strictly followed those used in previous publications in which TIMPs, MMPs, and VEGFs were analyzed [21].

2.8. Statistical analysis

All results were presented as the mean ± SE. Statistical analyses were conducted using Prism 5 software and included Student's *t*-tests and one-way or two-way ANOVA followed by Dunnett's test. $p < 0.05$ was considered as significant.

3. Results

3.1. Decreased TIMP2 expression was associated with DMM induced osteoarthritis

To investigate the possible role of endogenous TIMP2 expression in OA progression, immunofluorescence staining was performed for TIMP2 in samples of WT mice. Expression levels of TIMP2 were detected in articular chondrocytes but absent in other tissues around knee joints and were considerably decreased at 1 month post DMM. Additionally, minor disorganized articular chondrocytes and reduced thickness of articular cartilage were also noted (Fig. 1A and B).

3.2. Loss of TIMP2 precipitated DMM induced osteoarthritis

To dissect the association between DMM-induced OA and TIMP2 expression, the causal role of TIMP2 deficiency in the OA phenotype was evaluated. The role of endogenous TIMP2 expression in long term OA progression was investigated by comparing knee joints morphology of WT and *Timp2*−/− mice 4 months after DMM. Histological analysis showed that *Timp2*−/− mice presented a more severe OA-like phenotype with obvious morphological

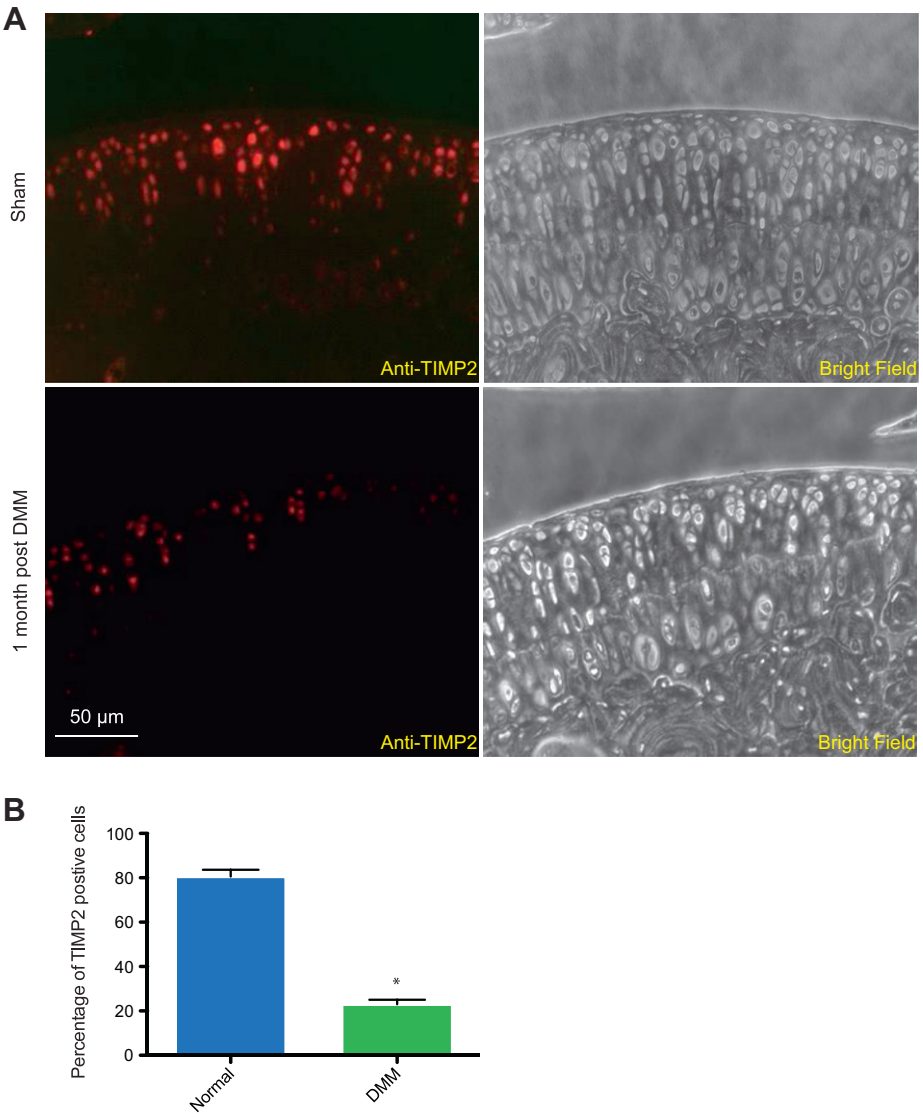

Fig. 1. Decreased TIMP2 expression on the articular surface is associated with surgical induction of osteoarthritis. (A) Immunofluorescence staining of TIMP2 in knee samples of WT mice 1 month post DMM showed that TIMP2 expression was significantly decreased. Minor disorganization of articular chondrocytes and reduced articular cartilage thickness were observed in DMM mice. Scale bar represents 50 μm; (B) Histomorphometric quantification confirmed a significant decrease in TIMP2 positive cells in the articular surface 1 month post DMM. ($n = 5$ each group, $^{*}p < 0.05$).

changes, including degradation of articular cartilage with significant fibrillation of the articular surface, decreased collagen II content, osteophyte formation, and elevated OARSI score compared with wide-type mice (Fig. 2A and B).

3.3. The expression pattern of angiogenesis-related factors

To ensure that loss of TIMP2 did not affect MMPs at the transcriptional level, mRNA levels of MMPs, VEGFs and TIMP1, 2, 3 and 4 were analyzed in both WT and *Timp2−/−* mice 4 months after DMM or sham surgery. DMM led to OA progression and the expression of MMPs and certain pro-angiogenic factors such as VEGF-A and B significantly increased; however, loss of TIMP2 did not exert obvious effects on these expression patterns (Fig. 2C).

3.4. Increased angiogenesis in post-DMM Timp2−/− mice

Given the unchanged levels of other TIMPs and MMPs, the more severe OA phenotype developed in *Timp2−/−* mice must occur through an alternative mechanism. As TIMP2 is a known anti-angiogenic factor, vascular structures in articular areas of WT and *Timp2−/−* mice were evaluated by micro-CT [14,22]. At an early stage of OA (1 month post DMM), WT mice showed few morphological changes; however, some indications of trabecular bone resorption and cystic degeneration of the joint were found in *Timp2−/−* mice (Fig. 3A). Vascular volume and connectivity density were significantly increased in the knee region of *Timp2−/−* mice compared to WT mice (Fig. 3B and C). This suggests that an increase in periarticular vasculature may accompany the morphological transformations seen in OA progression.

3.5. Increased angiogenesis in cartilage of Timp2−/− mice

To determine the role of TIMP2 on angiogenesis in a physiological relevant model, a well characterized fetal mouse metatarsal assay was performed. Metatarsal from E14.5 mice were dissected. At this time point, chondrocytes have already differentiated from mesenchymal cells and are surrounded by extracellular matrix.

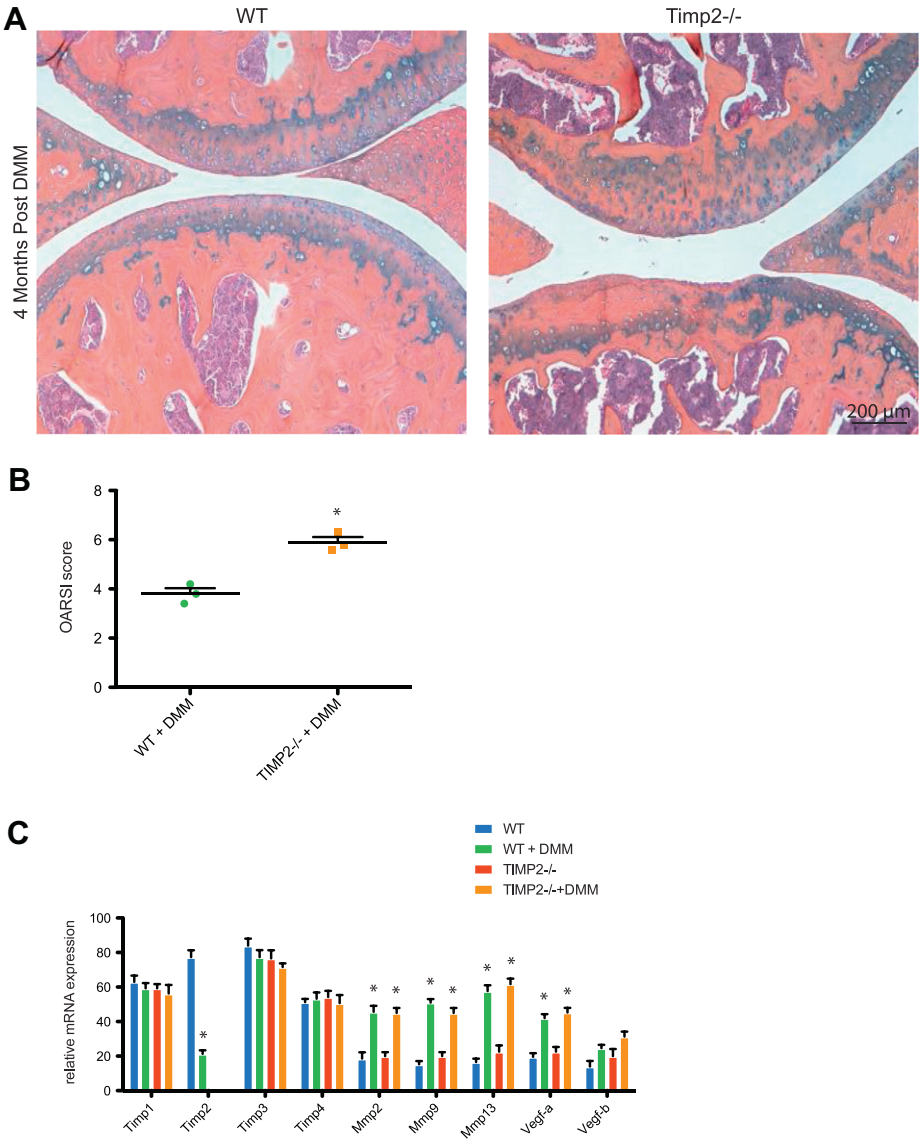

Fig. 2. Loss of TIMP2 precipitated DMM-induced osteoarthritis. (A) Representative histological sections from knee joints of mice 4 months post DMM were stained with Alcian blue. WT mice showed no obvious morphological changes. TIMP2 deficient mice showed severe osteoarthritis-like characteristics, with the articular surface exhibiting an irregular shape and decreased collagen. No significant changes in articular morphology were observed in WT or *Timp2*−/− mice with sham surgery (data not shown). Scale bar represents 200 μm; (B) The severity of osteoarthritis was described by OARSI scores ($n = 5$, $^*p < 0.05$); (C) Real-time PCR investigated Timp1, Timp2, Timp3, Timp4, Mmp2, Mmp9, Mmp13, Vegf-a, Vegf-b levels in articular cartilage from WT and *Timp2*−/− mice 4 months post DMM or sham surgery; Loss of *Timp2*−/− did not change the pattern of expression of the aforementioned mRNAs ($n = 5$, $^*p < 0.05$ versus WT).

Angiogenesis was found to be dramatically elevated in the absence of TIMP2 as indicated by anti-CD31 endothelial labeling and quantitative image analyses of angiogenic tube formation (Fig. 4A and B). No significant changes were observed in the expression levels of VEGFs in medium or whole metatarsal bone tissues of *Timp2*−/− mice compared to samples of WT mice (Fig. 4C). These findings indicate a putative anti-angiogenic function of TIMP2 in cartilage.

4. Discussion

This study identifies a mechanism by which articular chondrocytes may resist vascular invasion: high level expression of TIMP2, a known inhibitor of angiogenesis. It also suggests that angiogenesis may be an essential biological event in the initiation and propagation of OA.

TIMP2 is constitutively expressed in all mouse tissues at all developmental stages [23]. As with other TIMPs, it can bind the entire spectrum of MMPs and ADAMTs with varying affinity. TIMP2 inhibits MMPs by forming a non-covalent complex with the active site at a 1:1 M ratio and thereby preventing substrate cleavage. Previously, qPCR has shown that the expression of TIMPs was not strictly correlated with the expression levels of MMPs. In tissues such as heart, TIMP2 was expressed at high levels whereas MMPs levels were minimal [24]. Using immunofluorescence, we demonstrated that TIMP2 protein was present in normal articular chondrocytes at high levels. This expression of TIMP2, however, did not correlate with active MMP levels [25]. In normal joints, no active MMPs can be detected. This suggests that the primary function of TIMP2 in normal articular chondrocytes is something other than the inhibition of MMP activity.

Decreased TIMP2 expression was observed in articular chondrocytes 1 month post DMM without significant changes in

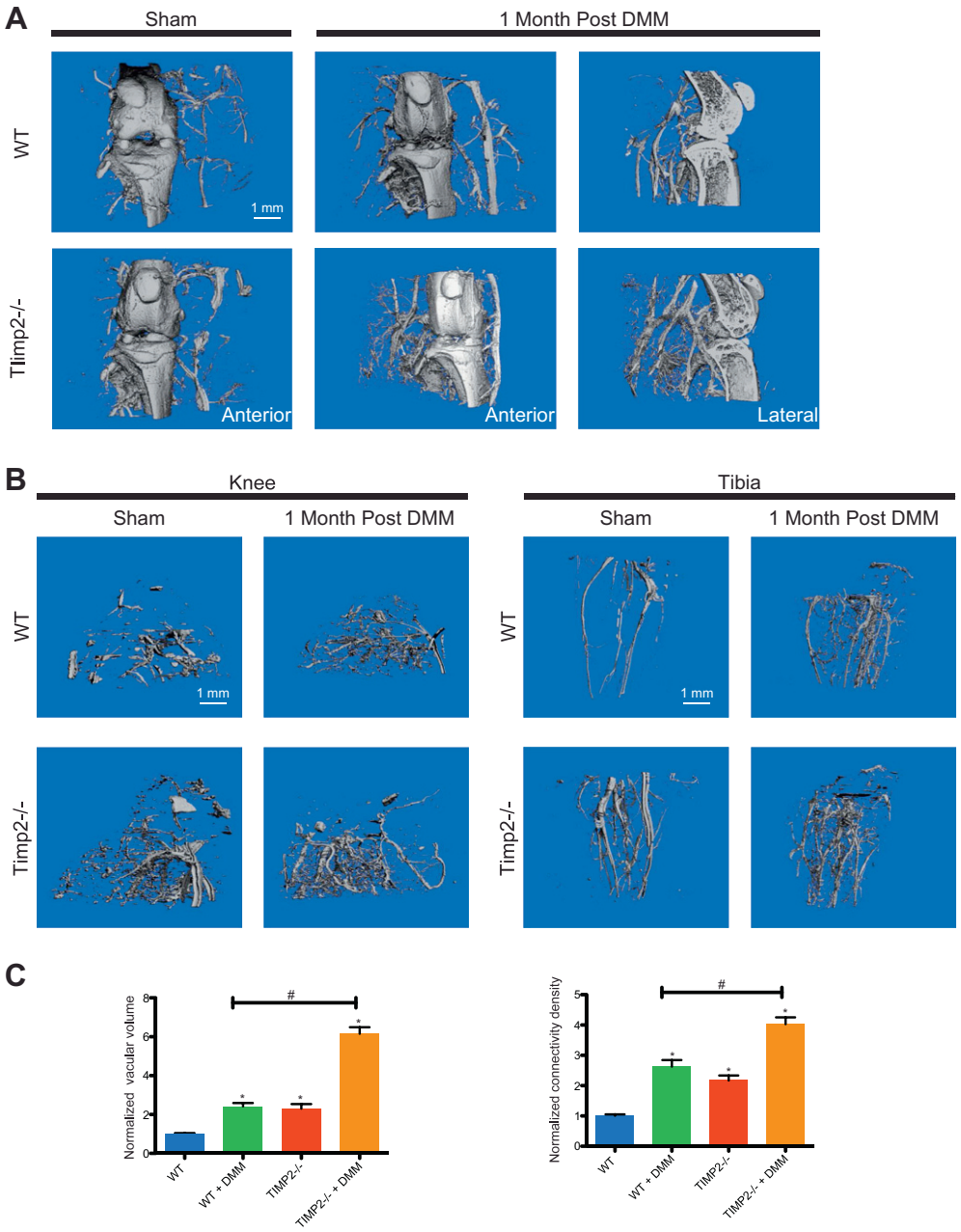

Fig. 3. Post-DMM *Timp2*−/− mice exhibit increased angiogenesis. Mice were subject to vascular perfusion and micro-CT reconstruction 1 month post DMM. (A) Samples from *Timp2*−/− mice displayed increased vessel formation and subchondral degeneration; (B) Micro-CT scans of decalcified samples confirmed the increased vasculature around knee joints in response to DMM. Scale bar represents 1 mm in A and B; (C) Vascular volume and capillary connectivity density were quantified by micro-CT. *Timp2*−/− mice post DMM treatment showed significant differences in these parameters compared to WT mice ($n = 4$, $^{*}p < 0.05$ versus WT; $^{\#}p < 0.05$ *Timp2*−/− + DMM versus WT + DMM).

chondrocyte number or morphology. This suggests that the reduction in TIMP2 levels is a primary event occurring in response to acute injury and aberrant mechanical loading, rather than a secondary event subsequent to chondrocyte pathogenesis. In corroboration with this finding, *Timp2*−/− mice develop a more severe OA phenotype compared to WT at 4 month post DMM. Taken together, these data support the hypothesis that TIMP2 expression in normal articular chondrocytes serves as an anti-osteoarthritic factor.

The anti-OA role of TIMP2 is exerted via its anti-angiogenic function. Though traditionally viewed as MMP inhibitors, restoration of TIMP1 and TIMP2 in articular chondrocyte cultures has failed to ameliorate changes in extracellular matrix [26]. Here we provide evidence that the primary function of TIMP2 is to prevent cartilage vascularization. Micro-CT confirmed that increased vascularization caused by loss of TIMP2 preceded noticeable OA phenotypes. Without significantly changing expression levels of VEGFs in metatarsal bone tissue or its surrounding environment, loss of TIMP2 was sufficient to induce massive capillary tube formation in cartilage *in vitro*. These findings stress the importance of further studies to investigate the mechanism behind TIMP2 inhibition of angiogenesis.

The current therapeutic repertoire for OA is still limited to pain management and local treatments such as intra-articular injection and surgical arthroplasty. Although such interventions have been proven effective in improving mobility and quality of life, they are often associated with high cost and risk in joint revision. By employing the DMM model and TIMP2−/− mice, angiogenesis has been described as a critical event in OA development. Since

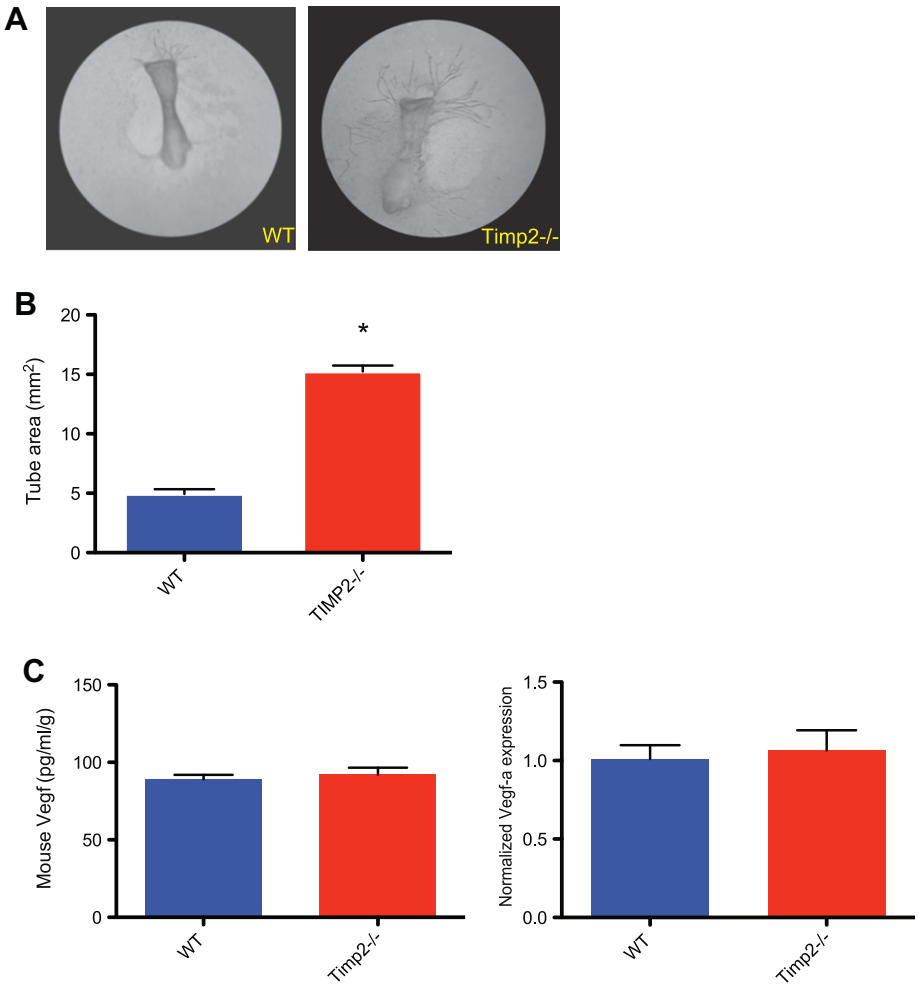

Fig. 4. Loss of TIMP2 enhanced angiogenisis. (A) Cultured metatarsals from *Timp2−/−* mice stained for CD31 demonstrated increased vascular outgrowth compared to WT mice; B) Loss of TIMP2 also resulted in significantly increased tube formation in the metatarsal angiogenesis assay ($n = 5$, $^{*}p < 0.05$); (C) ELISA and real-time PCR demonstrated no significant changes in the expression levels of VEGFs in medium or whole metatarsal bone tissues compared to samples from WT mice.

the discovery of the catabolic roles of MMPs in degradation of cartilage matrix, numerous pharmaceutical and genetic approaches have attempted to inhibit MMP activity as treatment for OA [27]. Our current study has designated angiogenesis as another critical event in OA development. In the development of more comprehensive OA prevention and treatment, the combination of anti-MMP and anti-angiogenic factors offer a promising area of investigation.

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