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FULL LENGTH ARTICLE

Aberrant expression of Twist1 in diseased articular cartilage and a potential role in the modulation of osteoarthritis severity



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Abstract The bHLH transcription factor Twist1 has emerged as a negative regulator of chondrogenesis in skeletal progenitor cells and as an inhibitor of maturation in growth plate chondrocytes. However, its role in articular cartilage remains obscure. Here we examine Twist1 expression during re-differentiation of expanded human articular chondrocytes, the distribution of Twist1 proteins in normal versus OA human articular cartilage, and its role in modulating OA development in mice. High levels of Twist1 transcripts were detected by qPCR analyses of expanded de-differentiated human articular chondrocytes that had acquired mesenchymal-like features. The induction of hallmark cartilage genes by Bmp-2 mediated chondrogenic differentiation was paralleled by the dramatic suppression of Twist1 in vitro. In normal human articular cartilage, Twist1-expressing chondrocytes were most abundant in the superficial zone with little to no expression in the middle and deep zones. However, our analyses revealed a higher proportion of deep zone articular chondrocytes expressing Twist1 in human OA cartilage as compared to normal articular cartilage. Moreover, Twist1 expression was prominent within proliferative cell clusters near fissure sites in more severely affected OA samples. To assess the role of Twist1 in OA pathophysiology, we subjected wild type mice and transgenic mice with gain of Twist1 function in cartilage to surgical destabilization of the medial meniscus. At 12 weeks post-surgery, micro-CT and histological analyses revealed attenuation of the OA phenotype in Twist1 transgenic mice compared to wild type mice.

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Collectively, the data reveal a role for Twist in articular cartilage maintenance and the attenuation of cartilage degeneration.

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List of abbreviations

AC	articular chondrocytes
bHLH	basic helix loop helix
Bmp-2	bone morphogenetic protein-2
DMEM	Dulbecco's modified Eagle's medium
DMM	destabilization of the medial meniscus
IHC	immunohistochemistry
OA	osteoarthritis
TGF β	transforming growth factor-beta
TG	transgenic

Introduction

Osteoarthritis (OA), the most common form of arthritis in middle aged and older individuals, is characterized by the progressive degeneration of articular cartilage, joint space narrowing, subchondral bone sclerosis, and the formation of bony outgrowths at the joint margins.¹ During the development of OA, the articular chondrocytes undergo distinct phenotypic changes, including the activation of hypertrophy and maturation, aberrant induction of cartilage matrix degrading enzymes, and cell death.^{2,3} The signaling pathways and molecular mechanisms that play an essential role in maintaining chondrocyte homeostasis and integrity of the hyaline cartilage matrix are becoming increasingly better understood. Aberrant signaling through developmental pathways controlling chondrocyte differentiation, such as the transforming growth factor-beta (TGF- β) pathway and the wingless MMTV integration (Wnt) pathway, are recognized as key contributing factors to the progressive degeneration of articular cartilage in OA (1–5). Thus, functional characterization of the common transcriptional regulators downstream of these fundamental signaling pathways may offer new insights into the pathophysiology of OA. In this regard, we previously identified the transcription factor Twist1 as a negative regulator of chondrocyte progression toward hypertrophy and terminal maturation in response to TGF- β and canonical Wnt signals.⁴

Twist1 is an evolutionarily conserved basic helix loop helix (bHLH) transcription factor that exerts pleiotropic effects as a mesoderm-determining factor, an epithelial-mesenchymal transitional regulator, and a critical regulator of the gene regulatory network in mesenchymal cell lineage allocation during skeletal development.^{5–13} Expression of Twist1 is abundant throughout the condensed mesenchyme that gives rise to both chondrocytes and osteoblasts of the vertebrate skeleton.⁶ In humans, haploinsufficiency of Twist1 results in an autosomal dominant inherited disorder

Saethre-Chotzen syndrome, characterized by craniosynostosis, short stature, and craniofacial defects.¹⁴ Moreover, several genetic studies examining the role of Twist1 in limb development previously demonstrated the requirement for Twist1 in mediating the outgrowth and patterning of the limb via modulation of the fibroblast growth factor (FGF) and sonic hedgehog (SHH) signaling pathways.^{8,15–18} Krawchuk et al further demonstrated significant forelimb patterning defects, tibial aplasia, and highly disorganized cartilage elements in mice with inactivation of Twist1 in Prx1-expressing mesenchyme.⁸ Collectively, these studies have provided substantial evidence of the requirement for Twist1 activity in defining multiple functions during limb development.

Through a combination of functional and mechanistic studies we, and others, previously examined the specific role of Twist1 in chondrogenesis and chondrocyte maturation.^{4,19,20} Using chondrocyte precursor cells, Reinhold et al demonstrated that Twist1 functions as a potent inhibitor of chondrogenesis¹⁹ and the suppressive effects of Twist1 are mediated, in part, through binding of the carboxyl-terminal Twist box to the DNA-binding domain of the master chondrogenic factor Sox9.¹⁹ Importantly, this direct physical interaction led to inhibition of Sox9-dependent activation of chondrocyte marker gene expression.^{19,21} Association of Twist1 to the 3'UTR of Sox9 has also been found to negatively regulate the chondrogenic initiation program in skeletogenic and mesenchymal murine cell lines.²² We previously determined that Twist1 expression is maintained in the proliferating, immature chondrocytes of the postnatal growth plate, whereas its expression is repressed in hypertrophic chondrocytes both in vitro and in vivo.^{4,23} Bialek et al had previously demonstrated that the direct interaction between Twist box with the DNA-binding domain of the transcriptional factor Runx2 led to inhibition of Runx2 transcriptional activity.⁶ Using in vitro models of chondrocyte maturation, we demonstrated that Twist1 acts as a potent repressor of hypertrophy in growth plate chondrocytes downstream of TGF-beta and canonical Wnt signaling⁴ and speculated that the inhibition of chondrocyte maturation is mediated through its repressive effects on Runx2, the master regulator of chondrocyte hypertrophy.²⁴ Thus, through its interaction with transcriptional regulators, Twist1 has the ability to regulate not only its direct targets, but also other transcription factor-mediated gene expression pathways involved in cartilage development and maturation. When we generated Twist1 transgenic (TG) mice with persistent expression in growth plate chondrocytes (Col2a1 expressing cells and progeny), we observed abnormal growth plate organization and postnatal longitudinal growth retardation attributed to impaired endochondral ossification.²³ While these collective studies

point to the importance of Twist1 for normal formation and growth of cartilage structures, the role of Twist1 in normal maintenance and homeostasis of adult cartilage has remained elusive.

Transcriptional analyses of healthy and OA human articular cartilage from age-matched donors revealed the significant elevation of Twist1 transcript levels in OA compared to normal articular cartilage.²⁵ However, no further analyses were performed to examine the expression and distribution of Twist1 proteins in normal versus OA human cartilage. In view of the inhibitory role of Twist1 in mediating the developmental progression of chondrocytes toward hypertrophy and terminal maturation, we speculated that aberrant regulation of Twist1 may contribute to some of the pathology-induced changes that arise in articular chondrocytes during the onset and progression of OA. In the present study, we examined whether Twist1 transcripts are regulated during the re-differentiation of expanded human articular chondrocytes *in vitro*. By immunolocalization, we further investigated the distribution of Twist1 proteins across the different zones of normal human articular cartilage, and determined whether the distribution of Twist1 proteins is altered in human OA cartilage. To evaluate the *in vivo* function of Twist1 in articular cartilage, we employed a well-established conditional Cre/Lox strategy for cartilage-specific gain of function in mice (23). Using this genetic model, we further explored whether elevated levels of Twist1 in cartilage alters the severity of surgery induced OA in mice.

Material and methods

Procurement of healthy donor and osteoarthritic human osteochondral tissue

We obtained macroscopically normal femoral hemicondyles isolated from three male deceased, de-identified human donors from the Musculoskeletal Transplant Foundation (MTF). Donors (ages 27, 48, and 52 years) had no history of joint disease. Osteochondral surgical discards were obtained from three male patients (64, 70, and 72 years of age) with end-stage OA undergoing knee replacement surgery at our clinic, in accordance with protocol approval by the UConn Health Institutional Review Board (IRB). Three to four cartilage-bone cylinders (6 × 6 mm) per sample were punched out perpendicular to the articular cartilage surface using an osteochondral delivery device (Arthrex).

Histologic and immunohistochemical analyses

Human osteochondral specimens were fixed in 10% formalin and decalcified in EDTA prior to paraffin embedding as we previously reported.^{23,26} Microtome sections (5µm) were deparaffinized in xylene, rehydrated through graded ethanol, and stained with 1% Alcian Blue or Safranin O/Fast Green (SO/FG). Immunohistochemical staining was performed following routine methods for antibodies against Twist1, Sox9, PCNA and VEGF.²³ Image analysis was performed using NIS-Elements BR 3.0 (Nikon, Melville, NY).

Primary human articular chondrocyte culture and re-differentiation

Normal human articular chondrocytes (NHAC-kn, #CC-2250) were obtained from Lonza (Walkersville Inc).²⁷ The cells were cultured in monolayer at 37 °C in a humidified atmosphere with 5% CO₂ in chondrocyte growth media consisting of DMEM/F12 supplemented with 10% FBS, 25µg/mL ascorbic acid and 1% penicillin/streptomycin solution. NHAC-kn cells (2.5 × 10⁵ cells) were sedimented (300 × g, 5 min) in polypropylene tubes to generate high-density pellets. Re-differentiation was induced by culturing the pellets in serum-free media consisting of DMEM-HG media (Gibco) supplemented with 1% ITS⁺ premix (containing human recombinant insulin, human transferrin, selenous acid, BSA and linoleic acid) (BD Biosciences), 40 µg/mL L-proline, 1 mM sodium pyruvate, 1% non-essential amino acids, 2 mM Glutamax, 50 µg/mL ascorbic acid 2-phosphate, 10⁻⁷ M dexamethasone, penicillin/streptomycin and 100 ng/mL human recombinant BMP-2 (Medtronic).^{27,28} Media and growth factor were replenished every other day for up to 21 days of differentiation.

Flow cytometric analysis

NHAC-kn cells (passage 5) were grown to near confluence, harvested by 0.25% trypsin/EDTA, washed with PBS and re-suspended in staining solution consisting of 2% FBS and 2% HEPES in PBS. Cell suspensions (1 × 10⁶ cells) were mixed with PE mouse anti-human CD90 (BD Pharmingen), PE mouse anti-human CD73 (BD Pharmingen), PE mouse anti-human CD29 (BD Pharmingen), PE mouse anti-human HLA-DR (BD Pharmingen), FITC mouse anti-human CD44 (BD Pharmingen), FITC mouse anti-human HLA-ABC (BD Pharmingen), FITC mouse anti-human CD105 (BD Pharmingen), FITC mouse anti-human CD45 (BD Pharmingen), and FITC mouse anti-human CD31 (BD Pharmingen).²⁸ Nonspecific fluorescence was determined by incubation of cell aliquots with isotype-matched monoclonal antibodies (IgG1-PE and IgG2b-FITC). Samples were run on a Becton–Dickinson LSR II Flow Cytometer (BD Biosciences) instrument using FACS Diva software (Becton Dickinson). For each analysis, a minimum of 10,000 cells was assayed. Data was analyzed using FloJo Software (Tree Star, Inc.).

Gene expression analyses

Total RNA was extracted from undifferentiated and differentiating NHAC cell cultures with TRIzol reagent (Invitrogen). RNA concentrations were determined by measuring the absorbance at 260 nm. Samples of 1 µg were treated with DNaseI (Biorad) to eliminate contamination with genomic DNA, then reverse transcribed to single stranded cDNA using iScript Reverse Transcriptase (Qiagen). 20 ng of cDNA was used for quantitative PCR analyses. Quantitative PCR was performed using SYBR Green I Mastermix (Roche) and analyzed using a 7600 real time qPCR machine (Applied Biosystems). Gene expression values are expressed as 2^{DeltaDeltaCt}, with DeltaDeltaCt defined as

the difference in crossing threshold (Ct) values between undifferentiated and differentiated NHAC samples, using *Gapdh* as an internal standard. Oligonucleotide primer sequences were as previously described in.²⁸

Surgical-induced osteoarthritis in mice

Female CAGCAT-Twist1 mice were bred with Col2a1-Cre males to generate offspring heterozygous for each transgene.²³ Genotyping for the Twist1 and Cre transgenes were performed by PCR analyses of genomic DNA isolated from tail clips.²³ Animal procedures were conducted according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Maine Medical Center Research Institute. We performed surgical destabilization of the medial meniscus (DMM) as described by Kamekura et al.²⁹ to model the development of OA in wild type and Twist1 transgenic mice. DMM surgeries were performed on skeletally mature, 10 week old mice, in accordance with our approved animal protocol. A 5 mm incision was made on the medial aspect of the knee joint and the medial collateral ligament was divided to expose the medial compartment of the joint. Attachment of the medial meniscus to the tibia was transected to destabilize the medial meniscus without damaging the underlying articular cartilage. Technically proficient surgery resulted in excessive opening of the medial side of the knee joint when stressed manually. Post-operatively, animals were allowed immediate unrestricted weight bearing. Mice were sacrificed at 12 weeks post-surgery.

Histological scoring of OA severity in mice

We employed the modified Chambers scoring system for histologic scoring of murine OA joints at 12 weeks post-DMM surgery.³⁰ Proteoglycans were stained using Alcian blue. A 0–6 subjective scoring system was applied to all four quadrants of the joint: medial femoral condyle (MFC), medial tibial plateau (MTP), lateral femoral condyle (LFC), and lateral tibial plateau (LTP). A score of 0 represents normal cartilage, 0.5 = loss of PG with an intact surface, 1 = superficial fibrillation without loss of cartilage, 2 = vertical clefts and loss of surface lamina (any % or joint surface area), 3 = vertical clefts/erosion to the calcified layer lesion for 1–25% of the quadrant width, 4 = lesion reaches the calcified cartilage for 25–50% of the quadrant width, 5 = lesion reaches the calcified cartilage for 50–75% of the quadrant width, 6 = lesion reaches the calcified cartilage for >75% of the quadrant width. OA severity for wild type and Col2-Twist1 transgenic mice is expressed as individual scores for each joint quadrant.

Statistical analyses

Experiments were repeated using a minimum of three biological replicates per condition. Data are presented as mean of three separate experiments \pm standard error. For all tests, $p < 0.05$ was considered significant.

Results

Expression of Twist1 is inversely correlated with chondrogenic marker expression in differentiating human articular chondrocytes

We initially assessed the relative levels of Twist1 transcripts in mesenchymal-like cells that arise from de-differentiation of human adult articular chondrocytes, and in cells re-differentiated to the chondrocyte phenotype. The expansion of human articular chondrocytes (hACs) following multiple passages (p5-6) in monolayer culture led to the acquisition of a characteristic fibroblast-like morphology (*data not shown*), consistent with previous reports in the literature.^{31–33} Flow cytometric analyses of cell surface immunologic markers confirmed the mesenchymal progenitor-like properties of the de-differentiated cells. As shown in Fig. 1A, the hACs expressed cell surface antigens indicative of a mesenchymal-like phenotype, including CD29 (99.4%), CD44 (84%), CD73 (99.9%), CD90 (70.6%), CD105 (80%), CD166 (92.2%) and HLA-ABC (81.6%). In contrast, the cells lacked expression of the definitive hematopoietic lineage marker CD45 (7.9%), the endothelial marker CD31 (8.8%), as well as the MHC class II cell surface receptor HLA-DR (2.2%).

We next used the well-established human articular chondrocyte pellet culture system in order to assess whether Twist1 transcript levels are altered during differentiation to a mature chondrocyte phenotype. Following induction of chondrogenic differentiation by high-density culture and treatment with human recombinant Bmp-2 for three weeks, the cells produced a proteoglycan-rich matrix that was visualized by Alcian blue staining of pellets (Fig. 1B). Microscopic analyses further revealed morphological features of a mixed population of immature chondrocytes and hypertrophic chondrocytes within the central core of the pellets (Fig. 1B). By quantitative PCR analyses, the de-differentiated hACs exhibited a low level of expression of hallmarks of the chondrocyte phenotype, including cartilage transcription factors (Sox9, Runx2) and cartilage matrix components (collagens types II, IX and X) (Fig. 1C). By five days of differentiation, the master chondrogenic transcription factor Sox9 was significantly up-regulated in Bmp-2 treated hAC pellets. This induction was followed by increased transcript expression of the chondrocyte hypertrophy marker Runx2 (Fig. 1C), reflecting the progression toward a more differentiated chondrocyte phenotype. Quantitative PCR analyses further revealed a significant induction in the expression of cartilage matrix genes Col2a1 and Col9a1 over the three-week differentiation assay (Fig. 1C). Consistent with the histological features of hypertrophic chondrocytes, the pellet culture conditions promoted expressions of ColXa1 transcript between days 14 and 21 of differentiation (Fig. 1C). In contrast, Twist1 transcript levels were highest in the de-differentiated, mesenchymal-like articular chondrocyte population, and were dramatically down-regulated by five days of chondrogenic differentiation. This repression was sustained at later time points (days 14–21), as the cells acquired a more mature phenotype. Similar results were obtained when hAC pellets were treated with TGF β 1 to

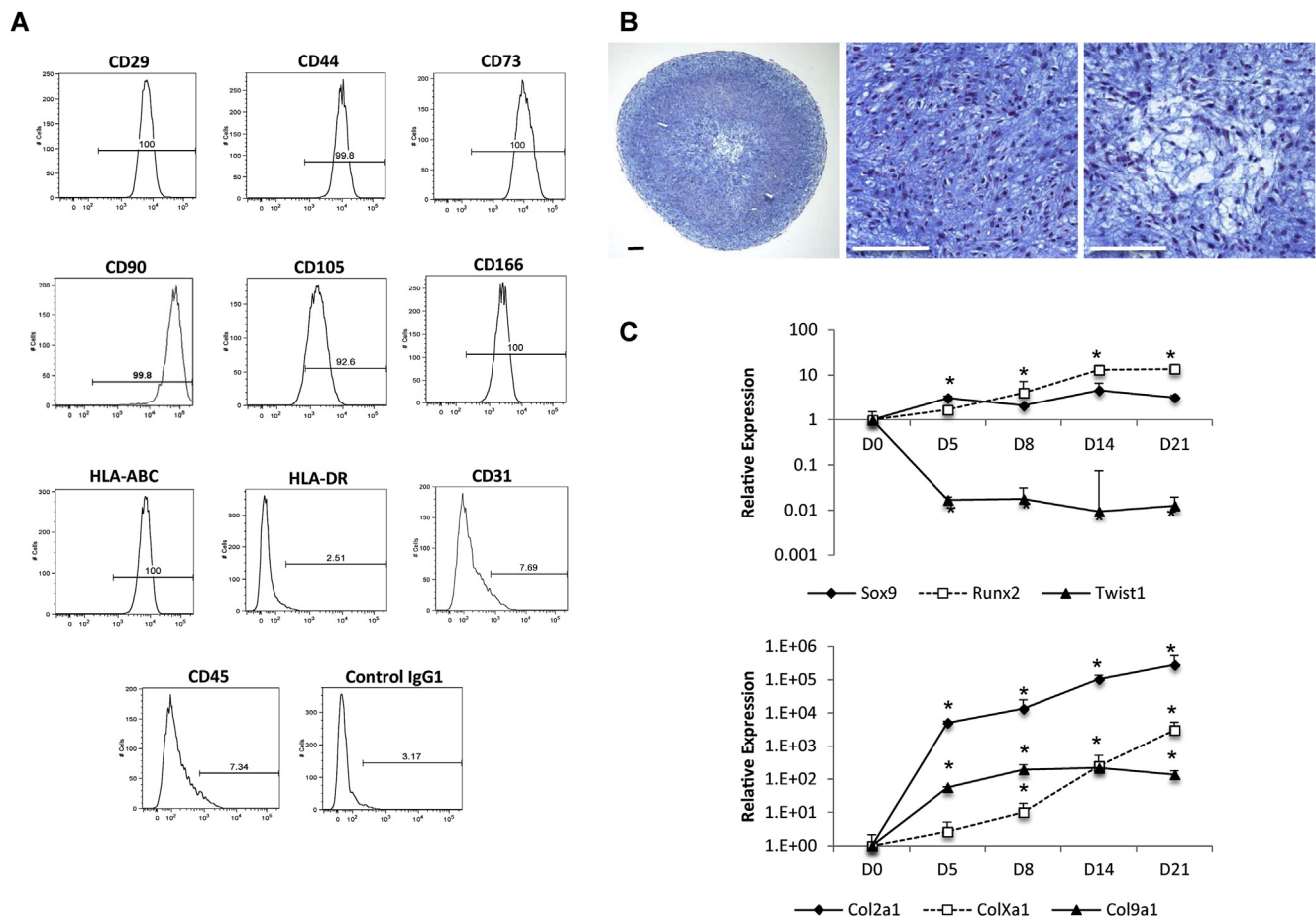


Fig. 1 Expression of Twist1 is repressed in differentiating human articular chondrocytes. (A) De-differentiated normal human articular chondrocytes (monolayer passage 5) exhibited features of mesenchymal-like progenitor cells. Cell surface flow cytometric analyses of the proportion of dedifferentiated human articular chondrocytes expressing CD29, CD44, CD73, CD90, CD105, CD166, HLA-ABC, HLA-DR, CD31, and CD45 receptors. IgG1 antibodies were included as a negative control. (B) Alcian blue staining of human articular chondrocyte pellets cultured in serum-free media and in the presence of Bmp-2 for 21 days. Higher magnification images in (ii) and (iii) revealed a heterogeneous population of immature and hypertrophic chondrocytes, respectively. Scale bar, 100 μ m. (C) RT-PCR-based gene expression analyses of Twist1 and chondrogenic markers Sox9, Runx2, Col2a1, Col9a1 and ColXa1 in normal human articular chondrocyte pellets treated with Bmp-2 for 5, 8, 14, and 21 days in culture. The relative mRNA level in each sample is normalized to its Gapdh content. The expression of Twist1 was suppressed as cells acquired a more differentiated chondrocyte phenotype. * $p < 0.05$. Abbreviations: D0 refers to the undifferentiated population on day 0 prior to onset of differentiation.

promote re-differentiation to the chondrocyte phenotype (*data not shown*). Together, these data indicate that the expression of Twist1 is negatively correlated with the induced expressions of chondrogenic markers during re-differentiation of human articular chondrocytes.

Twist1 exhibits differential distribution in normal versus osteoarthritic human articular cartilage

Our studies previously demonstrated that the expression of Twist1 proteins was restricted to immature, proliferating chondrocytes of the postnatal murine growth plate, whereas little to no expression was observed in chondrocytes undergoing terminal maturation.²³ However, no prior studies have evaluated the spatial distribution of Twist1 in normal versus OA human articular cartilage. Thus,

we performed IHC analyses to assess the distribution of Twist1 proteins across the discrete cartilage zones in intact, full-thickness articular cartilage isolated from the knee joints of three healthy male donors (ages 26, 48, and 52 years). Alcian blue staining of full thickness normal human articular cartilage of the femoral condyle revealed robust proteoglycan staining as well as the typical organization of articular chondrocytes across the superficial, middle, and deep zones (Fig. 2A). Scoring of these human donor condyles revealed no histological evidence of OA pathology (average Mankin score 0.48). IHC analyses using Twist1-specific antibodies showed presence of Twist1 proteins within the flattened articular chondrocytes of the superficial zone as well as some of the rounded chondrocytes within the middle zone of normal human articular cartilage (Fig. 2B). Semi-quantitative assessments shown in Fig. 2C showed enrichment of Twist1-expressing articular

chondrocytes at the superficial zone (SZ; upper 10% of full-thickness cartilage). The vast majority of the flattened, articular chondrocytes within the SZ exhibited Twist1

immunostaining ($85\% \pm 5.13\%$). By contrast, significantly fewer Twist1 immune-positive chondrocytes were observed within the rounded cells present in the middle zone (MZ) of

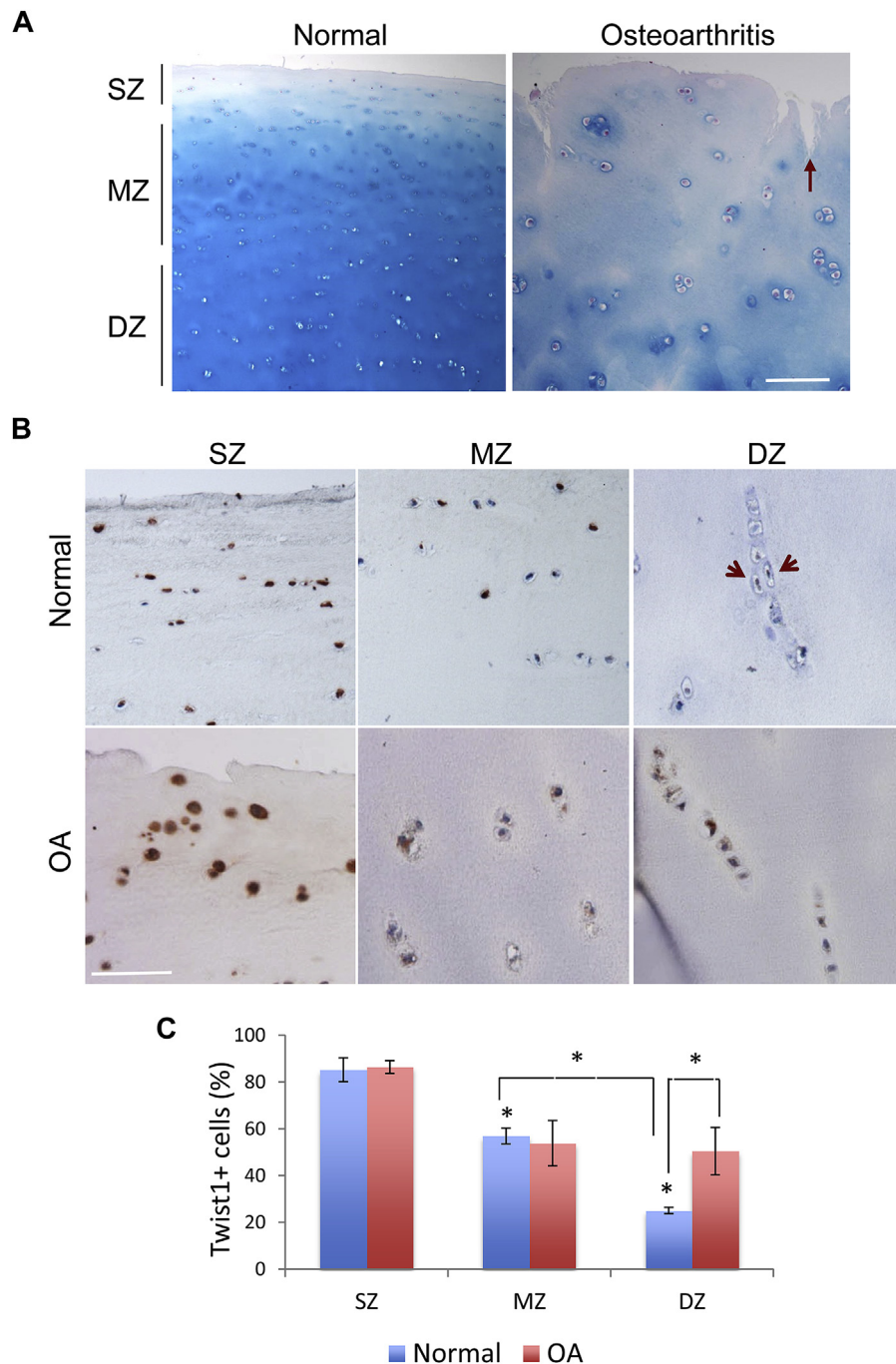


Fig. 2 The zonal distribution of Twist1 proteins is altered in human OA articular cartilage. **(A)** Alcian blue staining of normal and OA human articular cartilage. Discrete zones in normal articular cartilage are shown. SZ, superficial zone; MZ, middle zone; DZ, deep zone. OA cartilage exhibited diminished Alcian blue staining and histological evidence of cartilage degeneration (arrow). Scale bar = 50 μ m **(B)** Representative images of Twist1 protein distribution across the superficial, middle and deep zone articular chondrocytes in normal (i-iii) and OA (iv-vi) human articular cartilage. Immunostaining using Twist1-specific antibodies revealed localization within the nucleus of superficial zone articular chondrocytes (i, iv) in both normal and OA cartilage. Minimal Twist1 staining was detected within the deep zone chondrocytes in normal human articular chondrocytes (iii). Scale bar = 50 μ m **(C)** Assessment of the percentage of Twist1 positive cells detected via Twist1-specific nuclear staining in the superficial, middle and deep zones of normal and OA human articular cartilage. A significant increase in the number of deep zone chondrocytes exhibiting Twist1 nuclear staining was detected in OA cartilage.

the articular cartilage layer ($56.9\% \pm 3.33$) (Fig. 2C). As shown in Fig. 2C, the frequency of Twist1 positive cells was significantly lower in deep zone (DZ) articular chondrocytes, with only $25.1\% \pm 1.36\%$ of deep zone cells displaying nuclear Twist1 expression.

Next we performed immunohistochemical analyses to determine whether degeneration of human articular cartilage was associated with changes in the normal distribution of Twist1 proteins. For these experiments, we assessed Twist1 distribution across the cartilage layers in mildly eroded OA human specimens (lateral femoral condyles) in order to score a sufficient number of cells from the presumptive superficial zone. Articular cartilage tissue from OA specimens (male patients, ages 53, 64, and 72 years) displayed loss of proteoglycan staining, indicative of cartilage erosion (Fig. 2A). These patient-derived OA samples attained significantly higher histological grades of OA (Mankin score 2.3) as compared to the healthy donor control specimens (Mankin score 0.48). In OA cartilage, some articular chondrocytes exhibited a strong Twist1 immune signal, and variable distribution across the discrete zones. The frequency of Twist1 staining in OA chondrocytes was highest in the SZ ($86.4\% \pm 2.68\%$), and the percentage of SZ cells expressing Twist1 was similar to normal cartilage (Fig. 2C). The frequency of Twist1 nuclear staining in middle zone chondrocytes was also similar between normal and mild OA-affected articular cartilage (Fig. 2C). In contrast, there was a significant increase in the percentage of deep zone chondrocytes exhibiting Twist1 nuclear staining ($50.4\% \pm 10.1\%$) in OA articular compared to normal articular cartilage ($25.0\% \pm 1.36\%$) (Fig. 2C).

Activation of Twist1 expression in OA cartilage cell clusters

The distribution of Twist1 proteins was further examined in human patient cartilage surgical discards displaying a more severe OA phenotype. As shown in Fig. 3A, there was extensive loss of Alcian blue staining, as well as the extensive appearance of fissures within the articular cartilage of OA affected medial femoral condyles (Mankin score >5). The pathology-induced cartilage changes was further reflected by the presence of cell clusters, typically near fissures or clefts³⁴ in the severely affected human OA samples (Fig. 3). By immunohistochemical staining, we detected expression of Sox9 within the nuclei of the cartilage cell clusters as well as pathology-induced staining for vascular endothelial growth factor (VEGF) (Fig. 3). Furthermore, the cartilage cell clusters in OA-affected articular cartilage showed positive staining for the proliferating cell nuclear antigen (PCNA), suggestive of cartilage cell turnover and proliferation (Fig. 3). Nuclear Twist1 expression within the OA cluster cells (Fig. 2) was indicative of its abnormal activation during OA pathology.

Immunohistochemical analyses of Twist1 expression in the murine adult joint

Our previous studies revealed the localization of Twist1 within the proliferating chondrocytes in the postnatal

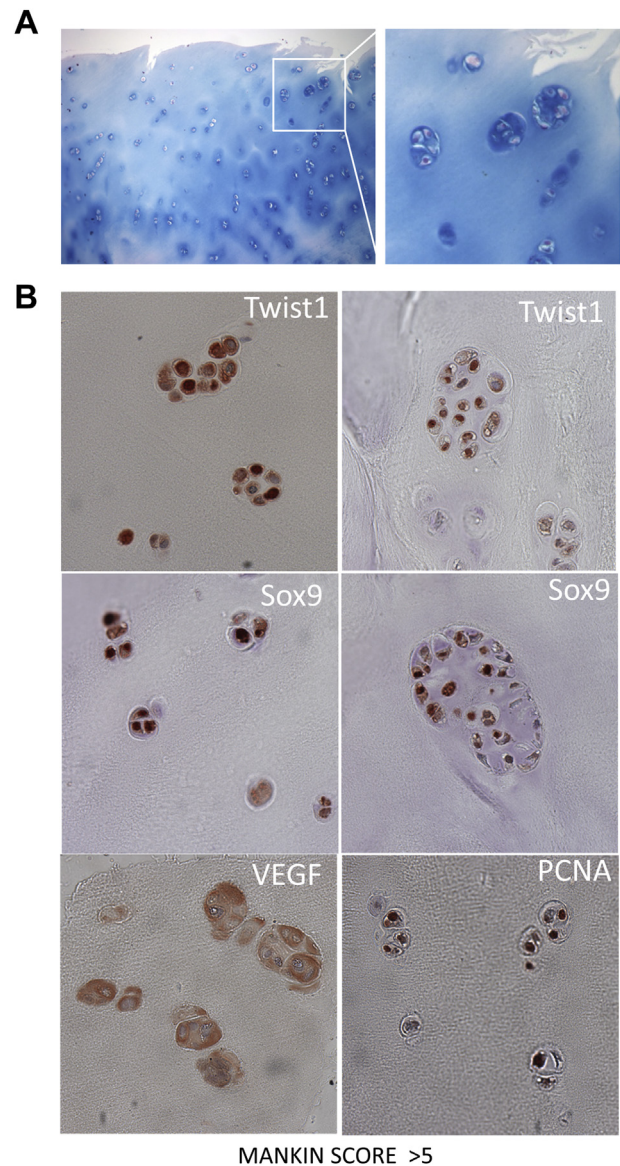


Fig. 3 Activation of Twist1 expression in cartilage cell clusters in OA cartilage. **(A)** Alcian blue staining shows presence hallmark cartilage cell cluster formation in severely affected human OA cartilage (Mankin score >5). **(B)** Representative images of Twist1-specific immunostaining in the OA cartilage cell clusters. Cells within the clusters were also positive for Sox9, VEGF, and PCNA. Scale bar = 50 μ m **(C)**

growth plate, and exclusion of Twist1 in the mature hypertrophic growth plate chondrocytes.²³ Immunohistochemical analyses of murine joints showed nuclear localization of Twist1 at the articular surface in wild type adult mice (Fig. 4). Expression of Twist1 proteins was observed in the superficial zone articular chondrocytes (Fig. 4e), whereas limited localization of Twist1 in chondrocytes was present in the large hypertrophic cells near the tidemark (Fig. 4f). The expression of Twist1 was not restricted to cells at the articular surface, as nuclear signal was also detected within cells in the typically fibrocartilage-like cells of the menisci (Fig. 4g).

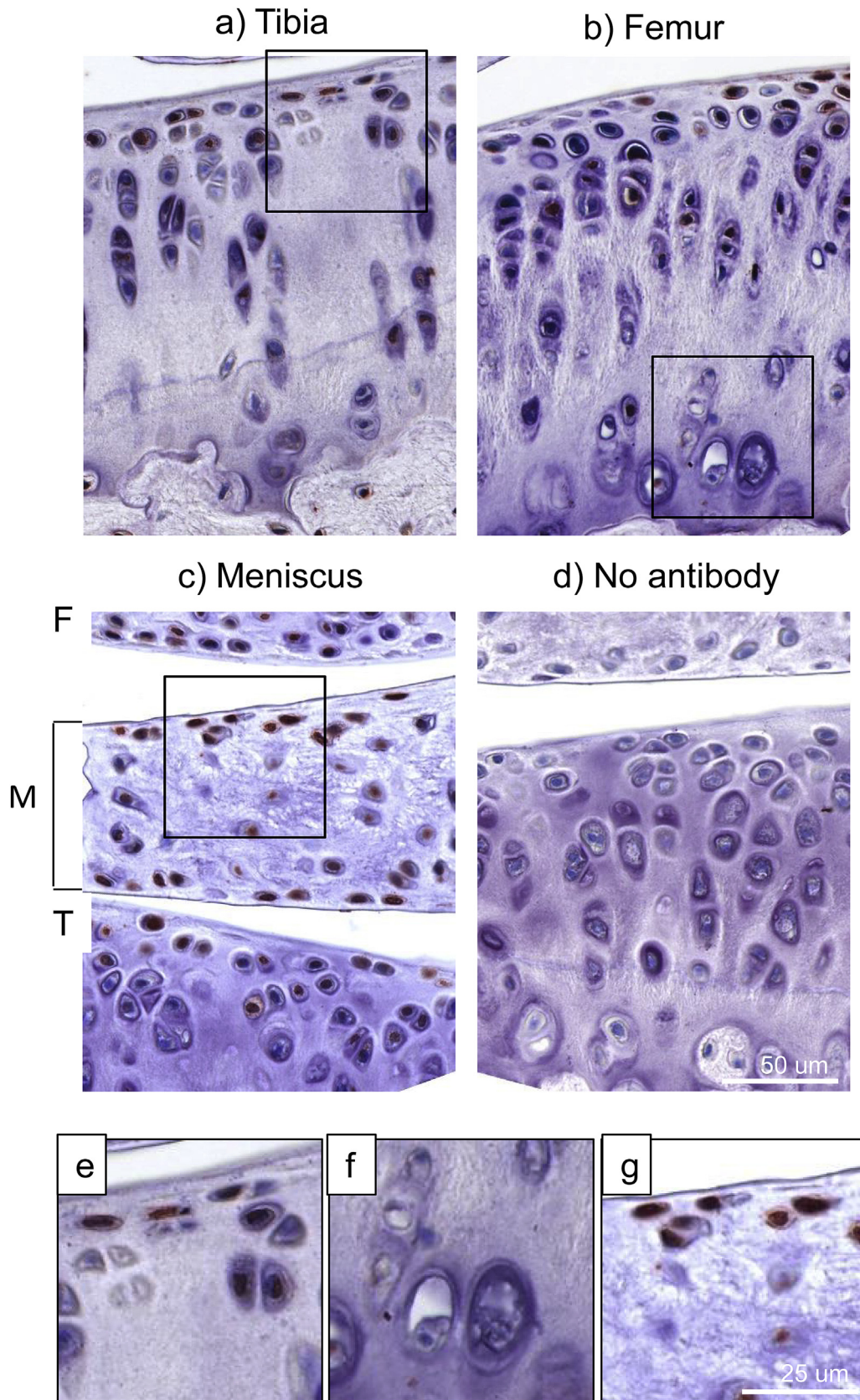


Fig. 4 Expression of Twist1 in murine articular cartilage. Immunohistochemical staining using Twist1-specific antibodies showed nuclear Twist1 expression within murine articular chondrocytes at the (a) medial tibial plateau and (b) the medial femoral condyle of adult mice. The expression of Twist1 appeared most prominent within the superficial zone chondrocytes (panel shown in e), and to a much lesser extent in the deep zone chondrocytes (panel shown in f). Twist1 positive cells were also detected within the meniscus (c, g). There was a lack of an immune signal in sections without primary antibody incubation (d). T, tibia, F, femur. M, meniscus.

Twist1 overexpression in cartilage attenuates cartilage erosion in a surgery-induced model of OA

Based on the association between Twist1 expression and the mesenchymal-like features of superficial zone articular chondrocytes, we speculated that elevated levels of Twist1 in cartilage may impact the severity of cartilage degeneration in mice following surgery-induced OA. For these studies, surgical destabilization of the medial meniscus (DMM)²⁹ was performed using skeletally mature wild type mice and Twist1 transgenic mice with forced expression of Twist1 in type II collagen expressing cells and their progeny.²³ The DMM knee joints from wild type and Twist1 transgenic mice were imaged by μ CT for the presence of osteophytes. μ CT imaging revealed enlarged and extensively ossified medial menisci as well as evidence of osteophyte formation within the surgical joints of wild type mice (Fig. 5A). Conversely, μ CT imaging of the DMM joints of Twist1 transgenic mice revealed limited evidence of osteophyte formation at 12 weeks post-surgery.

We further evaluated the changes that arise following DMM surgery in both phenotypes by histological evaluation. Knee joints from the nonsurgical, control limbs from both genotypes showed a smooth, intact articular surface (Fig. 5B). In contrast, Alcian blue staining of the medial compartment of the surgical DMM joints in wild type mice revealed extensive cartilage erosion marked by the presence of fissures within the medial compartment following 12 weeks of DMM-induced OA (Fig. 5B). In contrast, transgenic mice with sustained Twist1 expression in type II collagen-expressing cells and their progeny exhibited a remarkably well-preserved articular cartilage surface in the surgical DMM joints (Fig. 5). Histologic assessment of the severity of the OA phenotype within the four quadrants of joint (medial tibial plateau, lateral tibial plateau, medial femoral condyle, lateral femoral condyle) using a modified Chamber's scoring system revealed that the most significant damage occurred within the medial tibial plateau of the surgical limb in wild type mice. Notably, this semi-quantitative histological evaluation revealed a less severe phenotype in the medial tibial plateau of surgical joints from Twist1 transgenic mice compared to wild type mice at 12 weeks post-surgery. Collectively, these observations suggest that chondrocyte-specific over-expression of Twist1 may confer a protective effect against the development of articular cartilage degeneration in a translational OA murine model.

Discussion

In this study, we examined a previously unexplored role of Twist1 in human and murine articular cartilage. Our novel findings revealed that Twist1 exhibits a distinct zonal distribution in normal human articular cartilage, and that changes in its distribution arise during human OA pathophysiology. Enrichment of Twist1 proteins in articular chondrocytes at the superficial zone in normal articular cartilage was suggestive of a functional role in the maintenance of immature, progenitor-like cells. This was further supported by our *in vitro* studies demonstrating the high levels of Twist1 transcripts in the mesenchymal-like

chondrocytes that had undergone de-differentiation and loss of chondrocyte gene expression. We further determined the aberrant activation of Twist1 in cartilage cell clusters and within articular chondrocytes in deep zone cartilage is associated with human OA. Moreover, our *in vivo* studies suggested that the gain of Twist1 function in murine cartilage could attenuate the severity of cartilage degeneration at 12 weeks following surgical induction of OA. Together, these findings highlight a potentially novel role for this bHLH transcription factor in the preservation of articular cartilage *in vivo*.

Human articular cartilage is a highly organized tissue composed of three distinct layers above the tidemark ~ the superficial, middle, and deep zones. Each of these zones displays unique histologic features as well as important differences in proteoglycan content, gene expression profiles, and chondrocyte metabolism.^{35–37} Genome-wide expression profiling of the superficial, middle and deep zone of normal human articular cartilage by Grogan and colleagues showed that the greatest differences in gene expression occur between the chondrocytes in the superficial zone and the deep zone.³⁷ The superficial zone of adult human cartilage contains the highest percentage of cells that exhibit phenotypic and functional properties of mesenchymal-like progenitor cells.^{35,36,38–41} We speculate that the enrichment of Twist proteins within the superficial zone chondrocytes shown in our immunohistochemical analyses may be reflective of the involvement of Twist1 function in maintaining the immature differentiation status of chondroprogenitor cells in adult articular cartilage. In postnatal tissues, Twist1 is highly expressed in stem cell populations located in mesoderm-derived mesenchymal tissues, such as muscle, adipose tissue, and bone marrow, and functions as a regulator of mesenchymal stem cell differentiation.¹⁸ Our collective FACS and gene expression analyses demonstrated that immature, progenitor-like population arising from the de-differentiation of human articular chondrocyte *in vitro* exhibited high levels of Twist1 transcript expression. Twist1 levels were significantly down-regulated as cells turned on markers of chondrogenic differentiation (Sox9, Col2a1, Runx2, ColXa1) in response to Bmp-2 induced differentiation in high density cultures. We observed similar down-regulation of Twist1 transcripts at the early stages of chondrogenic induction in dedifferentiated human articular chondrocyte pellet cultures treated with TGF β 1 (data not shown). Our findings are consistent with prior studies showing the high level of Twist1 expression in murine limb bud mesenchymal progenitor cells at the onset of chondrogenesis.⁴² A number of studies including ours have indeed focused on the role of Twist1 as a chondrogenic repressor.^{4,19,22,42} Thus, the down-regulation of Twist1 transcripts during chondrogenic differentiation of human articular chondrocytes are reflective of the known repressive effects of Twist1 activity during chondrogenic differentiation of progenitor cells, chondrocytic cell lines, as well as growth plate chondrocytes.^{4,19,22,42,43} Several molecular mechanisms have been proposed for the repressive effects of Twist1 on chondrogenic differentiation as a downstream target of the canonical Wnt, transforming growth factor (TGF) and Notch signaling pathways,^{4,19,42} as well as a negative regulator of Sox9 transactivator function

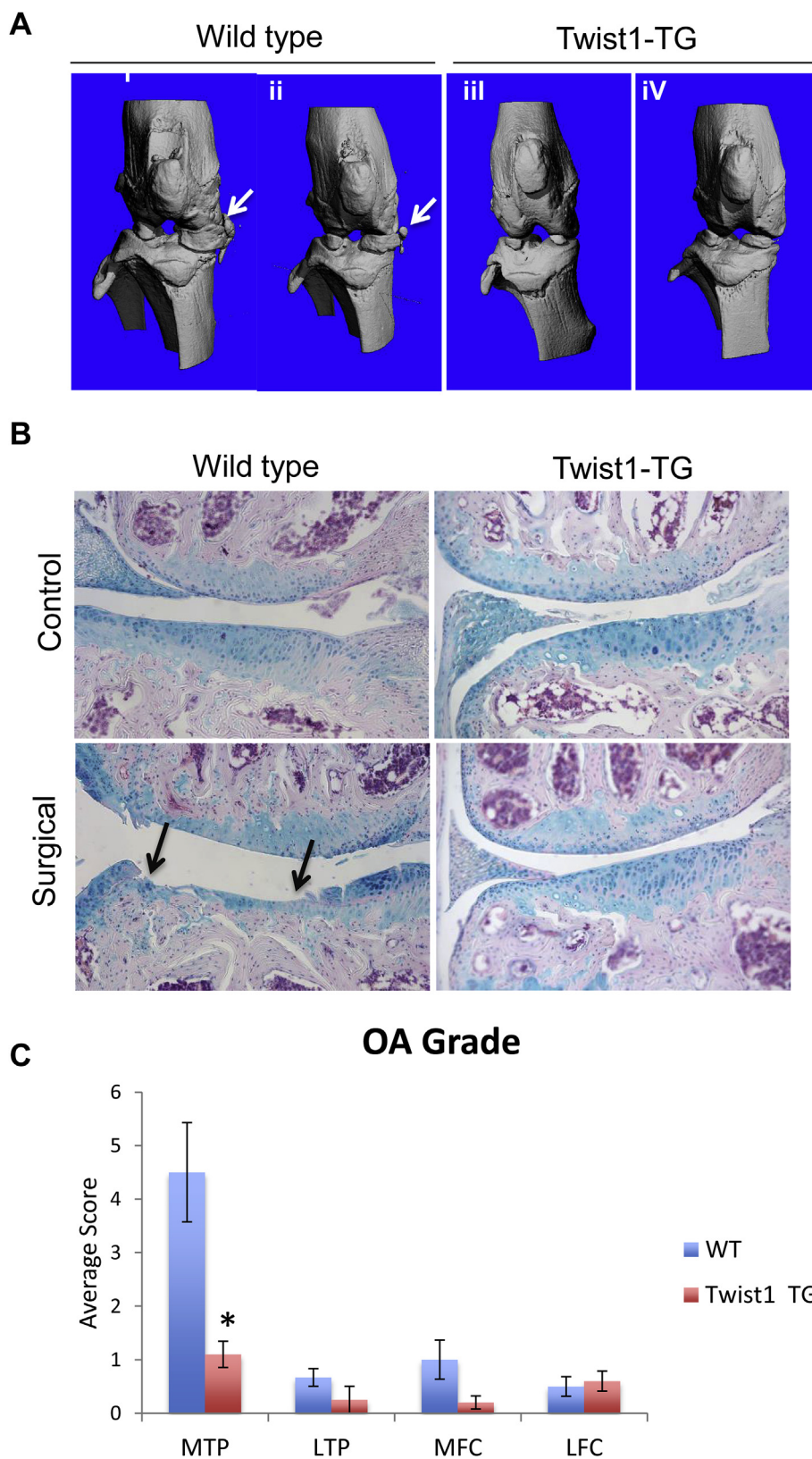


Fig. 5 Over-expression of Twist1 in articular cartilage confers a protective effect against DMM-induced cartilage degeneration in mice. (A) μ CT analyses of the surgical joints from representative wild type and Twist1 transgenic mice at 12 week post DMM surgery. (B) Alcian blue staining of sections of knee joints from wild type and Twist1 transgenic (TG) mice at 12 weeks post DMM surgery. Medial aspect of surgical limbs are shown for two animals of each respective genotype. Arrows demarcate cartilage erosion in wild type joints at 12 weeks post DMM surgery. Articular surface was well preserved in Twist1 TG mice at 12 weeks post DMM surgery. Scale bar, 100 μ m (C) Histological grading of OA severity in the medial tibial plateau (MTP), lateral tibial plateau (LTP), medial femoral condyle (MFC), lateral femoral condyle (LFC) from wild type and Twist1 TG mice at 12 weeks post DMM surgery. * $p < 0.05$.

and expression.^{21,22} Functional studies using bone marrow-derived mesenchymal stem cells (MSC) further showed that populations with high levels of Twist1 expression had a diminished capacity for osteogenic and chondrogenic differentiation in vitro, suggestive of a role as a mediator of MSC self-renewal.⁴³ Twist1 was shown to inhibit BMP2 signaling and osteogenesis in MC3T3-E1 via the recruitment of HDAC1 to Smad4, a common component of the BMP and TGF β signaling pathways involved in the regulation of bone and cartilage development.⁴⁴ However, the upstream mechanisms underlying the suppression of Twist1 expression and activity during the early stages of chondrogenesis have not yet been resolved.

Among the hallmark feature of OA include the depletion of chondrocytes within the superficial zone due to progressive erosion, and the abnormal activation of differentiation within the deep chondrocytes.⁴⁵ By semi-quantitative immunohistochemistry, we determined that changes in the pattern of Twist1 distribution across the discrete zones in human articular cartilage occur in OA. A significant increase in the number of deep zone articular chondrocytes exhibiting nuclear Twist1 signal was detected in OA articular cartilage compared to normal cartilage, indicative of aberrant activation of Twist1 in deep zone chondrocytes. Moreover, robust Twist1 expression was found in the nuclei of cartilage cell clusters arising in response to cell injury in the more severely affected cartilage specimens. Recent studies have indicated that such cartilage cell clusters arise adjacent to severe cartilage degeneration and exhibit progenitor markers such as Notch-1, Stro-1, VCAM-1, as well as proliferative potential.^{35,46} It remains unclear whether the aberrant activation of Twist1 in OA cartilage is a component of the remodeling process that occurs during degeneration or possibly, a failed attempt to repair the progressive damage to the articular cartilage during the pathophysiology of the disease. It is possible that the changes in the zonal distribution of Twist1 proteins could account, at least in part, for elevated Twist1 transcript levels detected in human OA articular cartilage.²⁵

Our in vivo studies suggest that the sustained expression of Twist1 in type II collagen-expressing cells reducing the severity of articular cartilage damage and proteoglycan loss during OA pathophysiology compared with control mice at 12 weeks post-DMM surgery. In human OA cartilage, Twist1 is aberrantly expressed in the deep zone cells, which may be detrimental to these cells, possibly contributing to further cartilage degeneration. The Twist transgenic mice turn on ectopic Twist expression in proliferating chondrocytes, thus its expression may aid in chondroprotection by expanding the progenitor population. Future studies will be geared towards evaluating the functional consequences of Twist1 deletion in postnatal cartilage. Specifically, one needs to address whether lack of Twist1 impairs articular cartilage homeostasis and/or promotes an accelerated OA phenotype in mice.

Authorship statement

R.M. Guzzo: concept and design, experimental studies, data analysis, manuscript preparation.

F. Alaei: experimental studies.

D. Paglia: experimental studies, data analysis.

J.D. Gibson: experimental studies.

D. Spicer: concept and design, experimental studies, manuscript editing.

H. Drissi: concept and design, experimental studies, manuscript editing.

Conflicts of interest

All authors have none to declare.

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