Accelerated development of instability-induced osteoarthritis in transgenic mice overexpressing SOST

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Abstract: Objective: Sclerostin (SOST), acting as a Wnt antagonist, has been shown to play a key role in regulating bone homestasis, and has also been linked to osteoarthritis (OA) development. Here, we investigated whether overexpressing SOST could affect OA development after destabilization of the medial meniscus (DMM) using SOST transgenic (Tg) mice. Methods: Bone and cartilage phenotypes of SOST Tg mice at 10 weeks of age were investigated by dual x-ray absorptiometry (DXA) and histology. Subsequently, 10-week-old SOST Tg mice and their wild-type (WT) littersmates were subjected to DMM or sham surgery. Knee joints were isolated to evaluate the cartilage damage and the subchondral bone plate thickness at 2 and 8 weeks post-surgery. The changes of chondrocyte anabolic and catabolic responses after IL-1β or TNFα stimulation, β-catenin signaling and apoptosis were also measured. Results: Ten-week-old SOST Tg mice were identical to their WT littersmate males except that they displayed digit abnormalities and osteopenic, whereas more severe OA was observed in SOST Tg mice at 2 and 8 weeks post-DMM. In addition, DMM resulted in significantly greater subchondral bone changes compared with sham surgery in SOST Tg mice at 8 weeks post-surgery. The accelerated OA in SOST Tg mice may be associated with reduced β-catenin signaling and increased chondrocyte apoptosis. Conclusion: Overexpressing SOST led to accelerated development of instability-induced OA. Our data further highlight that cartilage homeostasis requires finely tuned Wnt signaling.

Keywords: Sclerostin, osteoarthritis, cartilage, subchondral bone

Introduction

Osteoarthritis (OA) is a degenerative joint disease, characterized by cartilage degradation, osteophyte formation and subchondral bone sclerosis. The manifestations of the disease are pain, decreased mobility and disability [1]. Heart or metabolic diseases in these patients may be exacerbated due to lack of mobility. Unfortunately, there are no effective disease-modifying therapies for OA and great efforts are made on discovering molecular pathway and targets for drug development.

The Wnt/β-catenin signaling pathway is a key regulator in bone and cartilage homeostasis and has been linked to the cartilage degradation in OA [2]. Sclerostin (SOST) is a potent soluble antagonist of Wnt signaling and a regulator of bone homeostasis [3, 4]. It has been demonstrated that patients afflicted by van Buchem disease (MIM 239100) [5, 6] or sclerosteosis (MIM 269500) [7, 8] display a high bone mass phenotype owing to SOST loss-of-function mutations. Moreover, Sost knockout (KO) mice have a high bone mineral density [9], whereas transgenic mice overexpressing human SOST are osteopenic [10, 11].

While SOST has been widely viewed as a secreted peptide produced by osteocytes, recent studies have shown that chondrocytes are capable of producing SOST [10, 12, 13]. The number of SOST-positive chondrocytes increases in surgery-induced OA in mice [13, 14] and sheep [14], as well as late stage human OA [13-16]. What is more, interleukin-1α (IL-1α) upregulates the SOST mRNA [14].

Recently, studies have documented that increased SOST could protect against cartilage damage. The initial
SOST effects in OA

Study by Chan et al. demonstrated that SOST dose-dependently inhibited IL-1α-stimulated aggrecanolysis in vitro [14]. A separate study confirmed the in vitro results and showed that Sost KO mice have accelerated surgery-induced cartilage damage [17].

The number of SOST-positive osteocytes decreased in femoral neck of hip OA, which was associated with increased cortical bone density [18]. Another study further demonstrated that SOST expression was the lowest under full thickness cartilage defects with the thickest subchondral bone in hip OA [19]. In addition, SOST in subchondral bone osteocytes was the lowest with the thickest subchondral bone plate in late stage knee OA [20]. SOST expression was also low in sclerotic subchondral bone in a mice model of knee OA [14]. These studies suggest the inhibition of osteogenesis in the subchondral bone by systematically increasing the SOST level may serve as a therapeutic strategy for OA.

As upregulating the SOST level in OA cartilage and subchondral bone may play a protective role [14, 17-20], we performed the present study to assess whether overexpressing SOST could decelerate the progression of OA using the SOST transgenic (SOST Tg) mice. SOST Tg mice were bred and subjected to the destabilization of medial meniscus model (DMM) of OA, a model offering an excellent translational perspective on OA [21].

Materials and methods

Ethics board approval

All animal procedures and protocols in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the Model Animal Research Center of Nanjing University (Animal Care and Use Protocol Permit Number: JQ01).

Animals

SOST Tg mice were kindly provided by Professor Gabriela G Loots, who generated these mice [11, 22]. Generally, the limb phenotype is more severe in homozygous mice, whose ulna or fibula may be absent [23]. Missing fibula may affect joints due to loading changes. To obtain a consistent phenotype, hemizygous male mice were bred with WT females to get hemizygous and WT littermate mice for experiments. SOST transgene was genotyped by PCR. SOST transgene primer sequences: upper primer, 5'-ATGTCCACCTTGCTGGACTC-3' and lower primer, 5'-GTCTGTGGGCTGGTTTGCAT-3'. All mice were caged in groups (n = 3-6 mice per cage), allowed free access to water and a standard rodent chow and maintained under 12-hour light/dark cycle.
SOST effects in OA

Figure 2. Accelerated OA in SOST Tg mice following destabilization of the medial meniscus (DMM). A. Representative photographs of articular cartilage destruction in SOST Tg mice and their WT littermates at 2 and 8 weeks post-DMM. Sections were stained with Safranin O/Fast Green. Scale bars = 100 μm. B. The OARSI scores for the medial femoral condyle and the medial tibial condyle at 2 and 8 weeks post-DMM in SOST Tg mice and their WT littermates. The OARSI score in the medial tibia were significantly higher in DMM knees of SOST Tg mice compared with their WT littermate mice at 2 (P = 0.0176) and 8 (P < 0.0001) weeks post-surgery. For the femur, the OARSI score was only significantly higher in knees of SOST Tg mice compared to WT littermates at 8 weeks post-DMM (P < 0.0001). *P < 0.05. ****P < 0.0001. NS = not significant. Data are shown as means + 95% CI.

X-ray analysis and BMD measurement

X-ray images were obtained using Philips Buckeye Diagnosis CS DR System (Philips Medical System, Best, Netherlands) as previously reported [24]. BMD of the whole body and left femur were monitored in mice at 10 weeks of age, using DXA (PIXImus II; GE-Lunar, Madison, WI, USA) as previously described [24]. The region of interest (ROI) for whole body excluded the head while the ROI for the left femur included the whole bone.

DMM model

DMM was performed to introduce joint instability as previously described [21, 25]. Briefly, after anesthesia with 100 mg/kg ket-
amine and 5 mg/kg xylazine, the medial meniscotibial ligament, which anchored the medial meniscus to the tibial plateau of the right knee, was transected. Separate mice were sham operated with medial capsulotomy only. Only male mice were used in the present study because of sex-related differences in this murine DMM-induced OA model [26].

A total of 100 mice male mice at 10 weeks old were operated and these mice were sacrificed at 2 or 8 weeks post-surgery. The right knee joints were harvested for subsequent histologic analyses. Only one mouse in the WT sham group died during the study. The number of each group was as follows: 2 weeks post-surgery (1) WT sham (n = 10), (2) WT DMM (n = 10), (3) SOST Tg sham (n = 13), (4) SOST Tg DMM (n = 13); 8 weeks post-surgery (1) WT sham (n = 10), (2) WT DMM (n = 17), (3) SOST Tg sham (n = 10), (4) SOST Tg DMM (n = 16).

**Histology**

Mouse knee joints were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline.

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**Figure 3.** Histomorphometric analyses of subchondral bone in SOST Tg mice and their WT littermates. A. Representative photographs of subchondral bone in SOST Tg mice and their WT littermates at 2 and 8 weeks post-DMM. Black line delineates the subchondral bone area and the transition of the subchondral bone plate to trabecular bone was defined as yellow line. Sections were stained with Safranin O/Fast Green. Scale bars = 100 μm. B. Quantification of the subchondral bone plate. *P < 0.05. ****P < 0.0001. NS = not significant. Data are shown as means + 95% CI.
PBS) overnight at 4°C, decalcified with 0.5 M EDTA (pH 7.4) and then embedded in paraffin. For quantification of surgery-induced cartilage lesions, serial frontal sections (5 μm thick) were cut, with 3 sections one slide. Each paraffin block yielded about 60 slides. Every six slide was stained with Safranin O/Fast Green. Cartilage damage in the medial femoral condyle and tibial plateau was graded using the Osteoarthritis Research Society International (OARSI) recommended 0 to 6 scoring system [27]. All the slides stained were evaluated by two independent investigators (SZ and YXG) in a blinded manner. The highest OARSI score among all the slides stained was selected for the severity of cartilage damage. The OARSI scores of the two independent observers were averaged for each mouse prior to statistical analysis.

**Subchondral bone plate histomorphometry**

Histomorphometry was performed using a published method [28]. One slide per animal was analyzed and sections were chosen from the weight-bearing area of the knee joint. Digital image was analyzed using Image J software. For each Safranin O/Fast Green stained section, represented field with a fixed width (600 μm) was chosen based on the following: the upper limit was the transition of cartilage to subchondral bone while the lower limit was the transition of subchondral bone to growth plate (referred to as subchondral bone area, which was defined by black line in Figure 3A). The upper limit of the subchondral bone plate area was just the one of subchondral bone area and the lower limit was the transition of the subchondral bone plate to trabecular bone (yellow line in Figure 3A).

**Chondrocyte primary cell culture**

Mouse knee articular cartilage (femoral condyle and tibial plateau) isolated from 5-day-old SOST Tg mice and their WT littermates was digested with 1% collagenase II (Gibco), as previously described [29]. After reaching 80% confluence, chondrocytes were detached and plated in 6-well plates (5x10⁵/well). Cells were then stimulated with IL-1β (10 ng/ml; R & D Systems) or TNFα (10 ng/ml; R & D Systems) or vehicle for 24 h followed by RNA extraction. In the present study, only the first passage chondrocytes were used for assays.

**Real-time reverse transcriptase-polymerase chain reaction (q-PCR)**

Total RNA was extracted from murine chondrocytes using TRIzol reagent (Invitrogen). About 1 μg mRNA was used for reverse transcription using the PrimeScript RT Reagent Kit (TaKaRa). Q-PCR was performed using SYBR Green PCR Master Mix (Thermo) in an ABI Step One Plus instrument (Applied Biosystems). Relative transcript levels were calculated using the 2⁻ΔΔCt method. Data were normalized to the mouse β-actin (Actb) and all reactions were performed in triplicate. Primers names and sequences are list in Table S1.

**Immunohistochemistry (IHC) and TUNEL**

Anatomically equivalent sections from SOST Tg and their WT littermates were used for IHC or TUNEL. Sections were deparaffinized by xylene and rehydrated. After antigen retrieval in a sodium citrate (0.01 M, pH 6.0), sections were washed and deprived of endogenous peroxidase activity using 3% hydrogen peroxide. Then slides were blocked with 1% bovine serum albumin (Sigma) at room temperature and immunolabeled with primary antibodies against type II collagen (Col2a1, 1:100, Boster), Sox9 (1:100, Millipore) or β-catenin (1:100, Proteintech) at 4°C overnight. Subsequently, sections were incubated with biotinylated goat anti-rabbit secondary antibodies (Vector). Signals were amplified using a Vectstain ABC kit (Vector). After reaction with DAB (Vector), sections were counterstained with hematoxylin and photographed under a Leica light microscope.

TUNEL was performed with the In Situ Cell Death Detection Kit (Roche). Nuclei were counterstained with DAPI (Invitrogen) and scanned using a confocal microscope (Leica). The numbers of total chondrocytes and TUNEL-positive chondrocytes across the entire tibial plateau were determined with Image J software. Sections from 3 sections per animal were imaged. The final results were averaged and presented as the percentage of TUNEL-positive cells.

**Statistical analysis**

GraphPad Prism version 6 was used for all analyses. Data did not violate the assumptions of normality based on normality test. To derive
The knee joints of 10-week-old surgical destabilization of the knee SOST Tg mice exhibit exacerbated OA following with WT littermates, indicating that they are exhibited no obvious abnormalities compared their WT littermate males in the articular cartilage and proteoglycans in the articular cartilage by X-rays. No obvious abnormalities in knee joints were observed in SOST Tg mice relative to their WT littermates, as evaluated in vivo by DXA analyses.

Ten-week-old SOST Tg mice were indistinguishable from their WT littermates, except that they displayed digit abnormalities (Supplementary Figure 2A). Missing digits were only observed in the forelimbs of SOST Tg male mice grew to skeletal maturity with normal weight and body size but develop osteopenia (Supplementary Figure 1B). Total body and femur BMD and BMC were significantly lower in SOST Tg mice relative to their WT littermates, as evaluated in vivo by DXA analyses (Supplementary Figure 1B).

Ten-week-old SOST Tg mice were breed by the above strategy. SOST transgene was genotyped by PCR (Supplementary Figure 1A) and only hemizygous mice and WT littermates were used in this study. Consistent with previously reported findings [11, 22], 10-week-old SOST Tg male mice grew to skeletal maturity with normal weight and body size but develop osteopenia (Supplementary Figure 1B). Total body and femur BMD and BMC were significantly lower in SOST Tg mice relative to their WT littermates, as evaluated in vivo by DXA analyses (Supplementary Figure 1B).

Characterization of SOST Tg mice

SOST Tg mice were breed by the above strategy. SOST transgene was genotyped by PCR (Supplementary Figure 1A) and only hemizygous mice and WT littermates were used in this study. Consistent with previously reported findings [11, 22], 10-week-old SOST Tg male mice grew to skeletal maturity with normal weight and body size but develop osteopenia (Supplementary Figure 1B). Total body and femur BMD and BMC were significantly lower in SOST Tg mice relative to their WT littermates, as evaluated in vivo by DXA analyses (Supplementary Figure 1B).

We then measured the subchondral bone plate thickness in the medial tibial plateau of sham-operated and DMM-induced knee joints at 2 and 8 weeks post-DMM. Subchondral bone plate area/subchondral bone area ratio was significantly lower in SOST Tg mice than their WT littermates in the sham-operated knees at 2 and 8 post-surgery (Figure 2B; P = 0.0036; P = 0.0183; P = 0.0236 respectively). Subchondral bone plate thickness in the SOST Tg mice was significantly greater in the DMM group relative to the sham group at 8 weeks post-surgery (P < 0.0001). However, there was no significant difference between the DMM group of SOST Tg mice and littermates at 8 weeks post-surgery (P = 0.447).

Exacerbated OA in SOST Tg mice was associated with increased chondrocyte apoptosis

To further investigate the mechanism underlying the exacerbation of OA in SOST Tg mice, we performed the primary chondrocyte culture experiments. Isolated primary chondrocytes from 5-day-old SOST Tg mice and their WT littermates were cultured with IL-1β or TNFα for analysis. Chondrocytes from SOST Tg mice has a similar basal expression of anabolic genes (Col2a1, Aggrecan and Sox9) and cata-
bolic genes (MMP-3, MMP-13, Adamts4 and Adamts5) to those from their WT littermates. Next, we treated primary chondrocytes with IL-1β or TNFα for 24 hours. Both IL-1β and TNFα led to reduced Col2a1, Aggrecan and Sox9 and increased MMP-3, MMP13, Adamts4 and Adamts5. However, the more MMP-3 and MMP-13 mRNA levels via IL-1β were significantly less-
SOST effects in OA

Figure 5. A. Representative IHC images of β-catenin in the medial tibial plateau in SOST Tg mice and their WT littermates 2 weeks post-sham operation and DMM surgery. Scale bars = 20 μm. The cellularity of the section was confirmed with hematoxylin staining. B. Representative TUNEL images in the medial tibial plateau in SOST Tg mice and their WT littermates 2 weeks post-sham operation and DMM surgery. Green represents positive staining. Blue indicates DAPI staining. No intensity or background adjustments were made between sections. Scale bars = 50 μm. C. Quantifications of the percentage of TUNEL-positive cells are presented as percentages relative to cells stained for DAPI (n = 6/group) TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labelling; ****P < 0.0001. Data are shown as means + 95% CI.

ened in chondrocytes from SOST Tg mice (Figure 4; P = 0.0386; P = 0.0328 respectively).

Given the fact that imbalance between chondrocyte anabolism and catabolism was not aggravated in SOST Tg mice, the more severe cartilage degeneration in those mice must occur via an alternative mechanism. As SOST is a negative regulator of Wnt signaling, we then analyzed the β-catenin IHC staining of the medial tibial plateau of sham-operated and surgery-induced knee cartilage at 2 weeks post-DMM. The IHC results suggested that more β-catenin was present in WT mice compared with SOST Tg mice upon DMM surgery (Figure 5A). In the sham-operated knee cartilage, β-catenin was relatively low in both SOST Tg mice and their WT littermates (Figure 5A). As increased chondrocyte apoptosis may contribute to the articular cartilage lesions observed in chondrocyte-specific knockout of β-catenin mice [30], we then analyzed chondrocyte apoptosis by TUNEL and found that the percent of TUNEL-positive chondrocytes in SOST Tg mice was significantly more than that in WT littermates at 2 weeks post-DMM (Figure 5B and 5C; P < 0.0001).

Discussion

Multiple lines of evidence suggest that a certain level of Wnt/β-catenin signaling activity is critical important for maintaining the health of articular cartilage while the precise mechanism still remains uncovered [2]. Aberrant activation and inhibition of Wnt/β-catenin signaling both led to acceleration of articular cartilage degen-
eration [30, 31]. As a Wnt antagonist, SOST was mainly detected in osteocytes, suggesting that SOST might play a tissue-specific role in bone. However, increased SOST was detected in articular cartilage of OA [13-17] and Sost KO mice resulted in an acceleration of OA development upon DMM [17]. Unexpectedly, we found the exacerbated but not attenuated development of OA in SOST Tg mice compared with WT littermates upon surgery. We speculate that a certain level of SOST is required for regulating Wnt/β-catenin signaling activity in articular cartilage under pathological conditions.

Wnt/β-catenin signaling has been shown to be associated with not only chondrocyte survival but also differentiation [32, 33] and Lrp6 mutant mice display chondrocyte apoptosis and cartilage destruction [34]. Consistently, we found that increased chondrocyte apoptosis in SOST Tg mice compared with WT littermates upon DMM.

The reduced subchondral bone plate thickness observed in SOST Tg mice implied that sclerostin could also inhibit bone formation in the subchondral bone as described in the whole skeleton [9-11], whereas the DMM-induced knees exhibited a significant increase in the subchondral bone plate thickness compared with the sham-operated ones in the SOST Tg mice at 8 weeks post-surgery while their WT littermates exhibited a nonsignificant increase. Most probably, the severe cartilage damage upon DMM in SOST Tg mice had an effect on this subchondral sclerosis.

Previous study reported that injection of recombinant SOST (rSOST) did not alter the disease progression in the proteoglycan-induced spondyloitis (PGISp) mouse model [35]. The authors attributed the failure to the efficacy of rSOST, low stability of rSOST, insufficient dosage, etc. To avoid these multiple factors, we chose SOST Tg mice, in which human SOST showed effectiveness at blocking Wnt signaling, to perform this study.

In summary, our findings revealed that mice overexpressing SOST have accelerated OA development after DMM, which is associated with reduced Wnt/β-catenin signaling activity, and further highlight the fine-tuning and complexity of Wnt signaling in joint homeostasis and disease.

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Disclosure of conflict of interest

None.

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References

SOST effects in OA


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Table S1. List of primer names and sequences for q-PCR

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Supplementary Figure 1. A. Genotyping showing the presence of SOST transgene in SOST Tg mice and its absence in their WT littermates; SOST transgene band was detected at 208 bp. B. Body weight, BMD and BMC in 10-week-old SOST Tg mice and their WT littermates.
Supplementary Figure 2. A. Gross morphology of 10-week-old SOST Tg mice and their WT littermates. B. Radiographic assessment of whole body and knee joints in 10-week-old SOST Tg mice and their WT littermates.