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REVIEW

Sclerostin in the development of osteoarthritis: A mini review

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Abstract

Wnt signalling plays an important role in bone and cartilage metabolism. Activation of Wnt signalling promotes bone formation but cartilage degradation. Sclerostin (SOST) can inhibit Wnt signalling. It is expressed by chondrocytes in the articular cartilage and osteocytes in the subchondral bone. Since osteoarthritis (OA) is a joint degenerative disease involving both bone and joint compartments, SOST may have a role in mediating the progression of this disease. This review examined the current literature on the role of SOST in the pathogenesis of OA and its usefulness as a biomarker of OA. Most studies agree that SOST is upregulated as a rescue mechanism in OA to prevent further degenerative changes of the joint. It antagonises inflammation-induced cartilage catabolism while preserving chondrocyte anabolic activities. It also prevents abnormal bone mineralisation and osteophyte formation. However, studies on the performance of SOST as a biomarker to detect and stage OA are limited. Further studies are required to determine whether SOST can be a biomarker or therapeutic target for OA.

Keywords: cartilage, chondrocyte, inflammation, joint, subchondral bone, Wnt

INTRODUCTION

Osteoarthritis (OA) is a degenerative disease of the movable joints characterised by pain and swelling, thus limiting the range of joint motion. OA affects multiple compartments of the joint, causing cartilage degradation, synovitis, altered subchondral bone remodelling and infrapatellar fat pad.[1,2] A meta-analysis showed that knee OA affects 22.9% of adults aged > 40 years worldwide.[3] Global Burden of Disease Study also demonstrated that OA was the 15th highest contributor of years-live-with-disease (YLD) in 2020 (2.2% of total YLD).[4]

OA can be triggered by chronic imbalanced mechanical loading of the joint, leading to cartilage breakdown and generation of wear-and-tear particles in the joint space.[5] These particles form damage-associated molecular patterns that can bind with Toll-like receptors and stimulate macrophages and synoviocytes to release pro-inflammatory cytokines including interleukin (IL)-1, IL-6 and tumour necrosis factor-alpha (TNFα).[6] Synoviocytes can also release alarmins that further exaggerate the inflammation process.[7] Abnormal loading also promotes the expression of metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), and subsequent inflammatory response by chondrocytes which further degrade the cartilage. Apart from the mechano-inflammatory aspects, metabolic inflammation that occurs in obesity is speculated to play a role in the pathogenesis of OA. Obesity is a strong risk factor for OA. The excess adipose tissue can secrete pro-inflammatory cytokines which could enter joint space and promote cartilage degradation.[8,9] Coupled with the inability of chondrocytes to compensate for matrix loss, thinning of cartilage occurs. The imbalance in cartilage loading will cause osteolytic lesions at the subchondral bone. However, prolonged mechanical stress will favour bone formation and osteophyte growth.[10]

Among the many pathways that govern cartilage and bone homeostasis, the Wingless and Int-1 (Wnt) pathway is gaining attention in the recent decade. It is a critical pathway in governing cell proliferation, migration, function, death and body axis patterning.[11] It is activated by the binding of Wnt proteins with receptors

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on the cellular membrane and can be divided into canonical and non-canonical pathways. The canonical pathway is mediated by β-catenin and activated by the binding of Wnt1 or Wnt3a with Fizzled and low-density lipoprotein receptor-related protein 5/6 (LRP5/6). Without activation, β-catenin in the cytoplasm is phosphorylated by glycogen synthase kinase-3 beta (GSK-3β), adenomatous polyposis coli (APC) and Axin complex, subsequently ubiquitinated and degraded. GSK-3β activity is inhibited by the activation of the canonical pathway, leading to β-catenin accumulation in the cytoplasm, translocation to the nucleus and induction of gene transcription by forming a complex with T-cell factor/lymphocyte enhancer factor 1 and cAMP Response Element-Binding-binding protein. The non-canonical pathways are also activated by the binding of Wnt with Fizzled receptor and do not require the participation of β-catenin. In the calcium ion (Ca^{2+})-dependent pathway, elevated cytoplasmic Ca^{2+} activates calmodulin-dependent kinases and protein kinase C. In the planar cell polarity-dependent pathway, small GTPases are activated (Figure 1).[11-13] Wnt signalling, particularly the canonical pathway, is known to play a major role in the metabolic function of osteoblast and bone formation.[12,14,15]

Recent studies have discovered that Wnt signalling might play a role in the pathogenesis of OA. A functional variant of Frizzled-related protein B with attenuated ability to antagonize Wnt signalling was associated with the occurrence of hip OA.[16] Expression of β-catenin was found to correlate positively with disease severity of OA in patients.[17] In preclinical studies, blocking Wnt signalling by insertion of inhibitor of β-catenin and T cell factor gene into transgenic mice delayed chondrocyte proliferation, maturation and increased apoptosis. Inhibition of Wnt signalling also prevented vasculature development at the cartilage layer.[18] In another experiment, inhibition of β-catenin in a similar manner also resulted in chondrocyte apoptosis and OA phenotype.[19] Genetically modified mice overexpressing β-catenin at the cartilage upon tamoxifen induction experienced OA phenotype marked by premature chondrocyte differentiation.[17] OA occurred at the temporomandibular joint in mice expressing β-catenin but the effects were blocked by the deletion of gene coding MMP-13 and ADAMTS-5.[20] These studies demonstrated that Wnt signalling is involved in normal cartilage homeostasis and OA development.

Since the pathogenesis of OA involves the cartilage and subchondral bone compartments, and Wnt signalling mediates both bone and cartilage homeostasis, it is reasonable to speculate that inhibition of Wnt signalling

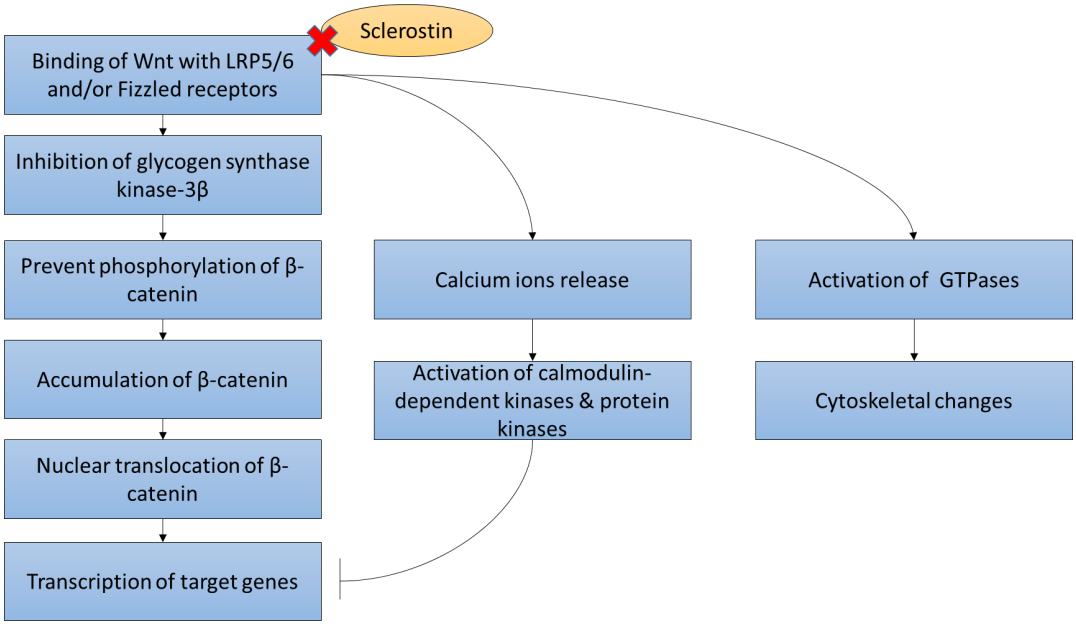

FIG. 1: Canonical and non-canonical Wnt signalling. The binding of Wnt with LRP5/6 and/or Fizzled receptor triggers canonical and non-canonical Wnt pathway, leading to transcription or inhibitions of target genes and cytoskeletal changes important for cellular function.

is a potential therapeutic target of OA. The endogenous suppressor of Wnt pathway is sclerostin (SOST). SOST is a monomeric glycoprotein with a cysteine-knot motif encoded by the SOST gene and it is synthesised by osteocytes but not osteoblasts and bone lining cells. It suppresses the canonical Wnt signalling pathway by binding to LRP5/6. It also binds to LPR4 which enhances its suppressive effects on the Wnt pathway.[12,13] Sirtuin 1 (SIRT1) has been shown to negatively regulate SOST gene expression by deacetylating histone 3 at lysine 9 at the SOST promoter.[21] The biological function of SOST in humans has been demonstrated by cases of SOST gene mutations, resulting in sclerosteosis and Van Buchem disease. Both diseases are autosomal recessive bone disorders featuring skeletal overgrowth (long bones, rib, skull and jaw) leading and increased bone mineral density.[22] Circulating bone formation marker levels like procollagen type 1 amino-terminal propeptide were high and correlated negatively with SOST levels in the patients with these conditions. Bone resorption marker levels including C-terminal telopeptide were normal in patients.[23,24] SOST-knockout (SOST-KO) mice recapitulate the skeletal phenotypes in patients with SOST mutations, such as increased bone mineral density, osteoblasts number, bone formation and mechanical strength.[25] Thus, the role of SOST as a negative regulator of bone mass is well established.

Currently, the involvement of SOST in OA development is an evolving topic. This review aims to summarise the current evidence on the role of SOST in OA progression and as a biomarker for OA.

SOST expression in cartilage and subchondral bone during osteoarthritis

The expression SOST was initially thought to be restricted to osteocytes. However, SOST has been detected in other tissues and variations of its expression have been associated with pathological conditions.[26] SOST was expressed by chondrocytes, especially osteophytic chondrocytes responsible for endochondral ossification, along with other bone formation genes coding bone Gla protein, collagen type 1 alpha 2 and runt-related factor 2 (Runx2). Its high expression in osteophytic chondrocytes is speculated to regulate the speed of ossification process. Meanwhile, normal articular chondrocytes express higher levels of bone morphogenetic protein (BMP)- and Wnt-signalling pathway inhibitors, among which was parathyroid hormone-related protein, a negative regulator of SOST.[27]

Several studies have demonstrated SOST expression at the cartilage and subchondral bone in animals and humans with OA.[28,29] Chan *et al*. noted only 30% of the human OA cartilage sample were SOST positive, probably due to varying degrees of synovitis and inflammation.[28] However, SOST expression by osteocytes in subchondral bone decreased in joints with OA.[28] Similarly, Power *et al*. found that the number of osteocytes/bone area and the expression of SOST was lower in osteocytes of patients with hip OA than controls.[30] This was associated with increased subchondral bone formation and cartilage mineralisation at the OA-affected joints.[30]

Other studies described different patterns of SOST in joint tissues. Abed *et al*. observed concurrent increased expression of SOST in subchondral bone and cartilage tissue of patients with OA compared to normal control.[31] In contrast, Jaiprakash *et al*. reported that expression of SOST was reduced and dentin matrix protein 1 was increased in osteocytes in subchondral bone from OA patients undergoing total knee replacement.[32] Additionally, SOST-staining was observed in calcified matrix of mice with OA induced with a destabilisation of the medial meniscus (DMM), but the staining was restricted to osteophytes, not chondrocytes.[33] Similarly, Zarei *et al*. reported a lack of SOST expression in chondrocytes from all parts of cartilage from patients with OA.[34] However, it was expressed in osteocytes from subchondral bone with partial defect compared to normal, full cartilage defect and osteophytes. In the same study, Dickkopf-1 (DKK1), another inhibitor of Wnt pathway was expressed by surface chondrocytes in normal cartilage and osteocytes, not in cartilage with partial defect and osteophyte.[34]

The spatial expression of SOST has been described by Roudier *et al*.[29] They observed that adult human, mouse and rat articular cartilage expresses SOST protein.[29] In mice, SOST-immunostaining was the most intense near the tide mark and least intense near the superficial zone. In humans, the SOST-staining varies in OA and normal cartilage. In human cartilage, it was generally least intense in the middle zone but the distribution and staining pattern did not differ from the OA cartilage. The mRNA and protein expressions of SOST were not significantly different between knee cartilage specimens of

humans with or without OA.[29] Similarly, Tarquini *et al*. found a similar SOST expression in both the cartilage and subchondral bone among the subjects with OA (from hip arthroplasty for primary OA) and/or osteoporosis (from femoral neck fracture).[35]

On the other hand, SOST expression might also depend on disease severity. Using fresh tibial plateaus from patients with primary OA undergoing total knee arthroplasty, Wu *et al*. demonstrated strong SOST-staining in bone cells at the subchondral bone plate and trabecular bone.[36] However, as the disease progresses, the SOST expression was attenuated. Furthermore, they showed that ratio of SOST positive osteocytes in the subchondral bone plate and trabecular bone correlated negatively with Mankin's scores that reflect the severity of OA. On the contrary, β-catenin protein expression in subchondral bone was positively correlated with OA severity. These results were confirmed by Western Blot analysis.[36]

The U-shaped expression pattern of SOST at the cartilage was observed by Wu *et al*.[37] They observed weakly SOST stained chondrocytes localised in calcified cartilage and deep cartilage near tidemark harvested from OA patients undergoing total knee arthroplasty. However, more SOST-stained chondrocytes were found in cartilage samples of patients with mid-stage OA. The authors suggest that the weight-bearing diversity of cartilage may contribute to different expression SOST in different regions.[37]

Overall, expression of SOST could increase in chondrocytes and decrease in osteocytes. However, the expression patterns could depend on different regions of the tissues and the severity of OA. These distinct patterns could explain the difference in findings among studies, and the confounding factors should be considered in further studies.

In vitro studies on the role of SOST in OA progression

Inflammatory cytokines trigger MMP synthesis by the chondrocytes, in turn, leading to cartilage breakdown.[38] The involvement of SOST in regulating this process has been explored by many researchers using chondrocyte cell lines, primary chondrocytes or cartilage explants harvested from animals or humans. Using mouse embryo teratocarcinoma-derived chondrogenic ATDC5 cells cultured with IL-1β and/or SOST for 3 days to 7 weeks (early stage) or 3 weeks to 7 weeks (late stage), Miyatake *et al*. showed that in the early stage culture, SOST could not reverse IL-1β induced increase in MMP-13 mRNA, suppression of WNT signalling and chondrogenic differentiation.[39] In the late stage culture, SOST reversed IL-1β-suppressed chondrogenic differentiation (marked by lower SRY-Box Transcription Factor 9 (SOX9), type II collagenalpha 1 (Col2A1) and type X collagen alpha 1 (Col10A1) mRNA levels), increased cartilage calcification markers (marked by Runx2 and BMP2 mRNA expression, and alizarin staining for calcium) and catabolic markers (marked by MMP-13, vascular endothelial growth factor (VEGF) and ADAMTS-5 mRNA expression). SOST also abolished the activation of Wnt signalling (lower mRNA expressions of LRP5/6, Axin 1 & 2, Wnt3a &5 and β-catenin 1) by IL-1β. The authors postulated that Wnt signalling was already inhibited by IL-1β at the early stage, so the inhibitory effect by SOST is not apparent.[39] These findings were replicated using chondrocytes from 6-day-old mice by Bouaziz *et al*.[33] The upregulation of catabolic markers and downregulation of anabolic markers induced by IL-1β or Wnt3a was prevented by SOST via c-Jun N-terminal kinase (JNK) pathway.[33]

Chan *et al*. reported that IL-1α, not TNFα, induced the expression of SOST mRNA and protein expression in ovine cartilage cultured ex vivo.[28] Recombinant human SOST exerted anti-anabolic (reduced type II collagen and aggrecan mRNA expression) and anti-catabolic (reduced tissue inhibitor of metalloproteinase 1) effects on normal ovine cartilage explants. However, SOST did not affect the mRNA expression of ADAMTS-4 in normal cartilage explants.[28] In the IL-1α stimulated explant, SOST prevented glucosaminoglycan release by explant into the media. SOST treatment also reduced Wnt1-inducible-signalling pathway protein 1 (WISP-1) and β-catenin-1 mRNA expression in the presence of IL-1α, indicating suppression of Wnt pathway. SOST also down-regulated mRNA expression of MMP-1, MMP-13, ADAMTS-4, ADAMTS-5, but did not prevent downregulation of aggrecan and type II collagen mRNAs induced by IL-1α.[28] These findings showed that SOST expression is increased as a response to cartilage damage to suppress activation of the Wnt pathway to prevent progression of OA. Similarly, Wu *et al*. showed that SOST exposure decreased LRP5/6 and β-catenin in chondrocytes from normal subjects and OA patients.[37] SOST also reduced RUNX-2, MMP-13, ADAMTS-4 & 5 and increased COL2A1 mRNA in normal

chondrocytes only. IL-1α also activated Wnt signalling (β-catenin and LRP5/6 mRNA expression increased) in normal chondrocytes, leading to increased catabolic markers (Runx-2, MMP13, ADAMTS-4/5) and reduced anabolic markers (Col2A1). This was not observed in OA chondrocytes. IL-1α also increased SOST in healthy but not OA chondrocytes,[37] suggesting SOST response to suppress activation of Wnt signalling during inflammation was attenuated in OA. However, SOST protein and mRNA expressions in chondrocytes were similar between patients with OA and osteoporosis.[35]

SIRT1 is a negative regulator of SOST. Expression of SIRT1 was reported to reduce osteoblasts from OA patients. SIRT1, transforming growth factor-beta 1 (TGF1β) and SOST have been suggested to form a complex loop in the bone formation in OA. TGF1β that works concordantly with BMP signalling in promoting osteoblasts formation,[40] did not influence the expression of SIRT1 in primary osteoblast culture from OA patients. In contrast, activation of SIRT1 using β-nicotinamide mononucleotide slightly decreased TGF1β.[31] Treatment with resveratrol stimulated SIRT1 activity and decreased SOST expression in osteoblasts from OA patients.[31] TGF1β, which was reported to increase in osteoblasts in OA, stimulated SOST expression in the primary culture of osteoblasts from OA patients. In conjunction, silencing TGF1β in osteoblasts reduced SOST expression.[31] Silencing of SOST led to activation of the Wnt pathway and BMP-2-dependent mineralisation in osteoblasts from OA patients. This observation could explain abnormal bone mineralisation in the subchondral bone.[31]

Papathanasiou *et al*. noted that SOST mRNA and protein expressions were higher in chondrocytes derived from OA patients undergoing knee replacement surgery than from normal controls undergoing knee fracture repair surgery, with Kellgren and Lawrence (KL) grade ≥3. They also identified two transcript starting sites of SOST, and Smad (an acronym from the fusion of *Caenorhabditis elegans* Sma gene and the *Drosophila Mad* gene) binding sites were found at the CpG island upstream of transcript start site. The CpG sites were more highly methylated in normal control than patients with OA.[41] Treatment of normal chondrocytes with demethylating agent 5'-Aza-2-deoxycytidine (5-AzadC) caused increased SOST mRNA and protein expression.[41] BMP signalling also has been reported to regulate SOST expression in bone.[42] Papathanasiou *et al*. further showed that BMP-2 upregulated the expression of SOST in chondrocytes from OA patients but not in chondrocytes from normal subjects.[41] However, BMP-2 could upregulate SOST expression in 5-AzadC-treated normal chondrocytes.[41] Smad1/5/8 binding was enhanced in OA than normal chondrocytes, and further enhanced by BMP-2 treatment in OA chondrocytes, and 5-AzadC treatment in normal chondrocytes.[41] The combination of BMP-2 and 5-AzadC also enhanced the binding better than individual treatment in normal chondrocytes.[41] Table 1 provides an overview of the role of SOST in OA progression in vitro.

Overall, the current evidence showed that SOST could be expressed in response to activation of the Wnt pathway in OA. It could help to lower cartilage degradation induced by inflammation and excessive osteophyte formation. The protective effects of SOST are mediated by JNK and TGFβ/BMP/Smad pathways (Figure 2).

In vivo studies on the role of SOST using animal models of OA and genetically modified animals

Earlier studies using SOST-KO mice have established that SOST depletion caused increased osteoblast number and bone formation, leading to a high bone mass phenotype.[25] Besides, Roudier *et al*. showed that knee joint histopathological characteristics of SOST-KO mice were similar to the wild-type despite having higher subchondral bone mass.[29] They also showed that SOST antibody (SOST-Ab) treatment given subcutaneously 25 mg/kg for 5 weeks (for male) or 15 mg/kg for 12 weeks (for female) subcutaneously (twice per week) did not reduce morphological characteristics of articular cartilage in aged male rats or ovariectomised female rats despite the increased bone volume. Systemic SOST-Ab (25 mg/kg; subcutaneous) or intra-articular injection of SOST fragmented Ab (385 μg in 50 μL) also did not affect OA development and severity in rats with OA induced by medial meniscus tear.[29] The authors postulated that SOST fragmented Ab cannot penetrate the cartilage to act on chondrocytes. They also suggested that during pharmacological inhibition, other Wnt inhibitors might be upregulated to replace SOST function.[29]

In a subsequent study, Bouaziz *et al*. induced OA in SOST-KO mice via DMM.[33] The SOST-KO mice did not have increased osteophyte compared to wildtype after DMM, casting doubts on the

TABLE 1: In vitro studies on the role of SOST in OA progression

Studies	Characteristics	Major findings
Chan *et al*. 2012 (28)	**Tissue/cell:** articular cartilage explants from the trochlear groove of 6-12-month-old ovine knee joints **OA model:** IL-1α-induced cartilage degradation **Treatment:** 25 or 250ng/ml rhSOST for 4h hrs **Control:** no treatment	↓ mRNA expression COL2A1, ACAN and TIMP1 in normal ovine cartilage explants. ↓ WISP-1, CTNNB1, LRP5 and LRP6 in normal ovine cartilage explants. ↓ expression of MMP13 at 25ng/ml; ↓ (dose-dependent) expression of ADAMTS-5 and TIMP3 in normal ovine cartilage explants. ↔ mRNA expression of MMP1 and ADAMTS-4 in normal ovine cartilage explants. ↓ mRNA expression of MMP-1, MMP-13, ADAMTS-4, ADAMTS-5 in IL-1α stimulated ovine cartilage explants. ↓ WISP-1 mRNA expression at the dose of 250ng/ml in IL-1α stimulated ovine cartilage explants. ↔ IL-1 α -induced inhibition of ACAN and COL2A1 in IL-1α stimulated ovine cartilage explants.
Abed *et al*. 2014 (31)	**Tissue/cell**: Ob cells from tibial plateaus of healthy and OA patients **OA model**: no **Treatment:** 25 ng/mL rhSOST for 48 hrs **Control**: no treatment	↑ SOST levels in OA Ob compared to normal Ob. A linear relationship between SOST and osteocalcin expression in ex vivo subchondral bone explants of OA patients. addition of rhSOST to OA Ob (post confluent cells) for 48 h stimulated osteocalcin expression. ↓ SIRT1 expression in OA Ob compared to normal Ob. ↑ TGF-β1 levels in OA Ob. TGF-β1 stimulated SOST expression in both OA and normal Ob. siTGF-β1 ↓ SOST expression in OA Ob. siSOST in OA Ob ↑ free β-catenin levels and BMP-2-dependent mineralisation.
Bouaziz *et al*. (33)	**Tissue/cell:** Primary chondrocytes from 6-day old mice **OA model**: WNT3a or IL-1β-induced chondrocyte apoptosis **Treatment**: 20 ng/mL rmSOST for 24hrs + 5 μg/ml SP600125 (JNK inhibitor) or 10 ng/ml staurosporine (PKC inhibitor) **Control:** no treatment	SOST pre-treatment partially restored the IL-1β-inhibited expression of GAGs. ↑ mRNA expression of COL2A1, and ACAN; ↓ mRNA expression of ADAMTS-5, MMP3 and MMP13 in IL-1β conditioned chondrocytes. ↑ mRNA expression of COL2A1, SOX9 and ACAN; ↓ mRNA expression of ADAMTS-5/4, MMP3, MMP13, COL10A1 and VEGF in Wnt3a conditioned chondrocytes.

Tarquini *et al*. (35)	**Tissue/cell:** HACs from femoral head samples of OA and healthy patients; femoral neck samples from OP patients. **OA model**: no **Treatment**: no **Control:** no	↔ SOST and OPN protein expression between OA, OP, AO+OP and healthy chondrocytes. ↔ SOST and OPN mRNA expression between OA and OP chondrocytes. ↑ TG2 and ↓ OCN protein expression in OA and OP+OA chondrocytes compared to control and OP chondrocytes. ↑ TG2 and ↓ OCN mRNA expression in OA chondrocytes compared to OP chondrocytes.
Wu *et al*. (37)	**Tissue/cell:** Primary chondrocyte culture from healthy and OA human cartilage **OA model:** IL-1α-induced inflammation **Treatment**: 250 ng/mL rhSOST for 48 hrs **Control**: No treatment	↓ LRP5/6 and β-catenin in SOST treated healthy and OA chondrocytes. ↓ mRNA expression of RUNX-2, MMP-13, ADAMTS-4 & 5; ↑ Col2A1 mRNA expression in SOST-treated healthy chondrocytes. ↑ SOST mRNA expression in IL-1α-treated healthy chondrocytes. ↑ β-catenin, LRP5/6 mRNA expression associated with ↓ COL2A1 and ↑ Runx-2, MMP13, ADAMTS-4/5 in IL-1α-treated healthy chondrocytes.
Miyatake *et al*. (39)	**Tissue/cell**: ATDC5 cells **OA model**: IL-1β-induced chondrocyte apoptosis **Treatment:** 200 ng/ml rmSOST for 3 days -7 weeks **Control**: no treatment	↔ mRNA expression of SOX9, RUNX2, COL2A1, COL10A, MMP13, VEGF and ADAMTS-5 at the early stage of chondrogenic differentiation compared to control. ↔ mRNA expression of WNT3A/5A, LRP5/6, AXIN1, AXIN2 and CTNNB-1 at the early stage of chondrogenic differentiation compared to control. ↓ mRNA expression of WNT3A/5A, LRP5/6, AXIN1/2 and CTNNB-1 at the late stage of chondrogenic differentiation compared to control. ↓ mRNA expression of SOX9, RUNX2, COL2A1, COL10A, MMP13, VEGF, ADAMTS-5 and BMP2 at the late stage of chondrogenic differentiation compared to control.
Papathanasiou *et al*. (41)	**Tissue/cell**: Primary culture of HACs from articular cartilage samples of hypertrophic OA patients and non-OA patients **OA model:** no **Treatment**: 50 ng/ml of BMP-2 for 24 and 48 h and/or 5 μM 5-AzadC **Control**: no treatment	↑ SOST mRNA and protein expression in OA chondrocytes compared to normal chondrocytes. ↑ SOST mRNA and protein expression in 5-Aza-dC-treated normal chondrocytes. ↑ SOST expression in BMP2-treated OA chondrocytes compared to normal chondrocytes. BMP-2 ↑SOST expression in 5-AzadC-treated normal chondrocytes. ↑ Smad1/5/8 binding in OA chondrocytes, BMP-2 treated OA chondrocytes, 5-AzadC treated normal chondrocytes and BMP-2 + 5-AzadC-treated normal chondrocytes.

Abbreviation: ↑, increase; ↔, no change; ↓, reduce; ATDC5, mouse embryo teratocarcinoma-derived chondrogenic cell line; SOST, sclerotin; rmSOST, recombinant mouse SOST; rhSOST, recombinant human SOST; siSOST, small interfering SOST; OA, osteoarthritis; PTOA, post-traumatic OA; OP, osteoporosis; OPN, osteoprotegerin Ob,

osteoblast; IL-1α/β, interleukin 1 alpha/beta; HAC, human articular cartilage; WISP-1, Wnt-induced signalling protein-1; TIMP1/3, tissue inhibitor of metalloproteinase 1/3; TG2, transglutaminase 2; ADAMTS-5/4, a disintegrin and metalloproteinase with thrombospondin motifs; BMP-2, bone morphogenic proteins 2; 5-AzadC, 5'-Aza-2-deoxycytidine; SOX9, SRY-Box transcription factor 9; RUNX2, runt-related transcription factor 2; COL2A1, collagen type 2 alpha 1; COL10A1, collagen type 10 alpha 1; MMP 1/3/13, matrix metalloproteinase 1/3/13; VEGF, vascular endothelial growth factor; LRP5/6, low-density lipoprotein receptor 5/6; CTNNB-1, catenin beta like 1; SIRT1, sirtuin 1; JNK, c-Jun N-terminal kinase; PKC, protein kinase C; GAG, glycosaminoglycan; ACAN, aggrecan; TGF-β1, transforming growth factor beta 1; siTGF-β1, small interfering transforming growth factor beta 1; WNT3A/5A, wingless and int-1 3A/5A

involvement of SOST in osteophyte formation. In contrast to Roudier *et al*.,[29] SOST-KO mice did experience more severe cartilage lesions, a higher ADAMTS-5 expression in the calcified lesion, and a higher ADAMTS-4 expression in non-calcified lesions than wildtype after DMM. However, SOST-KO did not influence DMM-induced chondrocytes apoptosis and type X collagen expression.[33] The increased OA severity, MMP-13 expression and loss of type II collagen were replicated by Li *et al*. using SOST-KO mice with OA induced by anterior cruciated ligament transection.[43] However, they also observed increased subchondral bone volume and invasion of subchondral bone into the cartilage layer, with the increased bone remodelling in these mice.[43] Jia *et al*. further demonstrated following DMM or DMM plus hemisectomy of the meniscus, SOST expression was lost in osteocytes at the medial not lateral site, which might promote new bone formation at the bone marrow side in cartilage-specific Egfr-knockout mice (ERGF-CKO).[44] These mice also developed more severe OA after DMM compared to wild-type. SOST-KO had the similar subchondral thickness and cartilage damage as the WT after DMMH (no

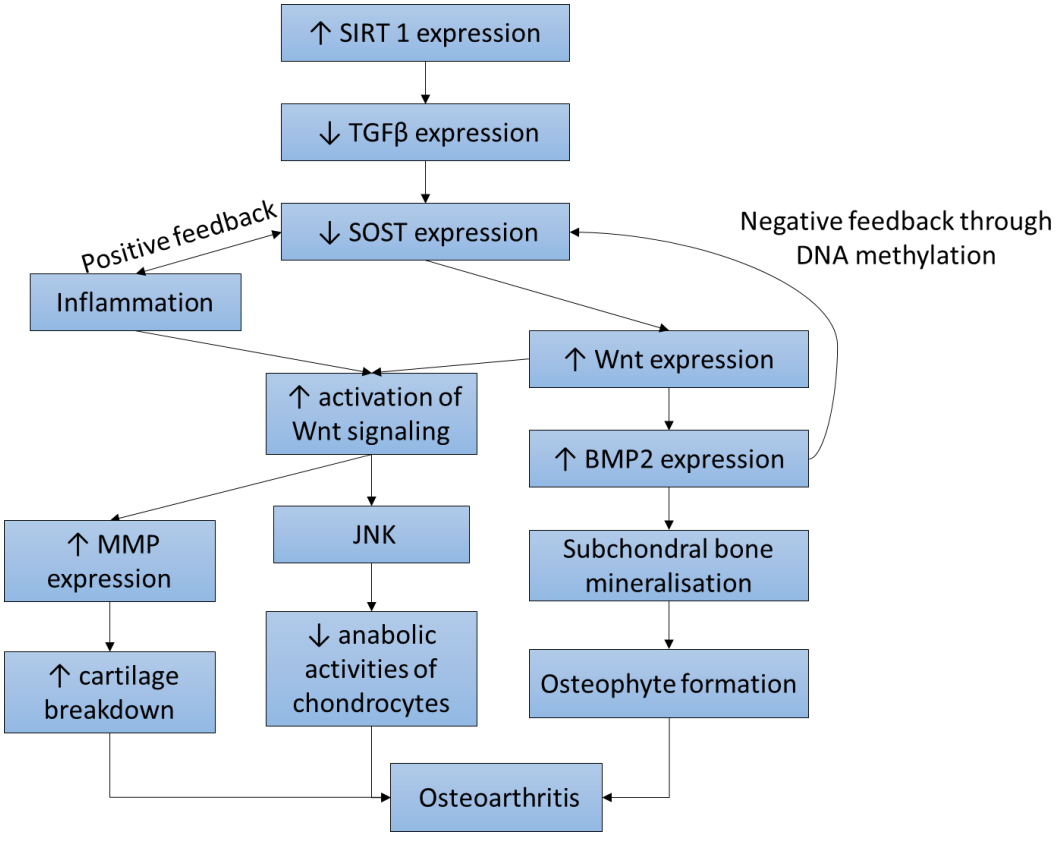

FIG. 2: The role of SOST in osteoarthritis progression. SOST exerts its joint protective function by inhibiting the activation Wnt pathway, thus preventing cartilage breakdown and osteophyte formation, as well as preserving the anabolic activities of chondrocytes.

increased subchondral bone thickness), although at the uninjured site, the subchondral bone was thicker.[44] These studies provide evidence that SOST depletion might aggravate cartilage damage and subchondral bone changes in OA, although the damage might be limited to damage sites.

Reduced autophagy has been postulated to be involved in the pathogenesis of OA as it is an important process to prevent cellular senescence and oxidative stress.[45] In line with this, Ma *et al*. demonstrated that the more severe joint degradation in SOST-KO following DMM was accompanied by activation of Wnt signalling, increased MMP-13, VGEF, and chondrocytes apoptosis, but reduced microtubule-associated protein 1A/1B-light chain 3 and autophagy-related 5 (both markers of autophagy).[46] Rapamycin, an autophagy inducer reversed all these changes in SOST-KO mice.[46]

SOST transgenic mice were also used to study the role of this protein in OA. Earlier studies showed that sustained overexpression of SOST induced trabecular bone loss in mice.[47] Zhou *et al*. demonstrated no difference in joint structure between hemizygous SOST transgenic mice and wildtype.[48] After DMM, the transgenic mice experienced an accelerated loss of cartilage compared to WT, and the entire layer was eroded after 8 weeks. The chondrocytes in the cartilage of transgenic mice experienced a higher rate of apoptosis and less expression of MMP3 and 13 compared to WT.[48] The levels of ADAMTS-4, 5 and aggrecan did not differ between transgenic mice and WT.[48] The subchondral bone plate was thicker in the transgenic mice than the wildtype only after DMM. Zhou *et al*. postulated that SOST is necessary to sustain Wnt/ β-catenin signalling, which is necessary for chondrocyte survival. Similarly, partially abolishing Wnt signalling as in LRP6 heterozygous mice caused a higher chondrocyte apoptosis rate and serum MMP9.[49]

However, the transgenic models of Chang *et al*. revealed opposite findings.[50] SOST transgenic mice suffer from less cartilage damage than SOST-KO mice and wildtype. SOST-positive chondrocytes were found in the deep zone cartilage of wild-type mice and SOST transgenic mice. After anterior cruciate ligament rupture due to tibial compression injury, SOST expression increased significantly in the deep zone cartilage but its expression in osteocytes was not altered. This increase could be associated with activation of NFκβ because intra-articular injection of NFκβ inhibitor and TNFα antibody prevented an increase in SOST in mice with joint injury. Besides, SOST-KO induced more while SOST transgene-induced less osteophyte formation post joint injury. SOST-KO also abrogated subchondral bone loss due to joint injury. They also showed that SOST transgene and intra-articular injection of recombinant mouse SOST protein (4 μg/kg in 10 μL) reduced the level of activated MMPs after injury. Overall, they showed that NFκβ/SOST/MMP form a negative feedback loop that prevents further joint degradation.[50] Administration of SOST might help to reduce joint injury and ectopic bone formation that could cost pain. The observations of Chang *et al*. were supported by that of Liao *et al*.[51] miR-218-5p inhibition and SOST overexpression in rats with OA prevented joint degenerative changes, chondrocyte apoptosis, overall inflammatory status and synovial superoxide dismutase activity. Besides, these two approaches also increased collagen expression and reduced β-catenin and MMP expression on the cartilage.[51] Table 2 provides an overview of the role of SOST in OA progression in vivo.

Overall, the effects of SOST depletion and overexpression on joints in genetically modified animals revealed heterogeneous findings. In undamaged joints, SOST expression does not affect phenotypic characteristics of the cartilage despite thicker subchondral bone. SOST depletion induces more while SOST overexpression prevents cartilage damage due to OA in most studies.

The use of SOST as a biomarker of OA

The usefulness of circulating and synovial SOST as a biomarker for identifying and staging OA has been examined. In the study by Mabey *et al*.,[52] plasma SOST levels were significantly lower in knee OA patients compared to the normal control. Within the OA group, plasma and synovial SOST levels also correlated negatively and significantly with OA severity. The synovial samples from normal subjects were not available due to difficulties in sampling.

In contrast, Theologis *et al*. showed no significant difference in serum and synovial fluid SOST level between OA and normal control group, or among patients with different disease stages indicated by KL grades.[53] On the other hand, DKK1 in synovial fluid was higher in OA and higher in patients with KL grade 4 compared to lower grades.[53] In their subsequent studies, synovial and serum SOST

TABLE 2: In vivo studies on the role of SOST in OA progression

Study	Characteristics	Major findings
Bouaziz *et al*.[33]	**Animal:** Male SOST-KO mice of C57BL/6 strain (10 weeks old; n=10) and male WT mice (10 weeks old; n=10) **OA model:** DMM (right knee); sham operation (left knee). **Treatment**: no	↑ cartilage damage and OARSI score in DMM-SOST-KO compared to DMM-WT mice. ↑ type X collagen expression in DMM-SOST-KO compared to DMM-WT mice. ↑ apoptotic cells in calcified and no-calcified cartilage cells of DMM-SOST-KO compared to DMM-WT mice. ↑ ADAMTS-5 expression in non-calcified cartilage of DMM-SOST-KO compared to DMM-WT mice. ↑ ADAMTS-4 expression in calcified and non-calcified cartilage of DMM-SOST-KO compared to DMM-WT mice.
Roudier *et al*. [29]	**Animal**: Male SOST-KO mice (12 months old; n=12/16 months old; n=16) and WT littermates (12 months old; n = 12/16 months; n=16) **OA model**: no **Treatment**: no	↑ SOST immunoreactivity in cortical and cancellous bone osteocytes and chondrocytes associated with the articular cartilage in the long bone of WT mice compared to SOST-KO mice. ↑ bone volume and bone formation rate in the distal femoral epiphysis of 12-month-old male SOST-KO mice compared to WT control mice. ↔ cartilage area, cartilage thickness and % cartilage surface covering the joint surface in SOST-KO compared to WT mice. ↔ loss of articular cartilage, roughening of the articular surface, and loss of proteoglycans in femoral condyles of 16-month-old male SOST-KO compared to age-matched WT mice. ↑ subchondral bone mass in SOST-KO mice compared to WT mice. ↔ lesion severity between SOST-KO mice and WT mice.
	Animal: Male Sprague-Dawley rats (16 months old; n=10) **OA model:** no **Treatment:** 25 mg/kg subcutaneous injection of SOST-Ab twice/week for 5 weeks **Control:** no treatment **Animal:** Female Sprague-Dawley rats (14 months; n=10) **OA model**: OVX **Treatment:** 15 mg/kg subcutaneous injection of SOST-Ab twice/week for 12 weeks **Control:** no treatment	↑ bone volume and bone formation rate at the proximal tibial epiphysis of aged male rats and OVX female rats treated with SOST-Ab compared to rats treated with vehicle. ↔ cartilage area and cartilage thickness of aged male rats and OVX female rats treated with SOST-Ab compared to rats treated with vehicle.

Study	Characteristics	Major findings
	Animal: Male Lewis rats (260-286 g; n=20) **OA model**: MMT (right knee) **Treatment:** 25 mg/kg subcutaneous injection of SOST-Ab twice/week for 1 day-preMMT to 3 weeks postMMT **Control**: vehicle	↔ OA lesions between the vehicle-treated and SOST-Ab–treated MMT-induced OA rats.
	Animal: Male Lewis rats (281-330g; n=20) OA model: MMT (right knee) **Treatment:** 385 μg/50 μl IA subcutaneous injection of SOST-Fab twice/week for 3 days preMMT to 3 weeks postMMT **Control:** **Negative:** Vehicle **Positive**: 385 μg/50 μl IA subcutaneous injection of KLH-Fab twice/week for 3 days preMMT to 3 weeks postMMT	↔ OA lesions between the vehicle-treated and SOST-Fab-treated MMT-induced OA rats.
Li *et al*. [43]	**Animal:** Male WT C57BL/6j mice (10 weeks old; n=20) and male SOST-KO C57BL/6j mice (10 weeks old; n=20) **OA model**: ACLT (right knee); sham operation (left knee). T**reatment**: no	↑ OARSI scores in SOST KO mice, compared to WT mice. ↑ bone mineral density, bone volume ratio, bone remodelling rate and TRAP-positive cell number in subchondral plate and subchondral trabecular bone of tibia in SOST-KO mice compared to WT mice. ↑ MMP-13 expression and ↓ type II collagen expression in SOST-KO mice compared to WT mice.
Jia *et al*. [44]	**Animal:** Male EGFR-CKO, CKO-WT, WT and SOST-KO mice (3 months old; n=6) **OA model:** DMM or DMMH (right knee); sham operation (left knee) **Treatment:** no	Total depletion of the articular cartilage layer at the medial posterior site of EGFR-CKO mice compared to WT and CKO mice after DMM. ↓ SOST expression in the medial and lateral site in EGFR-CKO compared to WT mice. ↔ subchondral thickness and cartilage damage between SOST-KO and WT after DMMH. SBP thickening in Egfr-CKO mice after DMM correlates with ↑ bone formation at the bone marrow side of the SBP and ↓ SOST level in the SBP.

Ma *et al*.[46]	**Animal:** Male WT C57BL/6j mice (10 weeks old; n=60) and SOST-KO C57BL/6j mice (10 weeks old; n=30) **OA model**: DMM (right knee) **Treatment:** 1mg/kg of rapamycin for 2 or 8 weeks **Control:** no	↓ cartilage degradation, chondrocyte apoptosis at weeks 2 and 8 compared to negative control in WT mice. ↓ expression of MMP13, VEGF, β-catenin and SOST at weeks 2 and 8 compared to negative control in WT mice. ↑ expression of LC3 and ATG5 at week 2 compared to negative control in WT mice. ↓ cartilage degradation and OARSI score at week 2 compared to negative control in SOST-KO mice. ↑ expression of LC3 and ATG5 at week 2 compared to negative control in SOST-KO mice. ↓ expression of MMP13, VEGF, β-catenin and chondrocye apoptosis at weeks 2 compared to negative control in SOST-KO mice. ↔ expression of SOST at weeks 2 compared to negative control in SOST-KO mice.
Zhou *et al*. [48]	**Animal:** Male SOST-Tg mice (10 weeks old; n=52) and WT littermates (10 weeks old; n=47) **OA model:** DMM (right knee) **Treatment:** no	↔ joint structure between SOST-Tg and WT mice. ↑ loss of chondrocyte cellularity, proteoglycans at 2 weeks post-DMM in SOST-Tg compared to WT mice. Complete loss of non-calcified cartilage in the medial femoral condyle and tibial plateau at 8 weeks post-DMM in SOST-Tg compared to WT mice. ↑ OARSI scores at 2 and 8 weeks post-DMM in SOST-Tg compared to WT mice. ↓ subchondral bone plate area/subchondral bone area ratio at 8 weeks post-DMM in SOST-Tg compared to WT mice. ↔ expression of COL2A1, ACAN SOX9 and ADAMTS-4/5 in chondrocytes from the cartilage of SOST-Tg and WT mice. ↓ expression of MMP3/13 and β-catenin in chondrocytes from the cartilage of SOST-Tg and WT mice. ↓ expression of β-catenin in the medial tibial plateau at 2 weeks post-DMM of SOST-Tg compared to WT mice. ↑ chondrocyte apoptosis at 2 weeks post-DMM of SOST-Tg compared to WT mice.

Joiner *et al*. [49]	**Animal:** Male LRP6$^{+/-}$ mice (8 weeks old; n=21) and WT littermates (8 weeks old; n=25) **OA model**: ACLT, PCLT, LMTLT and removal of medial and lateral menisci (right knee). **Treatment**: no	↓ articular cartilage thickness in surgery knee of LRP6$^{+/-}$ mice compared to WT mice. ↑ cartilage degradation and histological scores in surgery knee of LRP6$^{+/-}$ mice compared to WT mice. ↔ expression of COL 1/2/10A1, SOX9, ACAN, MMP3/13 in LRP6$^{+/-}$ mice compared to WT mice. ↑ serum MMP9 in LRP6$^{+/-}$ mice compared to WT mice. ↑ chondrocyte apoptosis rate surgery knee of LRP6$^{+/-}$ mice compared to WT mice.
Chang *et al*. [50]	**Animal:** male SOST-Tg mice (16 weeks old; n=5), SOST-KO (16 weeks old; n=5) and WT littermates (16 weeks old; n=5) (C57Bl/6 background) **OA model**: ACL rupture (right knee) **Treatment:** intra articular injection of rmSOST (10 µLof 4 µg/kg) + NF- κB inhibitor (10 µL of 4 µg/kg) or rmSOST (10 µL of 4 µg/kg) + TNFα inhibitor (10 µL of 4 µg/kg) for 3 days in WT mice. **Control:** vehicle (DMSO or PBS) in WT mice	↓ PTOA in SOST-Tg compared to SOST-KO and WT mice. ↓ subchondral bone loss in SOST-KO compared to SOST-Tg and WT mice. ↑ joint erosion below growth plate of the posterior tibial plateaus in WT and SOST-KO compared to SOST-Tg. ↓ OARSI score and cartilage loss in SOST-Tg compared to SOST-KO and WT mice. ↓ osteophyte formation in SOST-Tg compared to SOST-KO and WT mice. ↓ activated MMP 2/3 mRNA and protein expression in the injured joint of SOST-Tg and WT mice injected with rmSOST protein compared to SOST-KO mice. ↑ SOST-positive chondrocytes in deep zone cartilage of WT and SOST-Tg compared to SOST-KO.
Liao *et al*. [51]	**Animal:** SPF male Sprague Dawley rats (200-220 g; n=70) **OA model**: ACL, PCL, MCL and medial menisci removal **Treatment:** Injection of miR-218-5p inhibitor, miR-218-5p inhibitor NC, OE-SOST, OE-SOST-NC, miR-218-5p inhibitor + siSOST, miR-218-5p inhibitor + siSOST-NC at knee joint cavity (doses not specified)	↑ miR-218-5p and ↓ SOST in KOA rats. ↓ degenerative changes in OA, chondrocyte apoptosis, synovial SOD activity and serum level of IL-1β, TNFα, PGE2, COX2 and MDA due to miR-218-5p inhibition and SOST overexpression in KOA rats. ↔ COLII staining, ↓ MMP staining, ↓ β-catenin and ↑ Col2A1 expression in the cartilage due to miR-218-5p inhibition and SOST overexpression in KOA rats.

Abbreviations: ↑, increase; ↔, no change; ↓, reduce; WT, wild type; DMM, destabilisation of the medial meniscus; DMMH, DMM plus hemisectomy of the meniscus; SOST, sclerotin; SOST-Ab, SOST antibody; SOST-Fab, SOST antibody fragment; SOST-KO, SOST-knockout; SOST-Tg, SOST transgenic; rmSOST, recombinant mouse SOST; siSOST, small interfering SOST; OE-SOST, overexpressed SOST; MMT, medial meniscus tear; OA, osteoarthritis; PTOA, post-traumatic OA; OVX, ovariectomy; IL-1β, interleukin 1 beta; TNFα, tumour necrosis factor alpha; ACLT, anterior cruciate ligament transection; PCLT, posterior cruciate ligament transection; LMTLT, lateral meniscotibial ligaments transection; KLH-Fab, keyhole limpet hemocyanin–derived antibody fragment; EGFR-CKO, cartilage-specific EGFR knockout; SBP, subchondral bone plate; LRP6$^{+/-}$, low-density lipoprotein-related receptor heterozygous deletion; DMSO, dimethyl sulfoxide; PBS,

phosphate-buffered saline; ADAMTS-5/4, a disintegrin and metalloproteinase with thrombospondin motifs; OARSI, osteoarthritis research society international; SOX9,SRY-Box transcription factor 9; COL2A1, collagen type 2 alpha 1; COL1A1, collagen type 1 alpha 1; COL10A, collagen type 10 alpha; MMP 2/3/9/13, matrix metalloproteinase 2/3/9/13; VEGF, vascular endothelial growth factor;; ACAN, aggrecan; TRAP, tartrate-resistant acid phosphatase; PGE2, prostaglandin E2; COX2, cyclooxygenase; MDA, Malondialdehyde; SOD, superoxide dismutase; ACL, anterior cruciate ligaments; MCL, medial collateral ligaments; KOA, knee osteoarthritis; COLII, collagen type 2; ATG5, autophagy-related gene 5; LC3, Microtubule-associated protein 1A/1B-light chain 3; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B; SPF, specific pathogen free; MiR-218-5p, microRNA-218-5p; NC, negative control

levels were not correlated with the Knee Injury and Osteoarthritis Outcome Score (KOOS) of the patients. However, synovial but not serum DKK1 showed a negative correlation with KOOS.[54] Thus, DKK1 level in synovial fluid was a better predictor of OA disease stage and functional.

Min *et al*. observed higher serum SOST levels in OA patients with KL grade 4 than KL grade 2.[55] Increased SOST level was also associated with increased knee OA risk (odds ratio, OR: 1.001) after adjustment. Gender sub-analysis revealed that it was significantly associated with knee OA incidence in women (OR: 1.001) but not in men. The receiver operating characteristic curve of SOST in predicting knee OA gave a sensitivity of 60.9 % and specificity of 77.6% at cutoff 1733.5 pg/ml. Thus, SOST alone might not be a strong predictor of OA. Table 3 provides an overview of the use of SOST as a biomarker of OA in human studies.

TABLE 3: Human studies on the performance of SOST as a biomarker of OA

Study	Characteristics	Major findings	Limitations/Suggestions
Mabey *et al*. (52)	OA patients: n=95, age=69.5 ± 0.8 yrs, ♀/♂ = 79/16 Control: n=95, age=68.2 ± 0.7 yrs, ♀/♂ =77/18	↓ plasma and synovial SOST levels in patients with knee OA with higher severity. Negative correlation between SOST levels and OA severity.	Cross-sectional nature Subjects might have other OA sites Synovial fluid is not taken from control subjects.
Theologis, Masouros (54)	40 serum and synovial samples from knee OA patients, and 20 from controls undergoing arthroscopy.	No correlation between synovial/serum SOST levels and KOOS. Negative correlation between synovial DKK1 and KOOS. Serum DKK1 showed no correlation.	
Min, Shi (55)	Knee OA patients: ♀/♂ =122/26, age=68 (43-87 yrs) Non-knee OA patients: ♀/♂ =51/38, age=57.7 (41-79 yrs).	↑ serum leptin, osteopontin, SOST in the OA patients than the normal control. SOST was higher in KL4 vs KL2. ↑ SOST level was associated with ↑ knee OA incidence (OR: 1.001), especially in women. The receiver operating characteristic curve of SOST in predicting knee OA = 0.748, 95% CI 0.686–0.810. Sensitivity of 60.9% and specificity of 77.6% at cutoff 1733.5 pg/ml.	No data from snynovial fluid.

Abbreviation: ♀/♂, female to male ratio; CI: confidence interval, DKK1: Dickoff-1, KL: Kellgren and Lawrence, KOOS: Knee Injury and Osteoarthritis Outcome Score, , OA: osteoarthritis, OR: odds ratio, SOST: sclerostin, yrs: years.

Overall, studies examining SOST as a biomarker of OA remain scarce. The current evidence does not support the use of SOST alone as a marker of OA progression. Similarly, the performance of SOST in predicting osteoporosis and fracture is also inconsistent.[56] In certain studies, even a counter-intuitive positive relationship between SOST and bone mineral density has been reported.[57-59] The researchers attribute this pattern to a higher number of osteocytes in individuals of larger bone mass, but this speculation requires further validation.[57] As of current, SOST level may be combined with other markers of the Wnt signalling pathway like DKK1, and imaging results to enhance OA prediction and disease staging.

Potential of SOST as a target of OA treatment

From the literature, SOST is essential in maintaining the survival of the chondrocytes. In OA condition, its expression is increased to suppress Wnt signalling in preventing cartilage degradation and osteophyte formation. Since SOST inhibits bone formation, it is a target for osteoporosis therapy. Monoclonal antibody for SOST, romosozumab, has been approved by FDA as osteoporosis therapy among postmenopausal women with a high risk of fracture.[60] A phase III clinical trial on postmenopausal women showed that romosozumab can increase bone mineral density up to 24 months.[61] Since menopause is a major risk factor of osteoporosis and OA, some patients might suffer from both conditions at the same time. Whether inhibition of SOST could aggravate OA in patients with osteoporosis will be of interest. So far, joint pain was not listed as the side effect of romosozumab.[62]

Some natural compounds commonly used as health supplements could also regulate SOST expression. For example, tocotrienols, a subfamily of vitamin E have been shown to suppress SOST protein levels in the bones of rats subjected to ovariectomy and high-carbohydrate high-fat diet to achieve therapeutic effects against osteoporosis.[63,64] Tocotrienol supplementation has also been shown to prevent cartilage damage in animal models of osteoarthritis.[65,66] Chin *et al*. showed that serum osteocalcin level, a marker of bone formation, spiked at low-dose annatto tocotrienol and reduced dose-dependently.[66] This observation correlated with dose-dependent reduction serum cartilage oligomeric protein levels and improved cartilage structure.[66] However, whether these changes were mediated by SOST warrants further investigation.

CONCLUSION

SOST is expressed by chondrocytes in articular cartilage and osteocytes in the subchondral bone. Most studies agree that SOST expression is induced in OA as a rescue mechanism to suppress the activation of Wnt signalling that promotes the catabolism of articular cartilage and osteophyte formation. SOST upregulation in response to inflammation and mechanical damage might be site-specific and attenuated with increased severity of OA. Studies on the use of SOST in predicting and stage OA are limited, thus it is premature to be used alone for such purposes. Whether existing anti-osteoporosis agents that inhibit the expression or function of SOST would induce or exacerbate OA is an interesting question to be explored in future studies.

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