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Low sirtuin 1 levels in human osteoarthritis subchondral osteoblasts lead to abnormal sclerostin expression which decreases Wnt/β-catenin activity

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ABSTRACT

Introduction: Wnt/β-catenin (cWnt) signaling plays a key role in osteogenesis by promoting the differentiation and mineralization of osteoblasts, activities altered in human osteoarthritic subchondral osteoblast (OA Ob). Sclerostin (SOST) has been shown to alter cWnt signaling. Sirtuin 1 (SIRT1) acts as a novel bone regulator and represses SOST levels in Ob. However the role of SIRT1 and SOST in OA Ob remains unknown. Herein, we explored the role played by SIRT1 and SOST on the abnormal mineralization and cWnt signaling in OA Ob.

Methods: Primary human normal and OA Ob were prepared from tibial plateaus. SOST levels were evaluated by immunohistochemistry, the expression and production of genes by qRT-PCR and WB analysis. Their inhibitions were performed using siRNA. cWnt signaling was measured by the TOPflash TCF/lef luciferase reporter assay. Mineralization was determined by alizarin red staining.

Results: SOST levels were significantly increased in OA Ob compared to normal and were linked with elevated TGF-β1 levels in these cells. SIRT1 expression was significantly reduced in OA Ob compared to normal yet not modified by TGF-β1. Specific inhibition of SIRT1 increased TGF-β1 and SOST expressions in OA Ob, while stimulating SIRT1 activity with β-Nicotinamide mononucleotide reduced the expression of TGF-β1 and SOST, and increased mineralization in OA Ob. Resveratrol also reduced SOST expression in OA Ob. Reduced cWnt signaling, β-catenin levels, and mineralization in OA Ob were all corrected via reducing SOST expression.

Conclusion: These data indicate that high level of SOST is responsible, in part, for the reduced cWnt and mineralization of human OA Ob, which in turn is linked with abnormal SIRT1 levels in these pathological cells.

Introduction

Clinical and in vitro studies suggest that human subchondral bone sclerosis and altered bone remodeling, due to abnormal osteoblasts (Ob), are involved in the progression and/or onset of osteoarthritis (OA) [1,2]. Modifications of cell markers, differentiation, and mineralization were shown in OA subchondral bone tissue both in vivo [3–5] and in vitro [6–10]. Compared to normal, OA Ob demonstrate enhanced cell proliferation [11] and elevated markers of differentiation, such as alkaline phosphatase (ALPase), osteocalcin (OC), type 1 collagen [7,9,12], and growth factors such as transforming growth factor β1 (TGF-β1) [7,9,13]. An inappropriate osteogenesis of OA bone tissue would explain these abnormal markers and incomplete mineralization [8,13] as observed in vivo [4] and in vitro [7].

Sclerostin is a cysteine-knot protein of the DAN family [14] secreted mostly by osteocytes. Mutations in the SOST gene cause a high bone mass phenotype in SOST knock-out mice [15] and in humans [16]. SOST is a potent inhibitor of bone growth [17,18] and inhibits β-catenin signaling via its interaction with the Low Density Lipoprotein Receptor-related Proteins-5/6 (LRP5/6) receptor [19,20]. Interestingly, its antagonist role on BMP signaling is also linked with its interaction with the LRP5/6 receptor [14,21,22]. SOST is a mediator of the response to mechanical loading in bone [17], suggesting that SOST could be involved in mechanical transduction and indeed mechanical unloading increases SOST expression [23]. A potential role for SOST in OA is at present controversial. Indeed, Chan et al. reported that SOST expression was increased significantly in OA cartilage compared to normal [24]. However, a recent study by Roudier et al. [25] failed to demonstrate such an increase of SOST in human OA cartilage and bone samples whereas Jaiprakash et al. indicated that SOST levels were actually decreased in human OA samples [26]. Hence, the regulation of SOST expression in OA bone tissue and cells remains controversial.

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Recent reports indicated alterations of Wnt/β-catenin signaling in OA tissues. Targeted overexpression or a decrease of β-catenin expression in chondrocytes both lead to alterations of articular cartilage similar to those observed in OA [27,28]. In contrast, we recently showed that a decrease of Wnt/β-catenin signaling in OA osteoblasts was associated with an increase of DKK2 levels (Wnt/β-catenin inhibitor) [8] or reduced R-spondin 2 levels (Wnt/β-catenin activator) [13].

The pathophysiology of OA is believed to be linked with abnormal biomechanical alterations of bone tissue. Such alterations would compromise the overlying articular cartilage in joints leading to cartilage fibrillation, fissures and loss. Inasmuch as biomechanical alterations are observed in OA and contribute to its pathophysiology, and Wnt/β-catenin signaling is altered in OA bone tissue, SOST could then be playing a role in OA initiation and/or progression since it affects both pathways.

Sirtuins are widely distributed class III histone deacetylases. Sirtuins are involved in a number of processes ranging from cell cycle regulation, apoptosis/proliferation, metabolism, cellular senescence/aging, and inflammation. There are presently seven mammalian sirtuins family members, SIRT1 to SIRT7 [28–35], and SIRT1 is the best characterized in human studies [36–40]. SIRT1 is an important regulator of lifespan extension during caloric restriction, and affects cell survival, differentiation and proliferation [41,42]. Recent studies indicated a potential role for SIRT1 in mouse models of OA [43], and in human chondrocytes, it affects cartilage-specific gene expression [44]. SIRT1 also plays a key role on Ob regulation [45–47] and represses SOST expression in bone [48,49]. However, whereas no reports have described the levels of SIRT1 or SOST in OA bone tissue, SIRT1 activity was shown to be altered in human OA cartilage [44,50]. Therefore, the present study investigated if an alteration of SIRT1 and SOST expression as well as of SOST-induced Wnt/β-catenin signaling could be responsible in OA subchondral Ob for their abnormal mineralization.

Material and methods

Patients and clinical parameters

Tibial plateaus were obtained from OA patients undergoing knee replacement surgery and prepared as previously described [6,9,12]. A total of 30 individual patients (69.0 ± 7.8 years, mean ± SD; 10 males/20 females) classified has OA according to the criteria of the American College of Rheumatology were used [52]. No patients received medication that would interfere with bone metabolism. Moreover, thirteen specimens from normal individuals (61.2 ± 18.1 years, mean ± SD; 9 males/4 females) were obtained from autopsy within 12 h of death. Ethical approval was obtained for the use of all human material following a signed agreement by the patients undergoing knee surgery and for the autopsy specimens by relatives, in accordance with the CHUM ethical committee guidelines.

Preparation of primary subchondral bone cell culture

Isolation of subchondral bone plate and the cell cultures were prepared as previously described [12]. Osteoblasts from different patients are never pooled, and individual experiments are performed with cells from individual OA patients or normal individuals. Confluent cells were incubated in the presence or absence of $1,25(OH)_2D_3$ (50 nM) for 48 h for the determination of biomarkers. Supernatants were collected at the end of the incubation. Cells were prepared in ALPase buffer for phenotypic determinations, in TRIzol™ for qRT-PCR experiments, or Laemmli buffer for Western blot analyses. Protein determination was performed by the bicinchoninic acid method [53]. SIRT1 activity in OA Ob was stimulated using either 100 μM β-Nicotinamide mononucleotide (NMN, Sigma-Aldrich, Canada) for 48 h which is converted to nicotinamide adenine dinucleotide in the cytosol, or increasing doses (10 to 500 nM) of resveratrol (Res) [31].

Phenotypic characterization of human subchondral Ob cell cultures

ALPase activity was determined by substrate hydrolysis using p-nitrophenylphosphate, and osteocalcin in cell supernatants using an EIA as previously described [9,12]. Determinations were performed in duplicate for each individual cell samples prepared from normal individuals and OA patients.

Preparation of Wnt3a conditioned media (Wnt3a-CM)

Conditioned medium (CM) was prepared from Murine L cell lines transfected with either an empty vector (Parental) or with Wnt3a (Wnt3a) obtained from the American Culture Type Collection (Cedarlane Laboratories, Ontario) as described [12]. CM was added to cells at a 20% final concentration.

Evaluation of mineralization

Confluent cells were incubated in BGJb media containing 10% fetal bovin serum (FBS), 50 μg/ml ascorbic acid, 50 μg/ml β-glycerophosphate. This media was changed every two days until day 28. Mineralization of cell cultures was measured by quantification of alizarin red staining (ARS) with the procedure of Gregory et al. [54]. Briefly, cells were fixed in 10% formaldehyde, incubated with 40 mM alizarin red at pH 4.1, washed and air-dried. Cells are then extracted with 10% acetic acid for 30 min, scraped from the Petri dishes, heated at 85 °C for 10 min and then transferred on ice. An aliquot of the cell extract is incubated with 10% ammonium hydroxide and the color product read at 550 nm against a standard curve.

Inhibition of TGF-β1 and SOST in OA Ob by siRNA

TGF-β1, SOST and SIRT1 expressions were inhibited in OA Ob by specifics siRNA (si) as previously described [12]. siTGF-β1, siSOST and siScrambled (siScr) preparations were obtained from Dharmacon (Lafayette, CO). siSIRT1 was obtained from Qiagen.

Protein determination by Western blotting

Cell extracts were prepared for WB as previously described [12]. Rabbit anti-SOST (1:1000, R&D Systems, Minneapolis), rabbit anti-β-catenin (1:2000, Cell Signaling Technology, Massachusetts), and rabbit anti-human actin (1:10,000, Sigma-Aldrich) were used as primary antibodies, whereas goat anti-rabbit IgG (1:10,000, Upstate Biotechnology, NY) were used as secondary antibodies.

qRT-PCR assays

RT reactions were primed with random hexamers with 1 μg of RNA followed by PCR amplification with the Rotor-Gene 6® RG-3000A (Corbett Research, Australia) as described [6,10,11] using 20 pmol of specific PCR primers: SOST, F: AGAATGATGCCACGGAAATC, R: TCACGTAGCGGGTGAAGTG; TGF-β1, F: GCGTGCTAATGGTGGAAAC, R: GCTGAGGTATCGCCAGGAA; SIRT1, F: CCAAGGCCACGGATAGGTCCA, R: ACAGACACCCCAGCTCCAGTT; Dmp1, F: AGTGCCCAAGATACCACCAG, R: CATTCCCTCATCGTCCAACT; OC, F: ATGAGAGCCCTCACACTC, R: GAAAGCCGATGTGGTCAG; GAPDH, F: CAGAACATCATCCCTGCCTCT, R: GCTTGACAAAGTGGTCGTTGAG, added at a final concentration of 200 nM. The data were processed with the GeneAmp 5700 SDS software and given as threshold cycle (Ct). Ct values were converted to number of target gene molecules and values expressed as the ratio to GAPDH.

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TOPflash dual-luciferase reporter assays

Normal and OA Ob were plated in 24-well plates at a density of 1.5×10^5 cells/well containing 10% FBS in BGJb media and left overnight. Plasmid mixtures containing 2 μg TOPflash luciferase construct (Upstate Biotechnology, NY) and 0.05 μg Renilla luciferase driven by the SV40 promoter (Promega, Wisconsin) were transfected into cells overnight using the FuGENE 6 transfection Reagent (Roche) according to the manufacturer's protocol. Media was changed and cells were left to recover from transfection for 6 h prior to incubation for 24 h with Wnt3a-CM or Parental-CM. Cells were lyzed and luciferase activity evaluated using the dual luciferase assay kit (Promega). Values for TOPflash luciferase activity were normalized with Renilla activity.

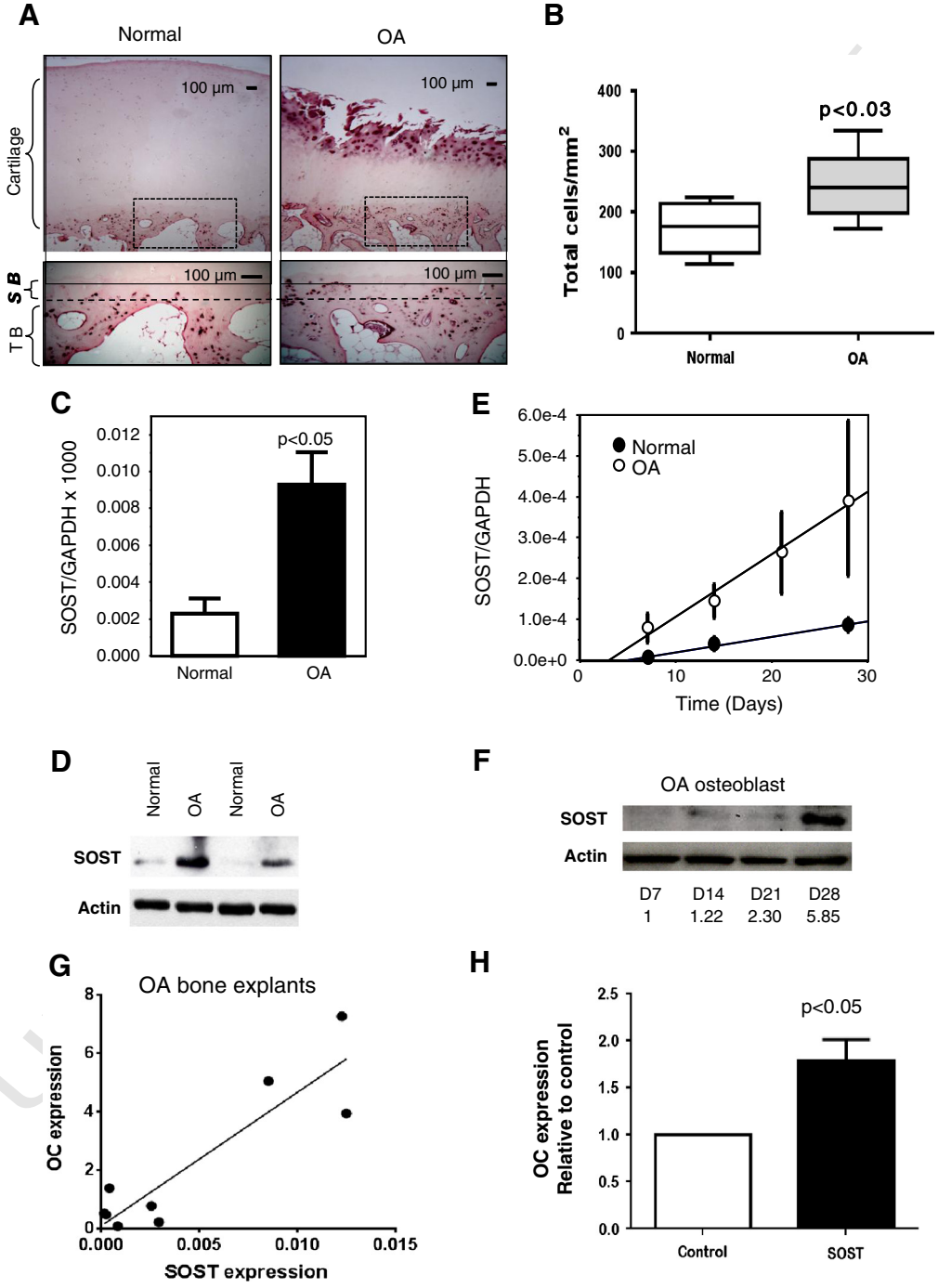

Fig. 1. Expression and production of sclerostin in normal and OA bone tissue and osteoblasts. A) Representative immunohistochemical determination of SOST protein in normal (n = 5) and OA (n = 13) joint tissues: cartilage (C), subchondral bone plate (SB) and trabecular bone (TB). B) Quantification of SOST immunostaining in normal and OA subchondral bone plate tissue (median ± percentile). C) Quantification of SOST mRNA in post-confluent normal (n = 4) and in OA (n = 6) osteoblasts (Ob). D) Representative WB of SOST production in post-confluent normal (n = 4) and OA (n = 6) Ob. E) Time-dependent expression of SOST in post-confluent normal (n = 5) and OA Ob (n = 12). F) Representative WB of time-dependent production of SOST by OA Ob (n = 4). Values at the bottom of the panel indicate the fold increase of SOST production at specific time points. G) Relationship between SOST and osteocalcin expression in OA bone explants (n = 15), r2 = 0.8473. H) Effect of recombinant human SOST (25 ng/ml for 48 h) on osteocalcin expression by OA Ob (n = 4).

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Immunohistochemistry

Full thickness specimens from the tibial plateaus were processed for immunohistochemical analysis as described [55]. Briefly, slides were incubated 60 min with a goat blocking serum (Vectastain ABC kit; Vector Laboratories, USA), blotted and then overlaid with the primary antibody against sclerostin (1:50, Santa Cruz) for 18 h at 4 °C in a humidified chamber. Slides were incubated in the presence of a biotin-conjugated secondary antibody (goat anti-rabbit, 1:1000) for 45 min at room temperature. This was followed by the addition of the avidin–biotin–peroxidase complex for 45 min (Vectastain ABC kit), and slides were counterstained with hematoxylin/eosin. Sections were examined under a light microscope (Leitz Orthoplan; Leica) and photographed using a CoolSNAP cf Photometrics camera (Roper Scientific, USA). Positive cells were counted and analyzed per surface area in 3 or 5 different fields.

Statistical analysis

Quantitative data are expressed as mean ± SEM. The data were analyzed by an ANOVA followed by appropriate subtest when significance was reached, and p values < 0.05 were considered statistically significant between subgroups.

Results

Phenotypic characterization of human subchondral Ob cell cultures

As we previously reported [12,56,57], ALPase and OC levels were high in all OA Ob compared to normal: 1195.1 ± 285.2 vs 316.6 ± 85.9 for ALPase, $p < 0.01$ and 273.3 ± 73.9 vs 138.7 ± 24.6 for osteocalcin, $p < 0.05$.

SOST expression and production

Sclerostin immunohistochemistry showed an increased cell staining in OA bone tissue compared to normal (Fig. 1A). As previously reported [24], OA cartilage also had a higher level of cells staining than normal (Fig. 1A). Quantitative analysis of total positive cells for SOST showed a significant increase ($p < 0.05$) of SOST distribution in OA bone tissue samples compared to normal (Fig. 1B). The data for bone concurred with the 4–5-fold increase ($p < 0.05$) noted for SOST expression in OA Ob compared to normal (Fig. 1C), and elevated protein levels (9.22 ± 0.86 fold increase, $p < 0.05$) (Fig. 1D). SOST expression progressively increased in post-confluent normal and OA Ob (Fig. 1E). This SOST expression in OA Ob remained higher at all times points compared to normal (Fig. 1E). An increased SOST production as a function of time was also observed at the protein level (Fig. 1F), and reached a maximum of 5.85 ± 2.51 folds at 28 days. As SOST is a maker of osteocytes, and as a relationship between SOST and osteocalcin has been reported [58], we next evaluated if such a relationship was present in OA bone tissue. A linear relationship was observed between SOST and osteocalcin expression in ex vivo subchondral bone explants of OA patients (Fig. 1G). Moreover, human recombinant SOST (25 ng/ml) addition to OA Ob (post confluent cells) for 48 h stimulated osteocalcin expression about 1.75 folds in these cells (Fig. 1H).

Regulation of SOST expression by TGF-β1 in OA Ob

As we previously reported the elevated TGF-β1 levels in OA Ob [7,9,13], we next determined if TGF-β1 could be responsible for the increase in SOST production. TGF-β1 stimulated SOST expression in both OA (Fig. 2A) and normal Ob (Fig. 2B). Conversely, siTGF-β1 in OA Ob for 48 h, which reduced TGF-β1 expression about 90% as previously reported [8] reduced SOST expression about 5 to 6-folds (Fig. 2C).

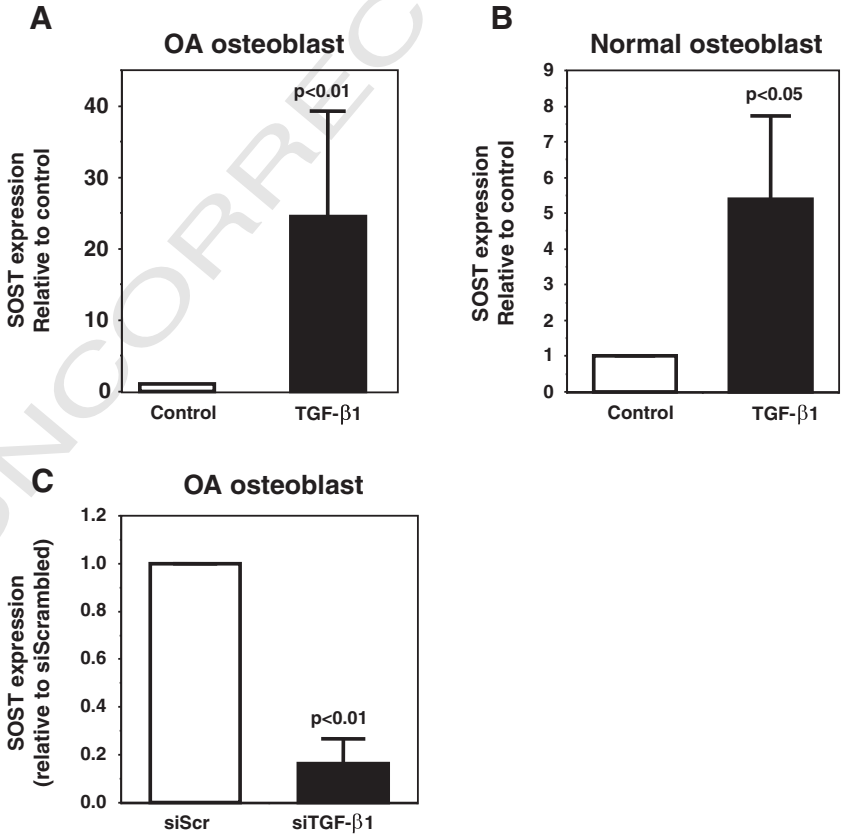

Fig. 2. Regulation of SOST expression by TGF-β1 in normal and OA Ob. SOST expression following TGF-β1 (10 ng/ml, 48 h) in: A) OA Ob (n = 6) and, B) normal Ob (n = 4). C) SOST expression following siTGF-β1 (n = 6).

Role of SOST on Wnt/β-catenin signaling and mineralization in OA Ob

Since SOST is an antagonist of Wnt/β-catenin signaling, and because SOST expression and production is elevated in OA Ob, we next looked at the effect of inhibiting SOST expression on the Wnt/β-catenin signaling. Firstly, data showed that Wnt3a stimulates TOPflash/Renilla activity by about 9 to 10-folds in normal Ob whereas it was only stimulated 4 to 5-folds in OA Ob (Fig. 3A). This activity was increased significantly by 2 to 3-folds in the presence of siSOST in OA Ob (Fig. 3A), at which point TOPflash activity in OA Ob was similar to normal Ob. As we previously reported that free β-catenin levels are reduced in OA Ob compared to normal [8,13], we then evaluated β-catenin levels. siSOST in OA Ob increased free β-catenin levels under basal condition (Fig. 3B, Parental). Moreover, whereas Wnt3a alone increased β-catenin levels directly in presence of siScr, siSOST treatments further increased β-catenin levels about 1.35 ± 0.07 folds ($p < 0.05$) (Fig. 3B).

We previously showed that the mineralization of OA Ob is reduced compared to normal Ob [7], and we showed that this could be due to an increase in TGF-β1 levels [8]. As TGF-β1 increased SOST expression in both normal and OA Ob (Figs. 2A and B), we next questioned if SOST could contribute to this abnormal mineralization. Fig. 3C shows that siSOST for 28 days in OA Ob increased about 2-folds their BMP-2 dependent mineralization.

Role of sirtuin 1 (SIRT1) on TGF-β1 and SOST expression in OA Ob

There is no information on the potential regulation of SOST in OA Ob. However, sirtuin 1 (SIRT1) represses SOST expression in bone [49,50]. As we observed an increase in SOST expression in OA Ob, we therefore questioned if SIRT1 expression could be altered in OA Ob. As illustrated at Fig. 4A, SIRT1 expression was reduced in OA compared to normal Ob. This reduction persisted in culture as a function of time post-confluence until day 28 (Fig. 4B). As SIRT1 expression is reduced in OA Ob (Fig. 4A), while SOST expression is elevated (Fig. 1C) and responds to TGF-β1 regulation (Figs. 2A and B), we next evaluated whether: i) differentiation of OA Ob towards osteocytes was different in these cells compared to normal Ob and, ii) if TGF-β1 could also regulate SIRT1 expression in Ob. Fig. 4C illustrates the expression of Dental matrix protein 1 (Dmp1), an osteocyte-specific gene [59], as a function of time post-confluence in both normal and OA Ob. The expression of Dmp1 progressively increased as a function of time post-confluence in both normal and OA Ob. However, no significant differences were noted between normal and OA Ob for Dmp1 expression at all times points. Next, as shown in Fig. 4D, TGF-β1 did not stimulate SIRT1 expression in normal Ob. In addition, reducing the elevated TGF-β1 expression in OA Ob by siTGF-β1 failed to modify SIRT1 expression in these cells (Fig. 4E). Conversely, inhibiting SIRT1 expression using a siSIRT1 approach in OA Ob, which reduced SIRT1 expression of about 50% (Fig. 5A), resulted in an increase in TGF-β1 expression by OA Ob (Fig. 5B). Under these siSIRT1 conditions, SOST expression in post-confluent OA Ob was also increased (Fig. 5C). Conversely, stimulating SIRT1 activity in OA Ob with NMN slightly inhibited TGF-β1 expression (Fig. 5D) while it completely inhibited the expression of SOST to undetectable levels in these cells (Fig. 5E). Increasing doses of resveratrol, a stimulator of Sirt1 activity [29], also significantly inhibited SOST expression by OA Ob (Fig. 5F). Last, NMN also increased the BMP-2-dependent mineralization of OA Ob (Fig. 5G).

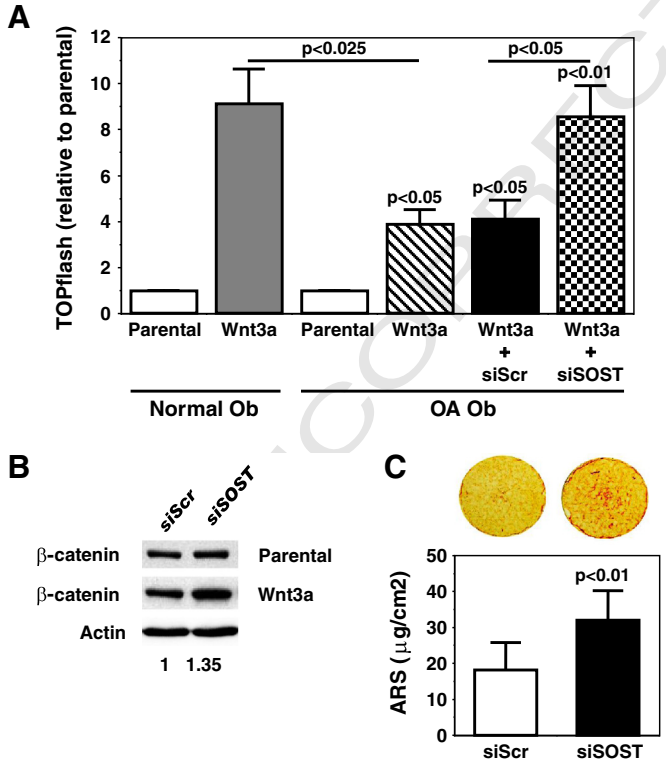

Fig. 3. Role of SOST on Wnt/β-catenin signaling and mineralization. A) TOPflash/Renilla activity in normal (n = 6) and OA (n = 7) Ob. Data are the mean ± SEM. B) Representative WB analysis of β-catenin in OA (n = 3) Ob in response to Parental or Wnt3a-CM following treatment with siScr or siSOST. Values at the bottom of the panel indicate the fold increase of β-catenin production in response to siSOST in Wnt3a-stimulated cells. C) Top: Representative ARS of OA Ob. Post-confluent cells were treated with a siScr or siSOST for 28 days prior to staining. Bottom: quantification of ARS.

Discussion

Wnt signaling is crucial for normal skeletal tissue homeostasis and function. Subchondral bone tissue is abnormal in OA patients [3–5], and we previously showed that OA subchondral osteoblasts have altered functions [7,12,60]. Indeed, we reported that the abnormal expression of phenotypic markers and reduced mineralization of OA Ob is linked with the stimulation of the Wnt antagonist DKK2 [8], as well as the inhibition of the Wnt agonist, R-spondin 2 [13]. Herein we show that another Wnt antagonist, SOST, is increased and involved in abnormal Wnt signaling and altered mineralization in OA Ob. Moreover, we observed that both the endogenous production of TGF-β1 and the reduced production/activity of Sirt1 by these cells are linked with this abnormal production of SOST.

The present study further demonstrates that abnormal regulation of SOST expression and release by OA Ob is playing a role on the terminal differentiation of these cells namely their osteocalcin expression and Ob mineralization. First, we observed an interesting linear relationship between SOST and osteocalcin expression in OA bone tissue extracts, indicating a potential link between the two genes. We further determined that the presence of recombinant SOST is driving the in vitro expression of osteocalcin in OA Ob. Second, SOST expression and release are increased in OA Ob compared to normal Ob, and this increased expression is due, in part, to elevated TGF-β1 expression by these cells since TGF-β1 inhibition in OA Ob reduced SOST expression, and to an abnormal activity of Sirt1 in OA Ob. The role of TGF-β1 here is similar to our observation for DKK2 in OA Ob [8] which is also linked with abnormally high TGF-β1 levels in OA Ob. Our observations therefore support the role of TGF-β1 on SOST expression in mature osteoblasts as previously described with rat osteoblasts [61]. Chan et al. reported that SOST expression was increased significantly in OA cartilage compared to normal [24], a situation we also observed herein (see Fig. 1A). However, a recent study by Roudier et al. [25] failed to demonstrate such an increase of SOST in human OA cartilage and bone. While this group used traumatic hip patients as control and either hip or knee as OA samples, those of Chan et al. [24] and ours only used knee samples, which could explain, in part, this difference. Jaiprakash et al. indicated that SOST levels were actually decreased in human OA samples [26]. However, there were no indications where bone samples were actually collected from in both

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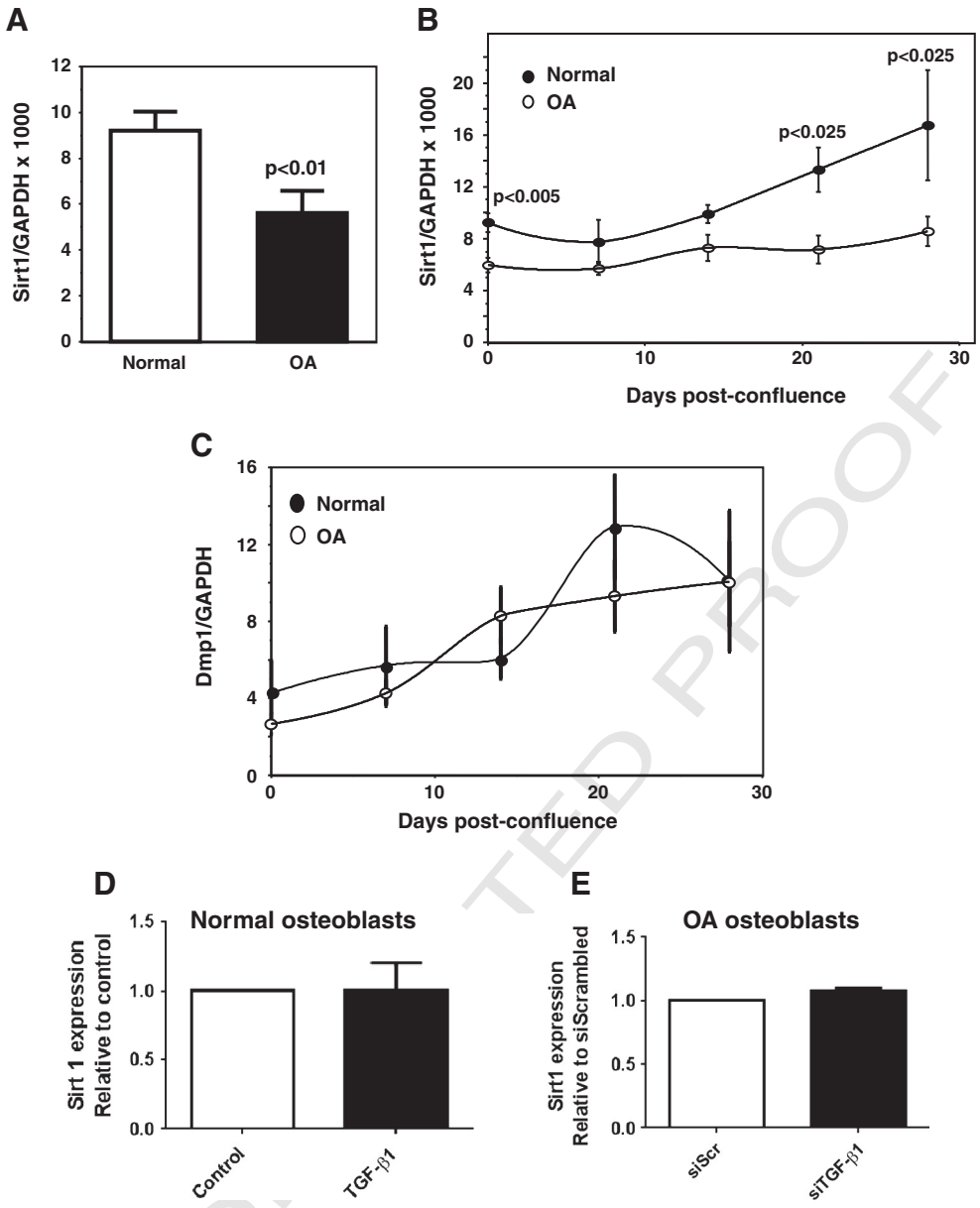

Fig. 4. Expression of SIRT1 in normal and OA osteoblasts. A) SIRT1 expression in normal (n = 8) and in OA (n = 8) Ob. B) Time-dependent expression of SIRT1 in normal (n = 5) and OA (n = 12) Ob. C) Time-dependent expression of Dmp1 in normal (n = 5) and OA (n = 12) Ob. D) Regulation of SIRT1 expression in normal Ob (n = 4) by TGF-β1. E) SIRT1 expression in OA Ob (n = 3) following siTGF-β1.

their normal and OA samples for the preparation of in vitro studies [26], whereas we only used samples from the subchondral bone plate of tibial plateaus, and we previously demonstrated that osteoblasts prepared from the subchondral bone plate or the subchondral trabecular bone are not similar [12,62]. Regardless of these differences, the observation of an increase at the tissue level (Figs. 1A and B) and in vitro level (Figs. 1C and D) for bone and osteoblasts samples in our study, and an increase at the OA cartilage level for the study of Chan et al. [24] and in our study, suggests that sclerostin may be playing a role in OA, albeit possibly at a key time point during the course of the disease. Indeed, episodes of pain and tissue deterioration follow resting periods in OA progression which could explain some of these differences. This specific situation will request further investigation to be fully assessed.

As SOST inhibits bone formation [17,21] and regulates bone mineralization [63], it was interesting to note it reduced the mineralization of OA Ob. Indeed, inhibiting the elevated SOST expression in OA Ob increased the mineralization of these cells as assessed by alizarin red staining. This would suggest that SOST can directly influence the mineralization process in in vitro osteoblasts. Moreover, our observation that inhibiting SOST promoted mineralization whereas addition of SOST reduced mineralization argues in favor of a unique role of SOST in bone mineralization. Although bone sclerosis and subchondral bone plate thickening are consistent clinical findings in OA, we now know this is not linked with an increase in bone formation per se but rather this is due to an increase in the formation of the bone type 1 collagen extracellular matrix with an imbalance in α1 to α2 chains leading to a reduced mineralization [7,64]. Therefore, SOST could participate in the abnormal mineralization of this matrix. In addition, recent studies have shown that SOST is involved in the normal response to mechanical loading whereby SOST expression goes up upon mechanical unloading whereas $SOST^{(-/-)}$ mice are resistant to mechanical unloading [65]. Moreover, the relationship we observed between SOST and osteocalcin could suggest that as OA Ob produce more SOST this contributes to increase their osteocalcin expression, a situation that we tested in vitro.

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As osteoblasts become osteocytes upon their embedment into their type 1 collagen extracellular matrix, and since OA Ob produce more type 1 collagen [7], this could suggest that OA Ob have a more osteocyte-like phenotype than normal Ob under similar culture conditions. However, the observation that the expression of Dmp1, a specific osteocyte-like marker [59], was similar in post-confluent differentiating normal and OA Ob (Fig. 4C), would suggest that the osteocyte potential of normal and OA Ob is similar in vitro and that other factors must key in to explain the alterations we observed for SOST expression between normal and OA Ob. This observation is also different from that of Jaiprakash et al. [26] who showed that Dmp1 expression was increased in OA samples compared to normal. As our experiments for SOST and Dmp1 expression were conducted in parallel, it concurs that as Dmp1 would indicate osteocyte maturation, the increased SOST expression in our OA Ob compared to normal Ob must be related to factors involved in SOST regulation per se, such as abnormal TGF-β1 levels and reduced Sirt1 activity, not on osteocyte maturation. This issue would, however, need more experiments to be clearly defined. In contrast, the link we observed between OC and SOST agrees with the recent hypothesis that as osteoblasts differentiate into osteocytes, they produce more osteocalcin and SOST, which in turn inhibits Wnt signaling and promotes further an osteocyte-like phenotype for these cells [58,63]. Our data would suggest that SOST alone, and not altered Sirt1 activity per se, directly affects osteocalcin expression in OA Ob.

In the present study, we observed that SOST inhibited Wnt/β-catenin signaling in OA Ob. Indeed, interfering with SOST expression by siRNA increased the Wnt3a-dependent Wnt/β-catenin activity of these cells which almost normalized Wnt/β-catenin signaling in these cells. Moreover, the inhibitory role of SOST on β-catenin signaling was observed both at the transcriptional level using the dual TOPflash/Renilla reporter assay and at the protein level using Western blot analysis of β-catenin levels.

In animal studies, a high fat diet reduces SIRT1 expression and levels [66] whereas nutrient starvation increases SIRT1 expression [67]. Of

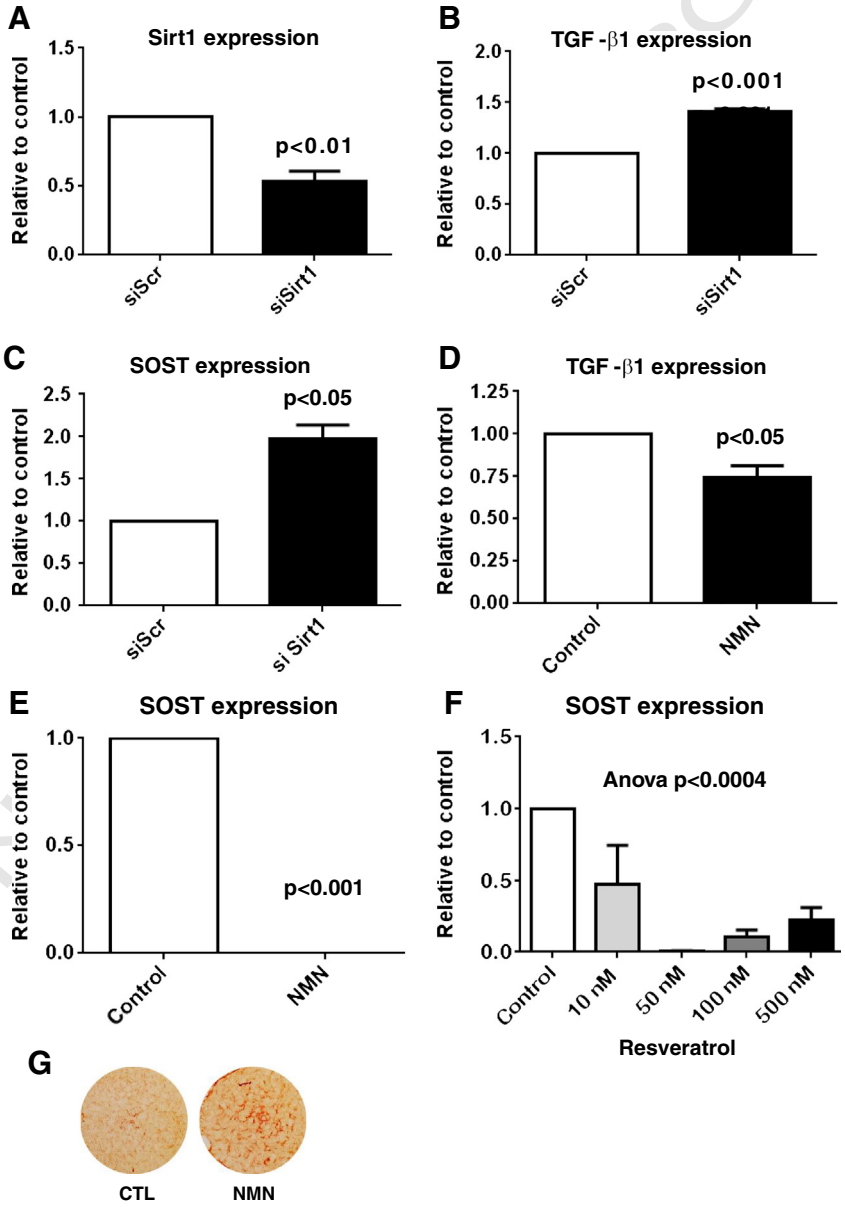

Fig. 5. Hierarchy of SIRT1, TGF-β1, and SOST expression in normal and OA Ob. Role of siSIRT1 on the expression of A) SIRT1 (n = 4), B) TGF-β1 (n = 8) and, C) SOST (n = 3). D) SIRT1 activation by 100 μM NMN in OA Ob on TGF-β1 expression (n = 6). E) SIRT1 activation by 100 μM NMN in OA Ob on SOST expression (n = 5). F) SIRT1 activation by increasing doses of resveratrol on SOST expression (n = 4). G) SIRT1 activation by NMN on ARS of OA Ob (representative of n = 4).

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note, obesity is a risk factor for OA patients [68–70] and a high fat diet enhances the OA burden [71,72]. Diet and nutrient reduction for OA patients have been considered to be beneficial via body weight reduction [73]. However, recent studies indicate this could also be linked with an increase in muscle strength in OA patients [74], whereas SIRT1 levels increase in muscle of starved animals [46]. These data suggest that promoting SIRT1 expression in affected joint tissues of OA patients, namely cartilage, bone and muscle, could potentially restore normal cell physiology in OA tissues. Previous studies have described the potential of SIRT1 in cartilage biology [45]. Reduced SIRT1 production in the heterozygous SIRT1 knock-out mouse model leads to increased apoptosis in chondrocytes and increased OA indices in these animals [44]. Moreover, a reduced SIRT1 activity in mice leads to a decreased collagen type II and glycosaminoglycan release by chondrocytes isolated from these animals, whereas it also increases the release of MMPs from these cells, indices of an OA-like phenotype. Therefore a key role for SIRT1 in OA pathophysiology is now suggested and may represent a potential target to treat OA. However, a direct assessment of the role of SIRT1 in either muscle and bone tissues has not been reported. In the present study, we show for the first time that SIRT1 is reduced in OA osteoblasts and leads to an alteration of osteoblast functions. Indeed, reducing SIRT1 expression increases the expression of TGF-β1 and SOST which can both alter the phenotype of OA osteoblasts. Last, we also confirmed that SOST production is increased in human OA cartilage as previously reported [24] and in human OA subchondral bone tissue.

Although the present data indicated a role for TGF-β1 on SOST expression and the potential role of Sirt1 on TGF-β1 expression, we also clearly demonstrated that SIRT1 can directly control SOST expression. Indeed, SIRT1 has been shown to promote osteoblast differentiation of mesenchymal stem cells [75] and to repress SOST expression [50]. Hence, reduced SIRT1 expression in OA Ob could be responsible for their elevated SOST. Interestingly, increasing SIRT1 activity inhibits TGF-β1 expression in diabetes [47]. We observed a similar situation for OA Ob upon stimulation of SIRT1 activity using NMN, although the effect of NMN on TGF-β1 expression was rather small whereas it totally inhibited SOST expression. Conversely, TGF-β1 could not regulate SIRT1 expression in normal and OA Ob. These data indicate that TGF-β1 is a downstream target of SIRT1 in OA Ob, a situation that could link reduced SIRT1 activity with a number of abnormal biomarkers in these cells [7,8,13]. In addition, we demonstrated that another stimulator of Sirt1 activity, resveratrol, also reduced SOST expression significantly. However, we must be careful to infer that reduced SIRT1 expression alone as observed in OA Ob could be sufficient to explain our observations for SOST expression. Indeed, SIRT1 expression does not fully reflect its activity which is controlled by an elaborate network of regulators such as aging, stress and nutritional variations, all variables that should be tested in OA.

Conclusion

The present study demonstrated that abnormal SIRT1 and TGF-β1 may be responsible for the increased SOST expression of OA Ob which contributes to reduce Wnt/β-catenin signaling and mineralization in these cells.

Abbreviations

cWnt	canonical Wnt/β-catenin signaling
OA	osteoarthritis
Ob	osteoblasts
SOST	sclerostin
SIRT1	sirtuin 1
qRT-PCR	quantitative reverse transcriptase-polymerase chain reaction
siRNA	silencing RNA
TOPflash	TCF/Lef luciferase assay
TGF-β1	transforming growth factor β-1
ALPase	alkaline phosphatase activity
OC	osteocalcin
LRP5/6	low density lipoprotein receptor-related proteins-5/6
BMP	bone morphogenetic protein
DKK2	Dickkopft-2
$1,25(OH)_2D_3$	active form of vitamin D_3
NMN	β-Nicotinamide mononucleotide
MMPs	matrix metalloproteinases
Dmp1	dental matrix protein 1

Uncited reference

[51]

Competing interests

The authors declare they have no competing interests.

Author's contributions

EA, DC and AD performed the experiments, participated in the statistical analysis and the interpretation of data, and drafted the manuscript. JMP and JPP participated in the immunohistochemical experiments, interpretation of data, and reviewed the manuscript. ND provided the human OA knee, participated in the interpretation of data, and reviewed the manuscript. DL participated in the design of the study, performed the statistical analysis and the interpretation of data, and drafted the manuscript. All authors read and approved the final manuscript.

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