

Original Article

Targeted and sustained Sox9 expression in mouse hypertrophic chondrocytes causes severe and spontaneous osteoarthritis by perturbing cartilage homeostasis

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Abstract: Sox9 is the master transcription factor essential for cartilage development and homeostasis. To investigate the specific role of Sox9 during chondrocyte hypertrophy, we generated a novel *Col10a1-Sox9* transgenic mouse model, in which Sox9 is specifically expressed in hypertrophic chondrocytes driven by a well-characterized 10-kb *Col10a1* promoter. These mice were viable and fertile, and appeared normal at birth. However, they developed dwarfism by ten weeks of age. The histological analysis of the growth plates from these transgenic mice demonstrated an abnormal growth plate architecture and a significantly reduced amount of trabecular bone and mineral content in the primary spongiosa. Real-time qPCR analysis revealed the reduced expression of *Col10a1*, and increased expressions of adipogenic differentiation markers in primary hypertrophic chondrocytes isolated from transgenic mice. Concomitantly, the transgenic mouse chondrocyte cultures had increased lipid droplet accumulation. Unexpectedly, we also observed an increased incidence of spontaneous osteoarthritis (OA) development in the transgenic mice by X-ray analysis, micro-computed tomography scanning, and histological examination of knee joints. The manifestation of OA in *Col10a1-Sox9* transgenic mice began by six-months of age, and worsened by eleven-months of age. In conclusion, we provide strong evidence that the proper spatiotemporal expression of Sox9 is necessary for normal adult hypertrophic cartilage homeostasis, and that the aberrant expression of Sox9 might lead to spontaneous OA development.

Keywords: Hypertrophic chondrocytes, adipogenesis, osteoarthritis, Sox9, Col10a1

Introduction

Osteoarthritis (OA) is a degenerative disease characterized mainly by articular cartilage degradations and alterations in subchondral bone and synovium [1]. Multiple factors contribute to the OA pathogenesis, including genetics, obesity, and age [2]. One of the leading causes of OA pathogenesis is excessive mechanical loading that results in articular cartilage damages [3, 4]. Several strategies, including cell-based approaches [5], biomaterial-guided gene deliv-

ery systems [6], and genetic manipulation methods [7] have been proposed to treat OA, but none have matured into standard clinical treatments. At present, the most acceptable surgical treatment for late-stage OA is total knee replacement (TKR). Over 650,000 TKRs were performed in the United States alone in 2008, and the number is projected to increase to over 3.5 million by 2030 as the population ages [8]. OA often begins affecting different joints long before middle age, but evades diagnosis until it becomes symptomatic decades

later, at which point the structural degradation is already advanced and irreversible [9]. Thus, it is critical to identify the key factors mediating the early-stage OA pathogenesis to improve its diagnostic and treatment outcomes.

Cartilage homeostasis depends on the controlled degradation of the matrix proteins such as type II collagen and aggrecan by proteolytic enzymes such as matrix metalloproteinases (MMPs), aggrecanase, and other extracellular proteases in the ADAMTS family. These catabolic events are followed by the deposition of newly synthesized extracellular matrix (ECM) proteins [10]. This complex and dynamic process results in a balance between catabolism and anabolism. In OA patients, this delicate balance tips toward degradation due to a variety of factors, including genetics, lifestyle, and differential expression of proteinases, growth factors, cytokines, and other inflammatory factors [9]. Furthermore, the synovium and subchondral bone are also involved in OA pathogenesis [11]. Human and mouse genetics studies have identified multiple factors that may be associated with OA, including, TGF- β /BMP receptors (*Bmpr1a* and *TgfbRII*), ECM molecules (*Col2a1*, *Aggrecan*), signaling molecules (*sFRP3*, *Gdf5*, *Opg*), transcription factors (*Runx2*), and microRNAs [12-15].

Long bones are created through a dynamic and well-regulated process called endochondral ossification where cartilage templates are replaced by bone [16]. Chondrocytes in the cartilage templates undergo successive steps of differentiation. These early chondrocytes actively proliferate and produce a highly specific cartilage extracellular matrix (ECM), and later become hypertrophic. These hypertrophic chondrocytes either undergo apoptosis or differentiate into osteoblasts and form bone during the course of chondrocyte maturation [16]. Chondrocytes are ultimately replaced by osteoblasts in the process of endochondral ossification. Sox9, the master transcriptional factor regulating chondrogenesis, is essential for chondrocyte condensation and the successive stages of cartilage formation by transactivation of chondrocyte-specific matrix genes, including *Col2a1* and *Aggrecan* [17, 18]. Moreover, SOX9 has been shown to directly regulate connective tissue growth factor (CTGF/CCN2), which is necessary for chondrocyte proliferation and

maturation [19]. Additionally, SOX9 regulates multiple genes encoding ECM proteins, ECM modification enzymes, receptors, and transporters in chondrocytes [20].

Sox9 haploinsufficiency in humans causes Campomelic Dysplasia, a severe skeletal malformation syndrome characterized by the generalized hypoplasia of endochondral bones [17, 21]. Interestingly, during mouse embryonic development, Sox9 is highly expressed in all chondrogenic precursor cells and in differentiated chondrocytes. However, the Sox9 mRNA and protein expression was extremely low in hypertrophic chondrocytes [22, 23]. Notably, Sox9 haploinsufficiency in mice also results in premature skeletal mineralization and an enlarged hypertrophic zone in the growth plates, with increased expression of type X collagen, the major and most specific extracellular component synthesized by hypertrophic chondrocytes [24].

Runx2, the master transcription factor required for osteoblast differentiation, also positively regulates chondrocyte maturation [25]. We have previously shown that Runx2 can directly bind to a consensus cis-element in the *Col10a1* promoter and contribute to its hypertrophic chondrocyte-specific expression [26]. Importantly, both Sox9 and *Runx2* are expressed in early chondrocytic cells, and we have demonstrated the dominance of Sox9 over Runx2 function during skeletogenesis [17]. Indeed, Sox9 can inhibit Runx2 binding to its target sequences in the *Col10a1* promoter and repress the transactivation of a hypertrophic chondrocyte-specific *Col10a1* reporter. Furthermore, in Campomelic Dysplasia patients, SOX9 haploinsufficiency results in the up-regulation of the both *RUNX2* and *COL10A1* [17]. Interestingly, two previous studies have demonstrated that the overexpression of Sox9 in hypertrophic chondrocytes leads to impaired terminal chondrocyte differentiation, with decreased *Vegf*, *Mmp13*, *Rankl*, and *Opn* expression, likely in part through the inhibition of Runx2 activity in the early postnatal stages [27, 28]. Furthermore, Sox9 was also reported to suppress *Vegfa* expression by directly binding to the cis-element in the *Vegfa* gene promoter. Thus, Sox9 is proposed to be a major negative regulator of cartilage vascularization and trabecular bone formation in the growth plates

[27]. However, the phenotype characterization in all previous Sox9-related studies was limited to embryonic development and early postnatal stages.

Despite many factors contributing to OA, the relationship of OA progression and altered hypertrophic chondrocyte regulation is largely unknown, due to the lack of animal models with targeted and sustained expression of the genes of interest specifically in hypertrophic chondrocytes. Thus, in this study, we aimed to determine the effect of hypertrophic chondrocytes-specific Sox9 overexpression on cartilage homeostasis. To address this question, we generated a novel *Col10a1-Sox9* transgenic (TG) mouse and characterized the consequences of Sox9 over-expression in hypertrophic chondrocytes in adult mice. We found that the sustained expression of Sox9 specifically in the hypertrophic chondrocytes of mice results in disorganized chondrocyte columns with reduced mineralization of hypertrophic cartilages. Furthermore, chondrocytes isolated from these mice showed elevated adipogenic markers. Unexpectedly, *Col10a1-Sox9* TG mice showed key OA features such as accelerated loss of cartilage matrix in the growth plates of knee joints, narrowed joint spaces, formation of osteophytes, and subchondral sclerosis. Thus, Sox9 overexpression specifically in hypertrophic chondrocytes results in severe osteoarthritis development in adult mice.

Materials and methods

Generation of Col10a1-Sox9 transgenic mice

The *Col10a1-Sox9* TG construct, in which flag-tagged Sox9 was specifically expressed in all hypertrophic chondrocytes driven by a 10-kb *Col10a1* promoter, was generated in a previously characterized pTYR-WPRE-hGHpA transgenic vector [17]. This 10-kb *Col10a1* promoter directed the β -galactosidase reporter expression specifically throughout the whole hypertrophic zone at high levels in a previous report [29]. The transgenic construct (**Figure 1C**) also includes a tyrosinase (*TYR*) minigene, which enables rapid genotyping of embryonic TG founders by eye color selection, and a chicken β -globin HS4 insulator to enhance the transgene expression by decreasing chromatin-mediated silencing effect [17]. A woodchuck hepatitis virus posttranscriptional regulatory

element (WPRE) was also included in the 3'untranslated sequence to increase the expression level of the transgene [17]. Transgenic founders were generated by pronuclear injection according to standard techniques as reported previously [17]. The transgenic mice were confirmed at birth by eye color change and verified by PCR using the transgene-specific primers as described [17]. Two independent transgenic lines were generated and maintained on a FVB/N background, and they displayed similar phenotypes. All animal protocols have been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Case Western Reserve University.

Isolation of chondrocytes from mouse knee joints

Hind limbs were collected from WT and TG mice upon euthanizing them with isoflurane. Knee joints were cut to separate the ends of the distal femur and proximal tibia under the dissection microscope. Primary chondrocytes were isolated from knee joints following a sequential digestion by pronase (2 mg/ml in PBS) for 1 hour at 37°C, collagenase D (3 mg/ml in DMEM) for 2 hours at 37°C, and overnight at 37°C in collagenase D (1.5 mg/ml in DMEM). Disassociated chondrocytes from WT and TG mice knee joints were collected to perform gene expression analysis.

Skeletal preparations and micro-computed tomography

Skeletons from ten-day-old mice were prepared as described and stained with Alcian blue 8GX for cartilage and Alizarin red S for bone [17]. The dissected hind limbs from six and eleven month old wild-type (WT) and transgenic (TG) mice were analyzed by radiography using a Faxitron radiographic inspection unit. Eleven-month old WT and TG mouse hind limbs were also scanned for micro-computed tomography (micro-CT) analysis on a Scanco μ CT40 scanner as described previously [30].

Histological analysis

Hind limbs from ten-week-old and eleven-month-old mice were fixed in 10% buffered formalin overnight and decalcified in 0.5 M EDTA for 3 months. After paraffin embedding, 5- μ m sections were prepared and stained with

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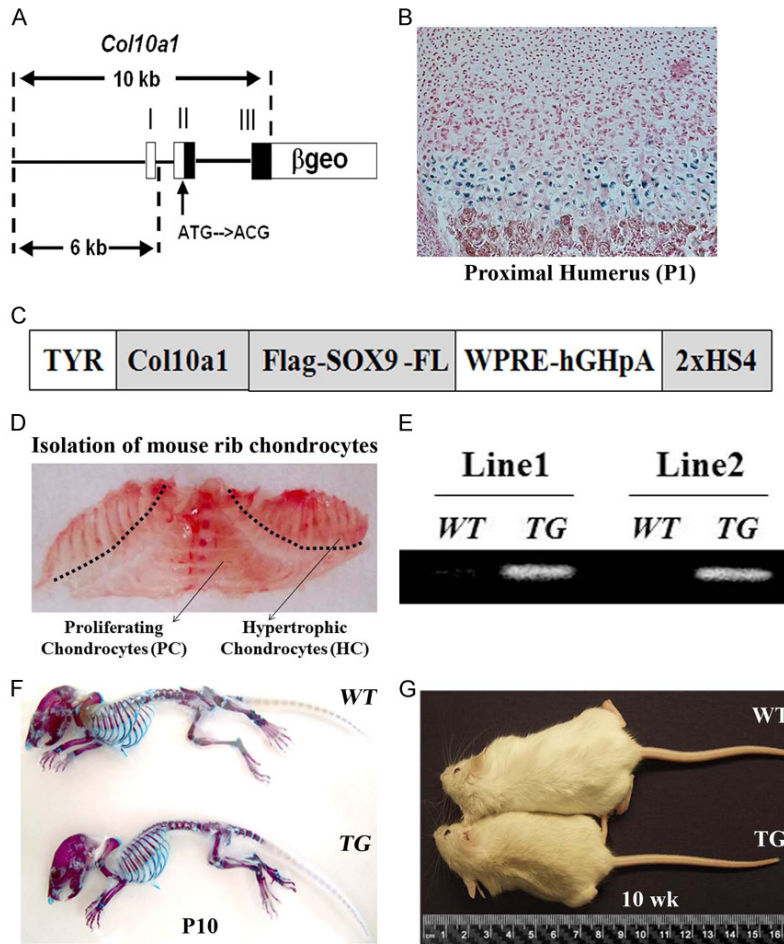


Figure 1. The generation of a novel *Col10a1-Sox9* transgenic mouse model. (A) A schematic representation of a *Col10a1-β-Gal* reporter construct driven by a 10-kb mouse *Col10a1* element utilized in this study. (B) The *Col10a1-β-gal* transgene drives high-level LacZ reporter activity specifically in the hypertrophic zone of transgenic mice. (C) A schematic representation of the *Col10a1-Sox9* transgenic construct that contains the full-length Flag-tagged Sox9 under the control of the same 10-kb *Col10a1* element as described in **Figure 1** (A). The *Col10a1-Sox9* construct was ultimately cloned into a coat color vector as described previously [17]. TYR, tyrosinase minigene; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element; HS4, chicken β-globin insulator. (D) The image of the separation of the hypertrophic and proliferating chondrocytes from a P6 mouse rib cage. The hypertrophic chondrocytes were isolated from the area above the dashed line. (E) The specific transgene expression in the hypertrophic chondrocytes was confirmed by RT-PCR with transgene-specific primers in two independent TG mouse lines. (F) The skeletal preparations of 10-day-old mice by Alizarin red/Alcian blue staining revealed no significant growth differences between WT and TG mice. (G) The gross appearance of 10-week old TG mice was clearly shorter than that of WT littermates.

Toluidine Blue and Safranin O/Fast Green (SO/FG). The standard immunostaining was performed on paraffin sections of ten-week and eleven-month-old hind limbs with anti-Sox9 antibody (Cat # AB5535, Millipore Sigma), anti-Type X Collagen antibody (Quartett, Berlin, Germany), anti-Flag antibody (Cat # 14793S,

CST), and ABC reagent (Vector Laboratories, Burlingame, CA) was used for detection as previously described [31, 32].

Bone histomorphometrical analysis

Undecalcified hind limbs from 10-week-old mice were embedded in methylmethacrylate, and 5-μm sections were generated on a rotation microtome. Sections were then stained with Toluidine Blue and von Kossa reagents. Histomorphometrical analysis was performed on proximal tibiae according to the American Society for Bone and Mineral Research (ASBMR) standards using the OsteoMeasure Analysis System (Osteometrix, Atlanta, GA) [17, 33].

Rib hypertrophic chondrocyte isolation and ex vivo chondrocyte culture

Hypertrophic chondrocytes from rib cages of six-day-old *Col10a1-Sox9* TG mice and WT littermates were isolated as previously described [31]. After the dissection, soft-tissue was cleaned-up and the hypertrophic portion of rib cartilage was cut at approximately 2-mm distal from the osteochondral junction of each rib, to separate it from the proliferating portion (**Figure 1D**). The rib cartilages were then digested with pronase (2 mg/ml in PBS) for 30 min at 37°C, collagenase D (3 mg/ml in DMEM) for 1.5 h at 37°C, and collagenase D (1.5 mg/ml in DMEM) overnight at 37°C. After washing with PBS three times, rib hypertrophic chondrocytes were plated at a density of 1×10^5 cells/ml in 24-well-plate for 9 days in DMEM supplemented with 2 mM GlutaMax,

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Table 1. Primers used in real-time RT-PCR analyses

Gene	Primers*	Size (bp)	Accession No.
Sox9	ACTCCCCACATTCCTCCTCCGGCAT GTGCTGCTGATGCCGTAAGTCCAG	343	NM_011448.3
Col10a1	GGCAGCAGCATTACGACCCAAGAT GAATAACAGACACCACCCCTCAC	563	NM_009925.3
Ppar γ 2	ATCATCTACACGATGCTGGCC CAAGGATTCATGACCAGGGAG	81	NM_011146.2
C/ebp α	CGCAAGAGCCGAGATAAAGC GCGGTCATTGCTACTGGTCA	81	NM_007678.3
Glut-4	ATGGCTGCTGCTGGTTTCTC ACCCATAGTATCCGCAACAT	81	NM_009204.2
Gapdh	ATGGGAAGCTCGTCATCAAC GTGGTTCACCCATCACAA	152	XR_030830.1

*All primer sequences are written from 5' to 3'; for each gene, the first sequence is the sense primer, and the second sequence is the anti-sense primer.

100 μ g/ml Penicillin/100 U Streptomycin, and 10% FBS (Invitrogen, USA). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. The medium was changed every other day. At day 9, half of the cells were fixed with formalin and stained with Oil Red O to detect adipogenesis [34], and the remaining cells were utilized for RNA isolation.

RNA isolation, cDNA synthesis, and real-time RT-PCR analysis

Using TRIzol reagent (Invitrogen) and Purelink Micro-To-Midi RNA Kit (Invitrogen), total RNA was extracted from rib hypertrophic chondrocytes after 9 days of culture ($n = 3-6$ /group) as described above. cDNA was then prepared from 1 μ g of total RNA with the iScript cDNA Synthesis Kit (Bio-Rad). Power SYBR Green PCR Master Mix (Applied Biosystems) and the 7500 Real Time PCR system (Applied Biosystems) were used for real-time RT-PCR analysis with the gene-specific primers listed in **Table 1**. The *Gapdh* gene was used as an internal control of the quantity and quality of the cDNAs. Real time RT-PCR analysis was quantified using the standard $\Delta\Delta$ CT method as described [35].

Statistical analysis

Results were presented as mean \pm standard deviations (SD). Statistical analysis was performed using the Student's t-test to compare the differences between two groups of age-matched *Col10a1-Sox9* TG mice and WT litter-

mates ($n = 3-6$). $P < 0.05$ was considered statistically significant.

Results

The sustained Sox9 expression specifically in hypertrophic chondrocytes in a novel Col10a1-Sox9 transgenic mouse model

We previously identified a mouse 10-kb *Col10a1* element that was able to direct a reporter activity specifically in hypertrophic zones in transgenic (TG) mice [29]. Indeed, this 10-kb element can direct high-level tissue-specific *LacZ* reporter activity throughout

the hypertrophic zone in *Col10a1- β geo* mice (**Figure 1A, 1B**). For this study, we first confirmed that the β -galactosidase staining revealed strong *LacZ* reporter activities in the hypertrophic chondrocytes of the proximal humerus of TG mice (**Figure 1A, 1B**). Then we generated a *Col10a1-Sox9* TG construct that contains the flag-tagged full-length Sox9 under the control of the same 10-kb *Col10a1* promoter in a previously described pTYR-WPRE-hGHpA TG vector (**Figure 1C**) [17]. We generated two independent lines of *Col10a1-Sox9* TG mice. To confirm Sox9 overexpression, we isolated hypertrophic chondrocytes from the rib cages of six-day-old *Col10a1-Sox9* TG mice and WT littermate controls (**Figure 1D**). Our RT-PCR analysis confirmed that both mouse lines exhibited high transgene expression in the hypertrophic chondrocytes (**Figure 1E**). Interestingly, Alizarin red S and Alcian blue staining of the whole skeleton preparations of ten-day-old mice displayed no gross abnormalities in TG mice compared with WT littermates (**Figure 1F**). However, by ten-weeks of age, TG mice were clearly shorter compared with the WT littermates (**Figure 1G**). These results demonstrate that the hypertrophic chondrocyte-specific overexpression of Sox9 in mice results in a postnatal growth delay by 10-weeks of age.

The sustained postnatal hypertrophic chondrocyte-specific Sox9 expression results in disorganized chondrocyte columns and reduced mineralization of hypertrophic cartilages

We performed immunostaining with an anti-flag antibody and observed strong transgene

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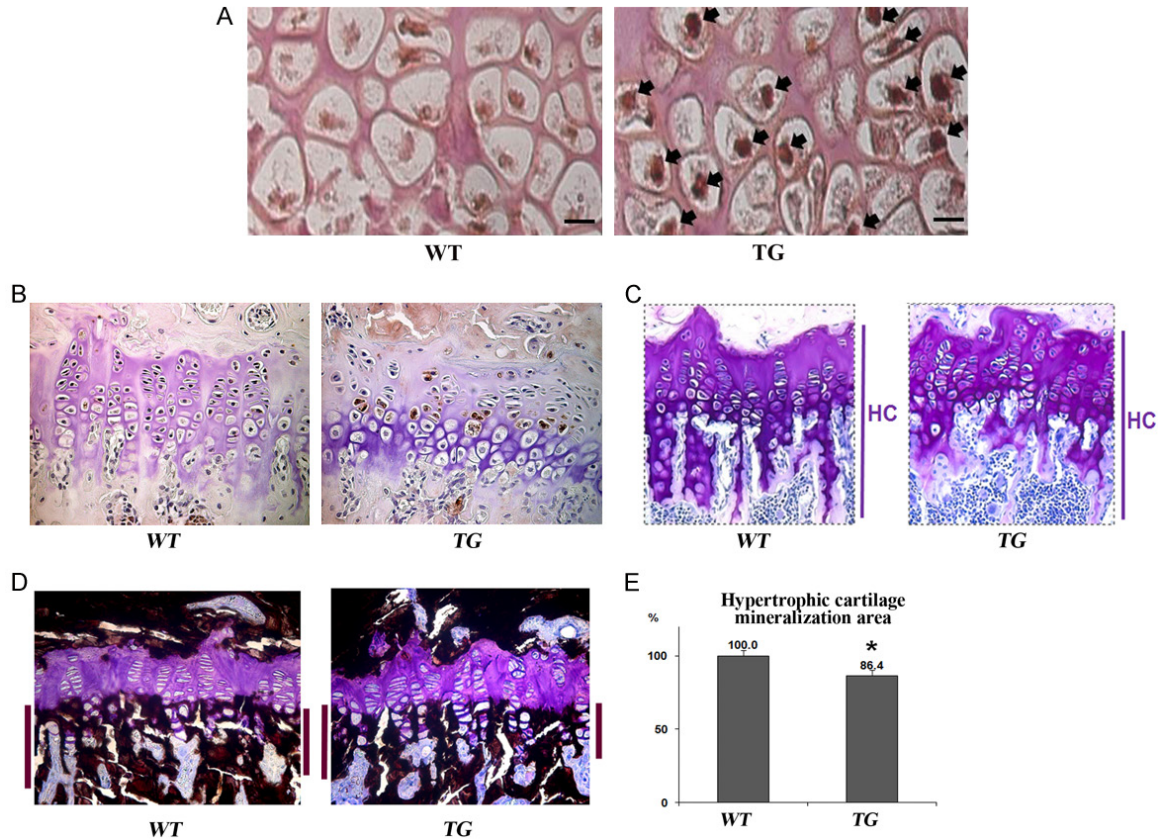


Figure 2. The histological analysis of hypertrophic cartilages in ten-week-old *Co/10a1-Sox9* transgenic mice. A. Flag-tag immunohistochemistry confirmed the hypertrophic chondrocyte-specific transgene expression in the proximal tibiae of six-day-old TG mice as indicated by black arrows (40 \times). B. Toluidine blue staining demonstrates disorganized chondrocyte columns and a reduced length of the trabecular bone in the primary spongiosa of TG mice (20 \times). C. Von Kossa staining demonstrates the slightly reduced mineralization of the primary spongiosa engulfing the hypertrophic zone in TG mice (20 \times). D, E. The histomorphometric quantification of von Kossa staining confirms a modest but significantly decreased mineralization of the primary spongiosa in TG mice. Error bars represent mean \pm SD. (p -value < 0.05).

expression specifically in the hypertrophic chondrocytes of the proximal tibiae of six-day-old TG mice, but not in the WT littermate controls (**Figure 2A**). Next, to confirm the sustained Sox9 expression in the cartilage, we performed immunostaining using an anti-Sox9 antibody in the proximal tibiae of 10-week-old WT and TG mice. The immunostaining confirmed Sox9 expression specifically in the hypertrophic zone of the TG mouse growth plates, but not in the WT littermate controls (**Figure 2B**). Additionally, we observed a severe growth plate disorganization in the TG mice (**Figure 2C**). Indeed, Toluidine Blue staining in the growth plates of proximal tibiae of 10-week old mice revealed that the chondrocyte columns appeared much more disorganized in TG mice, and that the primary spongiosa was also significantly shorter

in TG mice compared with WT controls (**Figure 2C**). Moreover, von Kossa and toluidine blue staining revealed a slight reduction in the mineralized area of the trabeculae of the primary spongiosa in *Co/10a1-Sox9* TG mice compared with WT controls (**Figure 2D**). The reduced mineralization of hypertrophic cartilage in *Co/10a1-Sox9* TG mice was further confirmed by histomorphometric quantification of von Kossa staining, which revealed over a 10% reduction in von Kossa staining in TG vs. WT mice (**Figure 2D** and **2E**). Together, these results indicate that the sustained overexpression of Sox9 specifically in hypertrophic chondrocytes results in a disrupted growth plate organization, and a reduction in the size and mineralization of trabecular bone in the primary spongiosa post-natally.

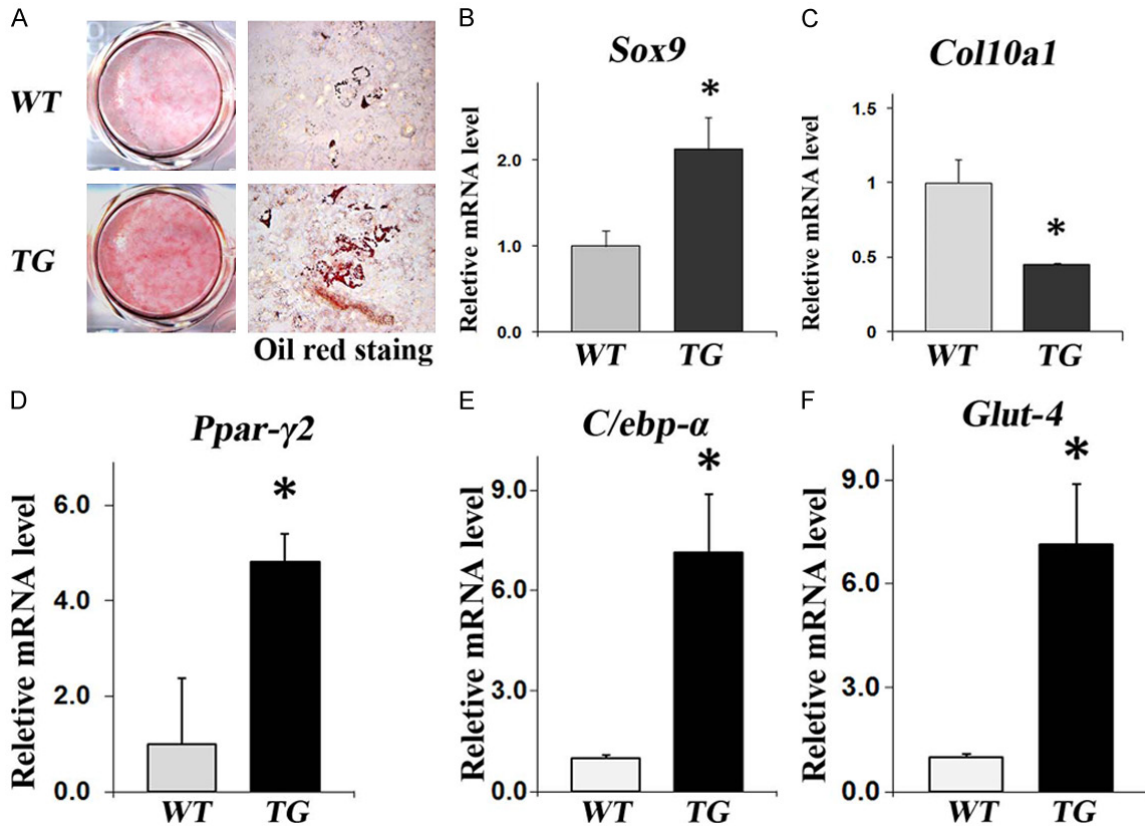


Figure 3. Enhanced adipogenesis in hypertrophic chondrocytes isolated from TG mice in comparison with WT control littermates after nine-days of ex vivo culture. (A) Oil red O staining demonstrates increased adipogenesis in TG mouse hypertrophic chondrocytes after 9-days of ex vivo culture (scale bar = 50 μ m). Real-time RT-PCR analysis demonstrated (B) increased *Sox9* expression, (C) decreased *Col10a1* expression in hypertrophic chondrocytes, (D-F) elevated expressions of key adipogenic markers *Ppar γ 2*, *C/ebp α* and *Glut4* in TG versus WT hypertrophic chondrocytes.

The increased adipogenesis in Col10a1-Sox9 transgenic mouse hypertrophic chondrocytes

Our histological analysis (Figure 2), which demonstrated altered growth plate morphology and reduced mineralization of primary trabeculae, suggests that chondrocyte differentiation may be perturbed in *Col10a1-Sox9* TG mice. Interestingly, a previous study demonstrated a negative correlation between chondrocyte and adipocyte differentiation [36]. Thus, we decided to examine the effect of *Sox9* overexpression on adipocyte differentiation ex vivo. To that end, we performed Oil red O staining on hypertrophic chondrocytes after 9 days of ex vivo culture. Interestingly, we found a much stronger Oil Red O staining in TG chondrocytes compared with WT controls, suggesting that sustained *Sox9* expression results in increased adipogenesis (Figure 3A). Moreover, our real-time RT-PCR analysis in hypertrophic

chondrocytes confirmed that the overexpression of *Sox9* was concomitant with the down-regulated expression of the most specific marker for hypertrophic chondrocytes, *Col10a1* (Figure 3B, 3C). Furthermore, real-time RT-PCR analysis also revealed significantly elevated expressions of adipocyte differentiation markers in TG hypertrophic chondrocyte ex vivo culture, including *Ppar γ 2*, *C/ebp α* , and *Glut4* (Figure 3D-F). Together, these results demonstrate that the sustained *Sox9* expression in hypertrophic chondrocytes enhances adipogenesis ex vivo.

The accelerated loss of cartilage matrix in the growth plates of knee joints in Col10a1-Sox9 transgenic mice

Next, we examined the hind limbs of WT and TG mice by X-ray analysis. Surprisingly, radiographs of six-month-old mice revealed some small

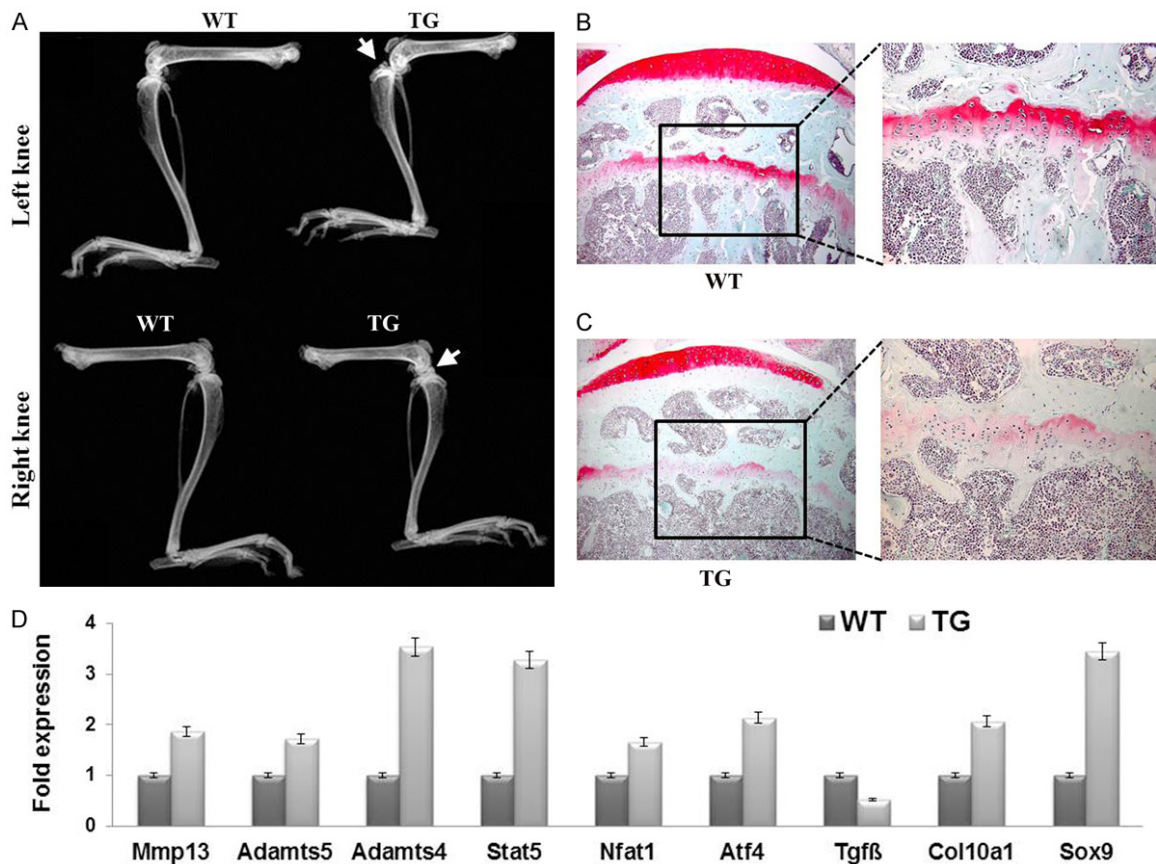


Figure 4. Premature cartilage degradation in the growth plates of knee joints of six-month-old *Col10a1-Sox9* transgenic mice vs. wild-type control littermates. **A.** Radiographs of six-month-old TG and WT mice. White arrows point to osteophytes in the legs of TG mice, which might be associated with the degeneration of cartilage in the TG mice. **B, C.** Safranin O (SO) staining in the growth plates of proximal tibiae of six-month-old WT mice and TG mice. A reduced SO staining in the TG mice suggests the likely early onset of osteoarthritis. The images on the right represent the enlarged boxed areas of the left images (20 \times). **D.** Real-time RT-PCR analysis of the knee chondrocytes isolated from WT and TG mice.

osteophytes at the knee joints in TG mice, but not in WT mice (**Figure 4A**, white arrows). We then further analyzed the knee joints of six-month-old WT and TG mice by Safranin O staining. Interestingly, we found a partial loss of Safranin O staining at the articular surface of TG mouse knees, indicating a reduced amount of articular cartilage in TG mice compared with WT littermates (**Figure 4B** and **4C**, red staining at the top). Real-time RT-PCR analysis revealed significantly elevated expressions of *Mmp13*, *Adm5*, and *Adm4* in the 9 months old TG mice knee chondrocytes. Moreover, pro-inflammatory cytokines including *Stat5*, *Nfat1* and *Atf4* were upregulated in TG mice. As expected the expression levels of *Col10a1* and *Sox9* were increased in the TG mice. Interestingly, *Tgfb β* , which has been shown to boost chondrogenesis, was downregulated in knee chondro-

cytes of the TG mice (**Figure 4D**). These results demonstrate that the increased *Sox9* expression in hypertrophic chondrocytes impaired cartilage homeostasis and led to the likely early onset of osteoarthritis in TG mice at six month-old.

The occurrence of severe osteoarthritis in eleven-month-old Col10a1-Sox9 transgenic mice

At eleven-months of age, 62.5% of *Col10a1-Sox9* TG mice developed spontaneous osteoarthritis (OA) (**Table 2**). In contrast, only 2.5% of WT mice developed spontaneous OA (**Table 2**), indicating that the TG mice had a much higher incidence of spontaneous OA at this stage. Indeed, X-ray analysis of the knee joints of 11-month-old TG mice showed many typical OA features, including narrowed joint space

Table 2. Percentage of OA developed in TG mice compared with WT at eleven-month-old

Genotype	Joints	OA	Percentage
WT	40	1	2.5%
TG	40	25	62.5%

(Figure 5B, black arrow), osteophyte development (Figure 5B, white arrow), and subchondral sclerosis (Figure 5D, asterisk) (Figure 5A-D). Next, the number of osteophytes were quantified as previously described [37]. WT mice do not show the presence of osteophytes, whereas 3 ± 1 osteophytes were observed in the TG mice (Table 3). Furthermore, micro-CT analysis of 11-month-old TG knee joints revealed rough bone surfaces and numerous osteophytes protruding into the joint cavity in TG mice, but not WT mice (Figure 5E, 5F). Next, we further characterized the OA phenotype of TG mice by performing Safranin-O/Fast Green (SO/FG) staining on knee joints of WT and TG mice (Figure 5G-J). SO/FG staining demonstrated that compared with WT controls, there was significant articular cartilage degeneration in TG mice (Figure 5G-J). OARSI Score analysis for the TG mice is two based on the loss of Safranin-O staining and the percentage of the calcified cartilage erosions extending to articular surface (Table 3). It is well-established that OA pathogenesis is associated with ectopic chondrocyte hypertrophy [15]. Thus, we next examined the expression of the most specific hypertrophic chondrocyte marker, Col10a1, in the articular cartilage by immunostaining. Interestingly, we observed high-levels of Col10a1 expression on the articular cartilage surface in the proximal tibiae of TG compared with WT mice (Figure 5K, 5L). Thus, the sustained Sox9 expression in the hypertrophic zone likely leads to ectopic Type X Collagen expression on articular surfaces, which might contribute in part to the OA occurrence in TG mice. Finally immunostaining confirmed elevated Mmp13 expression in the TG mice (Figure 5M, 5N). These data confirm that the overexpression of Sox9 in hypertrophic chondrocytes results in the accelerated, spontaneous development of OA.

Discussion

As the master transcription factor that is essential for chondrocyte differentiation, the expression of Sox9 was significantly lower in hypertrophic

chondrocytes compared with proliferating chondrocytes [23]. Interestingly, it was reported that Sox9 represses *Col10a1*, the most specific marker of hypertrophic chondrocyte differentiation, in proliferating chondrocytes by binding to its upstream elements [38]. On the other hand, Sox9 downregulation in hypertrophic chondrocytes is proposed to be necessary to initiate the cartilage-to-bone transition in the growth plate [39]. Therefore, the precise expressions of Sox9 and *Col10a1* must be tightly controlled in the growth plate to ensure proper chondrocyte differentiation, cartilage formation and endochondral ossification [16]. A previous study has demonstrated that the overexpression of Sox9 in hypertrophic chondrocytes during embryogenesis can lead to impaired terminal chondrocyte differentiation with decreased *Vegf*, *Mmp13*, *Rankl*, and *Opn* expression [27]. Thus, Sox9 is proposed to act as a major negative regulator of cartilage vascularization and trabecular bone formation in the growth plates by suppressing *Vegfa* expression [27]. In another mouse model, Kim and his colleagues demonstrated a delayed terminal chondrocyte differentiation as a result of Sox9 overexpression in hypertrophic chondrocytes [28]. Furthermore, a Sox9 conditional knockout mouse model demonstrated that the proper shutdown of Sox9 expression in hypertrophic chondrocytes is necessary for terminal chondrocyte differentiation and cartilage mineralization [23]. Recently a lineage tracing study revealed that the persistent Sox9 expression in hypertrophic chondrocytes hampers terminal chondrocyte differentiation, possibly by suppressing chondrocyte-to-osteoblast transdifferentiation [39]. Together, all these studies highlight the stage-specific and dose-dependent role of Sox9 during endochondral ossification. However, the phenotype characterization in all previous studies was limited to the embryonic and early postnatal stages, and their implication for adult cartilage homeostasis remains to be determined.

In this report, we examined the consequences of sustained Sox9 overexpression in the hypertrophic chondrocytes of adult mice by characterizing a novel *Col10a1-Sox9* mouse model. Consistent with the well-established role of Sox9 in repressing *Col10a1* expression *in vivo* [17], we observed that an elevated Sox9 expression was concomitant with the downregulation

Sox9 and adult mouse cartilage

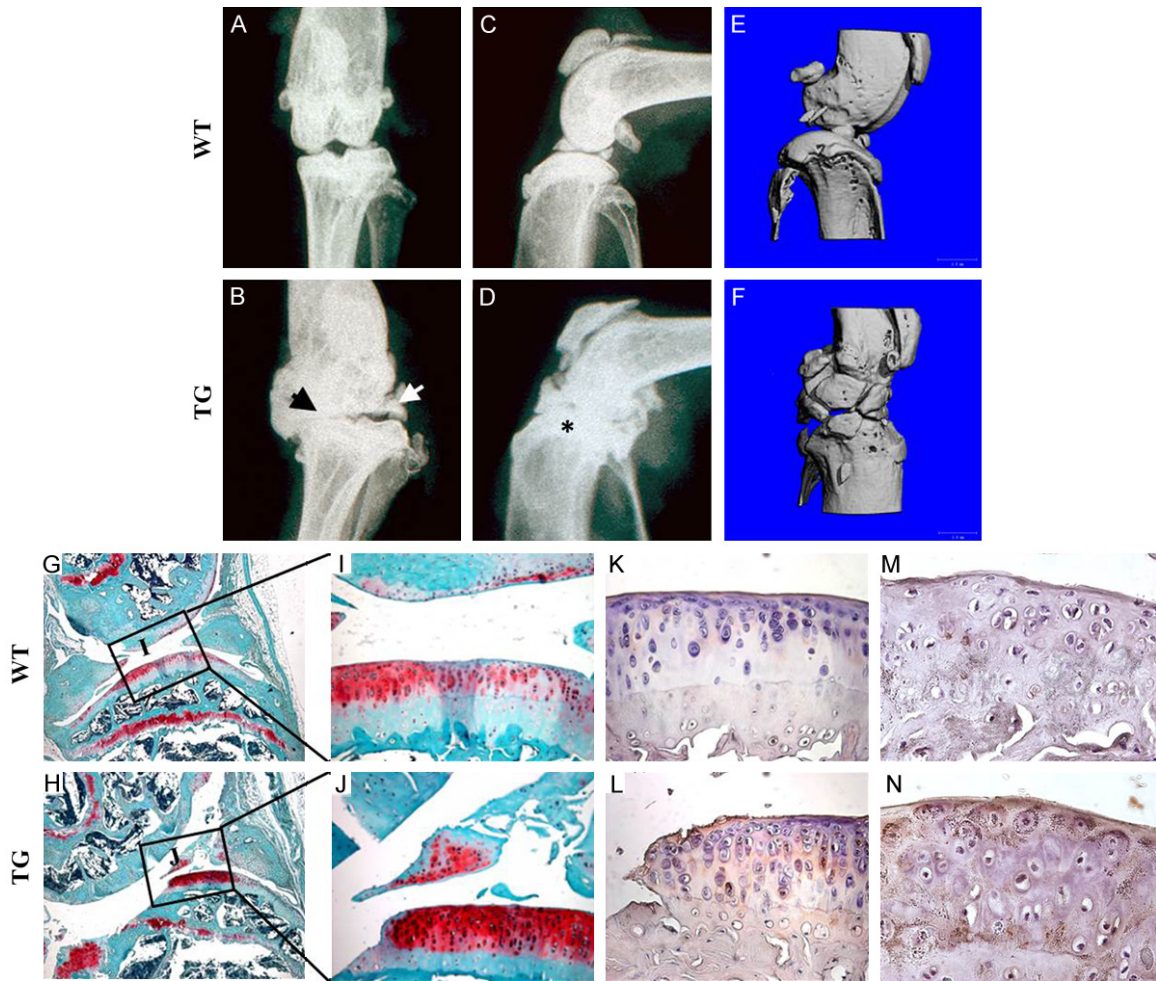


Figure 5. Spontaneous osteoarthritis development in eleven-month-old transgenic mice: (A-D) Radiographs of the knee joints of eleven-month old TG and WT mice. Note the characteristic osteoarthritis features in the TG mice: joint space narrowing (B, black arrow), development of osteophytes (B, white arrow), and sub-chondral sclerosis (D, asterisk). (E, F) The micro-CT analysis of the knee joints of eleven-month-old TG and WT mice revealed rougher bone surfaces and numerous osteophytes protruding into the joint cavity of the TG mice, but not WT mice. (G-J) SO/FG staining of the knee joints of the eleven-month-old TG and WT mice. Note the absence of Safranin-O staining at the articular surfaces in TG mice. (K, L) Type X collagen immunostaining revealed the ectopic Col10a1 expression in the articular cartilages of TG mice, but not of WT mice. (M, N) Immunostaining showed elevated secretion of matrix metalloproteinase-13 (Mmp13) in the articular cartilages of 11 months old TG mice.

Table 3. Osteophyte numbers and the Osteoarthritic Research Society International (OARSI) scores for the eleven-month-old WT and TG mice

Genotype	# of osteophytes	OARSI Score
WT	0	0 Normal cartilage
TG	3±1	2 Loss of Safranin-O Erosion of the calcified cartilage extending to < 25% of the articular surface.

lation of *Col10a1* expression in the hypertrophic chondrocytes of TG mice (Figure 3B, 3C). Interestingly, in a previous model of Sox9 over-

expression in hypertrophic chondrocytes, the mice exhibited dwarfism in the embryonic and early postnatal stages [27]. However, in our TG

mouse model, we did not observe any gross size difference at the early postnatal stage. However, we did observe a decrease in TG skeletal size at 10-weeks of age. Furthermore, 10-week-old TG mice had severe growth plate disorganization and reduced mineralization. These differences between ours and the previous report regarding the effect of Sox9 on the early postnatal stage might be due to the different *Col10a1* cis-elements used to target Sox9 expression, and/or due to the different levels of Sox9 transgene expression.

The transcriptional regulators *C/ebpα* and *Pparγ2* are considered master transcriptional factors required for both *in vivo* and *in vitro* adipogenesis [40]. Interestingly, our study also demonstrated an enhanced expression of *C/ebpα* along with the other key adipogenic markers, including *Pparγ2* and *Glut4*. Furthermore, we observed an elevated adipogenesis in *ex vivo* chondrocyte cultures derived from *Col10a1-Sox9* TG mice compared with WT littermates (**Figure 3**). Sox9 activity affects the expression of the key adipogenic transcription factor *C/ebpβ* in rat bone-marrow-derived mesenchymal stem cells (MSCs). The stable knock-down of Sox9 in rat MSCs resulted in *C/ebpβ* downregulation, which reduced their adipogenic potential [41]. Consistent with previous studies, we noticed that the increased Sox9 expression in hypertrophic chondrocytes results in elevated adipogenesis, reinforcing the importance of Sox9 in promoting adipogenic differentiation. A recent clinical study showed that the ectopic adipogenesis was a common pathological feature of advanced knee OA [42]. Interestingly, we observed OA pathogenesis in our *Col10a1-Sox9* TG mice at six-and eleven-months of age (**Table 2; Figures 4 and 5**). Because chondrocytes are essential for cartilage homeostasis by producing sufficient extracellular matrix in the knee joints, the increased adipogenic propensity of hypertrophic chondrocytes in our TG mice may render them more prone to OA. Thus, the potential link between increased adipogenesis and increased OA occurrences remains to be further determined.

Moreover, the dietary intervention also needs to be further investigated to reduce the risk of OA. It is important to note that OA occurred spontaneously and developed progressively in our *Col10a1-Sox9* TG mice, which recapitulates the natural course of OA progression to some

extent. Thus, our novel *Col10a1-Sox9* TG mouse model might be suitable for studying the natural course of OA pathogenesis *in vivo*.

In summary, our work demonstrates that sustained Sox9 expression in hypertrophic chondrocytes results in spontaneous OA. OA is a chronic disease with limited treatment options besides complete knee replacement and it is a huge health and financial burden for the general public [43]. One proposed strategy for improving cartilage tissue engineering and regenerative medicine to treat OA is the transduction of Sox9 into chondrocytes or mesenchymal stem cells to induce cartilage formation to reverse articular cartilage degeneration and to improve fracture healing [44]. Nevertheless, the long-term consequence of the Sox9 overexpression in the cartilage of animal models is still largely unknown. Our study cautions that the Sox9 expression has to be tightly controlled not only during skeletogenesis, but also at the adult stage, to ensure proper cartilage homeostasis. Thus, it is important to carefully design Sox9 overexpression experiments in cartilage tissue engineering and regenerative biology, especially when longer *Col10a1* promoter-intron elements are used for hypertrophic chondrocyte-specific expression of target genes as described recently [45]. In conclusion, we provide strong evidence for the crucial roles of Sox9 in adult hypertrophic cartilage homeostasis and OA pathogenesis. Moreover, this study has important implications for the early diagnosis of OA and for skeletal regenerative treatments in patients who might have abnormal and weakened hypertrophic cartilage.

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Disclosure of conflict of interest

None.

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