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Mice over-expressing salmon calcitonin have strongly attenuated osteoarthritic histopathological changes after destabilization of the medial meniscus

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SUMMARY

Objective: Calcitonin is well-known for its inhibitory actions on bone-resorbing osteoclasts and recently potential beneficial effects on cartilage were shown. We investigated effects of salmon calcitonin (sCT) on the articular cartilage and bone, after destabilization of the medial meniscus (DMM) in normal and sCT over-expressing mice.

Design: Bone phenotype of transgenic (TG) C57Bl/6 mice over-expressing sCT at 6 months and 12 months was investigated by (1) serum osteocalcin and urinary deoxypyridinoline and (2) dynamic and normal histomorphometry of vertebrae bodies. In subsequent evaluation of cartilage and subchondral bone changes, 44 10-week old TG or wild-type (WT) mice were randomized into four groups and subjected to DMM or sham-operations. After 7 weeks animals were sacrificed, and knee joints were isolated for histological analysis.

Results: Trabecular bone volume (BV/TV) increased 150% after 6 months and 300% after 12 months in sCT-expressing mice when compared to WT controls ($P < 0.05$). Osteoblast number, bone formation rate and osteocalcin measurements were not affected in TG mice over-expressing sCT. In WT animals, a 5-fold increase in the quantitative erosion index was observed after DMM, and the semi-quantitative OARSI score showed over 400% ($P < 0.001$) increase, compared to sham-operated WT mice. DMM-operated TG mice were protected against cartilage erosion and showed a 65% and 64% ($P < 0.001$) reduction, respectively, for the two histopathological evaluation methods.

Conclusions: sCT over-expressing mice had higher bone volume, and were protected against cartilage erosion. These data suggest that increased levels of sCT may hamper the pathogenesis of osteoarthritis (OA). However more studies are necessary to confirm these preliminary results.

Introduction

Hallmarks of osteoarthritis (OA) are progressive degeneration of articular cartilage, changes in the subchondral bone compartment, and subsequent joint space narrowing. Experimental and clinical observations suggest that the structural integrity of articular cartilage is dependent on normal subchondral bone turnover, intact chondrocyte function and ordinary biomechanical stresses[1,2]. Because there is a strong inter-relationship between the subchondral bone and the articular cartilage, an ideal therapeutic agent might logically be directed at regulating the metabolic activity of both bone and cartilage.

Calcitonin is a natural peptide hormone produced by parafollicular C-cells in the thyroid gland[3,4]. Calcitonin was discovered more than 40 years ago[5], and possesses potent anti-resorptive effects, which by Chambers *et al.* was shown to be mediated by direct binding of calcitonin to the calcitonin receptor (CTR) on the osteoclasts[6]. Recently, much attention has been drawn to the suggested effect of calcitonin on chondrocytes in addition to that on osteoclasts[7–9]. A range of studies has documented that salmon calcitonin (sCT) is associated with a shift in chondrocyte phenotype from a catabolic to an anabolic state. The initial study by Hellio *et al.* demonstrated that calcitonin dose-dependently inhibited collagenase and phospholipase A2 activity in isolated human OA articular chondrocytes cultured *in vitro*[10]. Others studies have shown anabolic effects of sCT under various settings in different types of

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articular cartilage[11–13]. Interestingly, in other experimental settings, calcitonin has been shown to stimulate growth and maturation of hypertrophic chondrocytes and cartilage matrix formation in pelvic cartilage from avian embryos[14,15]. Subsequently, the CTR in articular chondrocytes was identified[9] which may, in part, explain some of these pharmacological effects.

The effect of calcitonin has also been investigated in various *in vivo* models of cartilage degradation[9,16–21]. These principal experiments have provided important evidence for beneficial effects of calcitonin on both bone and cartilage by inhibiting the progression of OA, thus emphasizing calcitonin as a potential dual action treatment of OA[8]. However, all these experiments have been performed with either a nasal or oral pharmacological formulation, in which exposure was not reported. The effect of systemic continuous exposure has not been investigated. This is a critical distinction in the case of parathyroid hormone (PTH), which displays anabolic effects on bone when given intermittently, but catabolic and bone degrading effects with sustained increased exposure[22]. As both the PTH receptor and the CTR are type II G protein-coupled receptors, it is highly relevant to investigate how exposure patterns may result in divergent pharmacological effects, or whether exposure is directly proportional to efficacy. Furthermore, loss of functionality might be observed, as the CTR is known to be internalized after prolonged stimulation *in vitro*[23].

This study was performed to describe the bone phenotype and histomorphometric characteristics of the sCT over-expressing transgenic (TG) mice and to assess the effects on subchondral bone and articular cartilage of continuous systemic exposure to sCT in an OA pre-clinical model. Mice over-expressing sCT were bred and subjected to destabilization of the medial meniscus (DMM), which has previously been used as an appropriate traumatic OA model[24,25] and in pre-clinical murine models of OA[26–28].

Methods

Generation of TG mice

Mice over-expressing sCT, hereafter referred to as *ApoE-sCT* mice, were generated by injection of a construct into blastocysts of C57Bl/6 mice, where the complete open reading frame (ORF) encoding sCT was placed under the control of the apolipoprotein E (*ApoE*) promoter and liver-specific enhancer[29]. To ensure proteolytic processing by furin-like proteases and C-terminal amidation by peptidylglycine alpha-amidating monooxygenase two mutations were introduced into the sCT-encoding cDNA according to Takahashi *et al.*[30]

Of the TG mice bred using this methodology, six animals aged 6 months and a further six aged 12 months were used for the following investigations. Genotyping was performed by Southern Blotting using the SV40-pA as a probe. The same probe was used to monitor expression of the transgene in various tissues by Northern Blotting. Circulating serum levels of sCT were determined by ELISA (Diagnostic Systems Laboratories, Webster, Texas, US) and the endogenous mouse calcitonin levels were measured using radioimmunoassay (Immutopics, San Clemente, CA, US). Ethical Approval was obtained from the Amt für Gesundheit und Verbraucherschutz (09/03), Germany.

Biochemicalmarkers of bone metabolism

Serum concentrations of osteocalcin were quantified using radioimmunoassay (Immutopics, San Clemente, CA, US). To quantify osteoclastic bone resorption, we measured the urinary excretion of deoxypyridinoline (Dpd) cross-links using the Pyrilinks-D ELISA (Metra Biosystems, Santa Clara, CA, US). Values are expressed relative to creatinine concentrations as determined by a standardized colorimetric assay using alkaline picrate (Metra Biosystems, Santa Clara, CA). To rule out that observed differences are caused by diurnal variations, mice were generally sacrificed between 10 AM and noon after fasting.

Bone histomorphometry

Mice were sacrificed at 6 and 12 months of age, and the dissected skeletons were fixed in 3.7% PBS-buffered formaldehyde for 18 h, before they were stored in 80% ethanol. All skeletons were first analyzed by contact radiography using a Faxitron X-Ray cabinet (Faxitron X-Ray Corp., USA). For histology, the lumbar vertebral bodies L1 to L4 and one tibia of each mouse were dehydrated in ascending alcohol concentrations and then embedded in methylmetacrylate as described previously[31]. Sections of 4 μm thicknesses were cut in the sagittal plane on a Microtec rotation microtome (Techno-Med GmbH, Germany). These were stained by toluidine blue and von Kossa/van Gieson-staining procedures as described[31]. Histomorphometry, including the determination of cortical thickness, was performed according to the ASBMR guidelines using the OsteoMeasure histomorphometry system (Osteometrics Inc., USA)[32]. All parameters of static, cellular and dynamic histomorphometry were measured in two vertebral bodies (L3 and L4) for each animal, and the mean value was used for statistical analysis. Fluorochrome measurements for the determination of the bone formation rate were performed on two non-consecutive 12 μm-sections for each animal.

DMM model

Animals, housing, diet

The animals were housed in cages placed in Scantainer-plus both from Scanbur (Karlslunde, DK) at approximately 21–23°C and 55–65% relative humidity, and a 12 h light/dark cycle was maintained. Each individual cage had 530 cm^2 floor area, 1284 L EU standard type II L (Scanbur, Karlslunde, DK) with Tapvei 4HV bedding and as nest material Enviro-Dri (Brogaarden, Lynge, DK) along with two paper tissues and all cages were further equipped with a triangular red colored plastic mouse house (Tecniplast, Buguggiate, IT) and a $10 \times 10 \times 50$ mm aspen wood gnawing stick (Brogaarden, Lynge, DK). Only mice from the same litter of the same sex were housed in each cage, and maximum four male mice per cage. Food and water intake was allowed *ad libitum*, Altromin 1324 and 5–7 dried corn per mouse per week (Brogaarden, Lynge, DK). The animal welfare was checked daily, and the cages were cleaned once a week.

Microsurgery and study design

The experiment was conducted in parallel as the mice were born and the mice were weaned after 3 weeks, tagged, and blood samples were collected for genotyping, please see Fig. 1. The animals were randomly allocated to either the sham or DMM group. Prior to DMM or sham surgery, circulating serum levels of sCT were measured by an in-house biochemical ELISA to distinguish *ApoE-sCT* mice from wild-type (WT). Microsurgery was performed to introduce joint instability by DMM, using methodology previously described by Glasson *et al.*, 2007[24] or a sham operation in *ApoE-sCT* mice and controls. The experiment was approved by the ethics committee of the Danish Ministry of Justice and conducted in accordance with the European Standard for Good Clinical Practice (2008/561-1450). With mice under isofluran/CO_2 anesthesia, the knee joints were shaved free of fur, cleaned with chlorhexidin, and 2–4 drops of local anesthesia Xylocain were added

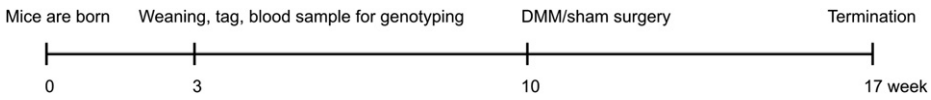

Fig. 1. Timeline and design of the experimental study.

before the joints were opened using a scalpel. The medial meniscotibial ligament was transected where it anchors to the anterior tibial plateau using a syringe needle under a microscope, and the meniscus body was left intact to move freely. Please see Fig. 2 for an illustration of the mouse joint. The wound was stitched by 0/8 vicryl suture and the skin closed by 3M Vetbond tissue adhesive (3M, Minnesota, US). The mice received Rimadyl® (carprofen) for pain relief after the operation and the two following days and the mice were observed daily. The DMM operation was performed bilaterally to avoid compensatory locomotion from the mouse.

A total of 44 male and female mice at 10 weeks of age were operated on as follows: (1) WT sham-operated ($n = 11$), (2) WT DMM ($n = 11$), (3) *ApoE-sCT* sham-operated ($n = 10$), (4) *ApoE-sCT* DMM ($n = 11$). One mouse in the *ApoE-sCT* sham group died during the study period. The distributions of male and female mice were 50% in each group. Seven weeks after surgery, all animals were terminated. The right knee joint was collected for histological purposes and fixed in 4% formaldehyde.

Histology

The formaldehyde-fixed knee joints were subsequently decalcified in 15% ethylenediaminetetraacetic acid (EDTA) and 0.3% formaldehyde and paraffin-embedded. The joints were sliced into 5 μm sections from the frontal plane towards the back and mounted on Superfrost plus glass (Thermo Scientific, Rockford, IL, US). After deparaffinization, all the serial sections were stained using Safranin'O and Fast green. Subsequently, the sections were dehydrated and mounted with Pertex and a covering glass. All sections were examined microscopically and the single section used for histological scoring representing the central weight-bearing region of the tibial plateau was selected based on the presence of the cruciate ligament and anatomy of the lateral meniscus. Histographs were captured on a 60× Olympus microscope equipped with a camera at 2× magnification.

Scoring of cartilage erosion

The same observer scored cartilage erosion and was blinded from treatment codes. For the quantitative erosion index, the length of eroded articular cartilage surface was measured and divided by the total length of the articular cartilage surface. The index has previously been validated as a measure of OA progression, although this was in the ovariectomized (OVX) rat model[33]. Additionally, the newly developed semi-quantitative scoring system for murine OA characteristics[34], the OARSI score (Table I), was applied. The two applied scores, the erosion index and OARSI score were used for the evaluation of all four condyles of the knee joint: medial tibial plateau, medial femoral condyle, lateral tibial plateau, and lateral femoral condyle, see Fig. 2.

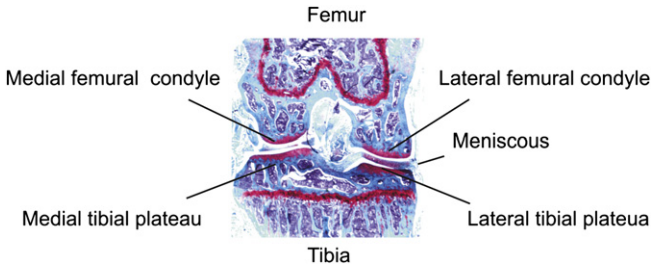

Fig. 2. The anatomy of a mouse joint. Histological section of a mouse knee joint stained by Safranin'O and Fast green.

Statistics

The effect of operation and sCT on cartilage erosion was statistically analyzed using a two-tailed non-parametric Mann–Whitney *U* test in the GraphPad Prism program. Only independent observations were used.

Results

Characterization of the ApoE-sCT mice

TG mice were generated to express bioactive sCT under the control of the *ApoE* promoter and liver-specific enhancer, as shown in [Fig. 3(A)]. The initial characterization of the TG mice by Northern blotting using the SV40-pA probe revealed that the gene was only expressed in the liver [Fig. 3(B)]. The circulating levels of sCT in the TG mice were in the range of 800 pg/mL, about eight-fold higher than the mouse calcitonin levels in WT mice [Fig. 3(C)].

Increased bone mass in ApoE-sCT mice

Since one of the well-established effects of calcitonin is its inhibitory action on bone-resorbing osteoclasts[6], an extensive bone histomorphometric analysis was performed to establish the bone phenotype of the TG mice. Figure 4(A) shows the non-decalcified von Kossa/van Gieson-stained bone sections of the vertebral bodies, and Fig. 4(B) shows the results of the histomorphometric quantification. The trabecular bone volume (BV/TV) of TG mice was more than 100% greater than that of WT mice ($P < 0.05$), at the ages of 6 and 12 months. Additionally, the trabecular number (Tb.N.) and thickness was increased in the TG mice compared to WT ($P < 0.05$). Accordingly, the trabecular spacing was significantly decreased by 50% and 70%, respectively.

As expected, histomorphometry analysis in mice aged 6 months revealed a significantly decreased osteoclast number but normal

Table I
The OARSI scoring system for murine OA[34]

Grade	Osteoarthritic damage
0	Normal
0.5	Loss of Safranin'O without structural changes
1	Small fibrillations without loss of cartilage
2	Fibrillation down to the layer immediately below the superficial layer and some loss of surface lamina
3	Fibrillation/erosion to the calcified cartilage extending to <25% of the width of articular surface
4	Fibrillation/erosion to the calcified cartilage extending to 25–50% of the width of articular surface
5	Fibrillation/erosion to the calcified cartilage extending to 50–75% of the width of articular surface
6	Fibrillation/erosion to the calcified cartilage extending >75% of the articular surface

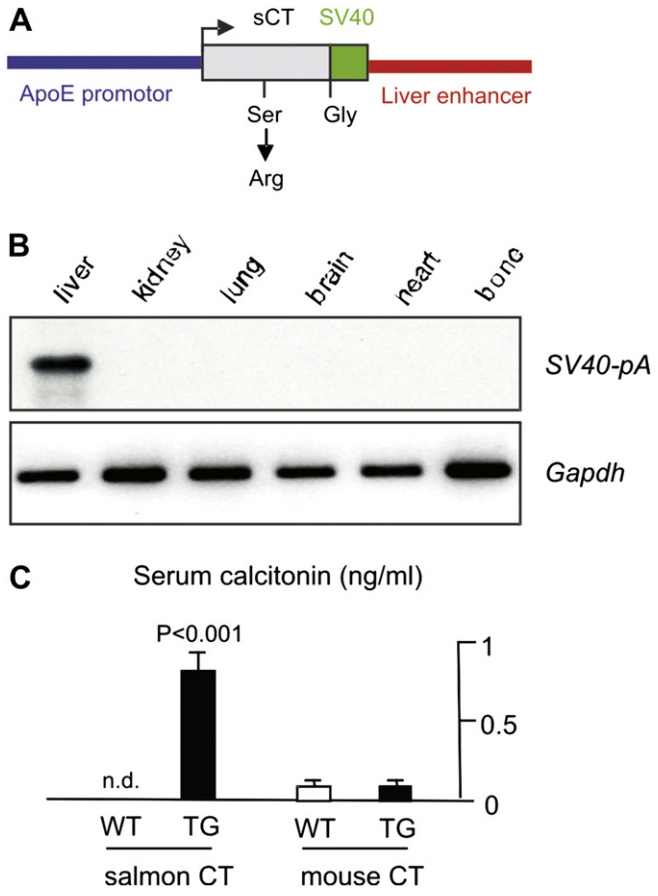

Fig. 3. Generation of *ApoE-sCT* TG mice. (A) Schematic representation of the injected sCT construct. (B) Northern Blot expression analysis with the SV40-pA probe revealed liver-specific expression. (C) ELISA determination of sCT and endogenous mouse CT serum concentrations in TG and WT mice. Not detectable, n.d.

bone formation rate (Table II). As presented in Table II, histomorphometric quantification of the osteoblast number (NOb/BPm) and function (BFR/BS), together with the determination of serum osteocalcin, revealed that bone formation was not affected in *ApoE-sCT* TG mice. In contrast, a decreased number of osteoclasts (NOc/BPm) and a reduced level of urinary Dpd cross-links, used as a bone degradation biochemical marker, were observed when the values were normalized to the increased bone mass.

ApoE-sCT mice are protected against OA progression

Pictures of representative sections of Safranin'O stained knee joints were used for histopathological evaluation [Fig. 5(A)]. The erosion index, in which the length of the eroded surface was divided by the total length of the articular surface, showed WT animals undergoing DMM had massive proteoglycan loss. Their erosion index was 5-fold higher ($P < 0.001$) than that of sham-operated controls. The DMM-operated *ApoE-sCT* TG mice, however, showed a 65% reduction in erosion, $P < 0.001$, compared to DMM-operated WT [Fig. 5(B)].

The OARSI semi-quantitative scoring system for murine OA models showed that in WT animals, DMM resulted in a 400% ($P < 0.001$) increase in histopathological changes compared to sham-operated mice [Fig. 5(C)]. For the DMM-operated TG mice, only a 20% non-significant increase in OARSI score was observed compared to TG sham-operated. However, in DMM-operated TG mice, histopathological changes was 64% less ($P < 0.001$) than in the DMM WT group.

Discussion

At present there are no disease-modifying drugs available for OA, although a number of compounds are currently under clinical investigation[35]. These compounds include aggrecanase inhibitors, interleukin-1 (IL-1) antagonists, selective estrogen-receptor modulators (SERMs), inducible nitric oxide synthase (iNOS) inhibitors, tissue inhibitor of metalloproteinases (TIMPs), and various MMP-13 inhibitors. This list gives an indication of the difficulty in finding an effective treatment.

Complicating matters further, is the emerging understanding that different forms and stages of OA are present[36], each with its own distinct pattern of initiation and progression. Consequently, one treatment may work in one stage of the disease but not in another. This highlights the need for testing possible intervention strategies in a range of animal models reflecting the different forms and stages of OA.

In this study we used TG mice to investigate the effect of systemic over-expression of sCT on bone remodeling and cartilage degradation as a function of traumatic induction of OA. Analyses of bones from TG, but non-operated, mice at both 6 and 12 months of age clearly showed increased bone volume in the trabecular bone. Histomorphometry confirmed that this was due to a reduction in osteoclast function, a finding which is in alignment with previous studies showing that sCT reduces bone resorption[6]. The previous data indicated that osteoclast numbers were reduced, a finding which was surprising, yet can be explained by a recent study showing that calcitonin reduces osteoclastogenesis in mouse osteoclasts[37]. Interestingly, no changes in bone formation were observed in the TG mice, despite the suppression of osteoclast function. We hypothesize that the induction of sclerostin expression by sCT induced in young rats, leading to reduction of bone formation, does not occur in these mice[38]. Although this will have to be investigated in more detail, there are also indications from human trials that bone formation is unaffected by sCT treatment, and thus calcitonin will not fall into the category of molecules showing a secondary inhibition of bone formation[39,40].

In the present study, we used the DMM model in mice since murine knees develop OA in a process highly similar to that in humans[34] and the model previously has been used to demonstrate the importance of ADAM-TS5 in murine OA[27]. We used animals of 10 weeks of age, having the operation for 7 weeks and in the model described by Glasson *et al.*, 2007 the mice is terminated 8 weeks post surgery[24]. While bone natural growth and development at that age may have influenced the OA parameters, we nonetheless clearly demonstrated that there was a significant reduction in cartilage damage in TG *ApoE-sCT* mice, compared to WT, following DMM. The reduction we found was in a comparable range of protection as previously observed using the DMM model in ADAM-TS5 deficient mice[27]. Previous studies using the DMM model have showed that male mice developed OA more consistently than female mice[25]. For the presented DMM study both female and male mice were included in all groups. We observed no difference of female or male mice in disease progression or severity, as previously has been described. It might be explained by the housing of littermates and the environmental enrichments described in the Methods section, which may have minimized the fighting incidences in the male housing groups.

A limitation of the study is that only one section from each animal was used for the assessment of the histopathological changes. The basis of the particular section was chosen from evaluation of all sections retrieved from the mouse joint as being the at the most

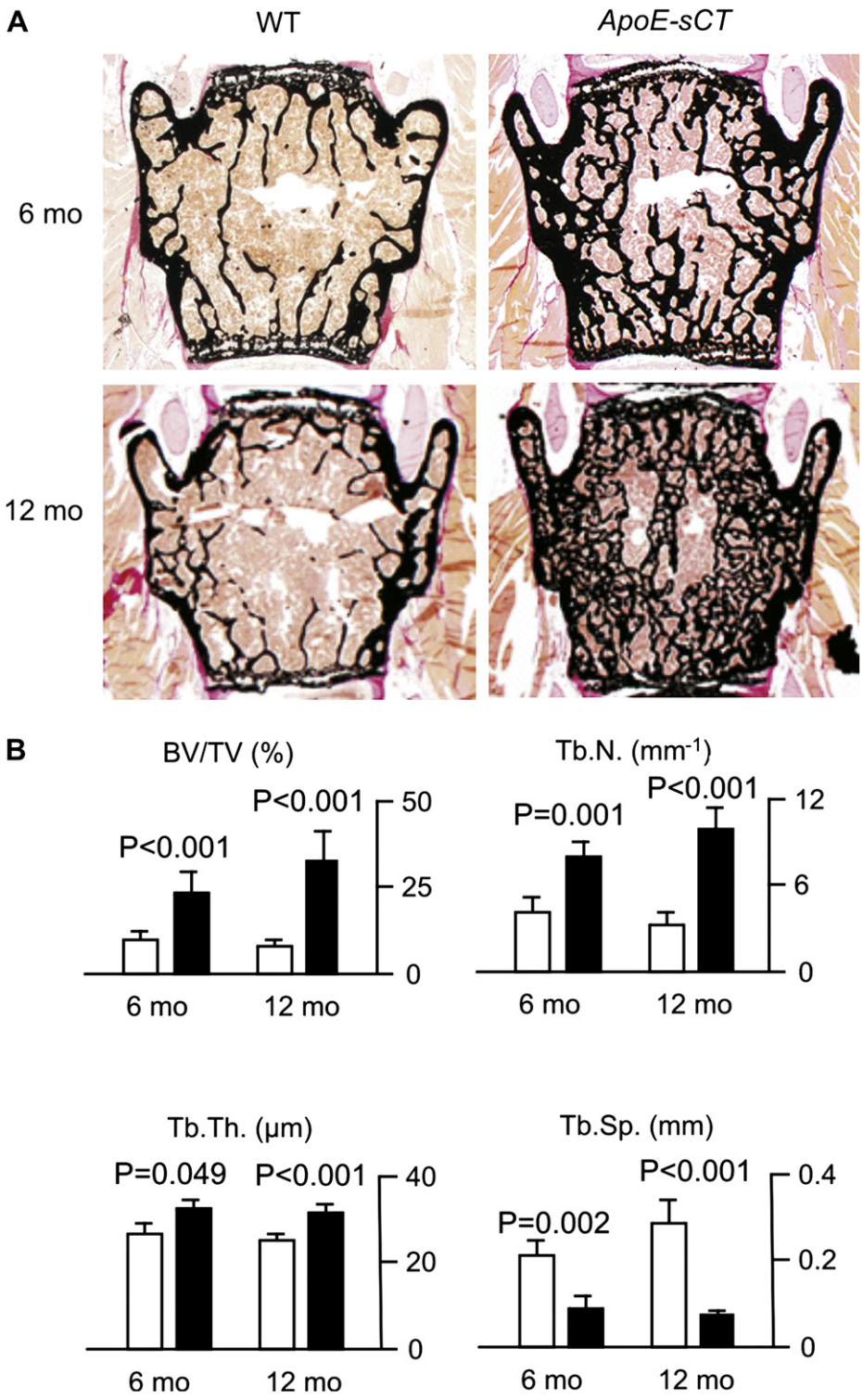

Fig. 4. Increased bone mass in TG mice over-expressing sCT. (A) von Kossa/van Gieson-staining of non-decalcified sections from vertebral bodies in *ApoE-sCT* TG mice at 6 and 12 months of age. Pictures were taken with a 12.5-fold magnification. (B) Quantification of the BV/TV, Tb.N., trabecular thickness (Tb.Th.) and trabecular separation (Tb.Sp.) by static histomorphometry. Bars represent Mean ± SD ($n = 6$).

weight-bearing region, and the section which had the anatomical landmarks as described in the methods section. The OARSI guidelines recommend scoring of multiple sections. The histopathological evaluations of the histological sections were carried out by a blinded observer, which may be considered biased to the transection of the menisci in the knee joints. As it can be seen in Fig. 5(A) the menisci was not entirely eliminated but simply transected and still present in the knee. Besides, in sections from sham animas the menisci were sometimes absent due to the quality of the sectioning. Furthermore advantages of the applied scores are that they primarily are quantitative and not biased by subjective qualitative quantifications. The erosion index is entirely quantitative.

Table II
Characterization of bone phenotype in 6 months old mice

	WT ($n = 6$)	*ApoE-sCT* ($n = 6$)	*P*-value
NOb/BPm [mm^{-1}]	24.81 (23.07–26.54)	22.62 (20.47–24.77)	0.073
NOc/BPm [mm^{-1}]	1.10 (0.99–1.21)	0.65 (0.5–0.8)	$P < 0.001$
BRF/BS [$\mu m^3/\mu m^2/y$]	187.85 (157.2–218.5)	183.69 (141.2–226.2)	0.843
Serum osteocalcin [μg/mL]	0.111 (0.088–0.134)	0.104 (0.078–0.130)	0.615
Dpd/Creatinie [nM/nM]	16.0 (13.02–18.88)	17.9 (13.47–22.36)	0.370
Relative resorption [ratio Dpd/BV]	1.36 (0.92–1.80)	0.69 (0.51–0.86)	$P < 0.01$

Values are reported as mean (CI 95%), *ApoE-sCT*: TG mice, NOb/BPm: number of osteoblasts/bone perimeter, NOc/BPm: number of osteoclasts/bone perimeter, BRF/BS: bone function, Dpd: urinary Dpd cross-links, BV: bone volume. *P*-values, WT vs *ApoE-sCT* TG mice.

The anatomy of the mouse knee resembles that of other species and is only notably different from other mammals by its extreme miniaturization, the cartilage layer being only 30 μm thick. The pathology of cartilage degeneration tends to progress rapidly from: (1) a loss of proteoglycan, to (2) superficial degradation, then (3) loss of non-calcified tissue extending to regions of full-thickness loss of non-calcified tissue[34].

It is well-known that OA is a highly heterogeneous phenomenon[41], and that there are both metabolic causes, such as the menopause, and traumatic causes such as meniscal tears. This heterogeneity will most likely influence the response to potential treatments of OA[41]. Interestingly, sCT has previously been tested in both traumatic[16,18,19] and non-traumatic animal models[21,42], where it, independent of OA model, has shown promise in preventing the development of OA.

An interesting aspect of treatment of OA with calcitonin is the potential effect on subchondral bone. It is well-described that accelerated turnover of this bone, i.e., due to menopause, is involved in the pathogenesis of OA, ultimately resulting in sclerosis[2,43–47]. The interest in the subchondral bone was further increased by a recent publication showing that bisphosphonate treatment, which is known to strongly reduce bone turnover[22], prevented cartilage loss, and importantly also reduces pain in the affected joints[48]. These data implicate the subchondral bone as an important part of the bone pain sensation. Since sCT has been shown to have musculoskeletal and bone pain-relieving effects[49] in patient with OA[39,50], these data suggest that calcitonin through several different mechanisms may provide benefits in OA patients.

The present study demonstrated that TG mice over-expressing sCT had higher bone volume, and were protected against cartilage damage associated with a traumatic induction of OA. Thus sCT in this model demonstrated dual activity in protecting both bone and cartilage. The precise mode of action remains yet to be fully understood but may be caused by combined effects on bone and cartilage cells. Validation in long-term clinical settings is needed to fully assess the potential utility of sCT in treating OA.

Author contribution

BCS performed the DMM experiments and drafted the manuscript, while PCL performed the histomorphometric analysis of the bone phenotype. SS and MH generated the mouse model, while AH assisted the characterization of their skeletal phenotype. TS and MA designed experiments and assisted the interpretation of data. RHN assisted the DMM animal trial and AJ and KH assisted the interpretation of data and drafting the manuscript and MK design the DMM experiment. MK owns stocks in Nordic Bioscience.

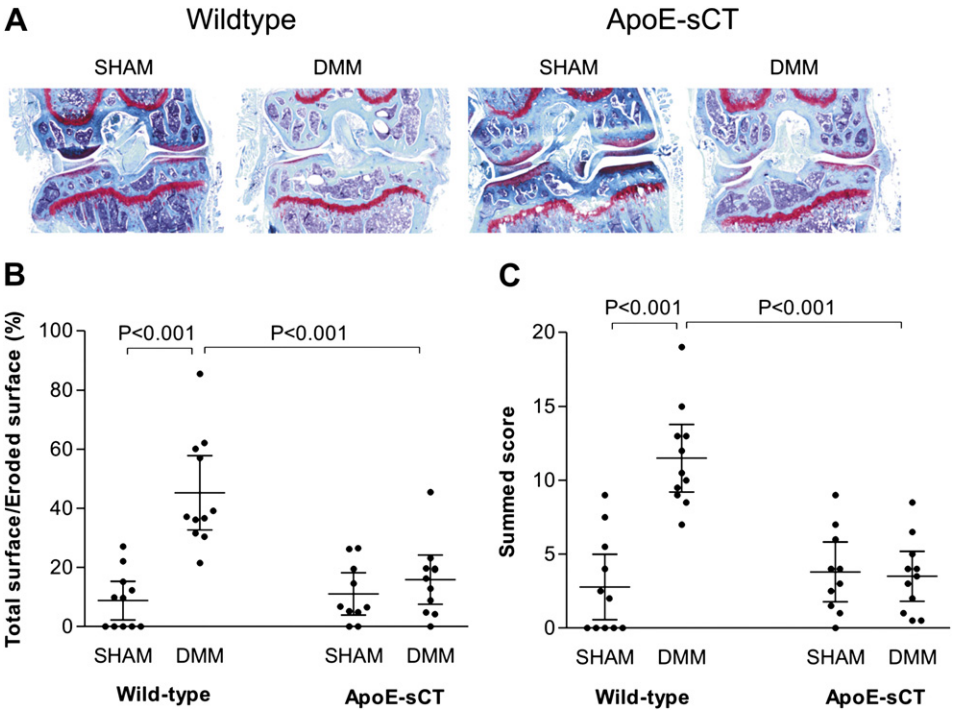

Fig. 5. *ApoE-sCT* mice are protected against OA progression. (A) Histological knee joint sections stained using Safranin'O and Fast green. Quantification of articular cartilage erosion in WT and *ApoE-sCT* TG mice using, subjected to either sham or DMM at 10 week of age until 17 weeks of age. WT sham-operated ($n = 11$), WT DMM ($n = 11$), *ApoE-sCT* sham-operated ($n = 10$), *ApoE-sCT* DMM ($n = 11$). All four condyles were evaluated for quantification of erosion and histopathological changes (B) an established erosion index score, sum of eroded length/sum of total length from all condyle surfaces (%) and (C) the newly developed OARSI scoring system applied to describe histopathological changes in the model. The error bars are mean ± 95% confidence intervals.

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Conflict of interest statement
MK owns stocks in Nordic Bioscience. All other authors have no competing interests to declare.

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