

Deletion of Cx43 from Osteocytes Results in Defective Bone Material Properties but Does Not Decrease Extrinsic Strength in Cortical Bone

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Abstract Deletion of connexin (Cx) 43 from osteoblasts and osteocytes (OCN-Cre;Cx43^{fl/-} mice) or from osteocytes only (DMP1-8kb-Cre;Cx43^{fl/fl} mice) results in increased cortical, but not cancellous, osteocyte apoptosis and widening of the femoral midshaft without changes in cortical thickness. Despite the consequent larger moment of inertia, stiffness and ultimate load, measures of mechanical strength assessed by three-point bending, are not higher in either model of Cx43 deficiency due to reduced Young's modulus, a measure of the stiffness of the material per unit of area. In OCN-Cre;Cx43^{fl/-} mice, this was accompanied by a reduced ratio of nonreducible/reducible collagen cross-links as assessed by Fourier transformed infrared imaging (FTIRI) in the femoral diaphysis. On the other hand, DMP1-8kb-Cre;Cx43^{fl/fl} mice did not show a significant reduction in collagen maturation in the same skeletal site, but a small decrease in mineralization was detected by FTIRI. Remarkably, both

osteoblastic and osteocytic cells lacking Cx43 expressed lower mRNA levels of lysyl oxidase, a crucial enzyme involved in collagen maturation. These findings suggest that Cx43 expression in osteoblasts is involved in maintaining the quality of the bone matrix in cortical bone through the maturation of collagen cross-links. Osteocytic Cx43 expression is important also to maintain the stiffness of the bone material, where Cx43 deficiency results in local reduction in mineralization, possibly due to osteocyte apoptosis.

Keywords Connexin 43 · Young's modulus · FTIR · Osteocyte · Osteoblast · Lox

Introduction

Bone tissue homeostasis is guaranteed by intercellular communication among osteoclasts, osteoblasts, and osteocytes. Important mediators of this function are gap junctions, channels that allow coupling between neighboring cells through the passage of small molecules [1]. The most abundant gap junction protein in osteoblastic cells is connexin 43 (Cx43) [2]. Gap junctions result from the juxtaposition of two hexameric hemichannels formed by Cx43, one contributed by each coupled cell. Hemichannels can also function independently of gap junctions, by mediating the exchange of small signaling molecules between the cytoplasm and the extracellular space, in bone and other tissues [3–6].

Cx43-mediated functions may be involved in cell survival or death, depending on the cell type under investigation and the intracellular pathways activated [7]. We have shown that Cx43 is required for the preservation of osteoblast and osteocyte viability by bisphosphonates in

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vitro and in vivo [6, 8, 9]. The carboxy terminus of Cx43 also functions as a scaffold that interacts with β -arrestin and other proteins; we have shown that this function of Cx43 is specifically required for osteoblast survival induced by parathyroid hormone (PTH) [10]. The importance of Cx43 for the response of osteocytes to pro-survival stimuli is supported by the finding that osteocytes lacking Cx43 exhibit increased apoptosis in vitro and in vivo [11]. Moreover, recent evidence indicates that osteocyte survival is critical for the maintenance of bone tissue homeostasis. In particular, osteocyte apoptosis has been suggested as the trigger for targeted bone remodeling upon immobilization, fatigue loading, or ovariectomy [12–14].

We previously reported that OCN-Cre;Cx43^{fl/-} mice, lacking Cx43 in osteoblasts and osteocytes [8], and DMP1-8kb-Cre;Cx43^{fl/fl} mice, lacking Cx43 only in osteocytes [11], exhibit increased prevalence of apoptosis of osteocytes, but not osteoblasts, selectively in the cortical compartment.

In this study, we investigated whether osteocyte apoptosis is also associated with decreased strength of the bone material. This connection has been previously proposed in a mouse model of glucocorticoid (GC)-induced bone loss. Specifically, in that study, blockage of GC action selectively in osteoblasts and osteocytes protected these cells from GC-induced apoptosis and maintained the strength of the bones in vivo in mice [15]. Moreover, lack of Cx43 from chondro-osteoprogenitors and osteoblasts has been shown to reduce bone strength [16, 17].

Here, we show that the increased apoptosis in the absence of osteocytic Cx43 is indeed accompanied by decreased stiffness of the bone material in the femoral diaphysis. Moreover, we found that Cx43 deletion in osteoblastic and osteocytic cells in vitro reduces the levels of lysyl oxidase, a crucial enzyme for collagen cross-linking. Thus, the decreased strength of the bone material observed in vivo in mice lacking Cx43 could be due to increased osteocyte apoptosis [11], consistent with a role of osteocyte survival in the maintenance of skeletal integrity, and to reduced levels of lysyl oxidase (Lox).

Materials and Methods

Mice

Mice with deletion of Cx43 from osteoblasts and osteocytes (OCN-Cre;Cx43^{fl/-}) or exclusively from osteocytes (DMP1-8kb-Cre;Cx43^{fl/fl}) were generated using the Cre/LoxP system [18, 19], as previously reported [8, 11]. OCN-Cre;Cx43^{fl/-} mice and Cx43^{fl/-} control littermates were generated by crossing mice homozygous for the floxed Cx43 gene (Cx43^{fl/fl} mice) [20] (generated by K. Willecke, Universität Bonn, Bonn, Germany) with Cx43^{+/-} mice [21]

(generated by J. Rossant, University of Toronto, ON, Canada) expressing Cre recombinase driven by the human osteocalcin promoter (OCN-Cre mice) [22] (generated by T. Clemens, Johns Hopkins University School of Medicine, Baltimore, MD). This resulted in mice of a mixed C57BL/6-C129/J/FVB background. DMP1-8kb-Cre;Cx43^{fl/fl} mice and Cx43^{fl/fl} control littermates were generated by crossing Cx43^{fl/fl} mice with mice expressing Cre recombinase under an 8 kb fragment of the DMP1 promoter (DMP1-8kb-Cre mice), both in a pure C57BL/6 background, previously characterized [11]. Mice were genotyped by PCR using specific primer sets [20, 22–24]. For both models, female, 4.5-month-old mice were investigated. Mice expressing green fluorescent protein (GFP) in osteocytes (DMP1-8kb-GFP mice) were generated by I. Kalajzic (University of Connecticut Health Center, Farmington, CT) [25]. All protocols involving mice were approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences and Indiana University School of Medicine.

Biomechanical Testing

Three-point bending of the femur was performed at 37 ± 0.5 °C using a miniature bending apparatus with the posterior femoral surface lying on lower supports (7 mm apart) and the left support immediately proximal to the distal condyles, as previously described [26]. The load-bearing properties of the sixth lumbar vertebra (L6) were measured using a single-column material testing machine and a calibrated tension/compression load cell (model 5542; Instron, Norwood, MA), as previously described [27]. Cross-sectional moment of inertia and anterior–posterior diameter were determined by micro-computed tomography (μ CT) and used to calculate material-level properties, as previously described [28].

Fourier Transformed Infrared Imaging Data Collection and Analysis

Fourier transformed infrared imaging (FTIRI) was performed on the vertebrae and in the cortical portion of femora. Infrared data were collected using a Spectrum Spotlight 300 FTIRI system (PerkinElmer, Waltham, MA). Bone sections (5 μ m thick) were supported between two aluminum disks (13 mm diameter \times 0.5 mm thickness) each containing a narrow slit (9 \times 3 mm). FTIR images were collected in transmission mode from one vertebra as well as from the medial and lateral cortical shell of the mid-diaphysis of the femur from each animal, using a 6.25 μ m pixel size, four scans per pixel, at 8 cm^{-1} spectral resolution where the background was taken through an empty section of the sample holder. Protein content was

determined using the area under the amide I protein band (1,600–1,700 cm^{-1} , baseline 1,800 cm^{-1}). Mineral content was determined using the area under the ν_1 , ν_3 phosphate peak (1,200–900 cm^{-1} , baseline 1,200–900 cm^{-1}) [29]. The level of mineralization was determined as the mineral/protein ratio to account for any variations in sample thickness. The ratio of nonreducible/reducible collagen cross-links was determined from a peak height ratio in the amide I peak (1,660–1,690 cm^{-1} , baseline 1,800 cm^{-1}) [30], and crystallinity was determined from a peak height ratio in the mineral phosphate peak (1,020–1,030 cm^{-1} , baseline 1,200–900 cm^{-1}) [31].

Micro-CT

For μCT analysis, femora were dissected, cleaned of soft tissue, fixed, and stored in 70 % ethanol until imaging. Mid-diaphysis femora were scanned wrapped in parafilm using a 60 kV source, 0.5 mm Al filter, 0.7 degree rotation, and two-image averaging at 6 μm pixel resolution on the Skyscan 1172 (SkyScan, Kontich, Belgium) [32]. Reconstruction and analyses were conducted using SkyScan software. Bone was segmented from marrow for a single bone slice of the diaphysis using a similar threshold for all animals. Standard two-dimensional geometric properties as well as bone material density in pixels were obtained.

Cell Culture

Bone marrow cells were isolated from femora and tibiae of 5-month-old mice as previously described [33]. Cells were cultured in differentiation medium (α -minimal essential medium [α -MEM; Life Technologies, Carlsbad, CA] supplemented with 10 % fetal bovine serum [Thermo Scientific Hyclone, Logan, UT] and 50 $\mu\text{g}/\text{mL}$ ascorbic acid [Sigma, St. Louis, MO]). After 8 days in culture, cells were harvested and RNA was extracted as described below. Osteoblastic cells were obtained from calvarial bones of neonatal mice and cultured at an initial density of $5 \times 10^4/\text{cm}^2$ for 6 days in the presence of α -MEM supplemented with 10 % fetal bovine serum and 50 $\mu\text{g}/\text{mL}$ ascorbic acid, as described [8]. Half of the medium was replaced by fresh medium every other day. Osteoblastic OB-6 cells and osteocytic MLO-Y4 cells treated with scramble (scr) or Cx43 short hairpin RNA (shRNA) were cultured as previously described [10, 11].

Authentic Osteocyte Isolation

Calvarial cells were isolated from double transgenic DMP1-8kb-GFP;DMP1-8kb-Cre mice. GFP-expressing cells (osteocyte-enriched) were separated from GFP-negative cells (osteoblast-enriched) by sorting the cell suspension using a FACSAria flow cytometer (BD Biosciences,

Sparks, MD) at the Indiana University Flow Cytometry Core Facility [11].

RNA Preparation and Real-Time PCR

Total RNA was purified using Ultraspec reagent (Biotech Laboratories, Houston, TX), and Taqman quantitative RT-PCR was performed, as previously described [8]. Primers and probes for floxed-Cx43 and CRE recombinase were manufactured by the Assays-by-Design service from Applied Biosystems Life Technologies (Carlsbad, CA). Primers for Lox and Lox1 1–4 were designed using the assay design center (Roche Applied Science, Indianapolis, IN). Relative mRNA expression levels were obtained by normalizing to the housekeeping gene mitochondrial ribosomal protein S2 (*Mrops2*) or glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Applied Biosystems Life Technologies) using the ΔCt method [34].

Statistical Analysis

Significance was evaluated by *t* test using SigmaStat (SPSS Science, Chicago, IL). For FTIRI, independent Mann–Whitney *U* tests were performed because it is a nonparametric statistical approach to test for significance between small groups without the assumption of normally (gaussian) distributed data. Differences were considered significant at $p < 0.05$.

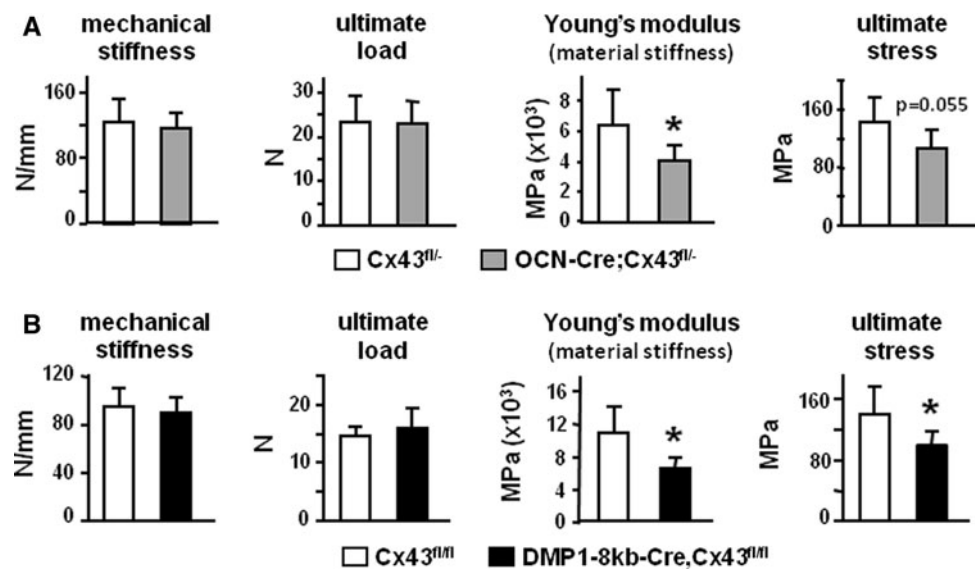
Results

Material Stiffness Is Decreased in Femoral Bone of OCN-Cre;Cx43^{fl/-} and DMP1-8kb-Cre;Cx43^{fl/fl} Mice

We have previously shown that OCN-Cre;Cx43^{fl/-} mice (lacking Cx43 in osteoblasts and osteocytes) and DMP1-8kb-Cre;Cx43^{fl/fl} mice (lacking Cx43 only in osteocytes) display high osteocyte apoptosis only in the cortical envelope of the vertebrae and in the diaphysis of the femora [11]. Increased osteocyte death was associated with changes in bone geometry at the femoral midshaft but not in the microarchitecture of cancellous bone in the distal femoral metaphysis.

Three-point bending of the femur was used to obtain the biomechanical properties of the bones in the two mouse models. Three-point bending showed that stiffness and ultimate load were not different between OCN-Cre;Cx43^{fl/-} and control mice (Fig. 1a), although the femoral midshaft of OCN-Cre;Cx43^{fl/-} mice had a higher moment of inertia compared to control animals, due to widening of the cross section, as previously shown [11]. Stiffness and ultimate

Fig. 1 OCN-Cre;Cx43^{fl/-} and DMP1-8kb-Cre;Cx43^{fl/fl} mice have decreased material properties in the femur. Mechanical properties of the femur from Cx43^{fl/-} and OCN-Cre;Cx43^{fl/-} mice (a) and Cx43^{fl/fl} and DMP1-8kb-Cre;Cx43^{fl/fl} mice (b) were determined by three-point bending. Bars are mean \pm SD. *Significant pairwise difference at $p < 0.05$, $n = 6-11$



load are also called “extrinsic” parameters because they depend on the size and shape of the bone [35]. Therefore, we determined the “intrinsic” strength values, to understand whether the benefit given by the geometry was canceled out by poor quality of the bone material, which also contributes to the overall resistance of the bone to bending. To do this, we applied engineering formulas [36] that correct the “extrinsic” parameters by the geometry of the femoral cross section to obtain the strength and stiffness per unit of area. We found that OCN-Cre;Cx43^{fl/-} mice displayed a 25 % lower material stiffness (Young’s modulus) and a lower, although not significant ($p = 0.055$), ultimate stress (Fig. 1a). This indicates that the lower bone material quality in OCN-Cre;Cx43^{fl/-} mice mechanically offsets the beneficial geometric adaptation of the femora. Similarly, femoral mechanical strength was not improved in DMP1-8kb-Cre;Cx43^{fl/fl} mice, despite the higher moment of inertia, due to reduced quality of the bone material. Indeed, DMP1-8kb-Cre;Cx43^{fl/fl} mice exhibited a marked reduction in material properties as Young’s modulus was decreased by approximately 50 % and ultimate stress was significantly lower than in control animals (Fig. 1b). Thus, Cx43 deletion in both animal models reduces the strength of the material in cortical bone.

OCN-Cre;Cx43^{fl/-} and DMP1-8kb-Cre;Cx43^{fl/fl} Mice Do Not Exhibit Common Changes in Microscopic Material Properties Assessed by FTIRI

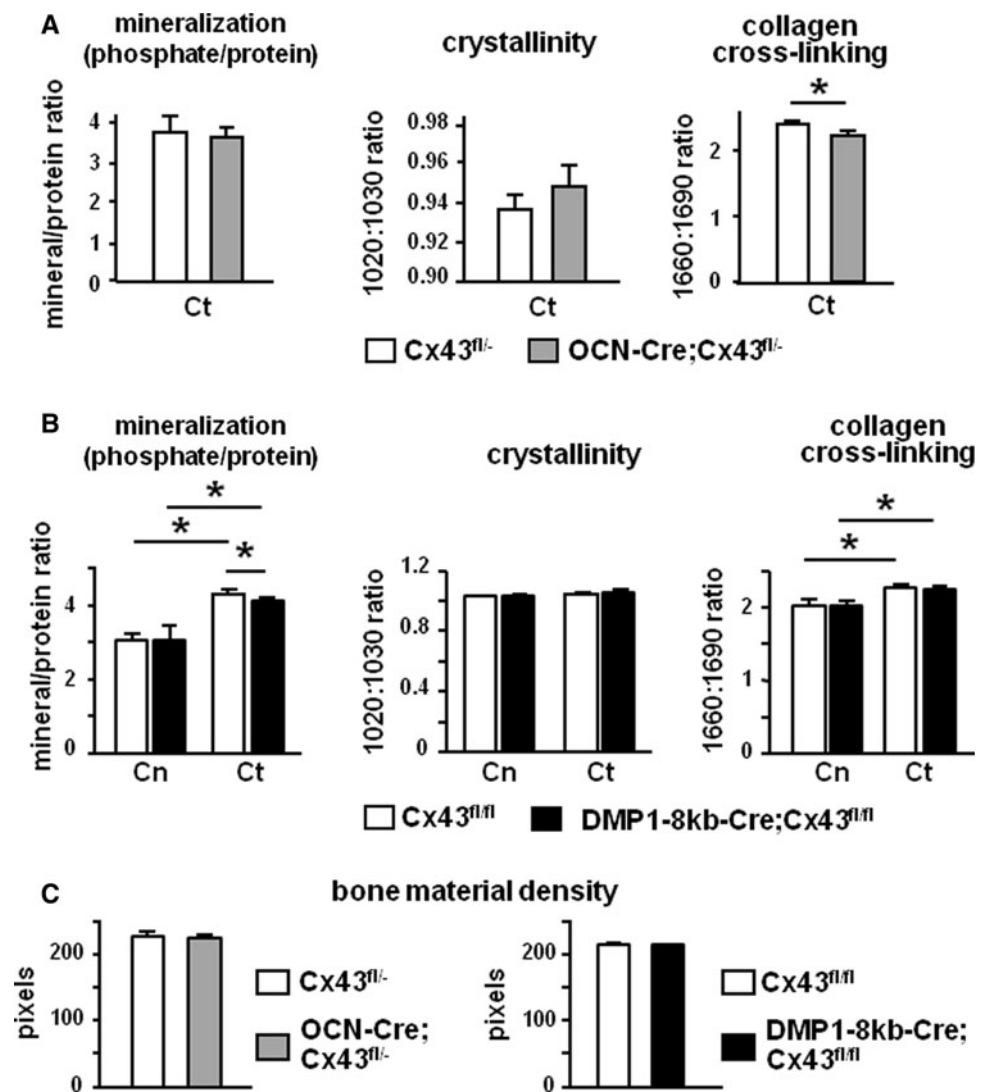
Young’s modulus is determined by the degree of mineralization as well as by the composition of the organic matrix [37]. We therefore analyzed the properties of the bone matrix in OCN-Cre;Cx43^{fl/-} mice by FTIRI in the appendicular skeleton (left femur, only cortical bone). A small, nonsignificant decrease in mineralization was

observed in OCN-Cre;Cx43^{fl/-} mice compared to control littermates (Fig. 2a). The ratio of nonreducible/reducible cross-links was selectively decreased in femoral cortical bone of OCN-Cre;Cx43^{fl/-} mice compared to control mice. On the other hand, in DMP1-8kb-Cre;Cx43^{fl/fl} mice collagen maturation was not different compared to control animals, while mineralization was significantly, although slightly, reduced in the femoral diaphysis (Fig. 2b). No differences were found in any parameter in cancellous bone of the distal femur between DMP1-8kb-Cre;Cx43^{fl/fl} and control mice. Mineralization and collagen cross-linking were higher in the femoral cortex compared to the cancellous bone in the femoral metaphysis, independently of the genotype under consideration. μ CT analysis showed no change in cortical bone material density in OCN-Cre;Cx43^{fl/-} or DMP1-8kb-Cre;Cx43^{fl/fl} mice compared to controls (Fig. 2c), in spite of the decrease in mineralization detected by FTIRI in DMP1-8kb-Cre;Cx43^{fl/fl} mice.

Deletion of Cx43 from Osteoblastic Cells Does Not Affect Vertebral Bone

Consistent with the lack of a phenotypic effect in trabecular bone, compression measurements of bone strength on the lumbar vertebra L6, which is mainly composed of cancellous bone, showed that the mechanical parameter ultimate load was not affected by Cx43 deletion in OCN-Cre;Cx43^{fl/-} mice (Fig. 3a). Moreover, strength/unit of area, determined by correcting this measurement by the cross-sectional area of the vertebral body, was not different between genotypes. Thus, in bones where strength is mainly determined by the cancellous compartment, neither mechanical nor material strength was affected by the lack of Cx43. Moreover, there was no difference between control and OCN-Cre;Cx43^{fl/-} mice in any parameter

Fig. 2 Deletion of Cx43 from osteoblasts and osteocytes results in reduced maturation of collagen cross-links in the femoral midshaft. Bone mineralization, crystallinity, and collagen cross-linking were assessed in femora by FTIRI in **a** the cortical (*Ct*) compartment of the diaphysis of $Cx43^{fl/-}$ and $OCN-Cre;Cx43^{fl/-}$ mice and **b** the cancellous (*Cn*) compartment of distal femora and the cortical compartment of the diaphysis of $Cx43^{fl/fl}$ and $DMP1-8kb-Cre;Cx43^{fl/fl}$ mice. Bars are mean \pm SD. * $p < 0.05$ by independent Mann–Whitney *U* tests, $n = 3$. **c** Bone material density was measured by μ CT in the femoral midshaft. Bars are mean \pm SD, $n = 7-10$



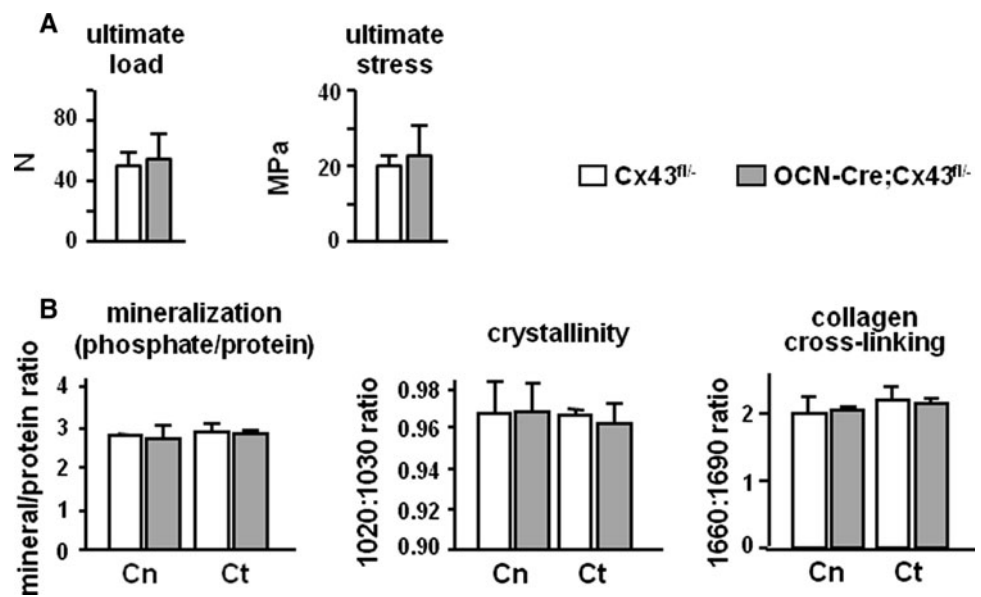
(mineralization, crystallinity, collagen cross-linking) in the cancellous or cortical bone of the vertebrae (Fig. 3b). Mineralization, expressed as the ratio phosphate/protein, was significantly higher in the cortical compartment of the femur compared to the same compartment in the vertebra, independently of the genotype (Figs. 2b, 3b). On the other hand, crystallinity, which is a measure of crystal size and maturation, was lower in the cortical bone of the femur compared to the vertebral cortex in $Cx43^{fl/-}$ mice. Collagen cross-linking did not differ between cancellous and cortical compartments in the vertebrae or between the axial and appendicular skeleton.

Cx43 Removal Reduces the Expression of Lox in Osteoblastic and Osteocytic Cell Lines in a Cell Autonomous Manner

The formation of collagen cross-links is initiated by conversion of lysyl or hydroxylysyl residues, present in

collagen telopeptides, to aldehydes catalyzed by Lox [38, 39]. This first step is followed by condensation with adjacent Lys or Hyl, to give immature, reducible cross-links [38]. Therefore, the decrease in the nonreducible/reducible cross-links ratio could be due to lower levels of Lox in Cx43-deficient animals compared to controls. The main form of lysyl oxidase is Lox; there are also four additional isoforms called Lox-like (Loxl) 1–4 [40]. First, we evaluated the expression levels of the different isoforms of Lox in authentic osteoblasts and osteocytes. To do this, we used RNA from calvarial cells isolated from DMP1-GFP mice. In these mice osteocytes are labeled with GFP, while osteoblasts are GFP-negative, thus allowing their separation by fluorescence-activated cell sorting [25]. We have previously shown that the GFP-positive fraction exhibits gene expression consistent with osteocytic cells, i.e., reduced levels of keratocan (a gene enriched in osteoblasts) and high expression of the osteocyte-specific gene sclerostin [11]. The GFP-negative

Fig. 3 Deletion of Cx43 from osteoblasts and osteocytes does not affect vertebral bone. **a** Mechanical properties of lumbar vertebra L6 from Cx43^{fl/-} and OCN-Cre;Cx43^{fl/-} mice were determined by vertebral compression. Bars are mean \pm SD, $n = 6-11$. **b** Bone mineralization, crystallinity, and collagen cross-linking were assessed by FTIRI in the cancellous (Cn) and cortical (Ct) compartments of lumbar vertebra L6. Bars are mean \pm SD, $n = 3$



fraction, on the other hand, expresses high levels of keratocan and lacks sclerostin, consistent with osteoblastic cells. We found that *Lox* was the most abundant lysyl oxidase in both cell types. The expression of *Lox* and *Loxl-1* was seven and two times higher in osteoblasts compared to osteocytes, respectively, whereas *Loxl-3* and *Loxl-4* were comparable in the two cell types and at least 10 times lower than *Loxl-1*, while *Loxl-2* could not be detected (Fig. 4a). These findings were confirmed in RNA purified from tibial diaphysis of 16 week-old wild-type C57BL/6 mice (Fig. 4b). Therefore, we focused our attention on *Lox* and *Loxl-1* since they are the main forms of *Lox* expressed by bone cells. We measured levels of *Lox* and *Loxl-1* in primary calvarial cells isolated from OCN-Cre;Cx43^{fl/-} and DMP1-8kb-Cre;Cx43^{fl/fl} mice and the respective control littermates and grown for 7 days in the presence of differentiation media. Cx43 was decreased in both primary calvarial cell preparations, although significantly only in cells isolated from DMP1-8kb-Cre;Cx43^{fl/fl} mice (Fig. 4c). We found that the levels of *Lox* and *Loxl-1* did not change significantly in response to lack of Cx43 in either culture. To determine whether deletion of Cx43 could have a cell autonomous effect on *Lox/Loxl-1* expression levels, we evaluated their levels in osteoblastic OB-6 cells and in osteocytic MLO-Y4 cells, in which Cx43 expression was silenced by shRNA [10, 11]. We found that both osteoblasts and osteocytes with reduced Cx43 exhibit decreased *Lox* and increased *Loxl-1* (Fig. 4d). Consistent with the relative levels of *Lox* and *Loxl-1* in primary osteoblasts and osteocytes (Fig. 4a), the enzymes were more abundant in osteoblastic cells compared to osteocytes. These findings suggest that *Lox* and *Loxl-1* transcription or mRNA stability is affected by deletion of Cx43 in osteoblastic cells.

Discussion

We have previously shown that Cx43 deletion from osteoblasts and osteocytes or only from osteocytes results in increased cortical osteocyte apoptosis and widening of femoral cross sections. We report herein that both mouse models of Cx43 deletion also exhibit decreased bone material properties selectively in cortical bone. Two recent reports also show widening of the marrow cavity and periosteal expansion in growing (1–2 months old) mice with deletion of Cx43 from chondro-osteoprogenitors using the *derm1* promoter [16] or from osteoblasts/osteocytes using the OCN-Cre transgene (the same model used in our studies) [17]. However, unlike our study, these reports show decreased bone mass and mechanical properties in the femoral midshaft. In particular, deletion of Cx43 from chondro-osteoprogenitors results in a strong phenotype, with decreased BMD and femoral length and an $\sim 50\%$ decrease in yield force [16]. Similarly, Zhang et al. [17] reported that OCN-Cre;Cx43^{fl/fl} mice exhibit decreased BMD and mechanical and material properties in the femoral midshaft. On the other hand, in the current study we did not find changes in mechanical properties when Cx43 was deleted from osteoblasts/osteocytes or from osteocytes. This apparent discrepancy could be explained by the fact that our study was performed in adult (4.5 months old) mice in which the growth of the femoral bone might have offset the deficient material properties, resulting in unaltered mechanical strength. Moreover, our data showing that DMP1-8kb-Cre;Cx43^{fl/fl} mice exhibit a similar decrease in material properties to OCN-Cre;Cx43^{fl/fl} mice, reported by Zhang et al. [17] and by us (this report), indicate that osteocytes contribute to the maintenance of the strength of the bone material. This finding

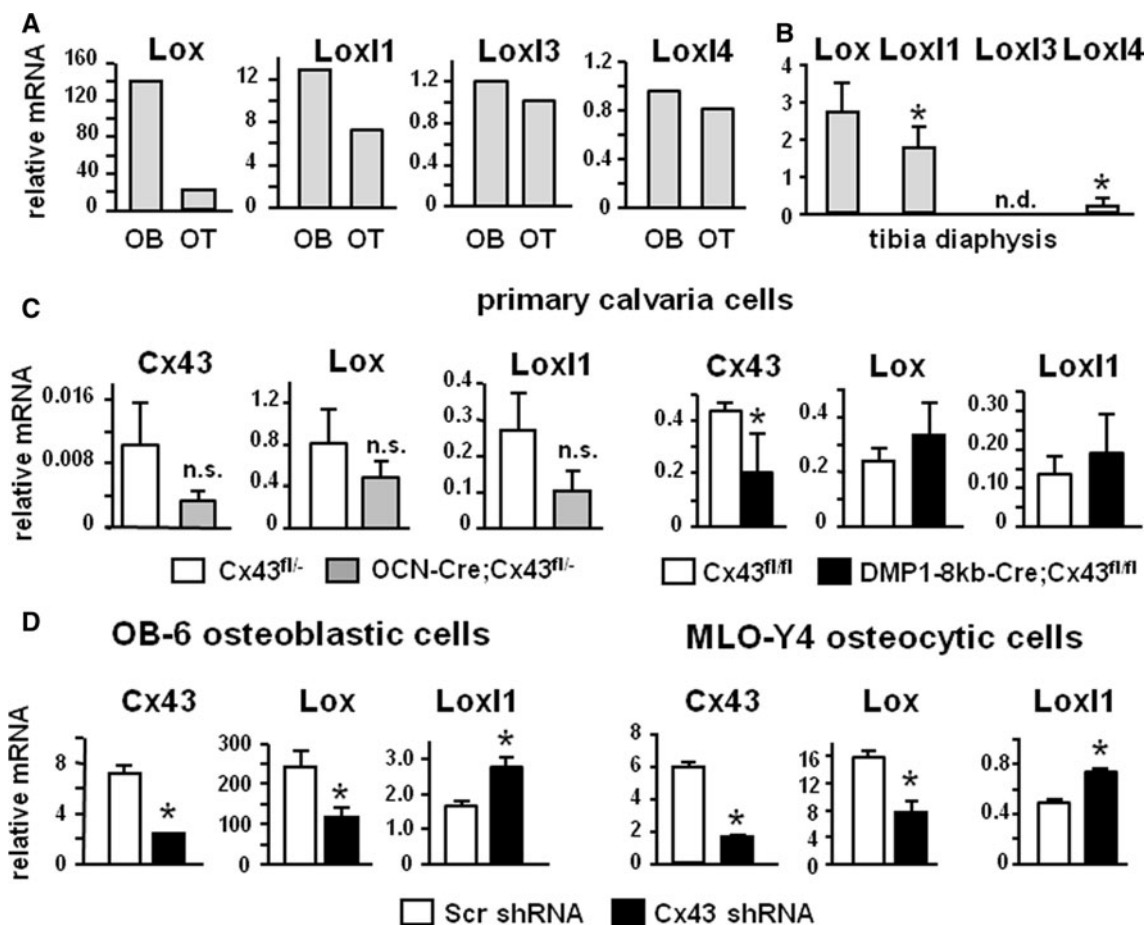


Fig. 4 Deletion of Cx43 results in decreased expression of *Lox*, *Cx43*, and *Loxl1* in calvarial cells, tibial diaphysis, and osteoblastic/osteocytic cells. **a** mRNA levels of the indicated genes in calvarial cells isolated from the progeny of DMP1-GFP;DMP1-8kb-Cre separated by FACS in GFP-negative and GFP-positive fractions. *Mrsp2* was used as a housekeeping gene. OB osteoblasts, OT osteocytes. **b** mRNA levels of the indicated genes in RNA purified from tibial diaphysis from wild-type C57BL/6 mice. *Mrsp2* was used as a housekeeping gene. Bars are mean \pm SD. *Significant difference at $p < 0.05$ versus *Lox* levels, $n = 6$. **c** Expression levels of *Cx43*, *Lox*, and *Loxl1* in

primary calvarial cells from *Cx43^{fl/fl}* and *OCN-Cre;Cx43^{fl/fl}* mice and *Cx43^{fl/fl}* and *DMP1-8kb-Cre;Cx43^{fl/fl}* mice after 7 days in culture with ascorbic acid to induce differentiation into the osteogenic lineage. Data are expressed relative to *Mrsp2*. Bars are mean \pm SD. n.s. not significant. **d** Expression levels of *Cx43*, *Lox*, and *Loxl1* in osteoblastic OB-6 cells and osteocytic MLO-Y4 cells with (scr shRNA) and without (*Cx43* shRNA) *Cx43*. Data are expressed relative to *Mrsp2*. Bars are mean \pm SD. *Significant difference at $p < 0.05$, $n = 3$

points to a higher complexity of osteocytic tasks than what was previously thought and is in line with a recent report showing that osteocytes can also exert typical osteoclastic functions, such as dissolution of matrix and mineral around their lacunae [41].

Mechanical testing shows phenotypic differences between *Cx43^{fl/fl}* and *Cx43^{fl/fl}* control mice, which could be due to the *Cx43* haploinsufficiency in the first model or to the different genetic backgrounds of the two mouse models (mixed C57BL/6-C129/J/FVB and pure C57BL/6 background for *Cx43^{fl/fl}* and *Cx43^{fl/fl}* mice, respectively). Nevertheless, in the femora, the lack of *Cx43* in both models significantly decreased Young's modulus, which is a measure of the rigidity or stiffness of the bone material that is determined by the amount of mineral and, to a lesser extent, by collagen [37]. However, mineralization

measured by μ CT was not different between the two genotypes, and it was only slightly, but significantly, reduced in *DMP1-8kb-Cre;Cx43^{fl/fl}* mice as assessed by FTIRI. Although the decrease in mineralization appeared to be very small, several studies have demonstrated that even a very small increase in mineral volume fraction will have a very large effect on modulus [42–45]. The finding that mineralization is not reduced in *OCN-Cre;Cx43^{fl/fl}* mice suggests that, besides mineralization, other properties of the bone material contribute to the reduction in Young's modulus in both animal models. Moreover, the reduction in mineralization might occur in spatially restricted areas of the cortical bone, likely associated with osteocyte apoptosis and remodeling, thus increasing the variability of the mineralization measurements and decreasing the difference between genotypes. Future studies will evaluate whether

changes in bone material properties correlate with areas that have increased osteocyte apoptosis.

Collagen cross-linking is an important determinant of bone biomechanical properties [46, 47] and could explain at least part of the decrease in bone material properties. During collagen maturation the content of reducible cross-links diminishes, whereas that of nonreducible cross-links increases, because with time the former likely matures into the latter [30]. Our FTIRI findings that the ratio of nonreducible/reducible cross-links decreases in OCN-Cre;Cx43^{fl/-} mice suggest that the lack of Cx43 hampers the proper maturation of collagen cross-links. Moreover, altered bone matrix with disorganized collagen fibers was also reported in mice lacking Cx43 early in the chondro-osteogenic lineage, in which osteoblast differentiation/function and lysyl oxidase levels are markedly reduced [16]. Although DMP1-8kb-Cre;Cx43^{fl/fl} mice also display a marked decrease in the strength of the bone material at the femoral diaphysis, we could not detect a significant decrease in collagen cross-linking by FTIRI. However, Cx43 downregulation lowers the levels of Lox and Loxl-1 in osteoblastic and osteocytic cell lines, indicating that Cx43 may be required in a cell autonomous manner for the expression of these enzymes in both cell types. Whether the modulation of Lox occurs through mRNA stabilization or active promotion of transcription by Cx43 is not known. We speculate that the effect of Cx43 deletion in osteoblasts, which express higher levels of Lox and Loxl-1 as shown by 10 times higher levels of Lox in OB-6 osteoblastic cells compared to MLO-Y4 osteocytic cells (Fig. 4c), has more dramatic consequences for bone and that the expression of lysyl oxidase in osteocytes is less crucial for collagen cross-linking. In addition, the lack of a reduction in Lox in calvarial cells isolated from the two mouse models points to the intriguing possibility that the decrease in Lox is a phenotypic feature exclusively of cortical bone of the appendicular skeleton and may be absent in other skeletal sites, such as the skull.

Some of the material properties we assessed by FTIRI were found to vary in different bone compartments and skeletal sites. Cortical bone of the femoral diaphysis had higher mineralization compared to cortical or cancellous bone of the vertebra and to cancellous bone of the femur itself. This could indicate that bone in the femoral cortex is deposited earlier and does not get remodeled as much as cancellous bone, due to the lower rate of bone remodeling of cortical bone.

As reported in a previous publication of ours [11], bones from OCN-Cre;Cx43^{fl/-} and DMP1-8kb-Cre;Cx43^{fl/fl} mice exhibit features that are found in aged subjects, including increased osteocyte apoptosis and widening of the femoral cross section. Remarkably, with age, both the enzymatic cross-links [48] and the activity of Lox [49] appear to

decline, thus further indicating that the loss of Cx43 accelerates the appearance of features of aging in bone. Further studies will be conducted to shed light on the contribution of reduced Cx43 expression to the aging of bone tissue.

In summary, although osteoblasts are likely to be the primary cells responsible for manufacturing matrix, our data point to a contribution of osteocytes to the regulation of the quality of the bone material. Moreover, Cx43 expression in both osteoblasts and osteocytes is important for maintaining the stiffness of the bone material, but the mechanism might be different in osteocytes, where it might depend also on Cx43-mediated control of osteocyte viability.

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