Altered distribution of bone matrix proteins and defective bone mineralization in *klotho*-deficient mice

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Running title: ***Bone matrix proteins in kl/kl bone***

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Abstract

In an attempt to identify the histological properties of the *klotho*-deficient (*kl/kl*) bone matrix, bone mineralization and the localization of Ca^{2+}-binding bone matrix proteins - osteocalcin, dentin matrix protein-1 (DMP-1) and matrix Gla protein (MGP) - were examined in *kl/kl* tibiae. While a widespread osteocalcin staining could be verified in the wild-type bone matrix, localization of the same protein in *kl/kl* tibiae seemed rather restricted to osteocytes with only a faint staining of the whole bone matrix. In wild-type mice, MGP immunoreactivity was present at the junction between the epiphyseal bone and cartilage, and at the insertion of the cruciate ligaments. In *kl/kl* mice, however, MGP was seen around the cartilaginous cores of the metaphyseal trabeculae and in the periphery of some cells of the bone surface. DMP-1 was identified in the osteocytic canalicular system of wild-type tibiae, but in *kl/kl* tibiae this protein was mostly found in the osteocytic lacunae and in the periphery of some cells of the bone surface. Mineralization of the *kl/kl* bone seemed somewhat defective, with broad unmineralized areas within its matrix. In these areas, mineralized osteocytes along with their lacunae and osteocytic cytoplasmic processes were found to have intense osteocalcin and DMP-1 staining. Taken together, it might be that the excessive production of Ca^{2+}-binding molecules such as osteocalcin and DMP-1 by osteocytes concentrates mineralization around such cells, disturbing the completeness of mineralization in the *kl/kl* bone matrix.

233 words

Key words: ***klotho deficient mice, osteocyte, DMP-1, osteocalcin, MGP***

Introduction

Klotho is a type I membrane protein with a single transmembrane domain near its C-terminal that may anchor the protein to the cell membranes [1]. The α form of the protein is a circulating hormone regulating mineral homeostasis; its homolog β, which relates to cell surface receptors, is involved in bile acid/cholesterol metabolism [2]. One of the suggested functions of α klotho is that of being a critical regulator of calcium (Ca) and inorganic phosphate (Pi) homeostasis or, more specifically that being a cofactor for the fibroblast growth factor (FGF) receptor 1c (FGFR1c) in FGF23 signaling [3-6]. While α klotho expression has been observed in the renal distal convoluted tubules [6], signaling linked to klotho/FGF23 is predominantly found in the proximal renal tubules, where it inhibits Pi reabsorption and 1α-hydroxylase activity, thereby reducing serum Pi and activation of vitamin D3 [3, 5, 7-9].

Mutations of the α *klotho* can produce a syndrome that seems to considerably accelerate aging. Mice carrying mutations of α *klotho* (*kl/kl* mice) grow normally until the third postnatal week, becoming less active afterwards and ultimately dying around 8-9 weeks of age [10]. The phenotype is one of osteoporosis, skin atrophy, ectopic calcifications, pulmonary emphysema, gonadal dysplasia, defective hearing, hypervitaminosis D, hypercalcemia, hyperphosphatemia [10, 11], and elevated osteoprotegerin levels [12, 13]. *Kl/kl* mice show increased renal expression of sodium phosphate cotransporter (NaPi) IIa and NaPi IIc protein, with concomitant hyperphosphatemia. Physical, biochemical, and morphological features are similar to those found in the FGF23 deficient (*Fgf23*$^{-/-}$) mice [10, 14, 15]. Bone abnormalities seen in *kl/kl* mice may result from the highly elevated serum Pi consequent to the defective klotho/FGF23 axis. Abnormal concentrations of serum Ca and Pi are a constant finding in *kl/kl* mice, but reports on the histology of *kl/kl* bone seem rather contradictory: while one described *kl/kl* bones as a low turnover, osteoporotic bone resulting from sharply reduced bone formation without a coupled decrease in bone resorption [11], others reported that *kl/kl* mice show elongated metaphyseal trabecular bone and increased trabecular volume, number, and thickness [16, 17].

Many non-collagenous proteins such as osteocalcin, dentin matrix protein-1 (DMP-1), matrix Gla protein (MGP) and osteopontin have high affinity with Ca^{2+}, and are therefore likely involved in bone mineralization. Assessing the distribution of these proteins in *kl/kl* mice, with their dysfunctional bone, may be an interesting research avenue to pursue. DMP-1 is a bone matrix protein expressed by osteocytes, and it is believed to influence bone mineral homeostasis due to

its high Ca^{2+}-binding capacity [18]. A recent report demonstrated that the DMP-1 absence leads to rickets or osteomalacia in mice as a result of FGF23 overproduction [19]. Osteocalcin and MGP, two other non-collagenous bone matrix proteins, are known for their γ-carboxyglutamic acid content and for binding to mineral crystals [20-22]. Post-transcriptional maturation in the presence of vitamin K permits osteocalcin γ-carboxylation, enabling further binding of hydroxyapatite molecules to Ca^{2+} [22-24]. Osteopontin is also well known to bind mineral crystals in bone [25]. Hunter *et al.* suggested that osteocalcin inhibits the nucleation of calcium phosphate crystals, while osteopontin was shown to slow crystal growth down [26]. MGP was reported to inhibit mineralization in bone, teeth and aorta [27-29]. Thus, it is reasonable to think that these matrix proteins may regulate bone minerals in *kl/kl* mice.

In addition to mineral-to-protein regulation, some sort of cellular control may be important for maintaining bone mineral homeostasis. Osteocytes are the bone cells most likely involved in mineral exchange, preservation and redistribution. Mature, well-mineralized bone develops an orderly arranged osteocytic lacunar canalicular system (OLCS), while immature bone features irregular, disorganized OLCS [30, 31]. OLCS, when well arranged, appear to form a functional syncytium [32-34]. Regarding osteocytic participation on bone mineral homeostasis, Tatsumi *et al* demonstrated that, after experimental exposure to the diphtheria toxin, osteocytes expressing diphtheria toxin-receptor were damaged and enlarged their lacunae and surrounding bone mineral crystals upon exposure to diphtheria toxin [35]. Osteocytes, therefore, appear to be involved in bone mineral preservation, an activity that complements that of non-collagenous proteins.

In this study, we have histochemically assessed bone mineralization, osteocytes' distribution and morphology and non-collagenous matrix proteins (osteocalcin, DMP-1, MGP and osteopontin) distribution to contribute to a more thorough understanding of the *kl/kl* bone matrix.

Materials and methods

Tissue preparation

Seven-week old male wild-type and *kl/kl* mice (n=8 each, Japan CLEA, Tokyo, Japan) were used in this study, which followed the principles for care and research use of animals set by Hokkaido University (research proposal approved under NO. 11-0096). Mice were anesthetized with an intraperitoneal injection of chloral hydrate and perfused with 4% paraformaldehyde diluted in 0.1 M cacodylate buffer (pH 7.4) through the cardiac left ventricle. Tibiae were immediately removed and immersed with the same fixative for 8 hr at 4 °C. Some samples were decalcified with 10% ethylenediamine tetraacetic disodium salt (EDTA-2Na) solution (for light microscopy) or 5% EDTA-2Na solution (for electron microscopy). Undecalcified specimens were used for RT-PCR analysis and transmission electron microscopy (TEM) examinations. For light microscopy, decalcified specimens were dehydrated in ascending alcohol solutions prior to paraffin embedding and sectioning. For TEM observations, specimens decalcified with 5% EDTA-2Na were dehydrated with dimethylformamide and embedded in glycolmethacrylate (GMA) prior to ultraviolet polymerization under -20 °C. Other decalcified and undecalcified tibiae were post-fixed with 1% osmium tetraoxide with a 0.1M cacodylate buffer for 4 hr at 4 °C, dehydrated in ascending acetone solutions, and embedded in epoxy resin (Epon 812, Taab, Berkshire, UK). Ultrathin sections were prepared with an ultramicrotome, and then stained with uranyl acetate and lead citrate or left unstained for TEM examination (Hitachi H-7000 Hitachi Co. Ltd, Tokyo, Japan) at 80 kV.

Double staining for tissue nonspecific alkaline phosphatase (TNALPase) and tartrate-resistant acid phosphatase (TRAPase)

After inhibition of endogenous peroxidase activity with methanol containing 0.3% hydrogen peroxidase for 30 min, dewaxed paraffin sections were pretreated with 1% bovine serum albumin (BSA; Serologicals Proteins Inc. Kankakee, IL, USA) in PBS (1% BSA-PBS) for 30 min. Sections were then incubated for 2-3 hr at room temperature (RT) with rabbit polyclonal antisera against human tissue nonspecific alkaline phosphatase (TNALPase) [36] diluted at 1:300 with 1% BSA-PBS. The antisera were reported to cross react with mouse TNALPase, and also work on paraffin sections [37]. The reacted sections were followed by incubation with ALPase-conjugated anti-rabbit IgG (Jackson Immunoresearch Laboratories Inc., West Grove, PA).

For visualization of ALPase immunoreaction, reacted sections were incubated with an aqueous solution containing 2.5 mg of naphthol AS-BI phosphate (Sigma, St. Louis, MO) and 18 mg of fast blue RR salt (Sigma) diluted in 30 mL of a 0.1M Tris-HCl buffer (pH 8.5) for 15 min at 37 °C. Following immunostaining, tartrate-resistant acid phosphatase (TRAPase) was detected as previously described [38]; in short, slides were rinsed with PBS and incubated in a mixture of 2.5 mg of naphthol AS-BI phosphate (Sigma), 18 mg of red violet LB (Sigma) salt, and 100 mM L (+) tartaric acid (0.76 g) diluted in 30 mL of a 0.1M sodium acetate buffer (pH 5.0) for 15 min at 37 °C.

Immunohistochemistry for DMP-1, matrix Gla protein, osteocalcin, osteopontin and E11, and double immunofluorescence for DMP-1 and E11

Detection of MGP, DMP-1 and osteopontin, as previously reported [39, 40], started with endogenous peroxidase inhibition for dewaxed sections. After pre-incubation with 1% BSA-PBS for 30 min at RT, the sections were incubated with 1) rabbit polyclonal antibody against MGP (TransGenic, Inc., Kobe, Japan) at 1: 50 for 1hr, 2) rabbit antibody against DMP-1 (Takara Bio Inc., Otsu, Japan) at a dilution 1: 500 overnight at 4 °C, or 3) rabbit anti-osteopontin antisera (Cosmo Bio, Co., Ltd., Tokyo, Japan) at 1:2000 for 1 hr. Sections treated with MGP, DMP-1 or osteopontin antibodies were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (DakoCytomation, Glostrup, Denmark). For osteocalcin and E11 immunohistochemistry, sections were incubated with goat anti-rat osteocalcin (Biomedical Technologies Inc. St. Stoughton, MA) at 1: 500 at RT for 1 hr, or with goat anti-mouse E11, also referred to as gp38, podoplanin, T1 alpha [41] (R&D Systems Inc, Mckinley Place, Minneapolis, MN) at 1: 100 for 1 hr at RT. The sections were subsequently incubated with HRP-conjugated anti-goat IgG (American Qualex, San Clemente, CA) at 1:100 for 1 hr at RT. For visualization of all immunoreactions, diaminobenzidine tetrahydrochloride was employed as a substrate. All sections were counterstained with methyl green, and observed under light microscopy (Eclipse E800, Nikon Instruments Inc. Tokyo, Japan).

Double immunofluorescent staining of DMP-1 and E11 was conducted as follows. Histological sections were reacted against anti-DMP-1 antibody following incubation with FITC-conjugated anti-rabbit IgGs (MP Biomedicals LLC-Cappel Products, Solon, OH) at 1: 100 at RT. Treated sections were, then, reacted against the E11 antibody as described above and subsequently incubated with RITC-conjugated anti-goat IgGs (Santa Cruz Biotechnology Inc.,

Santa Cruz, CA) at 1: 100 at RT. After mounting with DAPI-containing medium (Vector Laboratories Inc., Burlingame, CA), the double stained sections were observed under immunofluorescence microscopy.

Immunoelectron microscopy for DMP-1 distribution and silver enhancement for DMP-1, osteocalcin and MGP detection

Ultrathin section of GMA-embedded decalcified specimens were incubated with DMP-1 antibody at a dilution of 1: 500 with 1% BSA-PBS for 1 hr as described above, and then reacted against anti-rabbit Ig G conjugated with gold particles (10 nm in diameter) at a dilution of 1: 30 for 1 hr. After staining with lead citrate, immune-labelled sections were observed under TEM. For silver enhancement, GMA sections reacted against osteocalcin, DMP-1 and MGP antibodies followed by gold particle-conjugated second antibodies were incubated with an aqueous solution of 0.85% hydroquinone, 0.06% maleic acid, 50mM sodium citrate buffer (pH 3.5-4.0), 0.11% silver nitrate and 10% gum Arabic [25].

Von Kossa staining

Epoxy resin sections of undecalcified specimens were incubated with an aqueous solution of silver nitrate until black staining of the bone tissue was discernible under light microscopy [42].

Silver impregnation

Silver impregnation was performed as previously described [30]. In brief, dewaxed sections were soaked in a 1% Protargol-S solution diluted in borax-boric acid (pH 7.4) for 12-48 hr at 37^{o}C. After rinsing in distilled water, the reaction was enhanced by an aqueous solution containing 0.2% hydroquinone, 0.2% citric acid and 0.7% silver nitrate. After additional rinsing with DW, the sections were reacted for 5 min with a solution of 2.5% anhydrous sodium sulfite, 0.5% potassium bromide and 0.5% amidol diaminophenol dihydrochloride. Sections were then treated with 1% gold chloride, and subsequently with 2% oxalic acid amidol until the osteocytic canaliculi were stained black.

Imaging analyses for the indices of areas positive for osteocalcin, MGP and DMP-1, and the number of E11-positive cells on the metaphyseal trabecules in the wild-type and kl/kl tibiae

Osteocalcin, MGP or DMP-1 immunopositive areas, and the total epiphyseal and metaphyseal

areas were quantified using the ImagePro Plus 6.2 software (Media Cybernetics, Silver Spring, MD). For imaging analysis, pictures of immunostained areas were obtained from both groups (n=8 per group), and the index was expressed as a percentage of the total area of the epiphyseal and metaphyseal trabeculae. The number of E11-reactive cells located on the trabecular bone surfaces with 100μm in length was counted in the metaphyses of the wild-type and *kl/kl* mice (n=8 per group). The mean value is presented for each group.

Isolation of osteoblast-rich and osteocyte-rich fractions from the wild-type and kl/kl tibiae

For isolation of osteoblast-rich and osteocyte-rich fractions from 7-week old wild-type and *kl/kl* bone (n=5 for each), we followed the methods of Gu *et al* [43]. In brief, tibiae were dissected and dipped into 70% ethanol for 15 seconds in order to kill cells in the superficial layer. Then, tibial epiphyses were cut off, and the remaining shafts were cut longitudinally to allow for bone marrow removal by scraping and extensive washing. Pieces of bone were incubated in collagenase solution at 37 °C for 20 min, four times. Supernatants were collected and centrifuged at 200G for 5 min. Pellets were suspended in cell culture medium as the osteoblast-rich cell culture. Residual bone pieces were cut into smaller pieces, treated with EDTA at 37°C for 20 min, washed with HBSS and PBS, and treated with collagenase solution at 37°C for 20 min. Supernatants were centrifuged at 200G for 5 min. Pellets were suspended in cell culture medium and used as the osteocyte-rich fraction. These cells were employed for real time PCR after one day of culture.

Polymerase Chain Reaction and real time PCR for Osteocalcin, Mgp and Dmp-1

Total RNA was isolated from mouse tibiae using our established bone RNA extraction procedure. Tissues were frozen in liquid nitrogen, crushed to powder using a homogenizer and dissolved in 5ml TRIzol reagent (Life Technologies Co. Carlsbad, CA) per 300-400mg tissue. Total RNAs of osteoblast-rich or osteocyte-rich fractions were isolated by dissolution in TRIzol reagent. The mixture was centrifuged at 15,000 rpm for 5 min at 4 ^{o}C, allowing for removal of small bone debris. The supernatant was transferred to a new tube, which was vortexed for 15 seconds after addition of 1 ml of chloroform. The lysate was then transferred to a new tube and incubated for 5 min at RT. After phase separation, the aqueous phase containing the RNA was transferred to a fresh new tube and RNA was precipitated by adding 2.5 ml isopropyl alcohol per 5 ml TRIzol reagent. After 10 min incubation at RT, the mixture was centrifuged for 60 min at 15,000 rpm at

4°C. The resulting RNA pellet was washed with 1ml 75% ethanol and briefly air-dried. RNA was dissolved in 30μl DEPC-treated water. First strand cDNA was synthesized from 2 μg of total RNA by SuperScript VlLO cDNA Synthesis Kit (Life Technologies). The primer sequences used for PCR were: mouse *Gapdh* sense - TGTCTTCACCACCATGGAGAAGG, antisense - GTGGATGCAGGGATGATGTTCTG; mouse *Osteocalcin* (*Ocn*) sense - CTCTGTCTCTCTGACCTCACAG, antisense - GGAGCTGCTGTGACATCCATAC; mouse *Mgp* sense - TGCGCTGGCCGTGGCAACCCT, antisense - CCTCTCTGTTGATCTCGTAGGCA; mouse *Dmp-1* sense - TCCTCCCCACTGTCCTTCTT, antisense - CCCAGAGGCACAGGCAAATA. The PCR was performed using a thermal cycler, as follows : denaturation at 94 °C for 30 seconds, annealing at 60 °C (for GAPDH), 60 °C (for DMP-1), 62 °C (for osteocalcin) and 63 °C (for MGP) for 30 seconds, extension at 72 °C for 30 seconds ,and a final incubation at 72 °C for 10 min. RT-PCR products were subjected to 2% agarose gel electrophoresis, stained with ethidium bromide, and detected using E-Gel Imager (Life Technologies).

Twelve tibiae of 7-week old wild-type and *kl/kl* mice (n=6 for each) were used for real-time PCR assays. They were performed in a final reaction volume of 15μl that consisted of 7.5μl of 2×Quantifast SYBER green PCR Master Mix (QIAGEN GmbH, Hilden, Germany), 1.5μl of each primer and 3.5μl of DEPC-treated water. The primer pairs used were: mouse *Mgp* sense - AAAGAGAGTCCAGGAACGCA, antisense - GCGTTGTAGCCGTAGACCAT, product of 105 bp; mouse *Ocn* sense - AGGTAGTGAACAGACTCCGG, antisense - GCGGTCTTCAAGCCATACTG, product of 133 bp; mouse *Dmp-1* sense - GCACAGGCAAATAGTGACCA, antisense - TACTGGCCTCTGTCGTAGCC, product of 71 bp. Cycling conditions involved an initial activation step of 95°C for 5 min followed by 50 cycles of 95°C for 10s and 60°C for 30 seconds using a DNA Engine OPTICON2 (Bio-Rad Laboratories, Hercules, CA). Melt-curve analysis was also conducted for all samples to ensure specificity of the amplified products. The mean value of the expression of these genes was subjected to statistical analysis.

Statistical analysis

Statistical analysis was performed using Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA), with differences among groups being assessed by unpaired Student's *t*-tests, and considered statistically significant at $p < 0.05$.

Results

Distribution of osteocalcin, MGP, DMP-1and osteopontin in wild-type and kl/kl bones

Osteocalcin was evenly distributed in the wild-type bone matrix without immunoreactivity in normal osteocytes (**Figs. 1A-D**). *Kl/kl* bone, in contrast, featured intense osteocalcin immunoreactivity at the periphery of some osteocytes, despite a very weak staining throughout the *kl/kl* bone matrix (**Figs. 1E-G**). Consistently, osteocalcin-positive areas were significantly larger in the wild-type bone (*wild-type*, 85.33±12.72 *vs. kl/kl*, 7.94±2.36, $p<0.001$).

In the wild-type tibiae, MGP was mainly seen at the junction between cartilage and bone in the epiphysis, as well as the insertion area of cruciate ligaments into the articular surface (**Figs. 2A-C**). In the *kl/kl* tibiae, the cartilage cores of metaphyseal trabecules and several cells on the bone surfaces were MGP-positive (**Figs. 2D-F**), but there were few MGP-reactive osteocytes. Significant differences in MGP-positive areas between groups were absent (*wild-type*, 2.45±1.06 *vs. kl/kl*, 4.25±1.63, N.S.)

DMP-1 immunoreactivity was rather erratic in the *kl/kl* epiphysis and metaphysis as opposed to the wild-type specimens (**Fig. 3**). Although wild-type osteocytes had broader DMP-1 reactivity along their lacunae and canaliculi (**Figs. 3A-C**), *kl/kl* mice revealed DMP-1 accumulated in the periphery of the osteocytes (**Figs. 3D-F**). Also, some cells located on the *kl/kl* bone surfaces showed a sheath of DMP-1 positive material (**Figs. 3E, F**). Despite the marked difference in DMP-1 distribution, the differences in the index of DMP-1-positive areas between the two groups did not reach statistical significance (*wild-type*, 9.30±1.70 *vs*. *kl/kl*, 9.20 ±6.36, N.S.)

On the other hand, osteopontin did not accumulate in *kl/kl* osteocytic lacunae, and its expression levels were not significantly different from those in the wild-type mice (data not shown).

Gene expression of osteocalcin, MGP and DMP-1 in the wild-type and kl/kl bones

Consistent with RT-PCR, real time PCR showed significant reductions in the expression of osteocalcin in *kl/kl* tibiae (*wild-type*, 1.00±0.39 *vs. kl/kl*, 0.06±0.05, $p<0.001$, **See Figs. 4A, D**). The gene expressions of MGP and DMP-1 seemed slightly higher and weaker in *kl/kl* tibiae, respectively, when compared with the wild-type samples (**Figs. 4B, E**). However, statistical analysis of real time PCR showed no significant difference in expression of these genes. When

assessed in the osteoblast-rich and the osteocyte-rich fractions, osteocalcin expression was markedly reduced in both fractions obtained from *kl/kl* tibiae (**Figs. 4G, H**). In contrast, MGP was increased in osteocyte-rich specimens of *kl/kl* mice when compared with the same fraction obtained from wild-type mice (**Figs. 4G, H**). RT-PCR shows slightly decreased and increased expression of DMP-1 in *kl/kl* osteoblast-rich and osteocyte-rich fractions, respectively, compared with that of wild-type fractions (**Figs. 4G, H**).

Altered mineralization in kl/kl bone and the presence of mineralized osteocytes and cells on the bone surfaces

Histochemistry did not exhibit obvious differences in the distribution of TNALPase-positive osteoblasts and TRAPase-reactive osteoclasts in the wild-type and *kl/kl* mice (data not shown). An interesting finding in *kl/kl* specimens in our study, however, was the presence of mineralized osteocytes located within or adjacent to the unmineralized matrix, while osteocytes embedded in well-mineralized matrix were not mineralized (**Figs. 5A, B**). Of notice was the presence of some cells on the bone surfaces that were encapsulated by mineralized material (**Fig. 5C**). TEM observation of undemineralized ultrathin sections consistently demonstrated that mineral crystals extended from the wall of osteocytic lacunae towards the osteocytes (**Fig. 5D**), and also that osteocytic lacunae in the close proximity of unmineralized matrix were filled with mineral crystals (**Fig. 5E**).

Von Kossa staining and TEM observations were used to further assess the mineralized osteocytes and the cells on the bone surfaces enclosed by a mineral sheath found in the *kl/kl* tibiae (**Figs. 6A, C**). It is rather peculiar that even the superficial layer of bone matrix was not mineralized (**Figs. 6B**), and also that the osteocytic cytoplasmic processes were mineralized in the unmineralized bone matrix (See the inset of **Fig. 6D**). Under TEM, decalcified sections showed that some osteocytes were pyknotic with condensed nuclei, and others were completely collapsed as an electron-dense organic component accumulated in their osteocytic lacunae (**Figs. 6E-G**). Some cells over the bone surface, which did not seem to belong to the osteoblastic lineage, were surrounded by a similar same electron-dense material (**Fig. 6H**). These cells on the bone surface were partially encapsulated by a mineralized sheath that corresponds to the electron-dense material (**Fig. 6I**).

Osteocalcin and DMP-1are localized in mineralized osteocytes in kl/kl bone

In order to verify the spatial association between of Ca^{2+}-binding matrix proteins and mineralized osteocytes in the unmineralized bone matrix of *kl/kl* mice, we used serial GMA sections for silver enhancement of osteocalcin, MGP and DMP-1 immunohistochemistry and von Kossa staining. While osteocalcin immunopositivity was absent in osteocytes embedded in mineralized *kl/kl* bone, it could be verified in mineralized osteocytes located within and close to the unmineralized bone (**Compare Figs. 7A, B and C, D**). Likewise, mineralized osteocytes in the unmineralized bone matrix showed intense DMP-1 immunoreactivity, while those in mineralized matrix showed hardly DMP-1 positivity (**Compare Figs. 7A, B and E, F**). In contrast to these matrix proteins, MGP, however, was absent in the osteocytes from both mineralized and unmineralized matrices (**Figs. 7G, H**).

When immunoelectron microscopy was employed for DMP-1 localization in the wild-type bone, the walls of normal osteocytic lacunae and canaliculi showed DMP-1 reactivity, and the pericellular space between these walls and osteocytes and their cytoplasmic processes did not possess DMP-1 positivity (**Figs. 8A-C**). In contrast, DMP-1 accumulated within the *kl/kl* osteocytic lacunae (**Fig. 8D**), and their canaliculi also featured an excessive amount of DMP-1 (**Fig. 8E**). Cell debris in the osteocytic lacunae were filled with DMP-1 (**Fig. 8F**), and the organic material sheath of some cells on the bone surfaces was also abundantly positive for DMP-1 (**Fig. 8G**).

E11 immunohistochemistry indicates premature differentiation of kl/kl osteocytes

Silver impregnation unveiled the disorganized distribution of *kl/kl* osteocytes and their canaliculi (**Compare Figs. 9A and B**). Immunolocalization of E11, a hallmark for early osteocytic differentiation [41], showed that wild-type osteocytes located in the bone matrix of metaphyseal trabecules were positive for this marker. However, *kl/kl* metaphyseal trabecules showed several E11-positive cells on the bone surfaces (**Compare Figs. 9C and D**). The index of E11-positive cells on the bone surface was significantly higher in *kl/kl* bones (*wild-type*, 0.66 ± 0.12 *vs. kl/kl*, 4.51 ± 1.29, $p<0.05$). Double fluorescence showed that those E11-positive cells on the bone surfaces were also positive for DMP-1 (**Figs. 9E-G**).

Discussion

In this study, we demonstrated that in *kl/kl* mice osteocalcin, MGP and DMP-1 were ectopically synthesized, and also that mineralized osteocytes and some cells on the bone surfaces were present within or in the vicinity of unmineralized bone matrix. Bone metabolic abnormalities of *kl/kl* mice have been reported to link to highly elevated serum Pi levels due to defects in the FGF23-klotho axis [9]. However, the focal accumulation of DMP-1 and osteocalcin in the periphery of osteocytes and the mineralized osteocytes shown here are not typical features of metabolic bone diseases such as osteoporosis or osteomalacia. Our findings suggest that histological abnormalities such as the unusual synthesis of matrix proteins and the defective mineralization are unique to klotho deficiency. Our observations may also permit the inference that klotho deficiency renders osteocytes dysfunctional, compromising their mineral controlling abilities.

Two previous reports described radically different findings for the *kl/kl* bone: one described the *kl/kl* bone as osteoporotic [11], while others found elongated metaphyseal trabeculae in *kl/kl* mice [16, 17]. In our point of view, that is due to the observation tools employed: while regular light microscopy would show abundant trabecular bone, micro CT can only envision mineralized bone matrix [11, 16, 17]. By employing electron probe microanalysis, we previously demonstrated that *kl/kl* metaphyseal primary trabeculae had a high concentration of Ca and P, while the secondary metaphyseal trabeculae possessed a lower content of Ca and P [44]. At that time, however, we were not able to offer a reasonable explanation for the defective bone mineralization in *kl/kl* bones. In this study, we demonstrated that many *kl/kl* osteocytes and some cells on the bone surfaces were surrounded by a mineralized material containing excessive DMP-1 and osteocalcin; therefore, we postulate that, due to its high Ca^{2+}-binding affinity, DMP-1 and osteocalcin would play a pivotal role in mineralization of osteocytes and the cells on the bone surfaces. If osteocytes and their canaliculi are indeed involved in bone mineral homeostasis, regulation and transportation [30, 31], mineralization of osteocytes and their canaliculi would lead to their dysfunction which might prevent proper matrix mineralization by sequestrating the minerals available and impeding their proper transportation to the mineralizing osteoid. The broad areas of unmineralized matrix seen in *kl/kl* tibiae and shown in Figs. 5 and 6 may be consequence of such mineral transport obstruction.

Recently, Rhee *et al*. found that both FGFR1c and klotho transcripts are expressed in

osteocytes and osteoblasts [45]. It is possible that the klotho/FGF23 signaling may act in an autocrine/paracrine manner in bone, rather than affecting osteoblasts and osteocytes indirectly via serum Pi levels. If so, klotho deficiency in bone may induce premature osteocytic differentiation. Our RT-PCR and real time PCR showing higher expression of MGP and DMP-1 in the *kl/kl* osteocyte-rich fraction, and an elevated number of cells positive for E11 (a hallmark of early stage osteocytes) on the *kl/kl* bone surfaces may suggest that osteocytic differentiation is somewhat accelerated in the circumstance of klotho deficiency.

Based on the findings presented here, we can assume that the cells on the bone surface surrounded by mineralized sheaths that contain DMP-1 may be some sort of incompletely and prematurely differentiated osteocytes. This postulation is supported by the fact that mineralized osteocytes were consistently found in the vicinity of such cells on the bone surface. The abnormal synthesis of DMP-1 and the premature differentiation of osteocytes in *kl/kl* bone may be the two sides of a same coin, for a recent report suggested that C-terminal fragment of DMP-1 is the functional domain that controls osteocyte maturation [46].

Although we focused here on the effects of klotho deficiency on the bone tissue, klotho has important systemic effects as well. Signaling of FGF23-klotho axis is essential for normal regulation of serum Pi and for $1,25(OH)_2D_3$ synthesis [8, 9, 47-49]. *Fgf23*$^{-/-}$ mice showed hyperphosphatemia and highly elevated serum $1,25(OH)_2D_3$ levels together with ectopic calcifications, growth retardation and organ atrophy [14, 15]. Thereby, *Fgf23*$^{-/-}$ and *kl/kl* mice share phenotypic similarities. Although the high concentrations of $1,25(OH)_2D_3$ may cause many detrimental consequences in *kl/kl* mice, the alterations observed in the osteocytes throughout this study were not replicated in $1,25(OH)_2D_3$-administered rodents, which excludes the high levels of $1,25(OH)_2D_3$ as a causing factor for the abnormalities shown here. Recently, the group of Feng demonstrated that *Dmp1*$^{-/-}$*kl/kl* mice dramatically improved rickets with an identical serum biochemical phenotype, but showed elevated levels of apoptosis in osteocytes [50]. Theirs and our findings support the existence of local effects of klotho/DMP-1 on osteocytes. Even though it is necessary to examine *Fgf23*$^{-/-}$ bone with histochemical and ultrastructural tools similar to the ones used here to confirm it, it seems likely that klotho is an osteocytic regulator not only via the FGF23-klotho axis, but also in an auto/paracrine fashion.

Acknowledgments

This study was partially supported by grants from Japanese Society for the Promotion of Science (Amizuka N, Toyosawa S, Yamamoto T, Suzuki R).

Conflict of Interest

The authors have no conflict of interest.

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Figure Legends

Fig. 1

Immunolocalization of osteocalcin in wild-type and *kl/kl* tibiae

Osteocalcin (light brown color) immunoreactivity is equally distributed in the wild-type tibia (**A-D**). Note the uniform staining in the epiphysis (**B**), metaphyseal primary trabeculae (**C**) and metaphyseal secondary trabeculae (**D**). In contrast, the entire *kl/kl* bone is hardly reactive for osteocalcin (**E**). Some osteocytes (arrows) display osteocalcin reactivity (brown) in both the epiphysis (**F**) and metaphysis (**G**). epi; epiphysis, meta; metaphysis, dia; diaphysis

Bars A, E: 250μm, B, C, D, F, G: 50μm

Fig. 2

Immunolocalization of MGP in wild-type and *kl/kl* tibiae

In wild-type tibiae (**A**), immunostaining for MGP (brown color) is mainly seen at the insertion of the cruciate ligaments into articular surface (**B**, arrows), and also at the boundary between the growth plate cartilage and the bone matrix (**C**, arrows). Alternatively, in the *kl/kl* tibiae, uneven MGP immunolocalization is seen in the trabeculae of *kl/kl* epiphyses and metaphyses (**D**). At higher magnification, the material surrounding some cells (arrowheads) located on the bone surface is intensely stained for MGP in the *kl/kl* epiphysis (**E**). The periphery of the cartilage cores (arrows) of *kl/kl* metaphyseal trabecules is also positive for MGP (**F**). epi: epiphysis, GP: growth plate, meta: metaphysis

Bars A, D : 250μm, B, E : 150μm, C, F : 50μm

Fig. 3

Immunolocalization of DMP-1 in wild-type and *kl/kl* tibiae

DMP-1 (brown color) is evenly distributed in wild-type tibia at a lower magnification (**A**). However, at higher magnification, DMP-1 immunoreactivity could be seen in osteocytes (arrows in panel **B** and **C**), including their lacunae and their canaliculi in both epiphysis (**B**) and metaphysis (**C**). *Kl/kl* tibiae display many patchy DMP-1-immunoreactive areas (**D**). DMP-1 reactivity (dark brown) intensely accumulates around osteocytes (arrowheads) and in some cells (arrows) on the bone surface of the epiphysis (**E**) and the metaphysis (**F**). epi: epiphysis, meta: metaphysis, bm: bone marrow

Bars A, D : 250μm, B, C, E, F : 50μm

Fig. 4

RT-PCR and Real time PCR for expression of osteocalcin, MGP and DMP-1

Statistical analysis of real time PCR shows significantly-decreased expression (*wild-type*, 1.00±0.39 *vs. kl/kl*, 0.06±0.05, $p<0.001$) of osteocalcin (*Ocn*) (**A**), but no significant differences in the expression of *Mgp* (*wild-type*, 1.00±0.46 *vs. kl/kl*, 1.33±0.23, N.S., **B**) and *Dmp-1* (*wild-type*, 1.00±0.28 *vs. kl/kl*, 0.70±0.46, N.S., **C**) in *kl/kl* mice compared with those of the wild-type tibiae (**C**). Consistently, RT-PCR revealed extremely-reduced expression of *Ocn* (**D**), slightly-elevated *Mgp* (**E**) and slightly-reduced expression of *Dmp-1* (**F**) in *kl/kl* tibiae. RT-PCR using osteoblast-rich and osteocyte-rich fractions demonstrates that *Ocn* expression was markedly reduced in *kl/kl* tibia-derived osteoblast-rich and osteocyte-rich fractions (Compare *Ocn* between panels **G** and **H**). *Mgp* was slightly expressed in osteoblast-rich fraction of both the wild-type and *kl/kl* tibiae, osteocyte-rich specimens of *kl/kl* mice shows more *Mgp* than that of wild-type osteocyte-rich fraction (Compare *Mgp* between panels **G** and **H**). RT-PCR shows slightly decreased and increased expression of *Dmp-1* in *kl/kl* osteoblast-rich and osteocyte-rich fractions, respectively, compared with that of wild-type fractions (Compare *Dmp-1* between panels **G** and **H**).

Fig. 5

Von Kossa staining and TEM observations on undecalcified sections of *kl/kl* specimens

Von Kossa staining verifies mineralized (dark brown) and unmineralized (light blue) areas of the *kl/kl* bone (**A**). There are mineralized osteocytes (**B**, white arrows) located within or adjacent to the unmineralized matrix, while normal osteocytes (**B**, white arrowheads) are seen in the mineralized matrix. Some cells (white arrows) located on the unmineralized surfaces (light blue color) are present with a mineralized sheath surrounding them (**C**). TEM observations show many mineral crystals (an asterisk) partially occupying the pericellular space between the osteocytes and the lacunar walls (**D**). In another region, osteocytic lacunae (see the circularly arranged arrowheads) are totally filled with mineral crystals (**E**). Please observe the broad unmineralized area overlying mature osteoblasts (ob). ocy: osteocyte

Bars A : 100μm, B, C : 40μm, D, E : 5μm

Fig. 6

Ultrastructural observations on mineralized osteocytes and cells on the bone surface

Von Kossa staining shows that broad unmineralized area (an asterisk) contains osteocytes (black arrows) and a cell (a white arrowhead) encapsulated by mineralized materials (**A**). Broad areas (an asterisk) of unmineralized bone matrix can be seen beneath osteoblasts with von Kossa staining (**B**). TEM observations demonstrate an osteocytes (an arrow) and a cell located on bone surface (white arrowheads) encapsulated by mineralized materials in a close proximity (**C**). Notice that osteocytic canaliculi are mineralized within the unmineralized bone matrix (See the inset in **D**). Under TEM observations using *kl/kl* decalcified sections, some osteocytes are shown to be shrunken (**E**), some are pyknotic with condensed nuclei and surrounded by an amorphous material (**F**, asterisks), and others are totally collapsed (**G**). Panel **H** shows a cell embedded in an electron-dense component (an asterisk), while panel **I** demonstrates a cell on the bone surface that is partially encapsulated by a mineralized material (arrows). ocy; osteocyte

Bars A, B: 15μm, C-H : 4μm, I : 2μm

Fig. 7

Silver enhancement of immunostaining of osteocalcin, MGP and DMP-1, and silver staining in serial sections of *kl/kl* tibiae

Panels **B**, **D**, **F** and **H** are highly-magnified images of boxed areas in panels **A**, **C**, **E** and **G**, respectively. Von Kossa staining clearly reveals mineralized (an asterisk, black) and unmineralized matrices (**A**, **B**). Notice osteocytes (ocy) in unmineralized matrix are mineralized (black color) and osteocytes in normally mineralized area are not mineralized (**B**). A serial section demonstrates osteocalcin-immunoreactivity (brown) in osteocytes located in the unmineralized matrix, but those in mineralized area (an asterisk) are not immunopositive (**C**, **D**). Unlike osteocalcin, there was no obvious immunostaining for MGP (brown) in osteocytes in mineralized and unmineralized areas (**E**, **F**). Consistent with osteocalcin, DMP-1 immunopositivity (brown) is seen in osteocytes located in unmineralized matrix but not in the mineralized portion (**G**, **H**).

Bars A, C, E, G: 10μm, B, D, F, H : 5μm

Fig. 8

Immunolocalization of DMP-1 in wild-type and *kl/kl* bones

Immuno-gold technique showed fine gold particles indicative of DMP-1 molecules in the periphery of the wild-type osteocytes (**A**). When observed at a higher magnification, circularly arranged gold particles (**B**, arrowheads) suggest that DMP-1 is also localized on the walls of the osteocytic canaliculi. At a higher magnification, the wall of the osteocytic lacunae is full of gold particles indicative of DMP-1 (**C**, arrows). Distinguish the osteocytic cell membrane (white arrowheads) from the wall of the osteocytic lacunae (black arrows). In contrast, numerous gold particles representing DMP-1 accumulates in the lacunae of *kl/kl* osteocyte (**D**, ocy), and in their canaliculi (**E**, arrowheads). DMP-1 is abundant in the cell debris inside the osteocytic lacunae (**F**). Notice that the gold particles are not deposited on the collagen fibrils (See an inset of panel **F**). A cell (an asterisk) on the bone surface is also surrounded by organic material abundant with DMP-1 (**G**). ocy; osteocyte

Bars A : 2μm, B, C : 1μm, D, F, G : 5μm, E : 0.5μm

Fig. 9

Silver impregnation and immunolocalization of E11 and DMP-1 in the wild-type and *kl/kl* bones

Dark field images of silver impregnation demonstrate well-arranged osteocytic canaliculi in the wild-type epiphysis (**A**) but unevenly-distributed canaliculi in the *kl/kl* epiphysis (**B**). Immunoreactivity of E11, a hallmark for early stage of osteocytes, is seen in osteocytes (arrows) in the bone matrix of the wild-type trabeculae (TB) (**C**). However, *kl/kl* specimens show many E11 positive cells on the bone surface (**D**, arrowheads). Double immunofluorescent staining shows the co-localization of DMP-1 positive (**E**, green color) and E11 positive cells (**F**, red color). Dotted lines indicate the bone surfaces. Merged image ratifies the localization of some E11/DMP-1 positive cells (**G**, white arrows). Osteocytes (ocy) in the superficial layer also showed E11 and DMP-1 positivity. bm: bone marrow, ocy: osteocyte

Bars A, B : 30μm, C, D : 50μm, E-G : 30μm