特　集

Bone-Orchestrating Cells, Osteocytes

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ABSTRACT : Osteocytes build up functional syncytia, *i.e.,* the osteocytic lacunar-canalicular system (OLCS). The osteocytes are interconnected through gap junctions between their cytoplasmic processes, which pass through narrow passageways referred to as osteocytic canaliculi. There are two possible ways, in which molecules can be transported throughout the OLCS: via the cytoplasmic processes and their gap junctions, and via the pericellular space in the osteocytic canaliculi. Transport of minerals and small molecules through a spatially well-organized OLCS appears to be pivotal for bone mineral homeostasis and bone remodeling control. Recently, osteocyte-derived molecules -- sclerostin, dentin matrix protein-1, fibroblast growth factor 23 (FGF23) -- have been put in evidence as they may be related to osteocytic functions such as regulation of bone remodeling and so forth. Osteocytes were shown to regulate phosphorus serum levels and osteoblastic activity through the expression of FGF23 and sclerostin. In our observations, FGF23 and sclerostin synthesis seemed to be associated with the spatial regularity of the OLCS: both proteins were consistently expressed by osteocytes in epiphyses and cortical bones showing regularly arranged OLCS. In contrast, mice bearing high bone turnover, *e.g.,* osteoprotegerin deficient mice, revealed markedly-diminished sclerostin. This review will introduce our recent studies on the regularity of OLCS and the osteocytic function.

Key Words : *osteocyte, OLCS, sclerostin, FGF23, bone remodeling*

Introduction

Osteocytes are the most abundant cells in bone. These cells are at the center of bone turnover's mainframe, since they establish the network through which osteoblasts and bone lining cells communicate. All osteocytes lie within osteocytic lacunae and connect to other osteocytes and to osteoblasts on the bone surface via thin cytoplasmic processes that pass through narrow channels named osteocytic canaliculi. Depending on the site and on the interval since the embedding, osteocytic ultrastructure can vary significantly. Aarden *et al.* termed osteocytes located in osteoid and those embedded in recently mineralized matrix as osteoid osteocytes and young osteocytes, respectively[1)]. In contrast, osteocytes embedded in the deeper portion of matrix or those that have been buried for a long time (mature osteocytes) show fewer cell organelles with the nucleus becoming more prominent.

The process of osteocyte embedding in bone is not at all random. Osteocytes act as a functional group, since their cytoplasmic processes are connected through gap junctions[2-4)]. Such network of cytoplasmic processes permits the passage of small cytoplasmic molecules from one osteocyte to the next. In addition, the pericellular space (annular space) in the osteocytic canaliculi may serve as an alternative transport path[5)]. Recently, the diffusion coefficient of fluorescein in the pericellular space was shown to be similar to diffusion coefficients measured for comparably sized molecules in cartilage matrix[6)]. Through these possible paths, embedded osteocytes communicate and establish the osteocytic lacunar-canalicular system (OLCS)[1, 7, 8)]. The three-dimensional OLCS has been examined *in vivo*[9)], and our group has recently demonstrated that, in mice, the OLCS becomes progressively more regular as the individual

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grows[10].

For the OLCS to function properly, its anatomic arrangement has to be correct. In mature, cortical bone, osteocytic bodies parallel the bone surface and extended their cytoplasmic processes perpendicularly to it[11]. This regularity may be related with the direction of the collagen bundles: while the longitudinal axis of the osteocytes parallels the direction of the collagen fibrils, their cytoplasmic processes are perpendicular to them. The distribution of osteocytes and their cytoplasmic processes appears to mimic the distribution of collagen fibrils in mature bone, which may enable the osteocytes to sense mechanical loading, and to efficiently transport small molecules via their cytoplasmic processes and the pericellular space in their canaliculi.

Bone disease, on the other hand, may significantly affect the arrangement of the OLCS. Knothe Tate *et al* [8] documented that human osteomalacia features highly connected, non-regular OLCS, while osteoporosis revealed remarkably decreased connectivity and regularity of that system. It has been postulated that the OLCS would serve as a conduit for bone minerals and other chemicals, and would take part in mechanosensing and bone turnover regulation. The canaliculi network guarantees nutrition to distant osteocytes, and also allows the transit of small molecules and minerals originated from the extracellular fluid as evidenced by tracer experiments[9, 10, 12–14]. Bone remodeling appears to have three different aspects: 1) balance of essential minerals in serum, 2) skeletal adaptation to its environment, and 3) repairing of load-related microdamage[15]. While the first aspect does not need site-dependent remodeling, the other two do require that specificity. This concept is named targeted remodeling[15, 16]. There are many reports that osteocytic apoptosis and accumulated microdamage are important factors in initiating new remodeling sites[17–22]. It seems likely that osteocytic apoptosis and microdamage may disturb the signals carried throughout the lacunar-canalicular system, leading to signaling misinterpretation by osteocytes and osteoblasts, thereby initiating the targeted bone remodeling.

In this review, we will introduce elaborate on morphological aspects of osteocytic function, especially the biological function of the regularly arranged OLCS and the pivotal roles of osteocyte-derived factors in bone metabolism.

Regional differences of OLCS regularity in long bones

Regularly-formed osteocytic lacunar-canalicular system, OLCS, is a functional syncytia of osteocytes, and therefore, its geometrical arrangement is an important parameter in bone biology. However, the geometrical regularity of OLCS shows a regional difference in long bone (Fig. 1). Round-shaped osteocytes embedded in primary metaphyseal trabeculae extended their cytoplasmic processes in multiple directions (Fig. 1A). In addition, the inner cartilaginous cores of primary trabeculae seemed to interrupt the connections among osteocytic processes (Fig. 1B). Therefore, not only the regularity of OLCS, but also the connectivity of osteocytes seemed to be disrupted in primary metaphyseal trabeculae. Such finding suggests that the osteocytic functional syncytia could not be optimally functioning in the primary trabeculae[10, 11]. In contrast, in cortical bone subjected to physiological remodeling, OLCS was regularly arranged and osteocytes were flat (Fig. 1C, 1D). Osteocytes' bodies paralleled the bone surfaces, while osteocytic canaliculi ran perpendicular to them. It seems that bone remodeling is the driving force of OLCS regularization. We showed in previous reports that, as observations progressed from the metaphysis towards the diaphysis, the endosteal cortical bone displayed narrower bands of calcein labeling, indicating slower bone deposition, with a related increase in OLCS regularity[10, 11]. It is possible that bone deposition rate during bone remodeling affects the OLCS regularity.

Possible function of OLCS in mineral transport and sensing mechanical strains

While the true function of the OLCS is still under the investigation, the fact that transport of minerals and small molecules is carried through the OLCS serves as a fundament for assuming that this cell type might be intimately involved in chemical transduction[6, 23, 24]. Regarding mineral transport, ablation of osteocytes in transgenic mice expressing osteocyte-specific HB-EGF, the receptor for diphtheria toxin, strongly indicated that osteocytes control mineral traffic in bone[25]. After diphtheria toxin injection, transgenic mice showed many empty lacunae, indicating osteocyte death. Curiously, trabeculae were shortened after toxin injection, and the cortical bones came to show demineralized patchy areas in the periphery of empty osteocytic lacunae. It seems likely that disruption of osteocytic function leads to some type of bone mineral shortage.

The most accepted theory for osteocytic function places these cells as transducers of mechanical strains

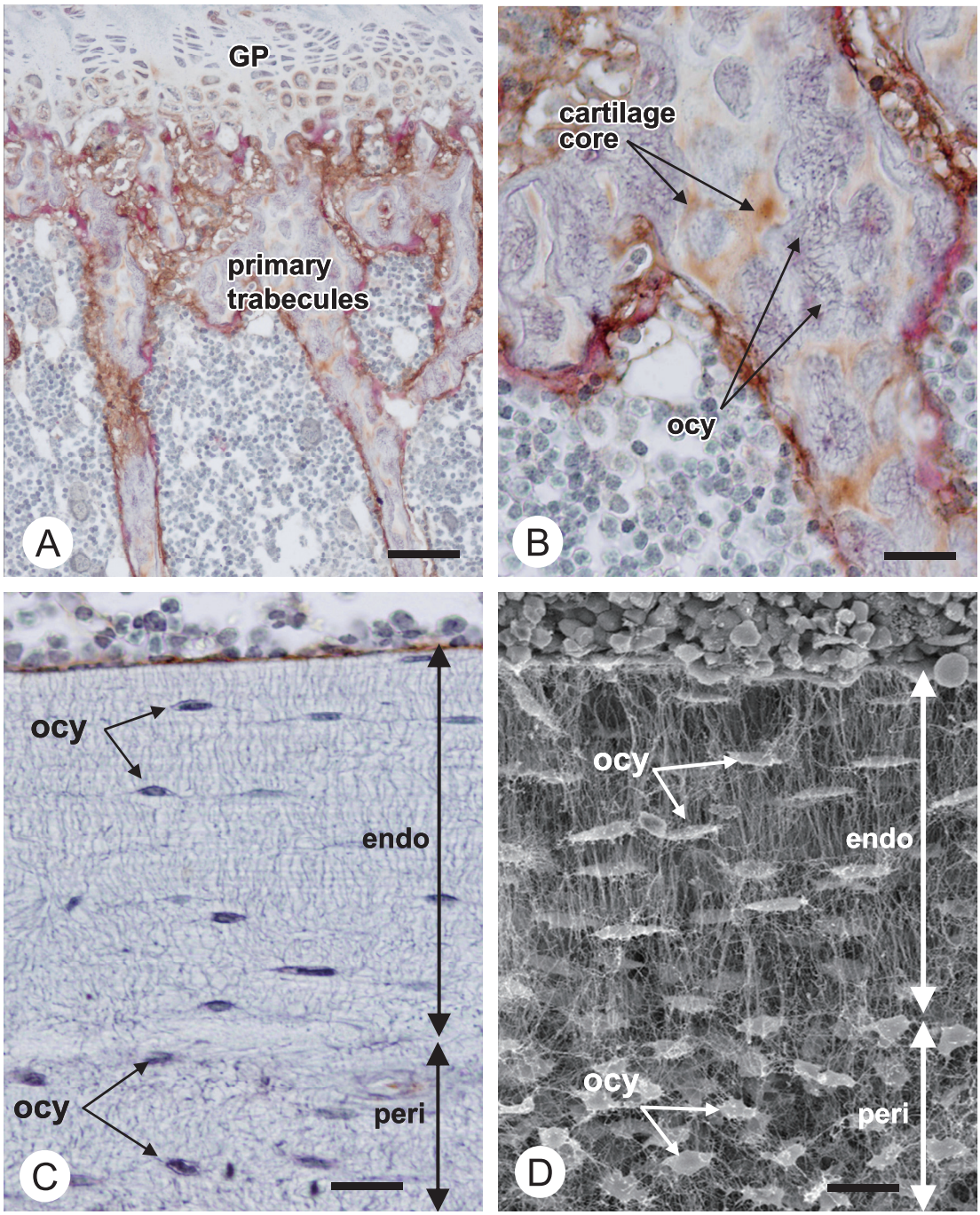

Fig. 1 Triple staining for silver impregnation, ALPase and TRAPase, and scanning electron microscopic image
A: alkaline phosphatase (ALPase)-positive osteoblasts (brown), tartrate-resistant acid phosphatase (TRAPase)-positive osteoclasts (red), and the silver impregnated traces representative of the OLCS (black) are depicted in the metaphysis and cortical bone. B: At a higher magnification, primary metaphyseal trabeculae displaying intensely ALPase-positive osteoblasts (brown) and several TRAPase-reactive osteoclasts (red) cover the trabecule. The cytoplasmic processes of ovoid osteocytes (ocy) spread in multiple directions, but the connectivity among these processes is interrupted by the presence of inner cartilage cores in the primary trabeculae. C: In the endosteal region of cortical bones (endo), all the osteocytes (ocy) localized their cell bodies parallel to the bone surface, and had their processes extended perpendicularly to the bone surface. In contrast, the periosteal region of the cortical bone (peri) revealed randomly-oriented osteocytes (ocy). D: Scanning electron microscopy demonstrates the three dimensional distribution of osteocytes (ocy). Please note regular arrangement of osteocytes (ocy) in the endosteal region (endo), while irregularly-oriented osteocytes in the periosteal region (peri).
Bar, A: 100μm, B-D: 30μm

into biochemical signals that affect communication among osteocytes and between osteocytes and osteoblasts[7, 23, 26, 27]. An osteocytic response to mechanical load has been suggested[28–30]; and it has been proposed that osteocytes may detect microdamage in bone[17, 22, 31] and undergo apoptosis, thus signaling for osteoclastic resorption of the damaged region[19, 32, 33]. Thus, the OLCS seems to be an appropriate network for transferring exogenous and endogenous signals, both mechanically and chemically. An intact OLCS seems crucial for molecular transport of bone minerals, and may be important for bone remodeling control.

If so, can an osteocyte regulate the mineralization of the bone matrix that encloses it? It is possible that osteocytes respond to parathyroid hormone (PTH) or to a low calcium diet by altering serum calcium levels. This phenomenon was reported a hundred years ago by Renckinghausen[34] and by Kind[35], and in the 60's Bélanger proposed a new notion of "osteocytic osteolysis"[36]. In our own observation, at six hours after injection of human PTH (1–34) into the jugular vein of mice, enlarged osteocytic lacunae in the cortical bone with regular OLCS were observed. Recent reports show some findings suggesting possible mechanisms for osteocytic osteolysis, *e.g.*, synthesis of tartrate-resistant acid phosphates by osteocytes[37], enlarged osteocytic lacunae and acid phosphates activity in osteocytes after continuous infusion of PTH for 4 weeks[38], as well as synthesis of matrix metalloproteinase-2 by osteocytes[39]. More recently, the groups of Teti and Bonewald reported osteocytic remodeling of the perilacunar and pericanalicular matrix[40, 41]. However, it seems necessary to carefully examine this possibility from various aspects.

Regarding mechanosensing, the geometrical regularity of OLCS appears to be essential. We have shown that osteocytes embedded in remodeled bone were flat and extended their cytoplasmic processes perpendicularly to the longitudinal axis of trabecular and cortical bones. Using biomechanical simulation analyses, McCreadie *et al*[42] demonstrated that strains were higher in an elongated cell compared to a less anisotropic one, when load parallels the long axis of the lacuna. Also, finite element models showed that longer, thinner cells have higher maximum strains[43]. Flattened osteocytes are found among collagen bundles, which run parallel to each other and, therefore, may not disturb the seam of collagen bundles in compact bones. Orderly distributed osteocytes and osteocytic processes, when geometrically harmonized with the surrounding collagenous architecture, may be very effective in recognizing mechanical loading. In addition, a regular OLCS may efficiently transport small molecules from one osteocyte to others, and to osteoblasts as well[7, 14, 23, 24]. The notion that bone remodeling occurs as the skeleton adapts itself to its mechanical environment[15, 16] supports our idea that osteocytes develop a well-organized OLCS as normal bone remodeling progresses.

Sclerostin, an osteocytes-derived factor, serve as a negative regulator for osteoblastic activity.

Osteocyte-derived molecules were recently highlighted because these molecules may reflect osteocytic function on bone remodeling and on regulation of serum concentration of phosphate. Sclerostin, is a glycoprotein encoded by the *SOST* gene[44], and was reported to bind the LRP5/6 receptor, thereby antagonizing Wnt signaling and increasing β-catenin degradation[45, 46]. Sclerostin secreted by osteocytes may pass through the osteocytic canaliculi and serve as a negative regulator of osteoblastic bone formation[44, 47–49].

Masuki *et al* have demonstrated the distribution of sclerostin and OLCS in long bones of mice lacking osteoprotegerin (OPG)[5, 50], a decoy receptor for the receptor activator of the nuclear factor κB ligand (RANKL) (Fig. 2). OPG has been characterized as an inhibitor of osteoclastogenesis[51]. OPG-deficient mice showed accelerated bone remodeling and an irregular OLCS. While dentin matrix protein-1 (DMP-1), which is another osteocytes-derived factor with a high calcium-binding affinity[52, 53], was found in all osteocytes in the OPG-deficient bone, sclerostin reactivity was significantly diminished in OPG-deficient epiphyses and cortical bone (Fig. 2). Sclerostin appears to be synthesized specifically once osteocytes possess a regular OLCS, for example in normal, mature cortical bone.

Recently, it was suggested that PTH administration inhibits sclerostin synthesis by osteocytes, thereby allowing for active bone remodeling[48]. Anabolic effect in bone caused by the intermittent PTH administration has been thought to be results from binding of exogenous PTH and its receptor in osteoblasts and preosteoblasts. However, recent hypothesis is that PTH binds to osteocytes and makes them reduce the synthesis of sclerostin, consequently activating osteoblasts. O'Brien *et al* have reported that transgenic mice expressing a constitutively active PTH receptor exclusively in

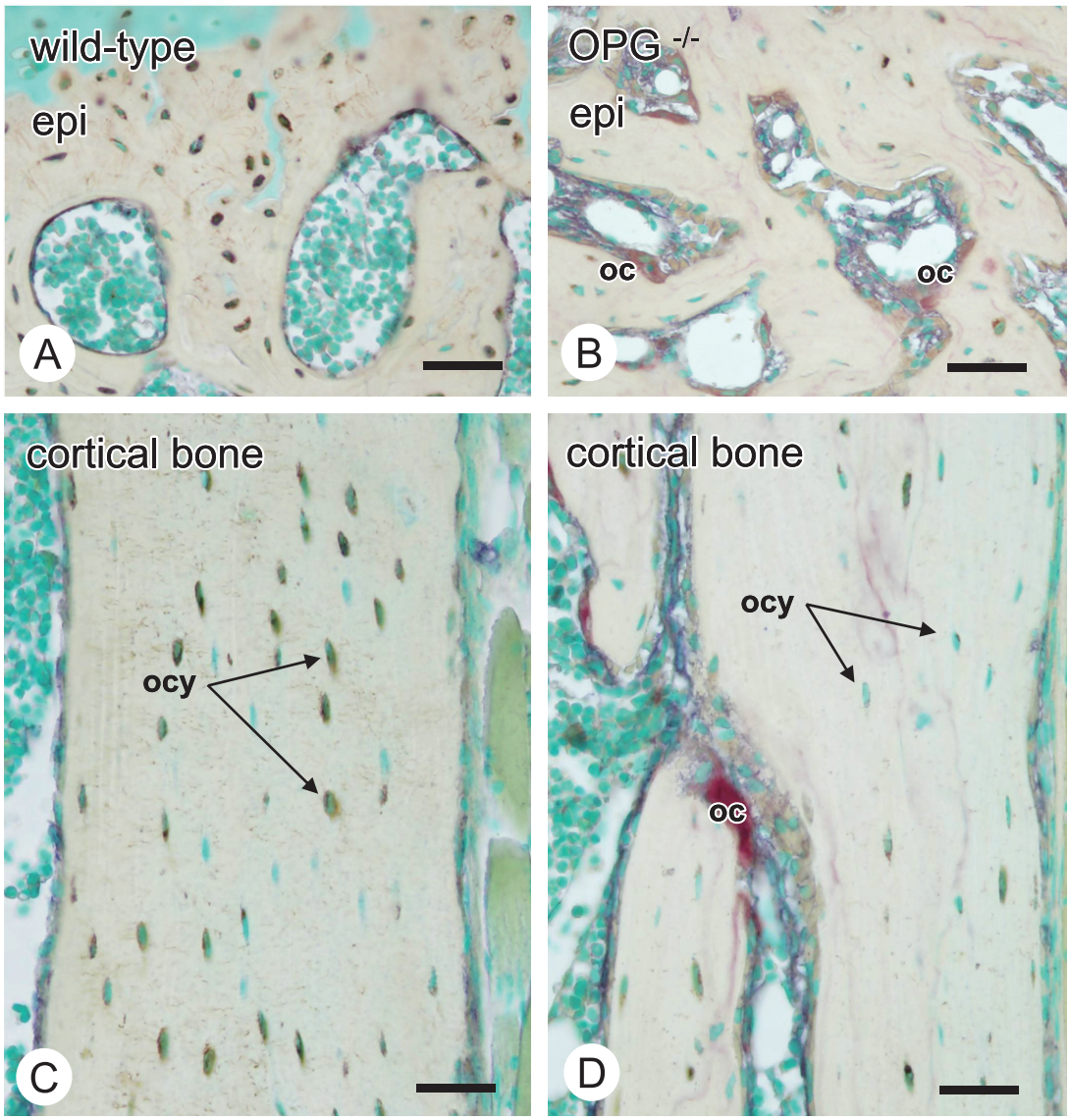

Fig. 2 Immunolocalization of sclerostin in the wild-type and OPG$^{-/-}$ bones

Histological sections were subjected to triple staining for alkaline phosphatase (ALPase, blue), tartrate-resistant acid phosphatase (TRAPase, red) and sclerostin immunohistochemistry (brown). A, C: Scrlostin-positivity (brown) was seen in many osteocytes of the wild-type epiphyses (epi, A) and cortical bone (C). B, D: However, OPG$^{-/-}$ epiphyses (epi, B) and cortical bone (D) revealed markedly-reduced sclerostin-immunoreactivity. Note weak immunopositivity of sclerostin in OPG$^{-/-}$ bone which show intense reactivity for ALPase and TRAPase. oc: osteoclast

Bar, A,B: 100μm, C,D: 70μm

osteocytes exhibited increased bone mass and bone remodeling, as well as reduced expression of the osteocyte-derived sclerostin, increased Wnt signaling, increased osteoclast and osteoblast number, and decreased osteoblast apoptosis[54]. Powell *et al* have generated osteocyte-selective PTH receptor deficient mice (Ocy-PPR^{cKO} mice), and had demonstrated a reduction in trabecular bone and mild osteopenia. They also reported that PTH administration failed to increase sclerostin synthesis in the Ocy-PPR^{cKO} mice, while the wild-type littermates revealed markedly-reduction of sclerostin by the PTH administration[55]. Consequently, we could see markedly-reduced sclerostin in the metaphyseal trabecular bones after PTH injection through murine jugular vein. However, it has been suggested that PTH/sclerostin axis is not involved in bone remodeling, but PTH stimulates bone turnover. Taken together, PTH affects bone cells in a dual pathway: via interplay between osteoblasts and preosteoblasts, and via osteocytic synthesis of sclerostin.

In the research field of dentistry, when examining the mesial region of the mandibular interradicular septum in ovariectomized rats, we attempted to elucidate whether estrogen deficiency would affect the synthesis of sclerostin by means of accelerating bone resorption or through a more direct effect on the estrogen receptors in osteocytes[56]. The mesial region of OVX interradicular septa showed an increased number of osteoclasts with intense RANKL labeling, as well as widespread absence of sclerostin-positive osteocytes. Accelerated bone remodeling induced by estrogen deficiency, rather than having a direct effect through the estrogen receptors in osteocytes, appeared to inhibit sclerostin expression by osteocytes in the mesial region of interradicular septa. These findings imply that bone remodeling or its participating cells, osteoblasts and osteoclasts, would primarily affect sclerostin synthesis.

Physiological function of FGF23 synthesized by osteocytes

Fibroblast growth factor (FGF) 23, which is also an osteocyte-derived factor, modulates serum phosphate concentration, by co-operating in kidney[57]. FGF23 was originally reported as a phosphaturic factor in autosomal dominant hypophosphatemic rickets[58], tumor-induced osteomalacia[59], McCune-Albright syndrome / fibrous dysplasia[60], familial tumoral calcinosis[61] and in X-linked hypophosphatemic rickets[62]. Although FGF23 mRNA is found in several tissues[58, 59, 61], this molecules is most abundantly expressed in bone[55] (Fig. 3). Investigations on the biological functions of FGF23 have broadened the understanding of the systemic regulation of phosphate homeostasis: FGF23 serves as a phosphaturic agent that inhibits $1,25(OH)_2D_3$ production and the function of sodium/phosphate co-transporter II (NaPi II), which inhibits reabsorption of phosphates in the proximal renal tubules[62-64] (Fig. 4).

We have observed FGF23-immunopositive osteocytes in disease-free secondary trabeculae and cortical bone with regularly oriented OLCS: FGF23 appears to be synthesized mainly by osteocytes forming regularly distributed OLCS in mature bone that had been remodeled[11] (Fig. 3A, 3C). Alternatively, intense immunopositivity for FGF23 in metaphyseal primary trabeculae with irregular OLCS could not be verified (Fig. 3B). Mature bone could, therefore, serve as an organ regulating serum phosphorus levels. One of our recent experiments hinted on enhancement of FGF23 synthesis by osteocytes after 2 weeks of PTH administration. However, PTH administration also accelerated bone remodeling and formation of an irregular OLCS. PTH may induce FGF23 synthesis in osteocytes even when the OLCS is malformed; also, PTH could directly attenuate phosphate reabsorption, but also by inhibiting NaPiII in the proximal renal tubules. These preliminary findings point to an important role for the osteocytes in phosphate homeostasis.

Recently, the transmembrane klotho was shown to be a main co-receptor for the FGF23 signaling pathways, though two klotho proteins are recognized: transmembrane klotho and circulating klotho[57, 65, 66]. Since klotho was shown to regulate phosphate homeostasis as an obligate co-receptor for FGF23 (FGF23-klotho axis), its deficiency could lead to defective FGF23 signaling, consequently accelerating phosphate reabsorption in kidney[57, 62-66]: *Klotho*-deficient mice feature high serum calcium, phosphorus[65, 67], and osteoprotegerin[68, 69] levels. Synthesis of FGF23 by osteocytes is modulated by PTH, $1,25(OH)_2D_3$, dietary and serum phosphate levels. Synthesis and secretion of FGF23 by osteocytes are positively regulated by $1,25(OH)_2D_3$ and serum phosphorus and negatively regulated, through yet unknown mechanisms, by the phosphate-regulating gene and by DMP-1. In turn, FGF23 inhibits the synthesis of $1,25(OH)_2D_3$, and it may negatively regulate the secretion of PTH from the parathyroid glands. However, FGF23 synergizes with PTH to increase renal phosphate excretion by reducing expression of NaPiIIa and IIc in the proximal tubules. Thus, most important insights gained into the regulation of phosphate homeostasis by these factors are derived from the investigation of osteocytes and its related human disorders.

Putative function of klotho in osteocytes

The klotho gene is involved in multiple aging phenotypes and age-related disorders. *Klotho*-deficient mice develop normally until 3 weeks of age, then become less active and ultimately die by 8-9 weeks of age[67]. The defect in *klotho* gene causes osteoporosis, skin atrophy, ectopic mineralization (vascular calcification), pulmonary emphysema, gonadal dysplasia, and defective hearing in mice -- all of which also appear in human aging. The abnormal histology found in *klotho*-deficient mice has been attributed for disrupted FGF23-klotho axis, and *klotho*-deficient mice feature high serum calcium, phosphorus[60, 68], and osteoprotegerin[68, 69] levels.

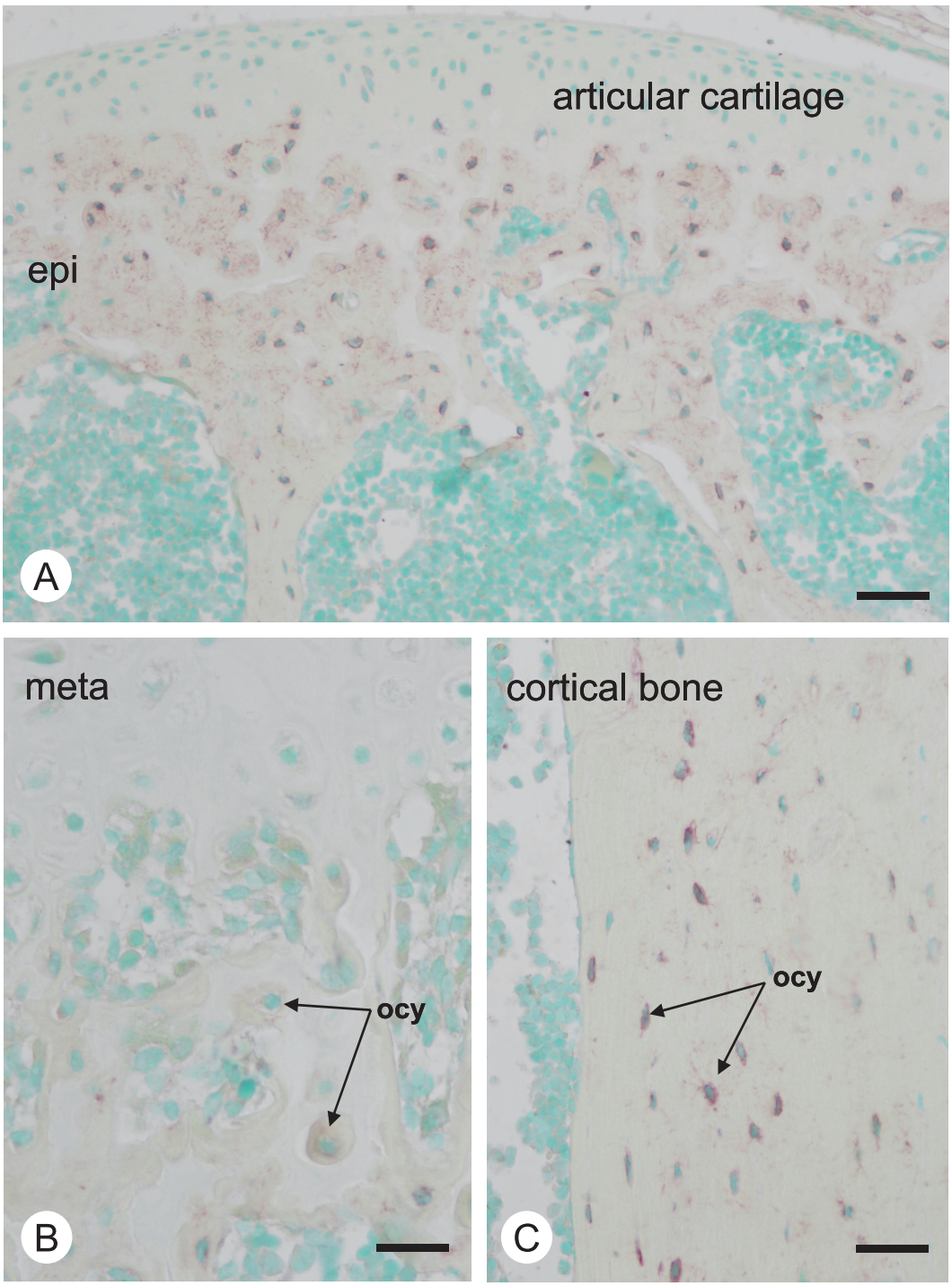

Fig. 3 Distribution of FGF23 in the epiphysis, metaphysis and diaphyseal cortical bone
A: FGF23-positive osteocytes (red) are prominent in the epiphysis (epi). B: In contrast, FGF23-reactive osteocytes (ocy) are hardly seen in the metaphysis (meta). C: In cortical bone, many FGF23-positive osteocytes (ocy) can be seen.
Bar, A: 80μm, B,C: 40μm

We, however, postulated that *klotho* deficiency might also affect bone in a way not linked to the FGF23-klotho axis. We have observed that *klotho*-deficient primary metaphyseal trabeculae were excessively mineralized, probably due to markedly increased concentrations of calcium and phosphate, while the secondary metaphyseal trabeculae showed defective mineralization[71, 72]. Using electron probe microanalysis, we demonstrated higher and lower contents of calcium and phosphorus in primary and secondary metaphyseal trabeculae of *klotho*-deficient

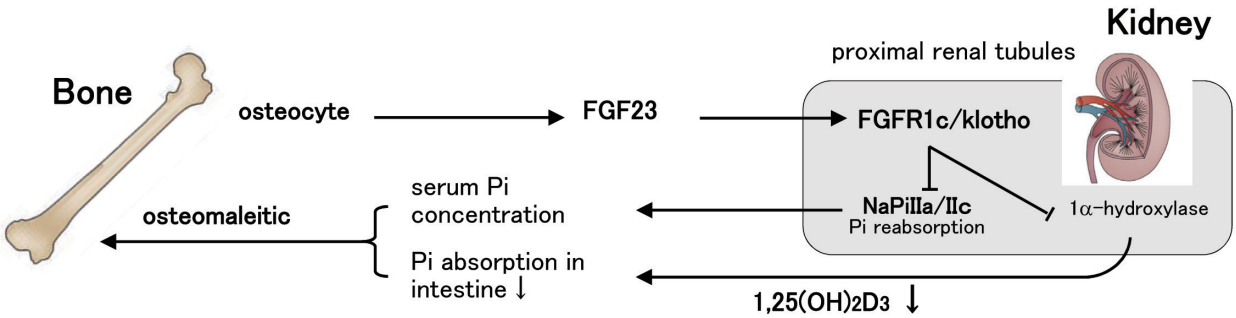

Fig. 4 Schmatic design of biological function of osteocytes controlling serum concentration of phosphate
FGF23 synthesized by osteocytes circulates, and bind to the FGFR1c/klotho complex in the proximal tubules of kidney. FGF23 serves as a phosphaturic agent that inhibits $1,25(OH)_2D_3$ production and the function of sodium/phosphate co-transporter II (NaPi II), which inhibits reabsorption of phosphates.

mice, respectively. More recently, we found matrix proteins -- matrix Gla proteins (MGP), DMP-1 and osteocalcin with a high affinity to hydroxyapatite -- localized in the excessively mineralized osteocytic lacunae of *klotho*-deficient bone, indicative of dying osteocytes[73]. These findings suggest that osteocalcin and MGP are ectopically synthesized in *klotho*-deficient osteocytes, which also synthesize a large amount of DMP-1. Consequently, the osteocytic lacunae are filled with mineralized crystals and osteocytic function is disrupted. It is feasible, therefore, that there is some degree of malfunction in *klotho*-deficient osteocytes in a manner independent from the FGF23-klotho axis.

Conclusion

The regularly arranged OLCS is a functional syncytia, where the osteocytes are interconnected through gap junctions between their cytoplasmic processes. Molecules in the OLCS can be transported throughout the cytoplasmic processes connected by their gap junctions, and through the pericellular space in the osteocytic canaliculi. Transport of minerals and small molecules through a spatially well-organized OLCS appears to be pivotal for mechanosensing, mineral homeostasis and bone remodeling control. In addition to the biological functions of OLCS, osteocytes synthesize sclerostin and FGF23, which have been reported to be involved in regulation of bone remodeling and serum concentration of phosphate. Thus, osteocytes were not merely varied cells in bone matrix, but actively orchestrate bone remodeling and phosphate homeostasis.

Acknowledgements

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