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Review

The discovery of α-Klotho and FGF23 unveiled new insight into calcium and phosphate homeostasis

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Abstract. The traditional view of calcium homeostasis is that it is maintained by two essential reactions. First, changes in extracellular Ca^{2+} are sensed in several distinct cell types, stimulating the secretion of parathyroid hormone (PTH), $1,25(OH)_2$ D and calcitonin in response to the body's requirement. Second, these calcitropic hormones then act on the calcium-translocating cells of the kidney, bone, and intestine to restore calcium balance. Recent progress indicates that α-Klotho and fibroblast growth factor (FGF) 23 are key players that integrate the multi-step regulatory system of calcium homeostasis that rapidly adjusts the extracellular calcium concentration and continuously maintains its concentration within a narrow physiological range. α-Klotho and FGF23 are also found to be major players in the regulatory system of phosphate homeostasis. Here, the demonstration of the molecular functions of α-Klotho and FGF23 has recently given new insight into the field of calcium and phosphate homeostasis.

Keywords. Calcium homeostasis, phosphate homeostasis, α-Klotho, FGF23, parathyroid hormone, vitamin D, calcium-sensing receptor, Na^+,K^+-ATPase.

Introduction

Calcium ion (Ca^{2+}) is a key mineral in the body because of its diverse intra- and extracellular roles [1]. Intracellular Ca^{2+}, particularly the cytosolic free calcium concentration ($[Ca^{2+}]_i$), is an important second messenger and is the cofactor for protein and enzymes that regulate a variety of cellular functions, including hormonal secretion, neurotransmission, muscle contraction, cell motility, glycogen metabolism and cellular proliferation [1]. The $[Ca^{2+}]_i$ in resting cells is ~100 nM. It is regulated by a series of channels, pumps, and other transport mechanisms that control the movements of Ca^{2+} into and out of the cell and between various intracellular compartments [2]. Consonant with its role as a second messenger, the $[Ca^{2+}]_i$ in activated cells can rise by 10–100-fold due to the uptake of extracellular Ca^{2+} and/or release of Ca^{2+} from cellular stores, such as the endoplasmic reticulum (ER). Despite the importance of intracellular Ca^{2+} in cellular metabolism, this compartment comprises less than 1 % of total body calcium content in mammalian species [3].

Extracellular Ca^{2+} ($[Ca^{2+}]_o$) serves as a cofactor (for adhesion molecules, clotting factors, and other proteins), regulates neuronal excitability, and is an essential part of mineral phase of bone. The cloning of a G protein-coupled calcium sensing receptor (CaR) from bovine parathyroid glands in 1993 [4] provided evidence that the calcium ion can, in fact,

serve as an extracellular first messenger. In contrast to intracellular Ca^{2+}, the extracellular free Ca^{2+} concentration is ~1–1.3 mM in mammals [3, 5]. The rigid control of the $[Ca^{2+}]_o$ ensures a steady supply of Ca^{2+} for its intracellular functions. The total amount of soluble extracellular Ca^{2+}, however, constitutes only a minute fraction of the total calcium content (*e.g.*, mammals ~0.1 %). The highest fraction of total body calcium resides as calcium phosphate salts within the skeleton where it provides a structural framework that protects critical body structures and facilitates locomotion, as well as a large reservoir of calcium and phosphate ions for times when intestinal absorption and renal conservation of these ions are not sufficient for maintenance of constancy of the extracellular Ca^{2+} and phosphate concentrations [3, 5, 6].

A complex homeostatic system has evolved in mammalian species, which is designed to maintain of the extracellular ionized calcium concentration nearly constant [3, 5–7]. This system includes two essential components. The first component comprises cell types that sense changes in the extracellular Ca^{2+} concentration and respond with appropriate alterations in their secretion of Ca^{2+}-regulating hormones. The parathyroid glands are key sensor of variations in the $[Ca^{2+}]_o$ in mammalian species, responding with changes in parathyroid hormone (PTH) secretion that are inversely related to the ambient ionized calcium concentration [8]. In contrast to the parathyroid cell, high $[Ca^{2+}]_o$ stimulate hormonal release from calcitonin (CT)-secreting parafollicular or C-cells of the thyroid gland [9]. The second essential component of the homeostatic system is the effecter systems, specialized cells in the kidneys, bone and intestine, that respond to these calcitropic hormones with changes in the transport of mineral ions so as to restore the extracellular concentrations of Ca^{2+} and phosphate toward normal.

Calcium homeostasis is largely regulated by the actions of three major hormones, *i.e.*, PTH, 1,25-dihydroxyvitamin D [1,25$(OH)_2$ D], and CT that trigger the response of target cells by binding to their receptors and subsequently control the intake, metabolism, and excretion of calcium. Furthermore, the synthesis and secretion of these hormones are mutually activated or suppressed by each other, and are also controlled by extracellular calcium concentrations monitored by the CaRs [10, 11].

In response to slight decrements in the level of the $[Ca^{2+}]_o$, PTH release is rapidly increased (within seconds) [12–14]. Renal actions of PTH include a reduction in tubular reabsorption of phosphate ions as well as an increase in distal tubular reabsorption of Ca^{2+} [2], which takes place within minutes. PTH also acts on bone cells to enhance the release of Ca^{2+} from the skeleton within 1–2 h [3, 5, 6, 15, 16]. If hypocalcemia is more prolonged, elevation of PTH level persists for several hours or more, and circulating 25-hydroxyvitamin D [25(OH)D] is activated by the renal 25-hydorxyvitaminD-1α-hydroxylase (Cyp27b1) to form 1,25$(OH)_2$D [17–19]. This acts on specific receptors in the intestine [20, 21] to promote gastrointestinal absorption of Ca^{2+} as well as phosphate ions. Hypercalcemia produces the opposite series of changes in the function of the Ca^{2+}homeostatic system. Reductions in the circulating levels of PTH promote renal Ca^{2+} wasting and phosphate retention as well as diminished skeletal release of mineral ions and, eventually, reduced gastrointestinal absorption of these ions through decreased synthesis of 1,25$(OH)_2$D. Therefore, as with hypocalcemia, the response of the homeostatic mechanism to hypercalcemia tends to restore both extracellular Ca^{2+} and phosphate concentrations toward normal.

Intensive study on calcium homeostasis over the past several decades has established a systematized understanding of its roles in living phenomena, leaving us with the impression that this field is fairly defined and understood. However, the currently demonstrated molecular function of α-Klotho (α-Kl) and FGF23 has given new insights into this field. Thus, the biological roles and molecular functions of α-Kl and FGF23 in Ca^{2+} and phosphate homeostasis are summarized here.

The discovery of α-Klotho

In Greek mythology, life span is controlled by the three daughters of Zeus and Themis, *i.e.*, Klotho who combs and spins the thread of life, Lachesis who determines the length of life by measuring the length of thread, and Athropos who cuts the string to bring a life to an end. In science, the name of Klotho was conferred to a gene that was fortuitously discovered in 1997 [22].

α-klotho (α-*kl*) mutant mice were originally described as a short-lived model that display a variety of accelerated aging-related disorders, including hypoactivity, sterility, skin thinning, decreased bone mineral density, vascular calcifications, ectopic calcification in various soft tissues (lung, kidney, stomach, heart, and skin), defective hearing, thymus atrophy, pulmonary emphysema, ataxia, and abnormality of pituitary gland, as well as hypoglycemia and severe hyperphosphatemia in association with increased concentrations of 1,25$(OH)_2$D [22] (Fig. 1).

The responsible gene for these phenotypes was termed α-*kl*, and was shown to encode a type I

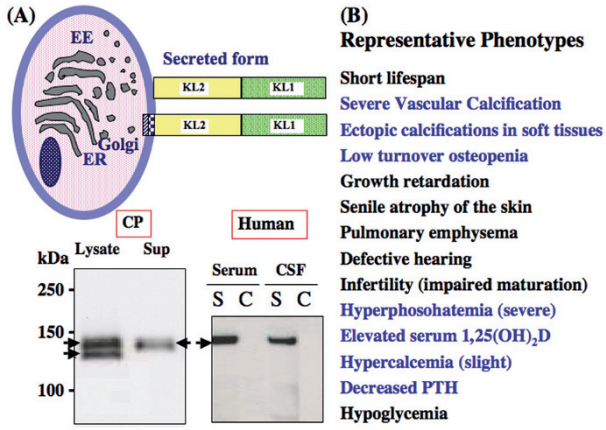

Figure 1. Cellular and secreted forms of α-Klotho (α-Kl) and representative phenotypes of *α-kl*$^{-/-}$ mice. (*A*) The *α-kl* gene encodes a type I membrane protein with considerable similarity to β-glycosidase. α-Kl has been predicted to be present on the cell surface. However, large amounts of α-Kl are detectable in the cytoplasm and gives rise to dual bands of 120 kDa (premature form) and 135 kDa (mature form). In addition, the extracellular domain is cleaved and secreted into blood, cerebrospinal fluid (CSF), and urine (data not shown). (*B*) Phenotypes of *α-kl*$^{-/-}$ mice are listed. Phenotypes related to the abnormalities of calcium and phosphate metabolisms are representative (blue).

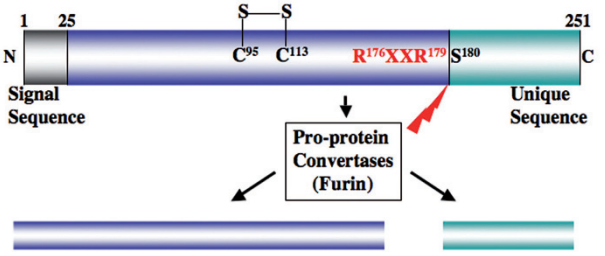

Figure 2. Schematic structure of fibroblast growth factor (FGF) 23. The FGF23 structure is schematically illustrated. FGF23 has a disulfide bond in the FGF-like sequence and internal cleavage site immediately after the $R^{176}X^{177}X^{178}R^{179}$ consensus sequence for convertase and cleaved into two peptides.

membrane protein (α-Kl) with an extracellular domain that exhibits significant similarity to β-glycosidases, enzymes involved in digestion of the sugar moiety of substrates [23]. The α-Kl has therefore been predicted to be present on the cell surface. However, large amounts of α-Kl are detectable as dual forms in the cytoplasm. The 120-kDa premature form of α-Kl resides mainly in the ER, while the 135-kDa form is a mature protein that is cleaved and secreted into the blood, cerebrospinal fluid (CSF), and urine [24, 25] (Fig. 1). This suggests α-Kl may have dual actions depending on its intracellular and secreted forms.

The *α-kl* gene is predominantly expressed in tissues that are involved in calcium homeostasis; *i.e.*, the parathyroid glands, kidney and the choroid plexus [22, 26]. The parathyroid glands play a key role in systemic calcium homeostasis by monitoring the concentration of $[Ca^{2+}]_o$ through the CaR and secreting appropriate levels of PTH to maintain normal $[Ca^{2+}]_o$ concentrations [4, 10, 27]. In the kidney, α-Kl is exclusively co-expressed with calcium-permeable transient receptor potential V5 (TRPV5) channels, Na^+/Ca^{2+}exchanger 1 (NCX1) and calbindin-D_{28K} (a vitamin D sensitive intracellular Ca^{2+}-transporting protein) in a specialized region of the nephron segments where transepithelial Ca^{2+} reabsorption is actively regulated. This co-localization is believed to be important for the homeostatic control of Ca^{2+}. Indeed, mice lacking TRPV5 display diminished renal Ca^{2+} reabsorption, which causes severe hypercalciuria and compensatory intestinal uptake of dietary Ca^{2+} [28]. The choroid plexus in many respects functions as "the kidney of the brain" [29]. Its main function is the secretion of CSF, which involves the movement of water by osmosis and the unidirectional transport of ions including calcium [30]. The expression of α-Kl in tissues involved in calcium metabolism, led to the prediction that α-Kl might be involved in calcium homeostasis [31].

The discovery of FGF23

Fibroblast growth factors (FGF) share a common core region, and thus the homology-based screening method has enabled us to identify FGF homologues [32]. Among the various trails, Yamashita et al. [33] succeeded in detecting a novel genomic sequence that showed some homology to a sequence of mouse FGF15 (recently, the mouse FGF15 was determined to be the mouse ortholog of human FGF19). This led Yamashita and colleagues [34] to the cloning of mouse FGF23 and human FGF23. Based on phylogenetic analyses, human FGFs are classified into seven subfamilies and FGF23 belongs to the FGF19 subfamily. The recently determined crystal structure of FGF19 indicates that the members of the FGF19 subfamily, FGF19 (FGF15 in mice), FGF21, and FGF23, share some unique structural features that distinguish the FGF19 subfamily from other subfamilies. The FGF23 protein is relatively large (30 kDa) compared to other FGF family members and is processed by a proconvertase enzyme into two smaller fragments of ~18 kDa (N-terminal fragment) and 12 kDa, a unique extended sequence at the C-terminal (Fig. 2). Since the calculated molecular weight of the mature FGF23 polypeptide (MW; 25331.05) is smaller than the actual one [35], FGF23 appears to undergo some post-translational modification. Recent studies have demonstrated that the recombinant mature FGF23 protein expressed in CHO cells has *O*-linked sugar chains [36]; however, it did not have an *N*-linked glycosyla-

tion at the sole putative attachment site (Asn27-Ala28-Ser29). The N-terminal amino acid of the 12-kDa protein (C-terminal part) was identified as Ser^{180}. Since this residue is located immediately after the consensus sequence (Arg^{176}-His^{177}-Thr^{178}-Arg^{179}) recognized by some proprotein convertases, this 12-kDa protein is a likely product of the internal proteolytic cleavage of the mature FGF23 protein (Fig. 2).

The mode of action and biological activity of FGF23 are quite distinct from the members of other subfamilies. The *in vivo* biological activity and physiological role of FGF23 in phosphate and vitamin D metabolism have recently been clarified. Forced expression of FGF23 *in vivo* exhibited hypophosphatemia with an increased phosphate excretion and low or inappropriately normal concentration of $1,25(OH)_2D$ [37–41]. On the other hand, *Fgf23* knockout mice showed hyperphosphatemia with an increased renal phosphate reabsorption and elevated serum concentration of $1,25(OH)_2D$ [42, 43]. FGF23 induces altered gene expression of renal Cyp27b1 and 25-hydorxyvitamin D-24-hydoxylase (Cyp24a) within 1 h after injection in mice [44]. FGF23 down-regulates the abundance of both type IIa and type IIc sodium phosphate co-transporters (NaPi-IIa, NaPi-IIc) on the apical surface of the renal proximal tubular epithelial cells [44, 45]. The canonical FGFs are local factors that usually function around the producing cells. The possible direct effects of FGF23 are, however, unique and quite different from the typical effects of other FGFs, since any substantial amount of FGF23 is not expressed in the kidney, but expressed in the bone and transported to the kidney *via* blood stream. FGF23, together with FGF19 and FGF21, acts as a systemic factor.

Major cause of *α-kl*$^{-/-}$ and *Fgf23*$^{-/-}$ mice

Of significance, the *α-kl*-deficient phenotype largely overlaps with the phenotype of *Fgf23* null mice. In fact, both *α-kl* and *Fgf23* knockout mice develop hypercalcemia, severe hyperphosphatemia and increased serum levels of $1,25(OH)_2D$ [22, 42]. Furthermore, the overproduction of $1,25(OH)_2D$ and altered mineral ion homeostasis are the major cause of the premature aging-like phenotypes observed in *α-kl*$^{-/-}$ and *Fgf23*$^{-/-}$ mice, because the lowering of $1,25(OH)_2D$ activity by (i) dietary restriction (a regimen in which *α-kl*$^{-/-}$ mice were fed a vitamin D-deficient diet) [46], (ii) genetic ablation of 1α-hydroxylase in *α-kl*$^{-/-}$ mice (unpublished data) or in *Fgf23*$^{-/-}$ mice [47, 48], or (iii) genetic ablation of the *VDR* gene in *α-kl*$^{-/-}$ mice (unpublished data) were all able to rescue the premature aging-like phenotypes and enabled these mice to survive normally without obvious abnormalities. These independently established conclusions consistently indicate that α-Kl is the key regulator of calcium and phosphate homeostasis and clearly explain the major cause of phenotypes observed in *α-kl*$^{-/-}$ mice and the reason why α-Kl is expressed in the tissues related to calcium and phosphate regulation. Furthermore, the reduced blood glucose and insulin concentrations observed in *α-kl*$^{-/-}$ mice can be strikingly improved in both male and female *α-kl*$^{-/-}$ mice when they are fed a vitamin D-deficient diet, suggesting that the impaired glucose metabolism in *α-kl*$^{-/-}$ mice is a secondary effect caused by increased vitamin D activity [46, 49]. Of particular interest, the circulatory levels of FGF23 are about 2000-fold higher in *α-kl* null mice [50] than those in wild-type (WT) mice. Despite higher circulatory levels of FGF23 in *α-kl* mutants, these mice show physical, biochemical and morphological features similar to those of *Fgf23* null mice, but not of *Fgf23* transgenic mice [51], suggesting that widely encountered premature aging-like features and altered mineral ion homeostasis in the *α-kl* null mice are due to an inability of FGF23 to exert its bioactivities in absence of α-Kl [52]. The next question that needs to be resolved is how hypervitaminosis D and the subsequently altered mineral ion balance lead to the multiple premature ageing-like phenotypes, as documented in both *α-kl* and *Fgf23* null mice [22, 42, 48].

α-Klotho mutations in human

Familial tumoral calcinosis is characterized ectopic calcifications and hyperphosphatemia due to inactivating mutations in *Fgf23* or UDP-*N*-acetyl-α-D-galactosamine:polypeptide *N*-acetyl-galactosaminyl transferase 3 (*GALNT3*). FGF23 is a hormone that promotes renal phosphate excretion by decreasing phosphate reabsorption in the proximal tubule and also reduces circulating $1,25(OH)_2D$ by both decreasing biosynthesis and metabolism of $1,25(OH)_2D$. GALNT 3 is a Golgi-associated enzyme that selectively *O*-glycosylates a furin-like convertase recognition sequence in FGF23, thereby allowing secretion of intact FGF23 by preventing proteolytic processing of FGF23 [53]. Therefore, dysfunction of either FGF23 or GALNT 3 decreases circulating bioactive FGF23. As described, the *α-kl*-deficient phenotype largely overlaps with the phenotype of *Fgf23*-null mice. These indicate functional cross-talk between α-Kl and FGF23 and leads us to speculate that *α-kl* mutation(s) might be found in the genome of patients with tumoral calcinosis.

Ichikawa et al. [54] reported a homozygous missense mutation (H193R) in the *α-kl* gene of a 13-year-old

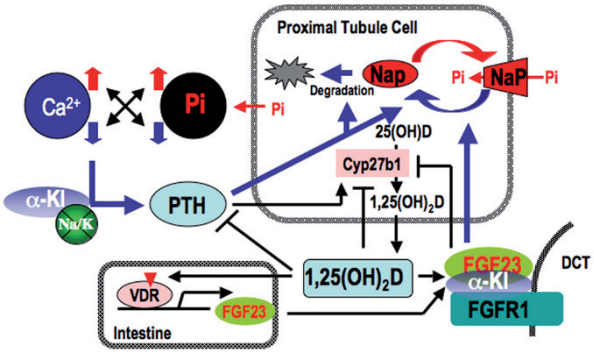

Figure 3. Parathyroid hormone (PTH) and FGF23/α-Kl suppress renal phosphate reabsorption. The majority of phosphate reabsorption occurs in the proximal tubule cells in the kidney. PTH and FGF23/α-Kl promote the rapid removal of NaPi-IIa from the membrane. An increase in serum phosphate promotes proliferation of parathyroid cells, PTH secretion and stabilization of PTH mRNA. The increased phosphate signal might be mediated by means of reduced serum Ca^{2+} levels.

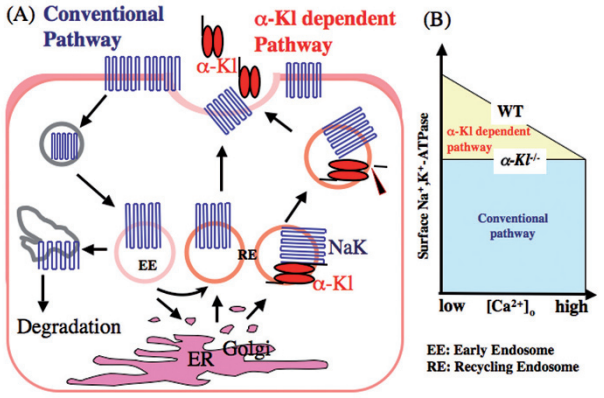

Figure 4. Surface recruitment of Na^+,K^+-ATPase in correlation with the cleavage and secretion of α-Kl. In α-Kl expressing cells, Na^+,K^+-ATPase is recruited to the cell surface by a combination of 'the conventional pathway' and 'the α-Kl dependent pathway'. Low Ca^{2+} induces the massive recruitment of Na^+,K^+-ATPase to the plasma membrane. In contrast, high Ca^{2+} leads to the decline of such additional recruitment. Accordingly, $[Ca^{2+}]_o$ regulates this additional recruitment of Na^+,K^+-ATPase to the plasma membrane in correlation with the cleavage and secretion of α-Kl.

girl who presented with severe tumoral calcinosis with dural and carotid artery calcifications. Mapping of H193R mutation onto the crystal structure of myrosinase, a plant homolog of α-Kl, revealed that this histidine residue was at the base of the deep catalytic cleft, and mutation of this histidine to arginine should destabilize α-Kl. Expression and secretion of H193R α-Kl were thus markedly reduced, resulting in diminished ability of FGF23. This patient exhibited defects in mineral ion homeostasis with marked hyperphosphatemia, hypercalcemia and subsequent ectopic calcifications in soft tissues, as well as elevation of FGF23 and $1,25(OH)_2D$ in serum.

Conversely, overproduction of α-Kl has also adverse physiological effects. Very recently, Brownstein et al. [55] discovered a new human disease featuring hypophosphatemic rickets with marked parathyroid hyperplasia and decrease in circulating $1,25(OH)_2D$ caused by a mutation that results in increased levels of circulating α-Kl. This disease is due to a *de novo* translocation with a breakpoint adjacent to *α-kl* gene resulting in marked increase in serum levels of α-Kl. Unexpectedly, despite of the increase in α-Kl, the circulating FGF23 level is markedly elevated in this patient. This is inconsistent with accumulated evidence that has implicated an elaborate mutual negative feedback regulation between α-Kl and FGF23. So far, circulatory levels of FGF23 have been shown to be increased in *α-kl* null mice [50] and conversely, the administration of FGF23 resulted in down-regulation of α-Kl expression. In this patient, higher circulatory FGF23, in combination with highly expressed α-Kl, led to remarkable suppressions of both $1,25(OH)_2D$ synthesis and renal phosphate reabsorption, resulting in vast renal wasting of phosphate and decreased serum $1,25(OH)_2D$ level. Furthermore, the increase in serum PTH led to severe hypophosphatemia by promoting the rapid removal of NaPi-IIa from the apical membrane and its subsequent degradation (Fig. 3). This case represents an example of a mutation that causes an increase in α-Kl activity. Importantly, the symptom of a gain-of-function mutation in human [55] is opposite to that found in a loss-of-function mutation in human [54] and in mice [22]. Discoveries in these patients provide a compelling evidence that α-Kl is a pivotal regulator of calcium and phosphate homeostasis not only in the mouse but also in humans, although the role of α-Kl in phosphate homeostasis is largely unknown.

α-Klotho-dependent surface recruitment of Na^+,K^+-ATPase in response to $[Ca^{2+}]_o$

The Na^+,K^+-adenosine triphosphatase (Na^+,K^+-ATPase) is a well-studied heteromer consisting of a larger α subunit responsible for ion transport and catalytic activity [56], and of a smaller glycosylated β subunit required for the regulation of the catalytic action of the α subunit and for the control of cell surface recruitment of Na^+,K^+-ATPase complex. Although Na^+,K^+-ATPase is ubiquitously expressed, its expression levels vary among tissues. It is notable that the expression level of Na^+,K^+-ATPase is significantly high in the kidney DCT cells, choroid plexus and the parathyroid glands, which suggests that highly expressed Na^+,K^+-ATPase in these α-Kl-expressing cells plays a specific role in cooperation with α-Kl. As

predicted, isolated Na^+,K^+-ATPase bound of two intracellular forms of α-Kl, the 120-kDa premature form and 135-kDa mature form (Fig. 1). Subcellular fractionation and the cell surface biotinylation analyses revealed that the complexes of premature α-Kl and Na^+,K^+-ATPase were located in the ER fraction, while mature α-Kl and Na^+,K^+-ATPase complexes accumulated in the endosome and Golgi apparatus fractions (Fig. 4). This suggests that a subset of Na^+,K^+-ATPase traffics from the ER to the cell surface in conjunction with α-Kl, and α-Kl/Na^+,K^+-ATPase complexes locate in ER and Golgi apparatus, and abundantly accumulate in the endosome fraction, ready for recruitment to the cell surface [31, 57] (Fig. 4).

The interaction of α-Kl and Na^+,K^+-ATPase raised a hypothesis that α-Kl directly affects Na^+,K^+-ATPase activity and/or its recruitment. Based on the following: (i) the α-Kl and Na^+,K^+-ATPase complexes are preferentially localized intracellularly and (ii) the catalytic action of Na^+,K^+-ATPase is an event that is regulated on the cell surface, the regulation of catalytic efficiency by α-Kl was deemed unlikely, and instead focus was put on its regulatory role in the recruitment of Na^+,K^+-ATPase to the cell surface. The surface expression of Na^+,K^+-ATPase is inversely correlated with extracellular Ca^{2+} concentration. When incubated in a low Ca^{2+} solution, the activity of Na^+,K^+-ATPase increased rapidly (it became detectable within 30 s after the shift of $[Ca^{2+}]_o$), and decreased in a high Ca^{2+} solution. Moreover, the increased activity of Na^+,K^+-ATPase in low calcium-media was associated with the increased presence of Na^+,K^+-ATPase in the plasma membrane. In contrast, high calcium led to a decrease of Na^+,K^+-ATPase in the plasma membrane (Fig. 4). Importantly, the evidence in *α-kl*$^{-/-}$ mice indicated that α-Kl is essential for the rapid recruitment of Na^+,K^+-ATPase to cell surface. Furthermore, secretion of α-Kl is induced in response to low $[Ca^{2+}]_o$ in the kidney, parathyroid glands and the choroid plexus, suggesting that the fluctuation of $[Ca^{2+}]_o$ mediates cleavage of α-Kl in conjunction with the rapid response that recruits Na^+,K^+-ATPase to the cell surface [31, 57].

The regulated recruitment of Na^+,K^+-ATPase into cell surface is summarized as follows (Fig. 4). The surface expression of Na^+,K^+-ATPase is usually controlled by the balance of recruitment to the plasma membrane and internalization of Na^+,K^+-ATPase (the conventional pathway) [58]. In α-Kl expressing cells, Na^+,K^+-ATPase is recruited to the cell surface by a combination of "the conventional pathway" and "the α-Kl-dependent pathway" (Fig. 4A). The latter is characterized by Ca^{2+} dependency and a rapid response (Fig. 4B). Under normo-calcemic conditions, a certain amount of Na^+,K^+-ATPase is additionally recruited by the α-Kl-dependent pathway. However, the surface recruitment of Na^+,K^+-ATPase in *α-kl*$^{-/-}$ mice is solely dominated by the conventional pathway and probably represents 'basal' recruitment. Therefore, the amount/activity of surface Na^+,K^+-ATPase in WT mice is significantly higher than that of *α-kl*$^{-/-}$ mice. Low $[Ca^{2+}]_o$ induces further recruitment of Na^+,K^+-ATPase to the plasma membrane. In contrast, high Ca^{2+} leads to the decline of such additional recruitment. Accordingly, $[Ca^{2+}]_o$ regulates this additional recruitment of Na^+,K^+-ATPase to the plasma membrane in correlation with the cleavage and secretion of α-Kl.

α-Klotho/Na^+,K^+-ATPase complex in the transepitherial Ca^{2+} transport

Na^+,K^+-ATPase is a membrane-bound pump that transports two K^+ in and three Na^+ out of the cell [56], resulting in the maintenance of an electrochemicnal gradient in the plasma membrane. Na^+,K^+-ATPase activity influences many kinds of cellular and physiological events that take place near the cell membrane by the regulation of electrochemical gradients in the plasma membrane, since the fluctuations of Na^+,K^+-ATPase activity reconcile the activities of other ATPases, ion channels, and ion exchangers including Ca^{2+}/cation antiporter superfamily, such as NCX and NCKX [58–60], whose main role is to control Ca^{2+} flux across the membranes. Na^+/Ca^{2+} exchangers comprise three members (NCX1, most extensively studied, and broadly expressed with particular abundance in heart, brain and kidney; NCX2 expressed in brain; and NCX3 expressed in brain and muscle) and the NCX proteins subserve a variety of roles depending upon the site of expression. These include cardiac excitation-contraction coupling, neuronal signaling and Ca^{2+} reabsorption in the kidney (these are summarized in [61]). This raises the possibility that the higher Na^+ gradient created by the Na^+,K^+-ATPase activity may drive the transepitherial calcium transport in the choroid plexus and the kidney.

In the choroid plexus, the importance of α-Kl/Na^+,K^+-ATPase in Ca^{2+} transport was suggested by by the facts that the surface amounts of Na^+,K^+-ATPase in *α-kl*$^{-/-}$ mice were lower than that of WT and that, consistent with this, the concentration of total calcium in CSF in *α-kl*$^{-/-}$ mice was considerably lower than that of WT mice. These results imply that calcium homeostasis is impaired in the CSF of *α-kl*$^{-/-}$ mice and strongly suggest that α-Kl is involved in regulating the calcium concentration of CSF.

Similarly, the increase of surface Na^+,K^+-ATPase expression may play a pivotal role in Ca^{2+} reabsorption in the kidney DCT cells. The calcium transport across various nephron segments was actively examined in the second half of the last century. In 1963, Lassiter et al. [62] hypothesized that the ascending limb of Henle may be a major site of Ca^{2+} and Na^+ reabsorption. Rocha et al. [63] demonstrated net Ca^{2+} absorption in the thick ascending limb perfused *in vitro*, and concluded that Ca^{2+} movement in this segment was not entirely a passive one. The renal handling of Ca^{2+} was then intensively investigated using advanced technologies such as micropuncture, microperfusion of isolated tubule segments, isolation of tubular cell plasma membrane, and electron microprobe analysis, resulting in the clarification of many aspects of complex renal functions [64]. In parallel, the involvement of Ca^{2+}-ATPase [65] and Ca^{2+}/Na^+ antiport [66] in Ca^{2+} transport was suggested, and Shareghi and Stoner found that Ca^{2+} absorption in the DCT and the granular portion of the cortical collecting duct was significantly enhanced by PTH [67]. In addition, Quamme and colleagues [68–70] reported the effects of CT and $[Ca^{2+}]_o$ levels on magnesium and Ca^{2+} transport in the nephron segments. In 1993, Brown et al. [4] proposed a molecular basis for the above observations by the discovery of CaR. CaR senses a high concentration of $[Ca^{2+}]_o$ and inhibits tubular reabsorption of calcium when $[Ca^{2+}]_o$ is increased. Thus, serum Ca^{2+} level is excessively elevated in CaR-knockout mice, because renal tubular Ca^{2+} reabsorption is not suppressed [71]. However, considering the transcellular Ca^{2+} transport in the DCT nephron and choroid plexus, the involvement of CaR seems unlikely or, if any, to be limited because the major site of intrarenal CaR expression is the thick ascending limb of Henle's loop but not in DCT cells [36], and because CaR is not detectable in the choroid plexus [10]. Furthermore, CaR is known to preferentially respond to increasing $[Ca^{2+}]_o$ concentration [27]. On the other hand, α-Kl and Na^+,K^+-ATPase are involved in the response to a wide range of $[Ca^{2+}]_o$. Indeed, transepithelial Ca^{2+} transport in the DCT nephron and choroid plexus is efficiently induced in response to low $[Ca^{2+}]_o$ concentrations and suppressed when Ca^{2+} concentrations are increased. This indicates that the α-Kl/Na^+,K^+-ATPase system regulates Ca^{2+} transport, at least in part, in a CaR-independent manner. Taken together, these findings imply the importance of a calcium sensor machinery that is distinct from CaR.

These studies suggest that Ca^{2+} reabsorption is enhanced directly in response to low $[Ca^{2+}]_o$ stimuli in Henle's loop and perhaps in the distal nephron as well in a cell autonomous manner independent of calcium-regulating hormones. Kronenberg et al. ([11] p. 1321) described their prediction as follows: "Ca^{2+} reabsorption is enhanced directly by tendency to hypocalcemia, which is detected by calcium-sensing receptors in Henle's loop and possibly also in the distal nephron that control transepithelial calcium movements independent of PTH or $1,25(OH)_2D$". Although its molecular basis has long remained unknown, the following newly proposed mechanism [31] may be the first satisfactory explanation for the prediction proposed by Kronenberg et al. The transepithelial transport of Ca^{2+} is directly triggered and processed by the increased Na^+ gradient in cooperation with TRPV5, calbindin D_{28k}, and NCX-1, all of which are exclusively co-expressed with α-Kl in the nephron segment responsible for the active and regulated Ca^{2+} reabsorption (Fig. 5A). Indeed, regulated reabsorption of calcium in the kidney is impaired in *α-kl*$^{-/-}$ mice, resulting in the excess excretion of calcium into urine [72].

α-Klotho/Na^+,K^+-ATPase complex in regulated PTH secretion

In the parathyroid glands, PTH secretion is stimulated by the decline of extracellular Ca^{2+}. Indeed, a substantial decease in serum Ca^{2+} concentration corresponds to a marked increase in serum PTH in WT mice. However, the serum PTH response in *α-kl*$^{-/-}$ mice is significantly lower than that of WT mice, suggesting that α-Kl is essential for the regulated secretion of PTH. Furthermore, the extent of PTH release in WT samples treated with ouabain, a specific inhibitor of Na^+,K^+-ATPase, is intriguingly similar to that seen in the specimens from *α-kl*$^{-/-}$ mice without ouabain, and ouabain treatment induced no additional inhibitory effects on PTH secretion in *α-kl*$^{-/-}$ cells [31]. Current and previous observations suggest the following regulatory scheme of PTH secretion. When $[Ca^{2+}]_o$ is lowered, Na^+,K^+-ATPase is quickly recruited to the plasma membrane through the"α-Kl dependent pathway" in correlation with the cleavage and secretion of α-Kl (Fig. 4A). An electrochemical gradient across the cell membrane created by increased Na^+,K^+-ATPase may cause the activation of unidentified signal(s), leading to release of PTH. In the PTH-secreting system, α-Kl and Na^+,K^+-ATPase act as the players of common signaling pathway, and thus, if this pathway is disrupted either by *a-kl* deficiency or by administration of ouabain, the regulated secretion of PTH is equivalently suppressed (Fig. 5A).

In turn, when parathyroid CaR is activated in response to the increase of $[Ca^{2+}]_o$, inositol-1,4,5-triphosphate

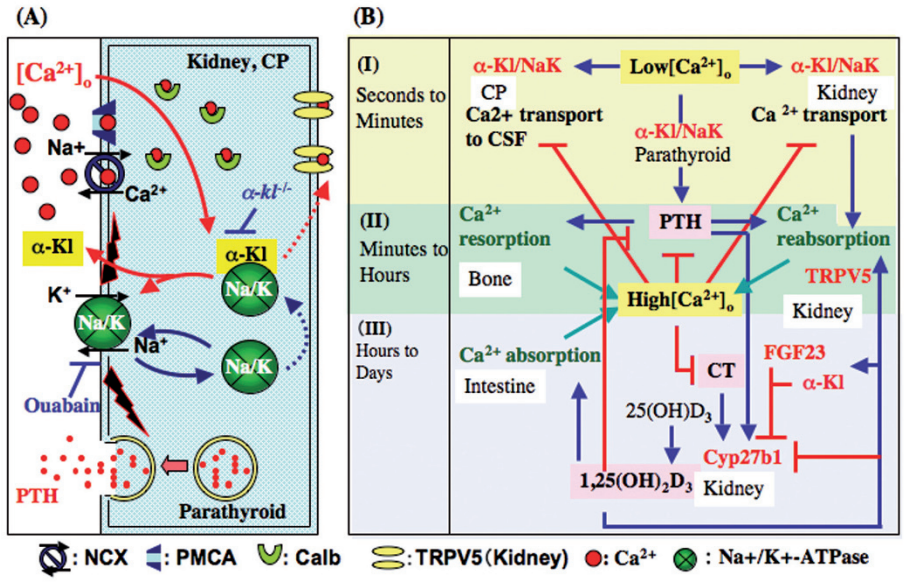

Figure 5. Roles of α-Kl in calcium homeostasis. α-Kl binds to Na^+, K^+-ATPase and regulates the recruitment of Na^+,K^+-ATPase to cell surface membrane in response to lowered $[Ca^{2+}]_o$. α-Kl controls calcium re-absorption in the kidney DCT cells, calcium transportation across the choroid plexus into CSF, and PTH secretion in the parathyroid glands. In contrast, α-Kl is involved in the signal transduction of Fgf23 that suppresses the gene expression of *Cyp27b1* in the DCT nephrons that leads to negative regulation of $1,25(OH)_2D_3$ synthesis. Calcium metabolism is governed by complicated reciprocal actions and feedback mechanisms and thus calcium concentration of serum, body fluid and CSF is maintained within strictly narrow ranges.

(IP_3) accumulates and intracellular calcium rises because of the release of calcium from intracellular stores and of the opening of plasma membrane calcium channels. This increase in the intracellular calcium subsequently leads to a decrease in PTH secretion [5]. Since Brown et al. [73] first suggested the importance of Na^+,K^+-ATPase in regulating the secretion of PTH, the crucial question of how Na^+,K^+-ATPase activity is regulated in response to low Ca^{2+} stimuli in the parathyroid glands has remained open. The identification of α-Kl as a key regulator for the surface recruitment of Na^+,K^+-ATPase in response to low Ca^{2+} stimuli offers an answer (Fig. 5A). However, the intracellular mechanism that actively induces PTH secretion when $[Ca^{2+}]_o$ is lowered remains to be fully understood.

α-Kl as a β-glucuronidase

Because of its high similarity with the β-glucosidase family, α-Kl may act as a β-glucosidase. However, this has been questioned because α-Kl lacks glutamic acid residues that are responsible for the catalytic activity of this enzyme family. Nonetheless, β-glucuronidase activity of α-Kl has been demonstrated and this enzymatic activity is reduced by addition of the specific inhibitors of β-glucuronidase [74]. In addition to naturally occurring β-glucuronides, α-Kl hydrolyzes extracellular sugar residues of TRPV5 [25]. As described, TRPV5 is exclusively co-expressed with calbindinD_{28k}, NCX-1 and α-Kl in the DCT epithelial cells responsible for the active and regulated Ca^{2+} re-absorption in the kidney. Ca^{2+} enters into the cell at the luminal membrane *via* the epithelial Ca^{2+} channel TRPV5 and/or TRPV6 and is sequestered by calbindinD_{28k} or -D_{9k}. Bound Ca^{2+} then diffuses to the basolateral cell surface where Ca^{2+} is extruded into the blood compartment *via* NCX1 and/or PMCA1b (Fig. 5A). The DCT epithelial cells are well equipped to transport and sustain high rates of Ca^{2+} influx. This high Ca^{2+} influx may be obtained by prolonged channel durability at the cell surface before inactivation or internalization, because, regardless of $[Ca^{2+}]_o$, both TRPV5 and TRPV6 are constitutively active as cation channels. Thus, a key component to create such an elevated Ca^{2+} influx is to increase the amount of TRPV5 and TRPV6 channel expression at the luminal cell surface. The question was how the TRPV5 and TRPV6 channel abundance is controlled? Since a large subset of TRPV5 is located in or near the apical (luminal side) membrane of DCT cells, it was hypothesized that these channels are shuttled from intracellular vesicles into the plasma membrane. This led us to focus on the regulatory mechanisms of intracellular trafficking, stabilization and internalization of TRPV5 and TRPV6. Chang et al. [25] reported a novel mechanism that regulates the abundance of TRPV5 at the luminal cell surface: α-Kl in urine, as a β-glucuronidase, increases TRPV5 channel abundance at the luminal cell surface by hydrolyzing the *N*-linked extracellular sugar residues of TRPV5 without affecting the Ca^{2+} uptake activity of the TRPV5 channel (Fig. 5A). This maintains a durable calcium channel activity and membrane calcium permeability in the kidney, resulting in an increased Ca^{2+} influx from the lumen to preserve normal blood Ca^{2+} levels by reduction of Ca^{2+} loss *via* urine (Fig. 5A). Ca^{2+} enters at the luminal membrane and is sequestered

by calcium binding proteins. The bound Ca^{2+} is then extruded into the blood compartment (Fig. 5A).
In this scheme, Ca^{2+} efflux at basolateral membrane is quickly controlled in response to the fluctuations of $[Ca^{2+}]_o$, and Ca^{2+} efflux at basolateral membrane might be mutually balanced by Ca^{2+} influx at apical membrane since the excessive influx of Ca^{2+} over the efflux ability results in the overflow of Ca^{2+} in the cytoplasm that is toxic to cells. Importantly, α-Kl plays the critical roles in both influx and efflux of Ca^{2+} by regulating the abundance of TRPV5 channels on the luminal cell surface [25] and the recruitment of Na^+,K^+-ATPase to basolateral cell surface [31], respectively (Fig. 5A). This leads to a prediction that α-Kl may play the critical role in the coordinate and balanced regulation of influx and efflux of Ca^{2+} that takes place on both sides of DCT cells.

FGF23, in combination with α-Kl, regulates vitamin D metabolism

FGF23, in combination with α-Kl, negatively regulates the metabolism of vitamin D in the kidney (Figs. 3, 5B). In this pathway, α-Kl is assumed to be necessary for the recognition of FGF23 by target cells. Urakawa et al. [50] reported that α-Kl binds to FGF23 and α-Kl converts the canonical FGF receptor 1c (FGFR1c) to a receptor specific for FGF23. This enables the high-affinity binding of FGF23 to the cell surface of the distal convoluted tubule where α-Kl is expressed. Subsequently, α-Kl enhances the ability of FGF23 to induce phosphorylation of FGF receptor substrate and extracellular signal-regulated kinase in various types of cells where exogenous α-Kl expression is induced. In summary, the interaction among α-Kl, FGFR1c, and FGF23 may be a new type of receptor modulation for signal transduction. As for the regulation of *1α-hydroxylase* (*Cyp27b1*) gene expression, it is reasonable to speculate that either (i) one or more signal mediators from the distal to the proximal tubule would be required, or (ii) some paracrine action of secreted α-Kl would be necessary for this signal transduction to occur, because the *Cyp27b1* gene is preferentially expressed in proximal convoluted tubule cells, but not in distal convoluted tubule cells where FGF23/α-Kl signal is transduced (Fig. 3).

FGF23, in combination with α-Kl, regulates renal phosphate reabsorption

The maintenance of normal phosphate homeostasis is critical for diverse physiological processes including energy homeostasis, formation of lipid bilayers, and bone formation. Its importance is illustrated by disorders featuring hypophosphatemia due to excessive renal phosphate loss. Affected patients develop rickets with diminished bone strength, deformity, short structure, and bone pain. Conversely, high phosphate levels may also have adverse physiological effects. Patients with chronic kidney disease develop hyperphosphatemia due to impaired renal clearance, and subsequent hyperparathyroidism and renal osteodystrophy.
The kidney plays a predominant role in the regulation of serum phosphorus levels. The majority of phosphate reabsorption occurs in the proximal tubule, and is mainly mediated by NaPi-IIa and additionally by the related protein NaPi-IIc [75, 76] (Fig. 3). PTH has been considered to be the major regulator of NaPi-IIa and NaPi-IIc abundance in the apical membranes of proximal tubule cells, promoting the rapid removal of NaPi-IIa from the membrane and its subsequent degradation [76–78]. As a feedback loop, an increment in phosphate is known to promote (i) proliferation of parathyroid cells, (ii) enhancement of PTH secretion, and (iii) stabilization of PTH mRNA, and thereby phosphate concentration can be approved. However, this evidence does not suggest the presence of phosphate receptor, since the effects of high phosphate is possibly indirect and may be mediated by means of reduced serum Ca^{2+} levels [79–82]. Notably, $[Ca^{2+}]_o$ and phosphate levels are inversely regulated, and thus the increase in serum phosphate results in the decrease in $[Ca^{2+}]_o$, which trigger the increase of PTH activity. Other than PTH, FGF23 down-regulates serum phosphate levels due to decreased NaPi-IIa abundance in the apical membrane (Fig. 3). In fact, homozygous loss of function of FGF23 results in hyperphosphatemia; conversely, gain of mutations of FGF23 result in hypophosphatemia [40, 41, 44]. α-Kl is essential for these two pathways since FGF23 signaling and regulated secretion of PTH are depend on the actions of α-Kl. As previously described, phenotypes found in *Fgf23*-deficient mice are commonly observed in *α-kl*-deficient mice, although the role of α-Kl in phosphate homeostasis *in vivo* and its detailed mechanism of effects remain to be solved.

The roles of α-Kl in multi-step regulation of calcium homeostasis

The comprehensive scheme of calcium homeostasis and a global image of α-Kl function in the regulatory network of calcium homeostasis are illustrated in Figure 5. As shown in Figure 5B, calcium homeostasis is maintained by a multi-step response system cate-

gorized according to the time course into (I) seconds to minutes, (II) minutes to hours, and (III) hours to day(s) order regulations. In response to hypocalcemic stimuli, transepithelial Ca^{2+} transport in the choroid plexus and the α-Kl-expressing nephron segments, as well as PTH secretion in the parathyroid glands are triggered by electrochemical gradients created by Na^+,K^+-ATPase (Fig. 5B-I) [31]. Because these responses begin immediately after a decline in Ca^{2+} concentration and persist for a short time, they can be placed into the 'seconds to minutes order regulation' (Fig. 5B-I). The PTH-mediated increase of Ca^{2+}, such as Ca^{2+} reabsorption in the kidney and Ca^{2+} resorption in the bone continues for hours [27] and thus belong to the 'minutes to hours order regulation' (Fig. 5B-II). The production of $1,25(OH)_2D$ and subsequent $1,25(OH)_2D$-mediated intestinal calcium uptake are in the order of 'hours to day(s) regulation' (Fig. 5B-III). In addition, $1,25(OH)_2D$ enhances the expression and function of TRPV5 present on the apical membrane of DCT cells, and thereby up-regulates Ca^{2+} reabsorption in the kidney [25, 28].

In turn, increased calcium suppresses the transepithelial Ca^{2+} transport and PTH secretion. The increased $1,25(OH)_2D$ suppresses *Cyp27b1* gene expression by the coordination of two types of feedback mechanisms: *in situ* self negative feedback regulation, and target tissue-mediated feedback loop. In the *in situ* self negative feedback regulation, $1,25(OH)_2D$ functions as a ligand of VDR and suppresses *Cyp27b1* gene expression. In the target tissue-mediated feedback loop pathway, $1,25(OH)_2D$ is transported to the target tissue (bone) and, as a ligand of VDR, also enhances the expression of FGF23. Subsequently, secreted FGF23 acts as the major regulator of the target tissue-mediated feedback loop in combination with α-Kl (Figs. 3 and 5B).

Calcium metabolism is therefore governed by complicated reciprocal actions along with feedback mechanisms operating over periods of seconds to minutes, hours and day(s). Thus, the extracellular calcium concentration is rapidly adjusted and continuously maintained within strictly narrow ranges. In this system, α-Kl is involved in the 'seconds to minutes order regulation' of transepithelial Ca^{2+} transport and secretion of PTH. Subsequently, α-Kl participates in the 'minutes to hours order regulation' and the 'hours to day(s) order regulation' through the action of secreted PTH and PTH-mediated production of $1,25(OH)_2D$, respectively. α-Kl also participates in the signal transduction of FGF23 to adjust calcium concentration by down-regulating the production of $1,25(OH)_2D$ (Fig. 5B). Furthermore, α-Kl in urine increases TRPV5 channel abundance at the luminal cell surface by hydrolyzing the sugar residues of TRPV5, which also increase Ca^{2+} reabsorption in the kidney [83]. Therefore, α-Kl is the key player that integrates a multi-step calcium control system, and should be categorized as a protein distinct from the known regulatory molecules [PTH, $1,25(OH)_2D$ and CT] and from the direct handling molecules for transepithelial calcium transport, such as TRPV5, $CalbindinD_{28k}$, and NCX-1.

Conclusions and perspective

Recent advances that have given rise to marked progress in clarifying actions of α-Kl and FGf23 can be summarized as follows: (i) α-Kl binds to Na^+,K^+-ATPase, and Na^+,K^+-ATPase is recruited to the plasma membrane by a novel α-Kl-dependent pathway correlated with cleavage and secretion of α-Kl in response to extracellular Ca^{2+} fluctuation. (ii) The increased Na^+ gradient created by Na^+,K^+-ATPase activity drives the transepithelial transport of Ca^{2+} in cooperation with the Na^+/Ca^{2+} exchanger in the choroid plexus and the kidney, this is defective in *α-kl*$^{-/-}$ mice. (iii) The regulated PTH secretion in the parathyroid glands is triggered *via* recruitment of Na^+,K^+-ATPase to the cell surface in response to extracellular Ca^{2+} concentrations. (iv) α-Kl, in combination with FGF23, regulates the production of $1,25(OH)_2D$ in the kidney. In this pathway, α-Kl binds to FGF23, and α-Kl converts the canonical FGF receptor 1c to a specific receptor for FGF23, enabling the high-affinity binding of FGF23 to the cell surface of the distal convoluted tubule where α-Kl is expressed. (v) FGF23 signal down-regulates serum phosphate levels, due to decreased NaPi-IIa abundance in the apical membrane of the kidney proximal tubule cells. (vi) α-Kl in urine increases TRPV5 channel abundance at the luminal cell surface by hydrolyzing the *N*-linked extracellular sugar residues of TRPV5, resulting in increased Ca^{2+} influx from the lumen. (vii) Human *α-kl* mutations cause tumoral calcinosis. (viii) The symptom of a gain-of-function mutation in human [55] is opposite to that found in a loss-of-function mutation in human [54] and mice [22]. (ix) Discoveries of human *α-kl* mutations provide a compelling evidence that α-Kl is a pivotal regulator of calcium and phosphate homeostasis not only in mouse but also in human.

Unveiled molecular functions of α-Kl and FGF23 provided answers for several important questions regarding the mechanisms of calcium and phosphate homeostasis that remained to be solved, such as: (i) what is the non-hormonal regulatory system that directly responds to the fluctuation of extracellular

Ca^{2+}, (ii) how is Na^+,K^+-ATPase activity enhanced in response to low calcium stimuli in the parathyroid glands, (iii) what is the exact role of FGF23 in calcium and phosphorus metabolism, (iv) how is Ca^{2+} influx through TRPV5 controlled in the DCT nephron, and finally (v) how is calcium homeostasis regulated in CSF.

However, several critical questions still remain to be solved. So far, α-Kl has been reported binds to Na^+,K^+-ATPase [31], FGF receptors and FGF23 [50], and α-Kl hydrolyzes the sugar moieties of TRPV5 [25]. Does α-Kl recognize these proteins directly or indirectly? Is there any common mechanism? How can we reconcile such diverse functions of α-Kl? What is the Ca^{2+} sensor machinery and how can we isolate it? How does hypervitaminosis D and the subsequently altered mineral ion balance lead to the multiple phenotypes? Is there a phosphate sensor molecule and how can we isolate it? How does the FGF23/α-Kl system regulate phosphorus homeostasis? How are serum concentrations of Ca^{2+} and phosphate mutually and inversely regulated?

Collectively, recent findings revealed a comprehensive regulatory scheme of mineral homeostasis that is illustrated by the mutually regulated positive/negative feedback actions of αKl, FGF23, PTH and $1,25(OH)_2$ D (Fig. 5). In this regard, α-Kl and FGF23 might play pivotal roles in mineral metabolism as the regulators that integrate calcium and phosphate homeostasis, although this concept requires further verification in the light of related findings. Recent α-Kl and FGF23 studies provide a new paradigm for the mechanisms controlling extracellular concentrations of Ca^{2+} and phosphate that may change current concepts in mineral homeostasis and give rise to new insights into this field.

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