Department of Biomedical Sciences
University of Veterinary Medicine Vienna

Institute of Physiology, Pathophysiology and experimental Endocrinology
(Head: Univ.-Prof. Dr.med. Dr.med.vet. Reinhold Erben)

The physiological interaction between fibroblast growth factor-23 (FGF23) and parathyroid hormone (PTH)

MASTER THESIS

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Vanessa Maria Knab

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Internal supervisor: Ass.-Prof. Dr.rer.nat. Olena Andrukhova

External supervisor: Harald W. Jüppner, MD

Reviewer: Ao.Univ.-Prof. Dr.rer.nat. Marina Karaghiosoff

Statutory declaration

I hereby assert that I authored this Master thesis myself using only the referred sources and support. I additionally confirm that this work has not been published elsewhere and has not been part of any other examination process in order to achieve an academic grade.

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1. Introduction

Parathyroid hormone (PTH) is a well-established regulator of calcium and phosphate homeostasis. Together with vitamin D it builds an axis which provides a conceptual framework in the regulation of the mineral metabolism. Fibroblast growth factor-23 (FGF23) is a recently discovered, bone-derived hormone which is predominately produced by osteoblasts and osteocytes (Yamashita et al., 2000). Hence, FGF23 belongs to the novel bone-kidney endocrine axis, exposing new insights into the regulation of the mineral homeostasis. Further understanding in hormonal interconnection between kidney and skeleton could have a great clinical importance for patients with bone and mineral disorders.
This introduction summarizes already known facts about FGF23 and PTH function and regulation, established animal models which were used and in the end discusses the aims of this thesis.

1.1. Fibroblast growth factor-23 (FGF23)

Origin and structure

FGF23 is a 32-kD (251 amino acids) protein with a N-terminal region, containing the FGF homology domain and a unique 71-amino acid C-terminal region (Yamashita et al., 2000).
The fibroblast growth factor family consists of 22 members involved in diverse functions. According to their action mechanism they are divided into three groups. *Fgf23* is one of three hormone-like *Fgf* genes and is located on the human chromosome 12 and the mouse chromosome 6. *Fgf21* and the orthologs *Fgf15* (absent in human) and *Fgf19* (absent in mouse) are also members of this hormone-like or endocrine FGF group (Fig. 1). They act systemically through FGF receptor (FGFR)-dependent mechanisms as endocrine factors (Itoh and Ornitz, 2004, 2008).

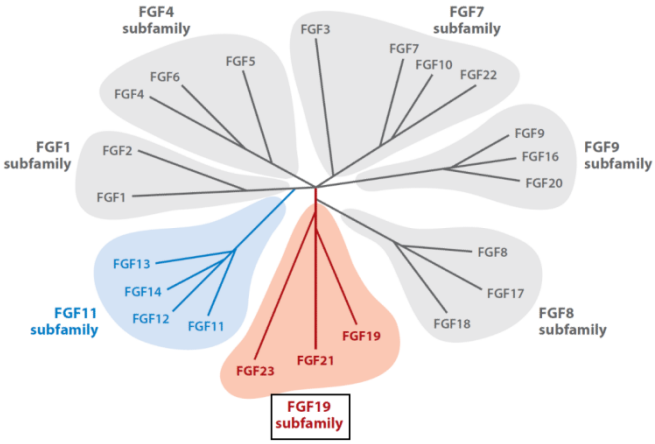


Figure 1: Phylogenetic tree of human FGF

Red represents the endocrine subfamily with FGF23 as a member. Image was adapted from Hu et al., 2013.

Klotho and FGF receptors (FGFR)

The *Klotho* gene was first identified in 1997 in transgenic mice inserted a mutation of a transgene and showing several age-related disorders. This includes a short life span, muscle atrophy, arteriosclerosis, ectopic calcifications - resembling human aging. Further severe hyperphosphatemia and hypercalcemia in association with increased concentrations of vitamin D ($1,25(OH)_2D_3$) (Kuro-o et al., 1997). In contrast, it has been shown that overexpression of *Klotho* extends the life span of mice (Kurosu et al., 2008).

αKlotho encodes a single pass, type-1 transmembrane protein with an extracellular domain which is composed of two homologous domains with homology to glycosidases (Tohyama et al., 2004). The extracellular domain of Klotho protein is shed and secreted into the blood and cerebrospinal fluid (Imura et al., 2004; Kurosu et al., 2008). Studies suggested that secreted Klotho function itself as well.

Klotho was named after the Greek goddess spinning the thread of life because it was originally thought to be an aging-like phenotype. Later studies revealed that αKlotho acts as a co-receptor for FGF23 converting canonical FGF receptors into specific receptors for FGF23 (Kurosu et al., 2006; Urakawa et al., 2006). Abnormalities in mineral, bone metabolism and the aging-related disorders seen in *Klotho*$^{-/-}$ mice could be shown to be very similar to FGF23 deficient mice and is due to vitamin D intoxication. This subsequently leads to hypercalcemia and hyperphosphatemia. Missing FGF23 or Klotho signaling in the renal proximal tubules leads to an overexpression of 1α-hydroxylase and therefore increased

production of vitamin D (Razzaque et al., 2006a; Shimada et al., 2004a; Tsujikawa et al., 2003).

Klotho is needed as a co-receptor for FGF23 and is therefore targeting the hormonal actions of FGF23 to Klotho expressing tissues, including predominantly the kidneys, the choroid plexus in the brain, parathyroid glands and sinoatrial cells of the heart (Martin et al., 2012).

The unique feature of members of the FGF19 family is that they require Klotho for FGF receptor (FGFR) activation. The C-terminal part of FGF23 forms a complex with Klotho and c isoforms of FGFR. Studies evidenced the c isoforms of FGFR1, 3 and 4 to bind with high affinity, FGFR2 binds lower than the other FGFR. Through binding of Klotho to FGFRs affinity of this complex to FGF23 increases. The maximum in FGF23 signaling could be seen when binding to FGFR1c-Klotho complexes. The mechanism is hypothesized leading to attract FGFRs to form heteromeric complexes that signal through the mitogen-activated protein kinase (MAPK) cascade (Fig. 2). Binding of FGF23 activates downstream signaling events (Hu et al., 2013; Kuro-o, 2006; Kurosu et al., 2006; Urakawa et al., 2006).

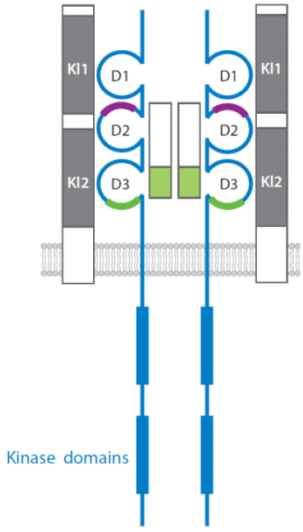


Figure 2: Complex of two FGF23, two FGFRs and two αKlotho

αKlotho consists of two homologous motifs KL1 and KL2 (grey). FGFRs have three immunglobulin-like domains (D1-D3) and two intracellular kinase domains that sustain signal transduction (blue). The binding region of Klotho in D2 is depicted in purple. The ligand-binding regions of FGFRs interact with the C-terminus of FGF23 (green). Image from Hu et al., 2013

Klotho is also present as a soluble, circulating form. Two different mechanisms can be responsible. Gene transcription of the alternatively spliced secreted isoform can be increased or ectodomain shedding of the extracellular domain from the cell surface by proteolytic cleavage can occur. The secreted isoform is a truncated gene product, encoding only the NH_2-terminal half of Klotho with its extracellular domain. The transmembrane form consists of the entire extracellular domain, including the second internal repeat (Imura et al., 2004) (Fig. 3A). Studies pointed at the sheddases A Desintegrin and Metalloproteinase (ADAM) 10 and ADAM17 to be responsible for proteolytic cleavage of Klotho on the cell surface (Chen et al., 2007).

Studies propose that αKlotho has functions independent of FGF23, serving as an endocrine regulator. As described beforehand shedding of Klotho generates cleaved Klotho (cKL). One model (Fig. 3B) proposes that cKL acts as a humoral factor and increases the production of FGF23 in osteocytes, which activates the FGFR1-mKL receptor complex in renal proximal tubular cells (PTC). cKL also directly deglycosylates NaPi-2a and NaPi-2c protein which leads to decreased expression of both transporters (Hu et al., 2010; Jüppner and Wolf, 2012; Martin et al., 2012; Smith et al., 2012).

Functions of soluble Klotho are not well understood, contradictory studies are existing. Investigating mouse models of FGF23 and αKlotho double-knockout provide evidence that αKlotho has no FGF23 independent role in the kidneys because their phenotype is similar to single FGF23 or αKlotho mutant mice in regard to actions on proximal tubules (Nakatani et al., 2009). A study of (Andrukhova et al., 2014a) further support this notion by demonstrating a direct effect of FGF23 on the calcium channel TRPV5, and no effect of αKlotho nor on TRPV5, neither FGF23.

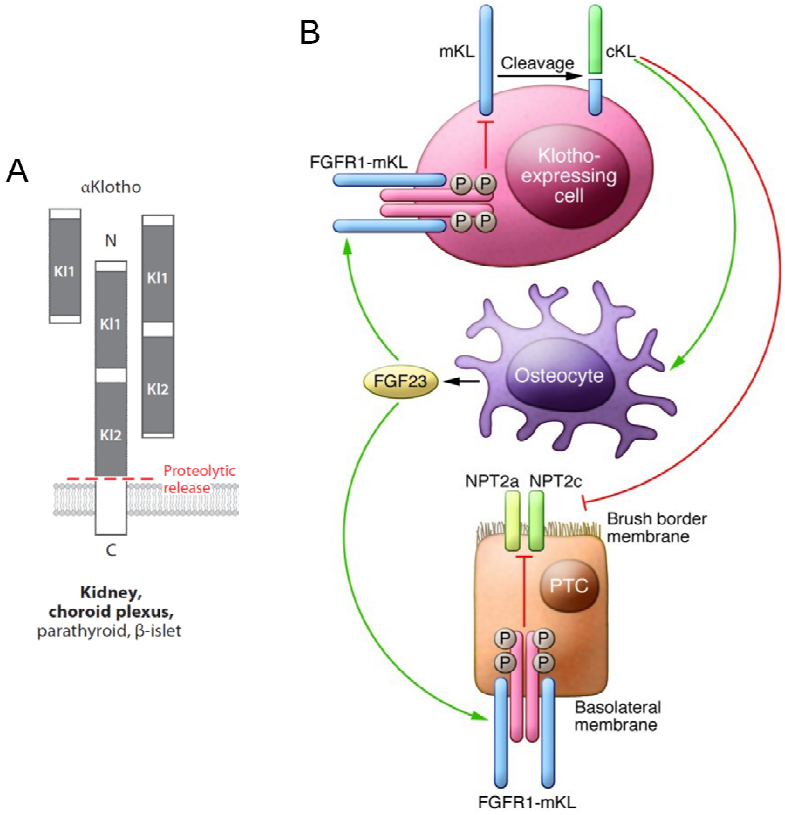


Figure 3: Soluble forms of αKlotho and model of their function

A: The three different possible forms of αKlotho. The soluble forms can be generated by either alternative splicing of its transcript or proteolytic cleavage of the transmembrane form. Adapted from Hu et al., 2013; **B**: Effects of the soluble Klotho (cKL) form acting as a humoral factor on osteocytes or proximal tubule cells in the kidney. Image adapted from Jüppner and Wolf, 2012.

Cleavage of FGF23

FGF23 circulates in a physiologically active, intact form (iFGF23) with a size of 32-35 kDa and in a cleaved, presumable inactive C-terminal form (cFGF23). The FGF23 protein (Fig. 4) is composed of 251 amino acids, containing a N-terminal 24 amino acid signaling peptide. Inactivation takes place in the Golgi by proteolysis at a highly conserved subtilisin-like proprotein convertase (SPC) site, R-H-T-R^{179}/S^{180}-A-E. Cleavage generates smaller N-terminal (~12 kDa) and C-terminal (~18 kDa) fragments. The Furin protease has been shown to be responsible for FGF23 cleavage. In this highly regulated FGF23 processing also O-glycosyl transferase N-acetylgalactosaminyltransferase 3 (GalNac-T3) plays an important role. GalNac-T3 O-glycosylates T^{178}, thereby stabilizing iFGF23 and preventing it from proteolysis by Furin (Bhattacharyya et al., 2012; Fukumoto, 2005; Mattoo, 2014; Wolf and White, 2014).

Further studies discovered the family with sequence similarity 20, member C (Fam20C), a secretory pathway-specific kinase which acts on S-x-E motifs, phosphorylates S^{180} to block O-glycosylation and promote FGF23 proteolysis by furin. The cross-talk between phosphorylation and O-glycosylation in FGF23 processing seems to be a physiologically important mechanism in the regulation of secreted intact to C-terminal FGF23 fragments (Tagliabracci et al., 2014).

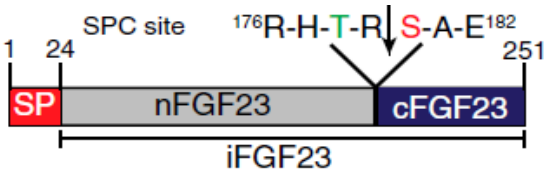


Figure 4: Schematic representation of human FGF23

The downward arrow indicates the SPC site with surrounding residues. iFGF23: intact FGF23; nFGF23: N-terminal fragment; cFGF23: C-terminal fragment; SP: signal peptide. Image from Tagliabracci et al., 2014.

FGF23 Function and Regulation

Tissue specificity of FGF23 is determined by distribution of the Klotho-FGFR complex as described previous. The best characterized functions of FGF23 are in the kidneys, FGF23 also has extrarenal targets, namely the parathyroid glands, the skeleton and presumably other organs.

FGF23 and Vitamin D

FGF23 acts as a vitamin D counter regulatory hormone. It reduces circulating $1,25(OH)_2D_3$ levels by two events. FGF23 decreases production by inhibiting the 1α-hydroxylase and enhances the catabolism of $1,25(OH)_2D_3$ by increasing 24-hydroxylase expression in the renal proximal tubular cells (Fig. 5). The latter and subsequent reduction of $1,25(OH)_2D_3$ levels by FGF23 has been shown to be a vitamin D receptor (VDR) dependent function (Inoue et al., 2005). Studies identified two vitamin D-responsive elements (VDREs) in the promoter region of the 24-hydroxylase (Kahlen and Carlberg, 1994). Effects on 1α-hydroxylase are not consistent in literature in this concern, the mechanism of action may differ from acute to chronic FGF23 excess (Martin et al., 2012; Shimada et al., 2005).

Contrary, $1,25(OH)_2D_3$ is the most significant regulator of FGF23 by acting in a classical hormonal loop. High vitamin D levels increase FGF23 production which in turn suppresses $1,25(OH)_2D_3$. Studies revealed a VDR dependent and independent action on FGF23 production. Analysis of the gene revealed a VDRE in the promoter region of *Fgf23* that is responsible for the direct vitamin D effects which are dependent on the VDR (Liu, 2006; Masuyama et al., 2006).

Learning from disorders unveiled factors like phosphate-regulating gene with homologies to endopeptidases on X chromosome (PHEX), Dentin Matrix Protein-1 (DMP1) and ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) to affect FGF23. PHEX has also been shown to be regulated by $1,25(OH)_2D_3$, which suggests an indirect mechanism of vitamin D to influence FGF23 acitivity in osteocytes by regulation of PHEX (Lanske et al., 2014; Martin et al., 2011).

FGF23 and Phosphate

The phosphate concentrations are regulated by adjusting its urinary excretion. This happens mainly by reabsorption in the renal proximal tubule through transcellular transport and is limited by the entry of phosphate. This process requires sodium-dependent phosphate cotransporters. The type 2 cotransporter family is of special interest, including three carrier, NaPi-2a, NaPi-2b and NaPi-2c (Martin et al., 2012).

FGF23 suppresses expression of NaPi-2a and NaPi-2c and as a consequence increases urinary phosphate excretion (Fig. 5). NaPi-2a is located at the apical membrane of the proximal tubule cells and has been proved to be the main transporter responsible for the effects of FGF23 on increased phosphate excretion. NaPi-2c is expressed on the brush-border membrane of the proximal convoluted tubules and effects of FGF23 on its expression in studies appear to be more variable. These actions of FGF23 on serum phosphate has been observed to be vitamin D independent and PTH independent, as they could be seen in parathyroidectomized rats as well (Gattineni et al., 2009; Inoue et al., 2005; Shimada et al., 2004b, 2005).

The regulation of FGF23 by phosphate still remains unclear, a phosphate sensor has not been identified yet. High phosphate increases FGF23 levels but with a low magnitude. Studies in rodents showed that, but this seemed to be an $1,25(OH)_2D_3$-VDR dependent mechanism (Shimada et al., 2005). In humans, regulation of FGF23 by phosphate showed variable results. Additionally in settings of renal failure phosphate restriction (using phosphate binders) failed to lower high FGF23 levels (Martin et al., 2012).

Other renal targets of FGF23

More recent studies additionally revealed targets of FGF23 action in the renal distal convoluted tubules (DCT). FGF23 binds to its FGFR-αKlotho complex and acts as a calcium-conserving hormone. In renal calcium transport the rate-limiting step is the apical membrane expression of the epithelial calcium channel transient receptor potential vannilloid-5 (TRPV5), a glycoprotein which is important for entry of calcium. FGF23 has shown to be a regulator of TRPV5 membrane abundance through an intracellular signaling cascade and therefore regulates renal calcium reabsorption (Andrukhova et al., 2014a).

Further FGF23 binding to its complex in renal distal convoluted tubules has been shown to regulate sodium (Na^+) reabsorption. FGF23 acts as a sodium-conserving hormone by directly regulating membrane abundance and activity of the Na^+:Cl^- co-transporter NCC in distal tubules (Fig. 5) (Andrukhova et al., 2014b).

The highest levels of FGFR-αKlotho complexes could be found in distal tubules, also in proximal tubules the presence of Klotho protein and mRNA was shown in more recent studies (Andrukhova et al., 2012; Hu et al., 2010). One proposed model for action of FGF23 in proximal tubules is the “distal-to-proximal tubular feedback mechanism” (Fig. 5) suggesting indirect action of FGF23, possibly through stimulation of the complex by FGF23 in distal tubules and subsequent release of paracrine factors that regulate the function in proximal tubules. With emerging evidence that αKlotho has no FGF23-independent effect no ideal candidate acting as paracrine factor in this case is known (Nakatani et al., 2009).

More recent studies question this model by elucidating a regulation of FGF23 binding to its FGFR-αKlotho complex directly on proximal tubules to down regulate membrane expression of NaPi-2a (Andrukhova et al., 2012).

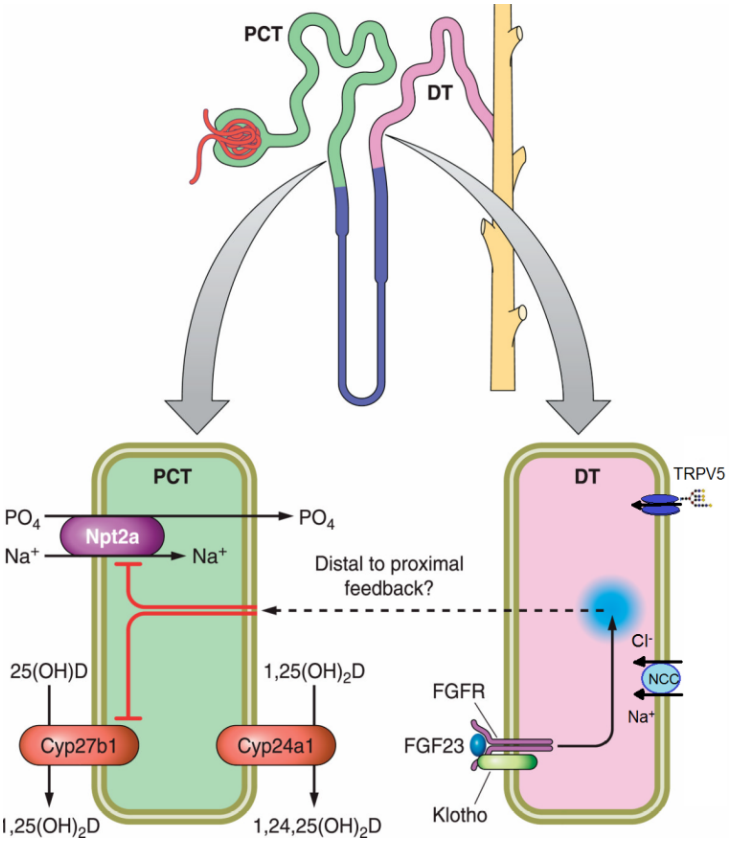


Figure 5: Renal functions of FGF23 on PCT and DT

FGF23 acts on NaPi-2a and on Cyp27b1 and Cyp24a1 in renal proximal convoluted tubules (PCT). In distal tubules (DT) FGF23 regulates the calcium channel TRPV5 and the Na^+:Cl^- co-transporter NCC. Model of FGF23 working in a possible distal to proximal feedback mechanism. Image adapted from Martin et al., 2012.

Extrarenal targets and regulators of FGF23

If FGF23 could have a Klotho-independent action in the extrarenal organs remains still elusive. Accumulating evidences identify bone and parathyroid glands as a target organ for FGF23 signaling.

It is clearly stated that PTH regulates FGF23 expression (Burnett-Bowie et al., 2009; Lavi-Moshayoff et al., 2010; Meir et al., 2014). There is a proposed feedback loop between PTH and FGF23 with direct expression regulation. However the exact mechanisms of this interregulatory action are not known and several studies show controversial results.

FGF23 is produced in osteoblasts and osteocytes (Yamashita et al., 2000) confirming that bone is an endocrine organ involved in the calcium and phosphate homeostasis (Bonewald and Wacker, 2014). Direct actions of FGF23 on the bone are debated. Studies in animal models and cell culture suggest direct effects of FGF23 negatively affecting bone mineralization (Wang et al., 2005, 2008). Moreover, it is still not completely clear whether FGF23 could act directly on the bone or only as a mediator of phosphate, $1,25(OH)_2D_3$ and PTH, which influence mineralization.

In turn, bone-derived factors regulate FGF23 expression. Molecules like PHEX and DMP1 are highly expressed in osteoblasts and osteocytes. They have been shown to regulate FGF23, mutations in PHEX and DMP1 increase gene transcription of FGF23 (Bonewald and Wacker, 2014; Liu and Quarles, 2007; Martin et al., 2012). The mechanisms for this regulation are still unclear.

Circulating levels of the biologically active FGF23 are further regulated at the protein level. FGF23 can be post-translationally regulated by Furin, GalNac-T3 and Fam20C. The cleaving of the intact form generates inactive C-terminal and N-terminal fragments of FGF23 (Bhattacharyya et al., 2013; Bonewald and Wacker, 2014).

1.2. Parathyroid hormone (PTH)

PTH is a well-established regulator of calcium and phosphate homeostasis. It is synthesized in the parathyroid glands and signals through a common G-protein coupled receptor, the PTH receptor 1 (PTHR1) which is abundantly expressed in renal proximal and distal tubules in the kidneys and in osteoblasts, osteocytes and chondrocytes in the bone (Lupp et al., 2010). Binding of PTH to its PTHRs activates cAMP/PKA and IP_3/PKC signaling pathways (Lavi-Moshayoff et al., 2010; Lee and Partridge, 2009; Nagai et al., 2011; Takasu et al., 1999). The mature PTH peptide consists of 84 amino acids and is derived from a longer pre-pro peptide. When entering the circulation, PTH is cleared with a short half-life of about 2 minutes (Christov and Jüppner, 2013).

Regulation and function of PTH

Parathyroid glands (PTG) detect fluctuations in blood ionized calcium with the calcium-sensing receptor (CaSR) at the cell surface of chief cells in the PTG. Under hypocalcemic conditions PTH secretion is increased to restore serum calcium to normal. This happens by increasing calcium efflux from bone, enhancing urinary reabsorption of calcium and by stimulating the production of vitamin D (Levine et al., 2014). There is a minute-to-minute release of parathyroid hormone into the circulation and therefore an almost immediate effect on urinary calcium excretion and efflux from bone. If serum calcium remains low for hours or days, vitamin D metabolism gets affected and thus, intestinal calcium absorption will be affected as well (Chen and Goodman, 2004; Filopanti et al., 2013).

PTH also gets directly stimulated by an increase in serum phosphate through a putative extracellular phosphate sensor (Fig. 6). Binding of PTH to its PTHR1 in the renal proximal tubules activates the PKC and PKA pathway leading to internalization of NaPi-2a and NaPi-2c and subsequently to renal phosphate wasting and lowering of serum phosphate (Komaba and Fukagawa, 2010; Silver et al., 2012).

PTH-Vitamin D axis

PTH and 1,25$(OH)_2D_3$ signaling pathways are linked within a tightly regulated feedback loop. High PTH, secreted in response to low calcium, stimulates renal vitamin D production. 1,25$(OH)_2D_3$ acts on the one hand on the small intestine, increasing dietary calcium and phosphate absorption and on the other hand it has an inhibitory effect on PTH expression in PTG closing a negative feedback loop in the PTH-1,25$(OH)_2D_3$ regulatory axis. These effects are mediated by binding of vitamin D to its specific VDR and acting on vitamin D response elements in the PTH promoter (Fig. 6) (Ritter and Brown, 2011).

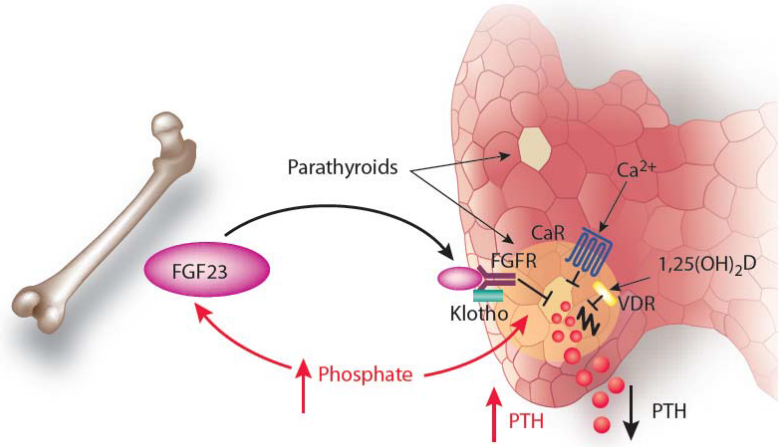


Figure 6: Regulation of PTH secretion in parathyroid glands

FGF23 is secreted from bone and acts on the parathyroid glands to decrease PTH synthesis and secretion. Calcium, phosphate and vitamin D action further contribute to normal mineral and bone metabolism. Image from (Silver and Naveh-Many, 2009).

FGF23-Vitamin D-PTH Axis

Klotho and FGFRs are expressed in parathyroid glands suggesting presence of FGF23 signaling. FGF23 binds to its FGFR1-Klotho complex on the parathyroid gland cells and activates downstream signaling which decrease PTH gene expression and secretion (Fig. 7). This was proved *in vitro* and *in vivo* as a direct effect of FGF23 (Ben-Dov et al., 2007; Krajisnik et al., 2007; Marsell et al., 2008). It was also shown *in vitro* that FGF23 concomitant with decreasing PTH, increased 1α-hydroxylase mRNA expression in parathyroid glands (where it is also expressed) in a dose-dependent manner (Krajisnik et al., 2007). The exact mechanism for this regulation still remains elusive. It was shown that FGF23 has a direct inhibitory effect on PTH production.

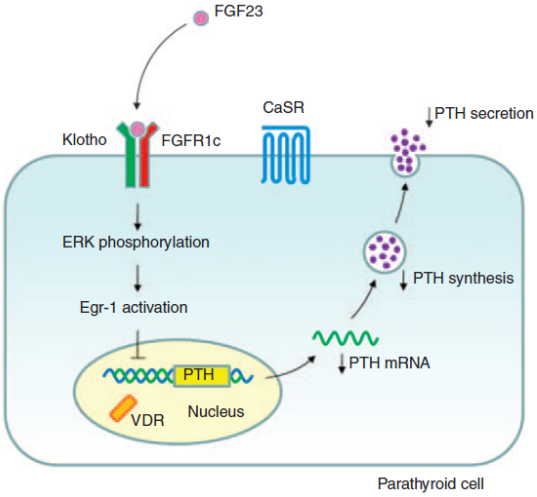


Figure 7: FGF23 signaling in the parathyroid cell

FGF23 binds to its FGFR1c-Klotho complex on parathyroid cells, activating downstream signaling. This leads to inhibition of PTH production and secretion. Image from Komaba and Fukagawa, 2010.

Recent studies are supporting a stimulating function of PTH as a feedback of the inhibitory action of FGF23 on PTH. PTH increases serum FGF23 by enhancing its expression, via two suggested mechanisms, the PKA and Wnt pathways (Burnett-Bowie et al., 2009; Lavi-Moshayoff et al., 2010). Additionally a recent study proposes regulation of FGF23 transcription by PTH through PKA pathway, which increases nuclear orphan receptor, nuclear receptor-associated protein 1 (Nurr1) expression in the bone. A functional region in the FGF23 promotor region containing sequence elements (NBREs) could be identified, where Nurr1 binds to induce FGF23 transcription. FGF23 in turn inhibits PTH production (Meir et al., 2014).

Figure 8 summarizes the endocrine feedback loops of mineral homeostasis discussed earlier. Taken together recent studies revealed a FGF23-Vitamin D-PTH axis (highlighted with red and green arrows). PTH stimulates renal $1,25(OH)_2D_3$ production and release into the circulation, which in turn has an inhibitory effect on PTH. FGF23 suppresses renal 1α-hydroxylase and therefore biologically active vitamin D. At the same time $1,25(OH)_2D_3$ stimulates FGF23 production closing the regulatory feedback loop.
Moreover, the feedback loop between PTH and FGF23 and the effects on calcium and phosphate, discussed in earlier chapters, are summarized in Figure 8.

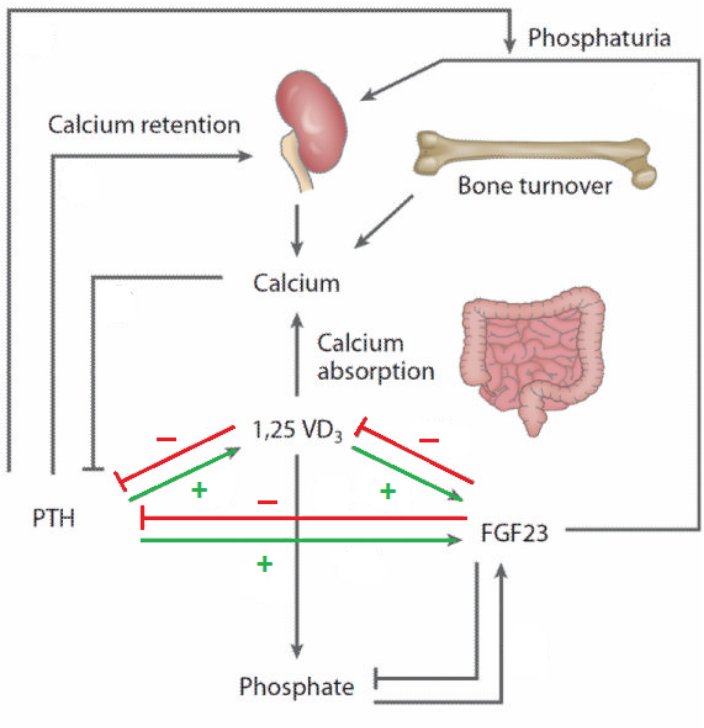


Figure 8: Endocrine feedback loops in mineral homeostasis

PTH, FGF23 and vitamin D form an axis, regulating each other. They further tightly regulate phosphate and calcium homeostasis as indicated by arrows and described in the text. Adapted from Hu et al., 2013.

1.3. Vitamin D

The metabolism of vitamin D starts with the production of vitamin D_3 in the skin and subsequent conversion to $25(OH)D_3$ in the liver. In the circulation it binds to vitamin D-binding protein (DBP), this complex is captured by renal proximal tubular cells. The enzyme CYP27B1 (1α-hydroxylase) converts $25(OH)D_3$ into the biologically active $1,25(OH)_2D_3$ (calcitriol). CYP24A1 (24-hydroxylase) is responsible for inactivating calcitriol (Prie and Friedlander, 2010). As already described in the previous chapters, PTH stimulates and FGF23 represses 1α-hydroxylase whereas only FGF23 can increase 24-hydroxylase expression (Fig. 9).

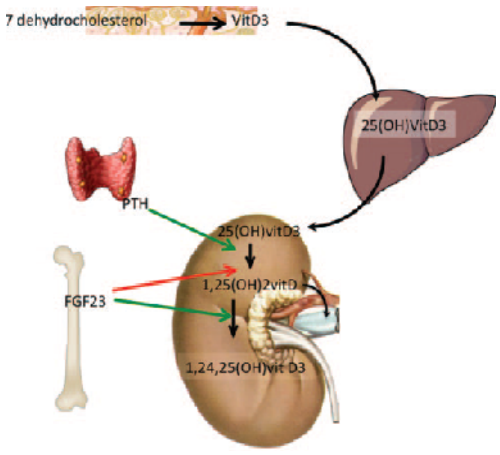


Figure 9: Vitamin D metabolism

In the kidneys PTH and FGF23 regulate 1α-hydroxylase by decreasing its expression and FGF23 further increases expression of 24-hydroxylase. Image adapted from Prie and Friedlander, 2010.

$1,25(OH)_2D_3$ works through the vitamin D receptor (VDR), which is a nuclear receptor that acts as a transcription factor when bound to vitamin D. After vitamin D entered the cell and bound the VDR, the receptor is activated and translocates to the nucleus where it acts on gene transcription. This is done by binding to vitamin D-responsive elements (VDRE) in the promoter region of target genes. The VDR further mediates nongenomic actions of $1,25(OH)_2D_3$ (Carrillo-López et al., 2008; Erben et al., 2002).

Regulation and function of vitamin D

$1,25(OH)_2D_3$ interacts with PTH, FGF23 and enhances gut phosphate and calcium absorption. 1α-hydroxylase is important for conversion of $25(OH)_2D_3$ into the biologically active vitamin D. Low phosphate, low calcium and PTH could induce 1α-hydroxylase expression whereas FGF23, high calcium and phosphate could reduce 1α-hydroxylase expression. The activity of 24-hydroxylase, responsible for inactivating $1,25(OH)_2D_3$, is regulated by FGF23 and also by $1,25(OH)_2D_3$ itself (Bergwitz and Jüppner, 2011).

1.4. Animal models

Vitamin D receptor ablated mice

Vitamin D receptor null mutant mice ($VDR^{\Delta/\Delta}$) are generated by gene targeting. They are lacking the first zinc finger necessary for DNA binding, but express a VDR with an intact hormone binding domain. Therefore the mutant receptor is nonfunctioning and homozygous mice display a phenocopy of mice totally lacking the VDR protein (Erben et al., 2002).

The mice show hypocalcemia, severe secondary hyperparathyroidism, growth retardation, rickets and alopecia. Keeping them on a rescue diet, enriched with calcium, phosphorus and lactose fully corrects blood calcium levels, which in turn partly prevents the increase in PTH. Further mice don't develop rickets, but alopecia is still observed in these mice on a rescue diet. Moreover, $VDR^{\Delta/\Delta}$ mutants display moderately elevated PTH levels and lower bone mineral density (Erben et al., 2002; Hesse et al., 2007; Li et al., 1998).

FGF23 deficient mice

FGF23 null mice (*Fgf23*$^{-/-}$) are viable but have a markedly short life span of 4-8 weeks. They show severe growth retardation as can be seen by their small body size and an abnormal bone phenotype. The mutant mice are hypercalcemic and have severe hyperphosphatemia due to increased renal phosphate reabsorption and in part due to their highly elevated $1,25(OH)_2D_3$ levels. These animals have increased 1α-hydroxylase expression and activity seen from 10 days of age. Additionally, mice have marked decreased PTH levels, exhibit ectopic calcifications, muscle and skin atrophy, organ atrophy, hypoglycemia and have a normal renal expression of Klotho. Heterozygous animals show no abnormalities (Hesse et al., 2007; Shimada et al., 2004a; Sitara et al., 2004; Yuan et al., 2011).

Fgf23$^{-/-}$/$VDR^{\Delta/\Delta}$ mutant mice

In order to dissect VDR-independent functions of FGF23, loss of FGF23 was induced in mice with VDR-ablation resulting in generation of *Fgf23*$^{-/-}$/$VDR^{\Delta/\Delta}$ compound mutants. Ablation of vitamin D signaling completely rescues the premature aging-like phenotype of *Fgf23*$^{-/-}$ mice, suggesting that alterations seen in FGF23 deficient mice are mainly caused by excessive vitamin D signaling. *Fgf23*$^{-/-}$/$VDR^{\Delta/\Delta}$ compound mutants on rescue diet have normal serum phosphate levels, normal renal tubular reabsorption of phosphate, show no difference in renal phosphate reabsorption, however have moderately elevated PTH levels and lower bone mineral density as compared to $VDR^{\Delta/\Delta}$ and WT animals (Fig. 10) (Hesse et al., 2007). Studies in FGF23 deficient mice with deleting the *1α(OH)ase* gene obtained similar results of

rescuing the aging like phenotype in *Fgf23*$^{-/-}$ (Razzaque et al., 2006b; Sitara et al., 2006; Streicher et al., 2012).

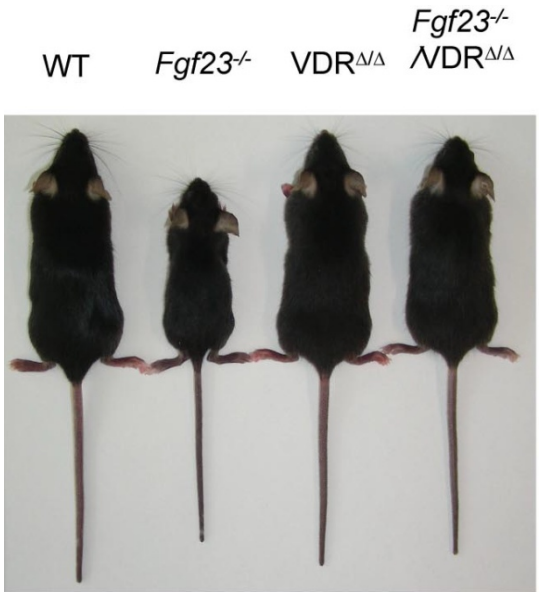


Figure 10: Body size of WT and mutant mice

Dwarfism in *Fgf23*$^{-/-}$ mice and normal body size in VDR$^{\Delta/\Delta}$ and *Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$ compound mutants. Image from (Streicher et al., 2012).

Klotho deficient mice

Klotho$^{-/-}$ mice lack both, the transmembrane and secreted form of the Klotho gene. The mice have some striking phenotypic similarities to *Fgf23*$^{-/-}$ as described in the previous chapter. They have a normal development until week 3 following by growth retardation, ectopic calcifications, elevated serum calcium and phosphate levels, discordantly high levels of 1,25$(OH)_2D_3$ and die prematurely (Tsujikawa et al., 2003). Klotho null mice also display marked elevation of serum FGF23, presenting evidence of Klotho as a regulator of FGF23 signaling (Kurosu et al., 2006; Urakawa et al., 2006).

Klotho$^{-/-}$/VDR$^{\Delta/\Delta}$ mutant mice

Klotho$^{-/-}$/VDR$^{\Delta/\Delta}$ compound mutants are generated by lack of *Klotho* in VDR ablated mice. *Klotho*$^{-/-}$/VDR$^{\Delta/\Delta}$ mice on rescue diet are normocalcemic, normophosphatemic, show no organ atrophy or premature aging-like phenotype and show similarities to *Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$ animals. This suggests that physiologically relevant functions of Klotho are dependent on vitamin D and FGF23 signaling (Anour et al., 2012).

1.5. Aim of this study

The parathyroid glands, producing PTH - a well-known regulator of mineral homeostasis, have been identified to be a target organ of FGF23, a recently discovered bone-derived hormone. Physiologic hormonal balance is maintained by interactions among the intestine, kidney, parathyroid glands and bone. Evidence suggests that FGF23 and PTH mutually regulate each other in a negative feedback loop, where PTH stimulates FGF23 production and FGF23 in turn suppresses PTH production and secretion.

Regulation and function of FGF23 is not fully understood yet and current findings are inconsistent.

Therefore, the aim of this study was to investigate whether FGF23 acts directly on the parathyroid glands to regulate PTH production. To study this regulation, FGF23 deficient mice were interbred with VDR ablated mice and kept on rescue diet. VDR ablated and wild type mice were used to conclude about direct effects of FGF23 on PTH regulation. Further, we used parathyroid gland organ culture in *ex vivo* experiments to verify *in vivo* data.

Next, we aimed to analyze whether PTH can directly regulate FGF23 production and cleavage, independently of $1,25(OH)_2D_3$ action. As FGF23 cleavage contributes to the circulating levels of FGF23 we used comparable assays for both, the intact, active form and the cleaved, inactive form of FGF23 to investigate PTH-mediated regulation of FGF23 cleavage. In this study we assessed the short-term effect of PTH as a single injection on FGF23 expression *in vivo* in wild type mice. To verify these results *in vitro* we further used primary osteoblast/osteocyte cell culture for PTH treatment.

2. Material and Methods

2.1. Animals

All animal procedures were approved by either the Ethical Committees of the University of Veterinary Medicine Vienna and the local government authorities or in accordance with the ethical guidelines adopted by Massachusetts General Hospital.

Heterozygous $VDR^{+/\Delta}$ (Erben et al., 2002) were mated with heterozygous *Klotho*$^{+/-}$ (Lexicon Genetics, Mutant Mouse Regional Resource Centers, University of California, Davis, CA, USA) and *Fgf23*$^{+/-}$ (Sitara et al., 2004) mutant mice to generate double heterozygous animals. *Klotho*$^{+/-}$/$VDR^{+/\Delta}$ and *Fgf23*$^{+/-}$/$VDR^{+/\Delta}$ mutant mice on C57BL/6 background were interbred to generate WT, $VDR^{\Delta/\Delta}$, *Klotho*$^{-/-}$, *Klotho*$^{-/-}$/$VDR^{\Delta/\Delta}$, *Fgf23*$^{-/-}$ and *Fgf23*$^{-/-}$/$VDR^{\Delta/\Delta}$ mutant mice. Genotyping of the mice was performed by multiplex PCR using genomic DNA extracted from tail as described (Hesse et al., 2007). The mice were kept at 24 °C with a 12h/12h light/dark cycle and were allowed free access to a rescue diet and tap water. The rescue diet (Sniff, Soest, Germany) containing 2.0 % calcium, 1.25 % phosphorus, 20 % lactose and 600 IU vitamin D/kg was fed starting from 16 days of age. This diet has been shown to normalize mineral homeostasis in VDR-ablated mice (Erben et al., 2002; Li et al., 1998).

All experiments on mutant mice were performed on 3-month-old males. At necropsy, the mice were exsanguinated from the abdominal V. cava under anesthesia with ketamine/xylazine (67/7 mg/kg i. p.) for serum collection.

Nine to 12-week-old male C57BL/6 mice (Charles River Laboratories, Wilmington, MA, USA) were used for PTH and Furin inhibitor treatment experiments. They were held in barrier and fed a standard diet. For all experiments C57BL/6 mice were randomized by their weight. Numbers of animals per group for each experiment are indicated on the respective graphs.

2.2. Treatment

C57BL/6 mice were injected subcutaneously with either PTH (1-34) (50 nmol/kg or as indicated), or vehicle (10 mM citric acid, 150 mM NaCl, 0.05 % Tween, pH 5.0). The peptide used was: PTH (1-34) (human sequence:
SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF). The peptide was C-terminally amidated and synthesized by the Massachusetts General Hospital Biopolymer Core Facility using solid phase, *N*-(9-fluorenyl)methoxycarbonyl (Fmoc)-based chemistry and an automated peptide synthesizer. Peptide purity and authenticity was verified by analytical

HPLC and MALDI-mass spectrometry. Further C57BL/6 mice were injected i. p. with Furin Inhibitor I (Decanoyl-RVKR-CMK, EMD Chemicals, Inc. San Diego, CA, USA) (10 µg/g), using 5 % DMSO (Dimethylsulfoxide, Sigma) as a vehicle. All biochemical analysis was performed using plasma samples collected via heparinized capillaries from mouse tail-vein. Blood was subsequent centrifuged and plasma separated. Samples were stored at -20 ° or -80 °C until analysis. Ionized calcium was measured using RapidLab348 (Siemens). Using Phosphorus Liqui-UV kit (Sanbio Laboratory, TX, USA) serum phosphorus was measured, reading the absorbance at a wavelength of 340 nm. Mouse/Rat C-terminal and intact FGF23 ELISA kit (Immutopics, San Clemente, CA, USA) were implemented for measurement of FGF23 levels and Mouse PTH (1-84) ELISA (Immutopics, San Clemente, CA, USA) for PTH levels in serum of mice.

Gain of function experiments were carried out on C57BL/6 mice injected i. p. with vehicle (phosphate-buffered saline with 2 % DMSO) or 10 µg/mouse recombinant FGF23 (FGF23 R176/179Q, rFGF23, kindly provided by Amgen, Thousand Oaks, CA, USA) for 5 days. For mRNA expression analysis trachea including thyroid and parathyroid glands were dissected from all animals and shock-frozen in liquid nitrogen.

2.3. *Ex vivo* – Parathyroid gland organ culture

Parathyroid glands were microdissected from 4-week-old mice. For *ex vivo* PTH secretion experiments parathyroid glands were incubated in 37 °C for 1 hour each with hypo-, normo- and hypercalcemic conditions (0.6 mM, 1.25 mM and 2 mM $CaCl_2$, respectively) in cultivation medium (Ham's F10 including 10 % FBS and 2 % Penicillin/Streptomycin). For treatment with recombinant FGF23 (10 ng/ml, Amgen, Thousand Oaks, CA, USA) parathyroid glands were incubated for 1 hour in 37 °C. Medium was collected and immediately frozen in liquid nitrogen till required. Mouse PTH (1-84) ELISA (Immutopics, San Clemente, CA, USA) was used.

2.4. *In vitro* – Primary osteoblast culture

Calvariae were dissected from 2-day-old mice for isolation of primary osteoblasts. Minced calvariae were incubated for 3 hours in serum-free medium (EMEM with 10 % FBS and 2 % Pen/Strep) containing collagenase II (2 mg/ml). After growing the cells confluent they were split into 12-well-plates. Differentiation was induced by addition of ascorbic acid and β-glycerophosphate in the medium. Cells were treated with vehicle or PTH after 14 days and

21 days of differentiation. Medium were collected and immediately stored at -80 °C for measurements. Cells were lysed and used for mRNA isolation.

2.5. mRNA isolation and quantitative RT-PCR

Cells, stored in TRI Reagent Solution (Molecular Research Center) were added BCP (1-Bromo-3-chloropropane; Sigma-Aldrich) to extract mRNA. Trachea samples were first homogenized with a pestle in TRI reagent solution. Precipitation was conducted using isopropanol. mRNA quality was assessed using Eukaryote Total RNA Nanochip assays (2100 Bioanalyzer, Agilent Technologies). Purity and concentration were determined spectrophotometrically (NanoDrop 2000, Thermo Scientific). Reverse transcription was performed using a reverse transcription kit (iScript cDNA Synthesis Kit, Bio-Rad) using 2 µg of mRNA. For the following quantitative RT-PCR cDNA samples were 1:1 diluted or used non-diluted. RT-PCR were carried out on a Rotor-Gene 6000 (Corbett Life Science) or a Viia7 (ABI) using 5x FirePol PCR Kit (Bio-Rad).

Table 1: Primers used for qRT-PCR (5' -> 3')

Primer	Forward	Reverse
Rplp0	AAGTGCTCGACATCACAGAGCAG	TTGTACCCATTGATGATGGAGTGT
GAPDH	CCGTGTTCCTACCCCCAAT	ATGTCATCATACTTGGCAGGTTTC
Pth	TCATGCTGGCAGTCTGTC	GTGTTTGCCCAGGTTGTG
Pthr1	TGAAGGCTAAAATGCAGTGTGAA	CCCATTCTGGGAAGCAATTG
Casr	GGTCCTGTGCAGACATCAAG	TCACCGCACTCATCGAAG
Klotho	AGCCCCTTGAAGGGACAT	TGCACATCCCACAGATAGAC
Fgfr1	AGATGATGACGACGACGATGACT	CATGCAGTTTCTTCTCCATTTTCTC
Fgfr4	TGCTGGGTGTCTGCACTCA	CCCTTGGCGGCACATTC
Fgf23E1	TGCTAGGGACCTGCCTTAGACT	GCTCTAGCAGTGCCCAAGCT
Furin	AGCCAAGAGGGACGTGTATC	CTCGCTGAGTGACACCAGAC
Fam20c	CGCGGGATAAGAAGCTATGG	AACACTCCCCGTAGAAACAGATG
Galnt3	TTCTCTGCACCGGGACCTT	GGCGGGCAGCGCTTA
Galnt3	GGTGGATGATGCTAGTGTAGAC	TCAGGCCTTTCCTTTCTTGC

To check for unspecific binding melting curve analysis was randomly performed. mRNA data were normalized to housekeeping genes, *Rplp0* (ribosomal protein, large, P0) or *GAPDH* (Glyceraldehyde-3-Phosphate Dehydrogenase).

2.6. Statistical analyses

Statistical analyses and graphical illustrations were carried out using SPSS 17.0. and GraphPad Prism 5 (La Jolla, CA, USA). Data were analyzed using Students unpaired ttest or one-way analysis of variance (ANOVA) followed by Tukey's test and Student-Newman-Keuls (SNK) post-hoc tests. P values of <0.05 were considered significant. All values are given as mean ± SEM or as indicated.

3. Results

3.1. *In vivo* – serum levels and mRNA expression of mutant mice

As it was previously suggested (Ben-Dov et al., 2007; Krajisnik et al., 2007; Yamazaki et al., 2008) FGF23 and PTH signaling may possibly interact. The exact mechanisms of this signaling association are not well understood. Current evidence (Ben-Dov et al., 2007) indicates that FGF23 may have a direct effect on the parathyroid glands to decrease PTH production and secretion. Genetic ablation of FGF23 leads to the decreased serum PTH levels in *Fgf23*$^{-/-}$ mice. However, since single *Fgf23*$^{-/-}$ mutants have high serum vitamin D levels and severe soft tissue calcification resulting in survival for only several weeks, it was still difficult to conclude about vitamin D-independent PTH regulation by FGF23 in those animals. Therefore, to further investigate if FGF23 and PTH signaling are interconnected in the present study we designed experiments using animals with loss of FGF23 in VDR ablated mice (*Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$). Wild-type mice and VDR$^{\Delta/\Delta}$ mutants were used as a reference.

Loss of FGF23 in VDR ablated animals leads to increased serum PTH levels and decreased Ca^{2+} levels

In order to identify the role of FGF23 in PTH expression regulation serum PTH level were measured in WT, VDR$^{\Delta/\Delta}$ and *Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$ mutant mice. Previous studies indicate that vitamin D inhibits PTH expression (Carrillo-López et al., 2008). In accordance with this in our study VDR ablated animals with non-functioning VDR show elevated serum PTH levels (Fig. 11A). Interestingly, loss of FGF23 in VDR ablated mice leads to further increase in serum PTH levels of *Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$ compound mutants suggesting importance of FGF23 in PTH secretion regulation (Fig. 11A).

Since PTH secretion is tightly regulated by serum calcium levels (Chen and Goodman, 2004), we next measured blood ionized Ca^{2+} levels in all experimental animal groups. We detected decreased blood ionized Ca^{2+} in VDR$^{\Delta/\Delta}$ and *Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$ mutant mice (Fig. 11B). The difference reaches statistical significance only in *Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$ mutants compared to WT mice (Fig. 11B). As it was already stated earlier, rescue diet used in our study normalized the calcium absorption defect of VDR mutant mice in the gut (Anour et al., 2012; Erben et al., 2002; Hesse et al., 2007). Therefore, in accordance with the existing data VDR ablated mice in our study were normocalcemic. Summarizing, these results suggest that lack of FGF23 leads to the simultaneous increase in serum PTH levels and hypocalcemia, pointing to the signs of secondary hypoparathyroidism in *Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$ mutants.

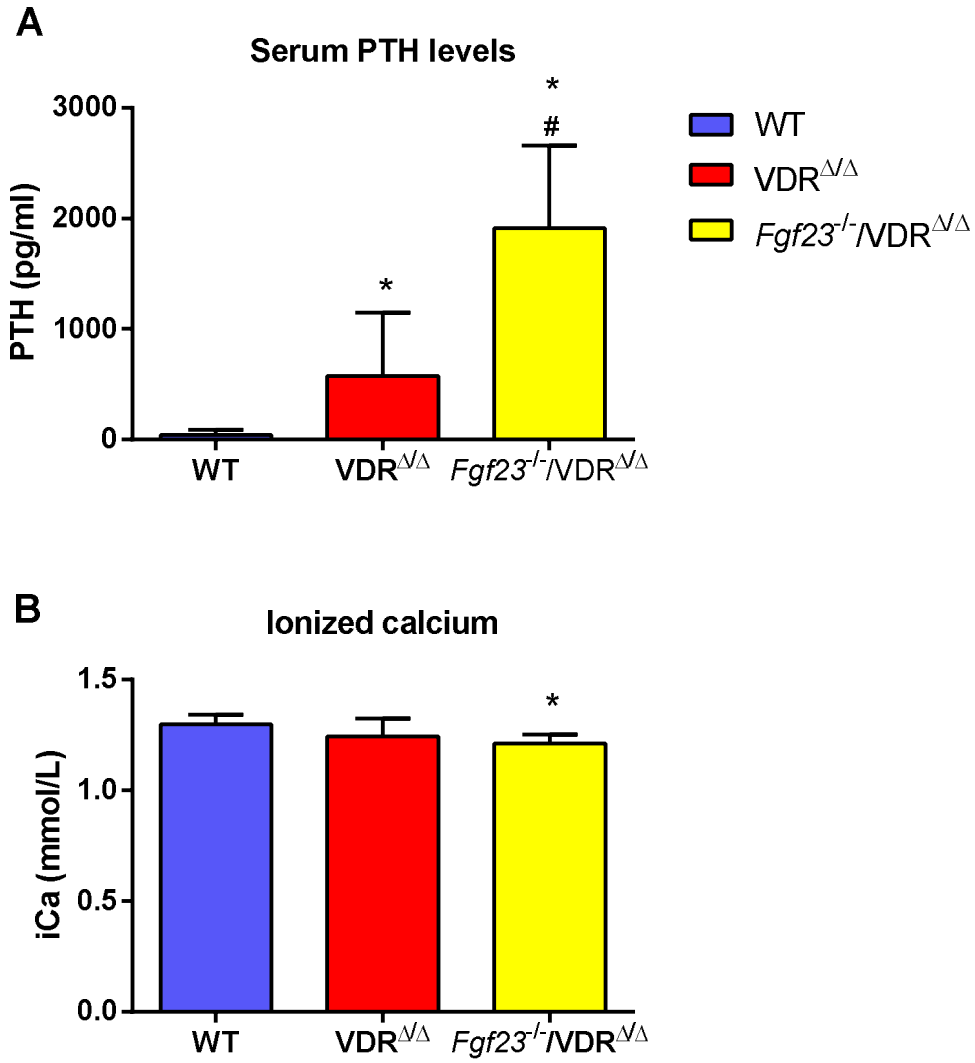


Figure 11: Serum PTH (A) and blood ionized calcium levels (B) of 3-month-old male WT, VDR$^{\Delta/\Delta}$ and *Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$ mice

Data represent mean ± SD. Each group consists of n=6-8 animals. *p<0.05 vs WT; #p<0.05 vs VDR$^{\Delta/\Delta}$

Parathyroidea profiling in FGF23 loss- and gain-of-function

To further investigate intracellular mechanisms of FGF23-mediated PTH expression regulation we next examined parathyroid mRNA expression levels of molecules principal for FGF23 and PTH signaling in loss-of-function experiments using WT, $VDR^{\Delta/\Delta}$ and *Fgf23*$^{-/-}$/$VDR^{\Delta/\Delta}$ mutants: PTH, PTH receptor 1 (PTHR1), calcium sensing receptor (CaSR), Klotho, FGFR1 and FGFR4. In addition, to examine the role of FGF23 co-receptor Klotho in FGF23-mediated PTH regulation we analyzed parathyroid glands of *Klotho*$^{-/-}$/$VDR^{\Delta/\Delta}$ compound mutant mice (Fig. 12). Additionally, parathyroid expression of the same molecules was also examined in gain-of-function experiments using 3-month-old WT males treated with recombinant FGF23 for 5 days (Fig. 13).

The results of loss-of-function experiments show a slight but not significant increase in *Pth* mRNA expression levels in *Fgf23*$^{-/-}$/$VDR^{\Delta/\Delta}$ and *Klotho*$^{-/-}$/$VDR^{\Delta/\Delta}$ compound mutants (Fig. 12). In comparison, mice treated with rFGF23 showed a significant down regulation of *Pth* mRNA expression levels (Fig. 13). Results indicate a direct regulatory effect of FGF23 on parathyroid PTH production. The PTH receptor 1 (*Pthr1*) mRNA expression levels in the loss-of-function experiment were elevated in both, VDR ablated mice and *Klotho*$^{-/-}$/$VDR^{\Delta/\Delta}$ compound mutants, whereas in *Fgf23*$^{-/-}$/$VDR^{\Delta/\Delta}$ mutants it was unchanged as compared to WT (Fig. 12). Since it is known that ablation of *Klotho* could play an important role in the regulation of PTH expression (Imura et al., 2007), these may explain observed changes in *Pthr1* mRNA expression in *Klotho*$^{-/-}$/$VDR^{\Delta/\Delta}$ compound mutant animals. In conformation to this notion, in our study *Pthr1* mRNA expression was also elevated in rFGF23 treated mice (Fig. 13). *Casr* and *Klotho* mRNA expression levels were enhanced in *Fgf23*$^{-/-}$/$VDR^{\Delta/\Delta}$ mice and correspondently significantly down regulated in the gain-of-function experiment, suggesting FGF23 regulation of these molecules (Fig. 12, 13).

Studies (Ben-Dov et al., 2007; Kurosu et al., 2006) already showed that the parathyroid glands are target organs of FGF23 signaling, also indicating the presence of Klotho and FGF receptors (FGFR) in parathyroid glands. Klotho has been shown to directly bind to FGFR with a higher affinity binding to FGFR1, 3 and 4 (Kurosu et al., 2006; Urakawa et al., 2006). Therefore we measured parathyroid mRNA expression of *Fgfr1* and *4* in both loss- and gain-of-function experiments. No change in *Fgfr1* was detected between all experimental groups and in both experimental settings (Fig. 12, 13). *Fgfr4* mRNA expression levels were slightly but not significantly upregulated in *Fgf23*$^{-/-}$/$VDR^{\Delta/\Delta}$ mutant mice (Fig. 12), whereas no regulation was observed in the mice treated with rFGF23 (Fig. 13).

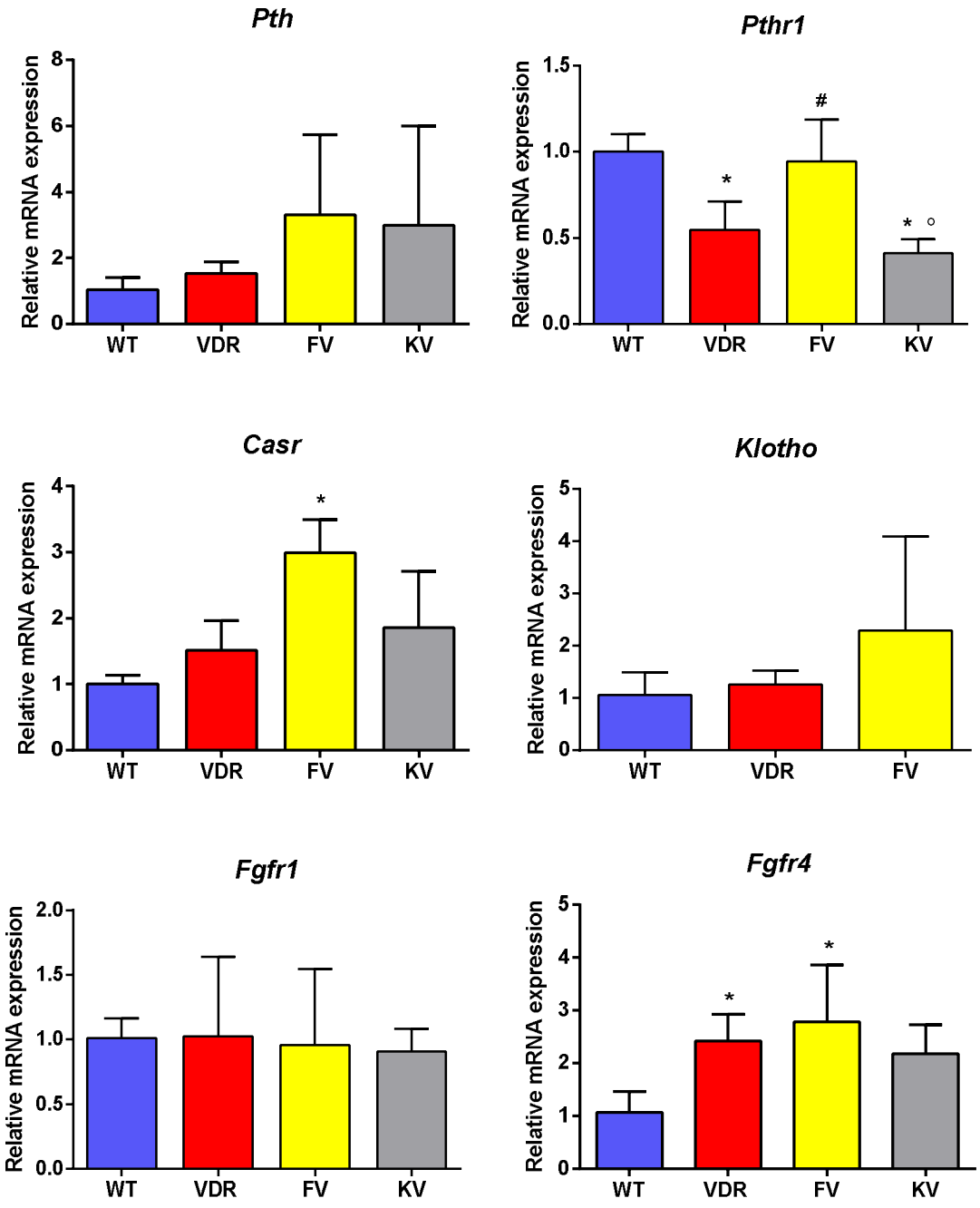


Figure 12: Relative mRNA expression of *Pth*, *Pthr1*, *Casr*, *Klotho*, *Fgfr1* and *Fgfr4* in parathyroid glands of WT, VDR$^{\Delta/\Delta}$, *Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$ (FV) and *Klotho*$^{-/-}$/VDR$^{\Delta/\Delta}$ (KV) animals

Standardized to *Rplp0*. Data represent mean ± SD. Each animal group was consisting of n=5-6 animals. *p<0.05 vs vehicle; #p<0.05 vs VDR$^{\Delta/\Delta}$; °p<0.05 vs *Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$

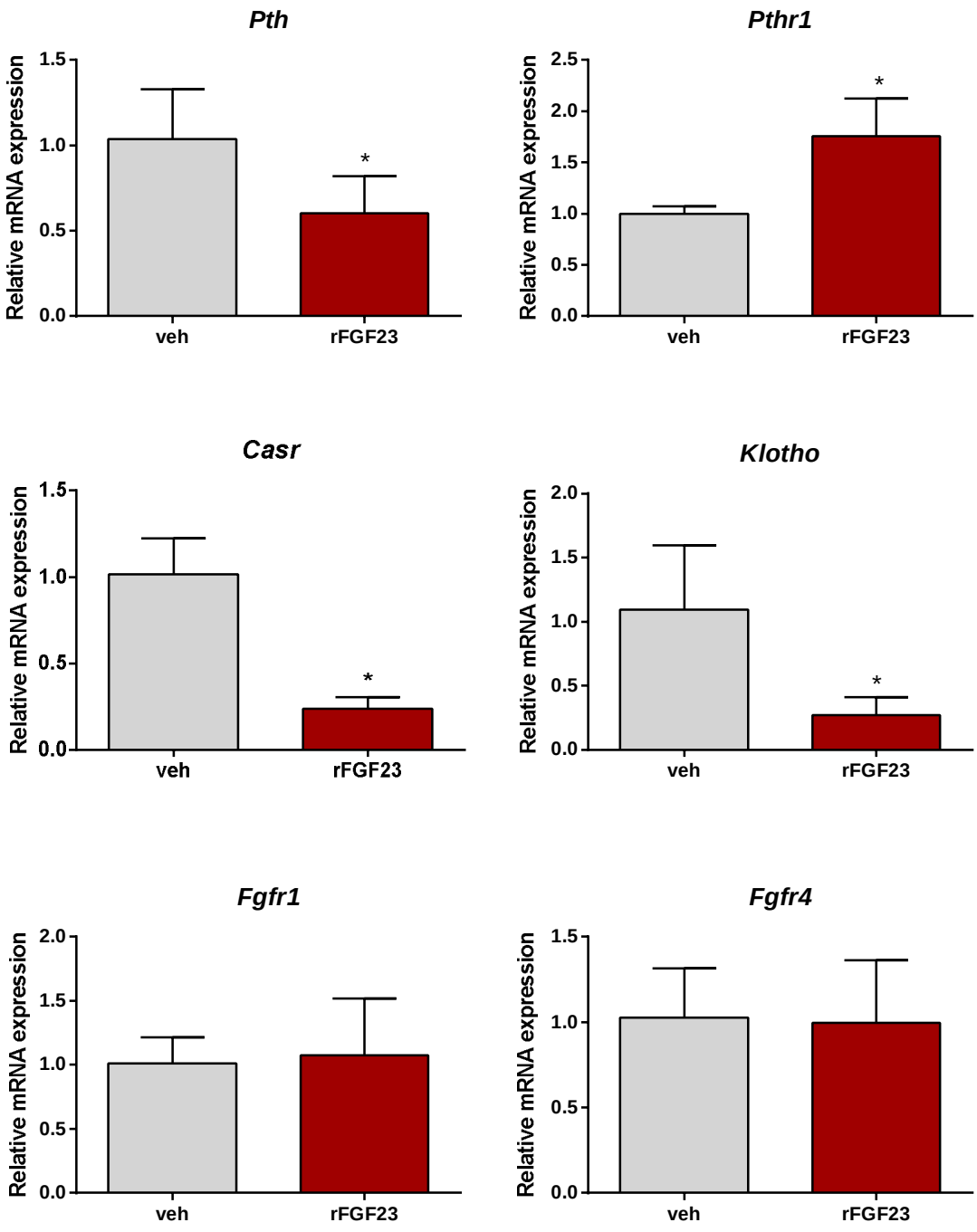


Figure 13: Relative mRNA expression of *Pth*, *Pthr1*, *Casr*, *Klotho*, *Fgfr1* and *Fgfr4* in parathyroid glands of vehicle (veh) or recombinant (rFGF23) treated animals

Standardized to *Rplp0*. Data represent mean ± SD. Treatment was performed for 5 days. Each animal group was consisting of n=5-6 animals. *p<0.05 vs vehicle

3.2. *Ex vivo* – Parathyroid gland organ culture

Since in our previous experiment we found that loss of FGF23 leads to hypocalcemia and increased serum PTH levels in *Klotho*$^{-/-}$/VDR$^{\Delta/\Delta}$ and *Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$ mutants at the same time with reduced *Pthr1* expression, we next ask the question if loss of FGF23 and Klotho could result in the disturbed parathyroid glands calcium sensitivity. Therefore, to prove this hypothesis we performed *in vitro* experiments using organ culture of parathyroid glands from WT, VDR$^{\Delta/\Delta}$ and *Klotho*$^{-/-}$/VDR$^{\Delta/\Delta}$ compound mutant mice. Moreover, a recent study (Ben-Dov et al., 2007) suggested that recombinant FGF23 may directly regulate PTH production and secretion. Therefore, we also monitored PTH production in parathyroid glands of WT animals treated with rFGF23.

PTH levels increase in hypocalcemic conditions in a Klotho and VDR independent fashion

Other groups already showed the sensitive control mechanism between PTH and calcium (Levine et al., 2014; Lin et al., 2014). In our study, treatment of isolated parathyroid glands from WT mice with low calcium induced an increase in medium PTH secretion (Fig. 14A). This result goes in accordance with already published data (Moallem et al., 1998; Rodriguez et al., 2005).

Interestingly, treating parathyroid glands of *Klotho*$^{-/-}$, VDR$^{\Delta/\Delta}$ and *Klotho*$^{-/-}$/VDR$^{\Delta/\Delta}$ mutant mice with different concentrations of calcium revealed no dependency of PTH production from Klotho and VDR signaling. These *ex vivo* data suggest that PTH secretion is regulated independently of Klotho and VDR signaling (Fig. 14B).

FGF23 inhibits the action of PTH

To find out if the regulation of PTH is a direct intracellular effect of FGF23, we next examined PTH secretion in parathyroid gland organ culture of WT mice in response to different calcium-concentrations in the presence of recombinant FGF23. PTH secretion was decreased in hypocalcemic conditions in the animals treated with rFGF23 as compared to the vehicle group (Fig. 14C). This signifies importance of suppressive FGF23 action on PTH expression.

Collectively, the results of the *ex vivo* experiments suggested FGF23 suppresses PTH expression under hypocalcemic conditions.

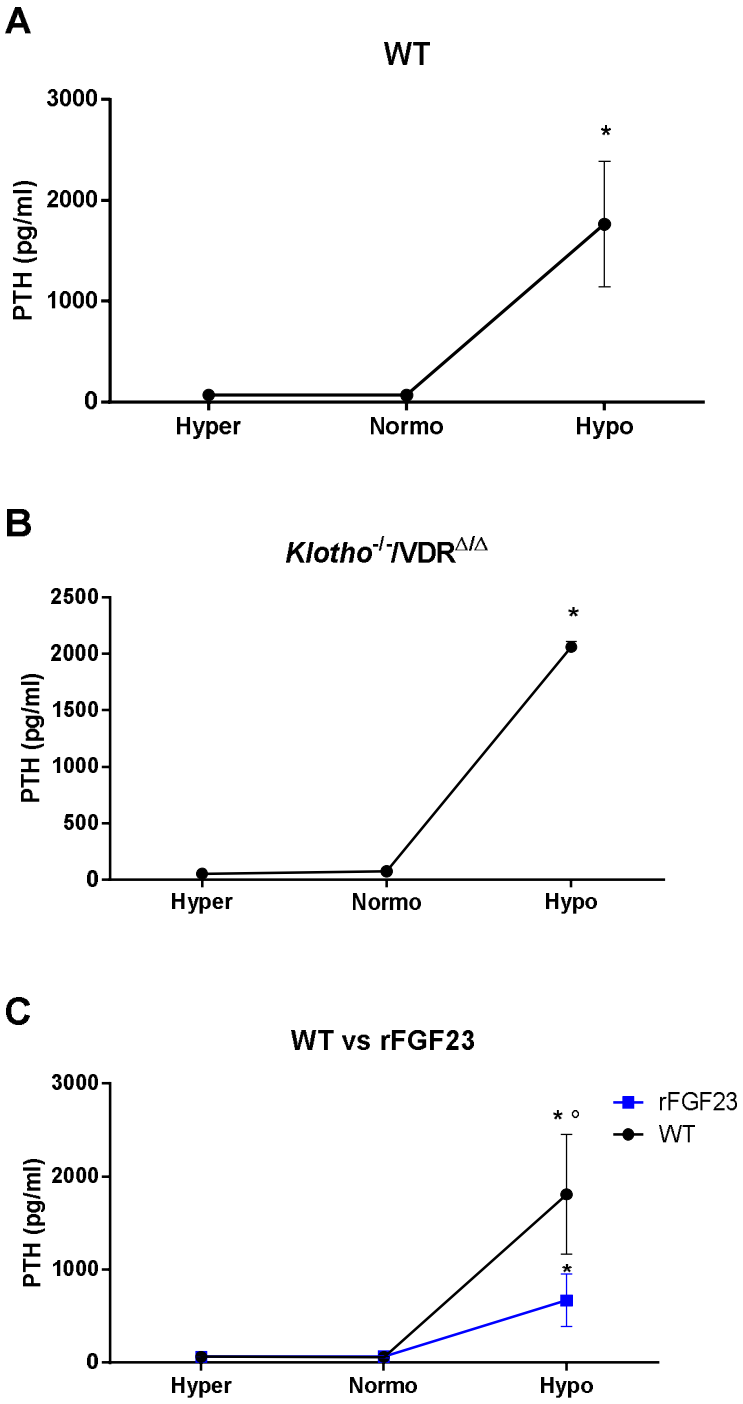


Figure 14: Medium PTH levels secreted by parathyroid glands of 4-week-old mice

Parathyroid glands were treated with hypercalcemic, normocalcemic and hypocalcemic medium for 1 hour each treatment. Data represent mean ± SD. **A**: PTH secretion of WT mice; **B**: PTH secretion of *Klotho*$^{-/-}$/VDR$^{\Delta/\Delta}$ mice; **C**: PTH secretion of WT mice compared to a group treated in combination with recombinant FGF23. Each group is consisting of n=4 animals. *p<0.05 vs normo; °p<0.05 vs rFGF23

3.3. PTH regulation of FGF23 *in vivo*

The circulating, intact form of FGF23 was described to have phosphaturic as well as calcium- and sodium conserving action in the kidneys (Andrukhova et al., 2014a, 2014b; Shimada et al., 2005). The C-terminus of FGF23 resulting from the proteolytic cleavage of the intact FGF23 was also proposed as a possible binding ligand to the FGFR1-Klotho complexes acting as a competitor for the intact FGF23 form (Goetz et al., 2010). The exact mechanism for FGF23 cleavage regulation is not well understood. It was previously suggested that PTH treated animals have increased circulating FGF23 level. If this action of PTH on FGF23 expression takes place at the transcriptional or at post-transcriptional level involving cleavage regulation is not yet described. Therefore, to investigate if PTH could regulate FGF23 cleavage *in vivo*, we treated WT animals with PTH (1-34) and measured the levels of intact and C-terminal FGF23 in the serum. The intact FGF23 assay captures only the intact form and the C-terminal FGF23 assay both forms, the intact and the C-terminal fragment of FGF23 (Wolf and White, 2014).

Acutely PTH increases FGF23 levels over 3-fold at 2 hours after injection, capturing mainly cleaved C-terminal FGF23

To monitor the effectiveness of the PTH injections in *in vivo* experiments we used blood ionized calcium levels as a read-out. As expected, measured 2 hours after injection the average in calcium levels of the experiments (shown in Fig. 15) were 1.25 mmol/L for the vehicle-group and 1.34 mmol/L for the group injected with PTH.

The data indicate an over 3-fold increase in total FGF23 measured with the cFGF23 assay, capturing the intact and the C-terminal FGF23 fragment in PTH treated animals 2 hours post-injection. Interestingly, 6 hours post-injection of PTH, levels of total FGF23 were normalized to the vehicle treatment (Fig. 15A).

To investigate if the increase seen in Fig. 15A is an effect of the intact form of FGF23 or if cleavage is involved in this short-term effect of PTH we measured serum level of only intact FGF23 (iFGF23) form for all experimental groups. A slight increase in iFGF23 was observed at 2 hours post-injection compared to vehicle treatment. Decreased iFGF23 was observed at 4 and at 6 hours post-PTH injection with further normalization to about vehicle levels at 24 hours after injection (Fig. 15B). Hence, the peak in the cFGF23 assay (Fig. 15A) at 2 hours post-injection displays mainly C-terminal FGF23, suggesting activated cleaved

iFGF23 post-PTH treatment. This is further depicted in a mathematical difference in intact FGF23 and total FGF23 (Fig. 15C).
These data demonstrate that PTH signaling participate in FGF23 cleavage *in vivo*.

To verify that the PTH dose used for this experiment give the optimal effect on FGF23 cleavage, we performed dose escalation experiments where we tested lower (12.5 nmol/kg) and higher doses (200 nmol/kg) of PTH (1-34) for the animal injections. We observed no significant difference in iFGF23 and cFGF23 levels as well as in blood ionized calcium levels when animals were injected with PTH concentrations of 50 nmol/kg and 200 nmol/kg (Fig. 16A, C). However, the animals injected with 12.5 nmol/kg of PTH showed a clear decrease in iFGF23 and cFGF23 levels, but not in the blood ionized calcium levels to those observed for 50 nmol/kg and 200 nmol/kg of PTH treatment (Fig. 16B, C). Therefore, it can be concluded that PTH treatment with 50 nmol/kg used in the previous experiments represents the optimal dose leading to the maximum effect on FGF23 cleavage.

Concomitant injecting of Furin inhibitor with PTH partially prevents FGF23 cleavage

Several studies suggested Furin as a regulator for FGF23 processing (Bhattacharyya et al., 2012). In order to investigate if Furin could be involved in the mechanism behind PTH-mediated FGF23 cleavage, we next treated WT mice with either vehicle or Furin inhibitor, 15 hours and 1 hour prior PTH injections. Treatment with only Furin inhibitor was used as a control. The data demonstrate that the inhibitor alone did not influence neither cFGF23 nor iFGF23 levels. On the other hand, animals treated with PTH after Furin inhibitor injections display an elevated peak in both intact and C-terminal FGF23 levels at 2 hours post PTH injections compared to animals injected with PTH but without inhibitor (Fig. 17A, B).

Taken together the data show that treatment with Furin inhibitor partially prevents FGF23 cleavage and moreover, slightly elevates total FGF23 expression.

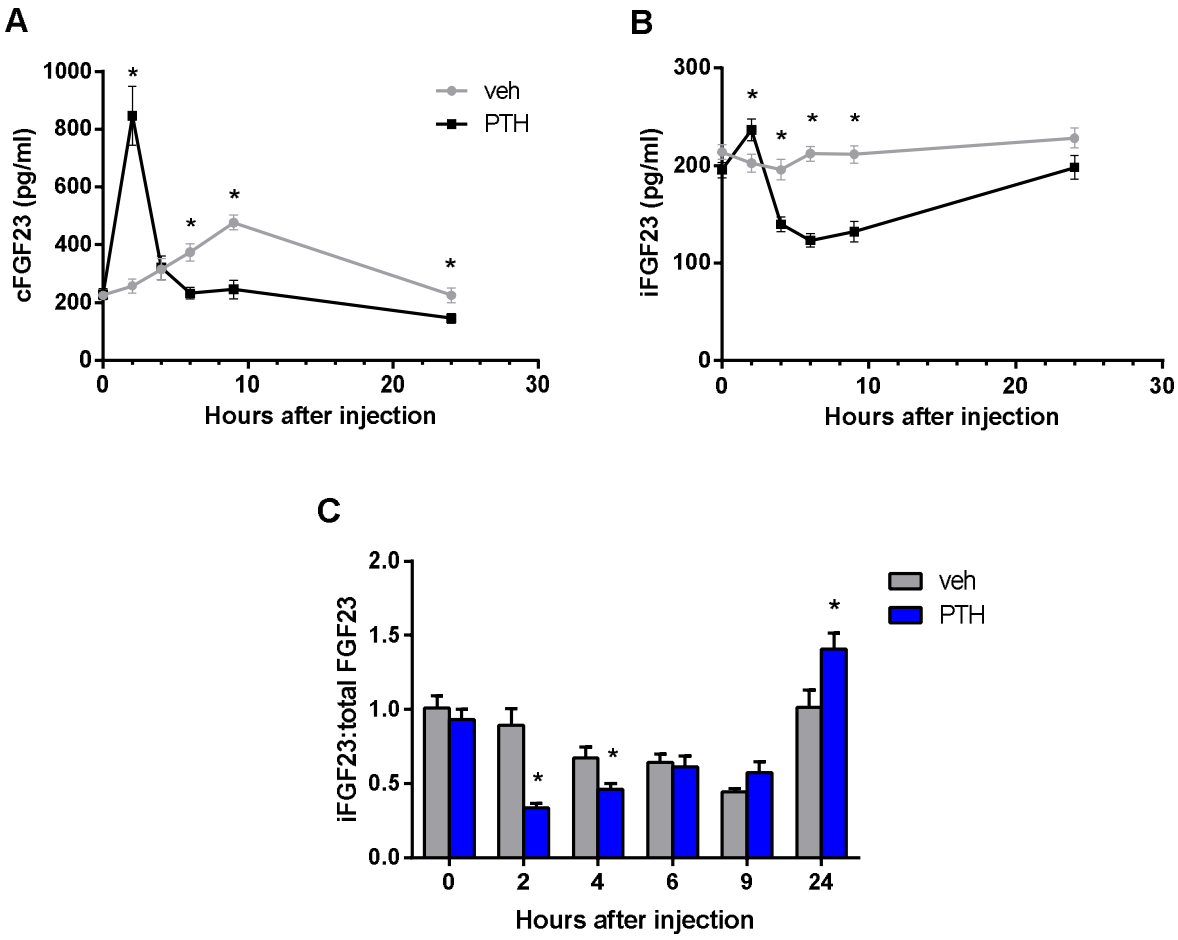
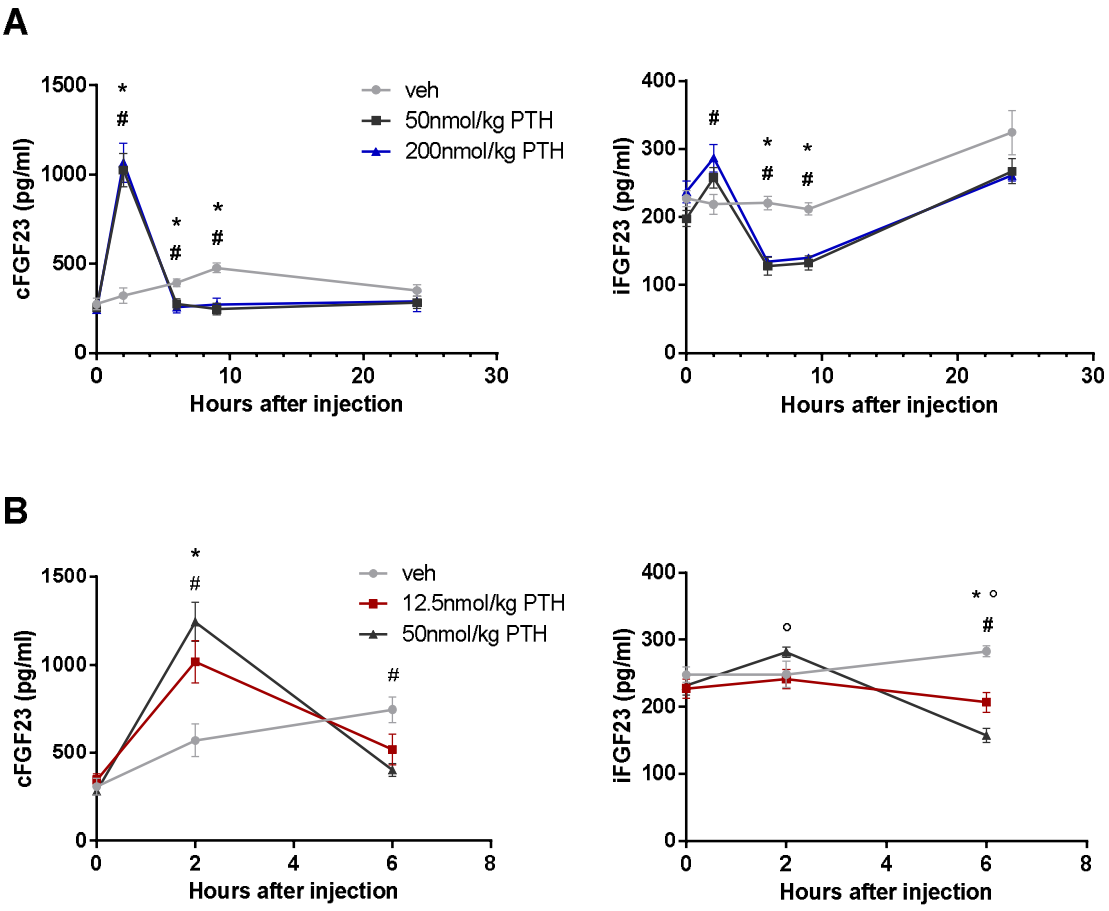


Figure 15: Total (cFGF23) and intact (iFGF23) levels (pg/ml) of WT mice injected with vehicle (veh) or 50nmol/kg PTH

Each graph shows combined data from three independent experiments. Groups are consisting of n=5-20 animals per group. Data represent mean ± SEM. **A, B**: Injection of 50 nmol/kg PTH or vehicle. **C:** Ratio intact (iFGF23) to total FGF23. *p<0.05 vs vehicle



C

iCa^{2+}	vehicle	50nmol/kg PTH	200nmol/kg PTH
I	1.26± 0.02	1.32± 0.01	1.33± 0.02
II	1.27± 0.01	1.37± 0.02	1.38± 0.02
	vehicle	12.5nmol/kg PTH	50nmol/kg PTH
III	1.25± 0.01	1.33± 0.01	1.33± 0.01

Figure 16: Total (cFGF23) and intact (iFGF23) (A, B) and ionized calcium levels (C) of PTH dose escalation experiments

A: Injection of 50 nmol/kg, 200 nmol/kg PTH or vehicle (veh). Each group consists of n=4-13 animals. *p<0.05 50 nmol vs veh; #p<0.05 200 nmol vs veh. **B**: Injection of 12.5 nmol/kg, 50 nmol/kg PTH or vehicle. Each group consists of n=5-7 animals. *p<0.05 12.5 nmol vs veh; #p<0.05 50 nmol vs veh; °p<0.05 12.5 nmol vs 50nmol. **C**: **I** and **II** are two independent experiments, summarized in graphs A. Experiment **III** is depicted in graphs B. Data represent mean ± SEM.

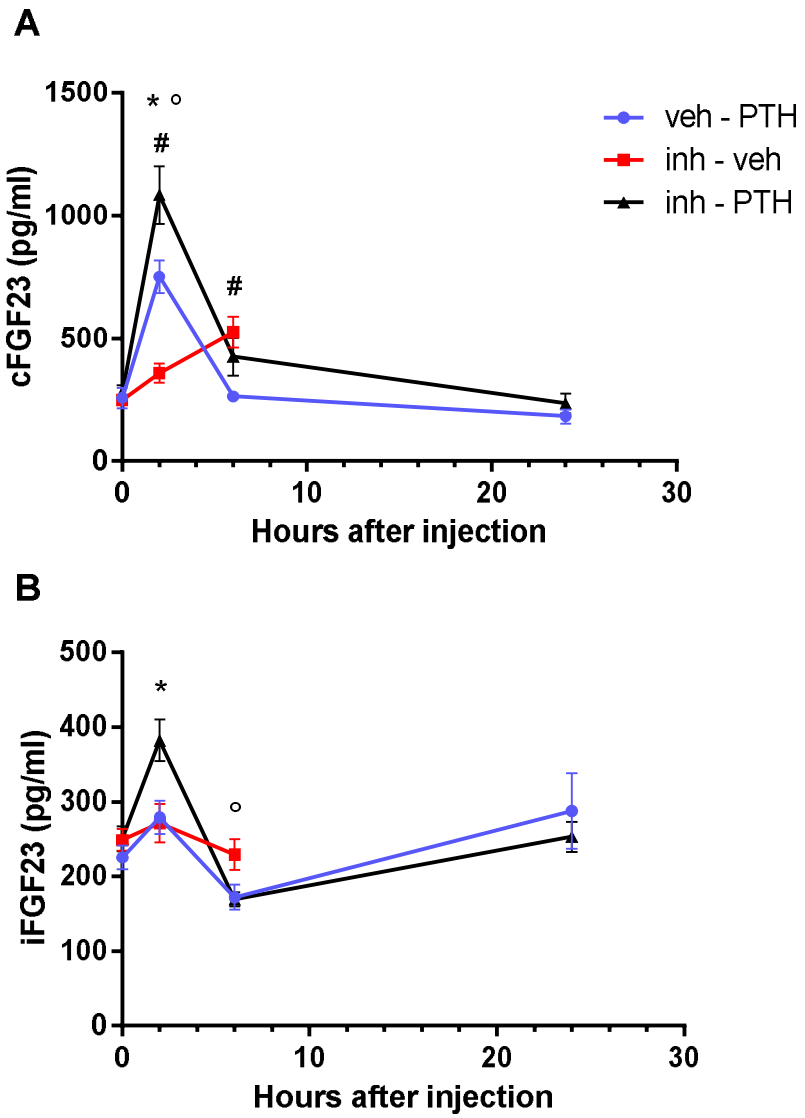


Figure 17: Total (cFGF23) (A) and intact (iFGF23) levels (B) of concomitant, PTH (50 nmol/kg) and furin inhibitor injected WT mice

Each graph shows combined data from three independent experiments. Data represent mean ± SEM. **A**, **B**: Injection of vehicle (veh) (5 % DMSO) or furin inhibitor I (Decanoyl-RVKR-CMK) followed by injection of vehicle or 50 nmol/kg PTH. Each group consists of n=3-13 animals.

*p<0.05 veh-PTH vs inh-PTH; #p<0.05 veh-PTH vs inh-veh; °p<0.05 inh-veh vs inh-PTH

3.4. Primary osteoblast/osteocyte culture

FGF23 is produced by osteoblasts and osteocytes. Osteocytes are embedded in the mineralized bone matrix and osteoblasts and lining cells are matrix-producing (Bonewald and Wacker, 2014). To further confirm previous *in vivo* results, and to reveal the mechanism of the involved pathway behind cleavage regulation of FGF23 we further carried out PTH treatment of primary osteoblasts. To depict effects of PTH on different differentiation stages we performed treatment on day 14 and day 21 of differentiation with an osteoblast and osteocyte-like phenotype correspondently.

PTH regulates FGF23 cleavage *in vitro*

Cells were treated on day 14 and day 21 of differentiation with vehicle and two concentrations of PTH (10ng and 100ng) for 2 and 6 hours and medium FGF23 was measured.

After 2 hours, 100 ng PTH treatment of 14 days differentiated cells significantly increased cFGF23, but unchanged iFGF23 levels were observed. This effect was not present for 10 ng PTH treated samples (Fig. 18A). The effects of treatment are enhanced in 6 hours showing an accumulation of medium FGF23 in 100 ng PTH treated cells. More osteocyte-like cells (differentiated for 21 days) show a regulation of FGF23 cleavage only at 2 hours with the 100 ng but not 10 ng PTH treatment (Fig. 18B). These date indicate that PTH stimulate FGF23 cleavage *in vitro* and that this effect is more pronounced in osteocyte-like cells at 21 days of differentiation.

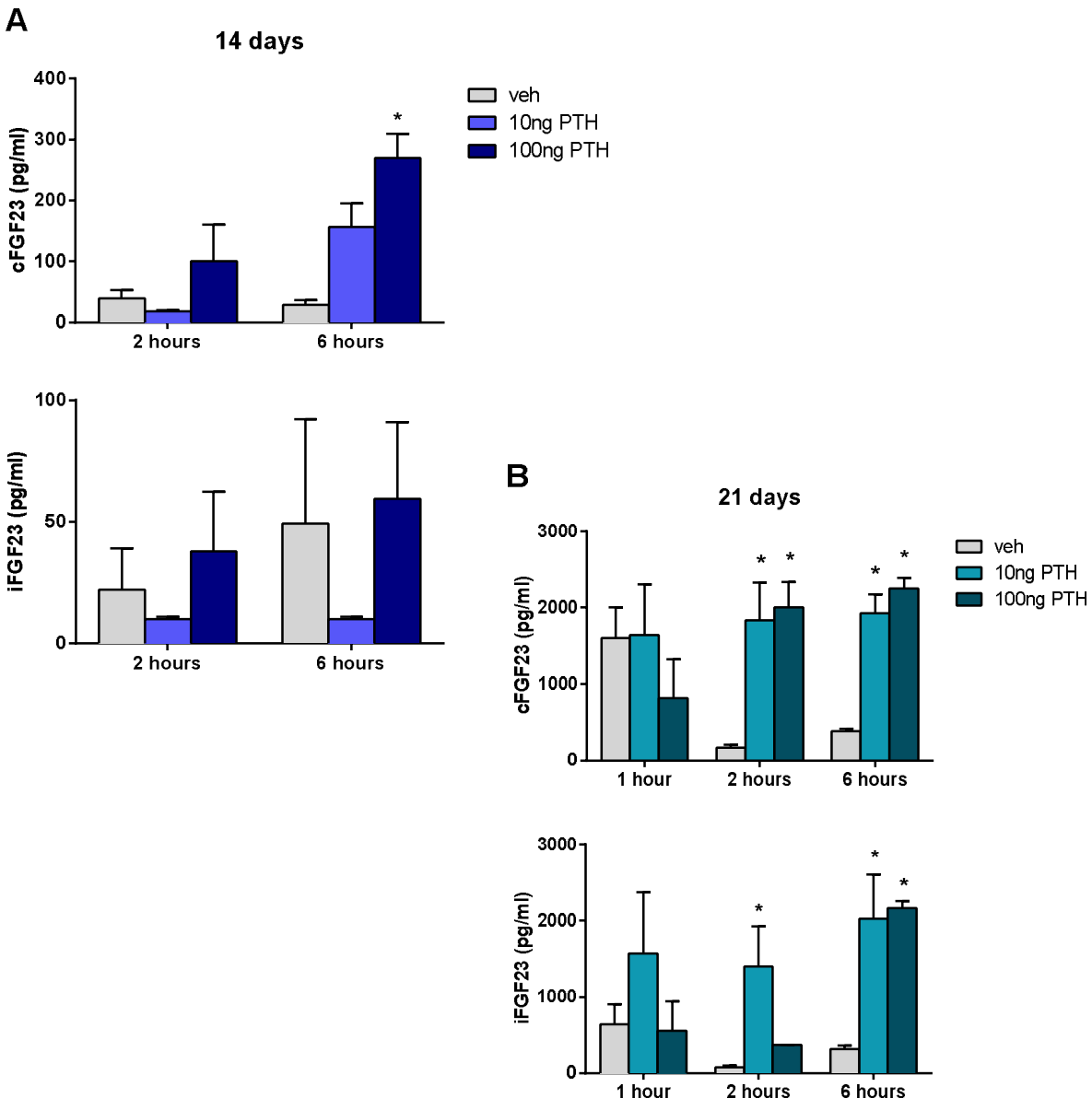


Figure 18: Total (cFGF23) and intact (iFGF23) levels (pg/ml) in primary osteoblast cell culture treated with vehicle (veh) or PTH (10 and 100 ng/ml) for 1, 2 or 6 hours

A: Cells differentiated for **14 days** prior treatment. **B**: Cells differentiated for **21 days** prior treatment. Data represent mean ± SEM. n=3 in each treatment group. *p<0.05 vs vehicle

PTH regulates FGF23 stability by targeting cleavage pathway in osteocytes *in vitro*

Previous studies (Bhattacharyya et al., 2012; Tagliabracci et al., 2014) showed that FGF23 protein normally undergoes O-linked glycosylation by GalNAc-T3, which stabilizes the intact FGF23 molecule. Phosphorylated FGF23 by Fam20C reduces glycosylation, thus making intact FGF23 more susceptible to cleavage by furin-like enzymes. Therefore, to further examine if PTH treatment alters *Fgf23* mRNA expression as well as enzymes Furin, GalNAc-T3 and Fam20C, involved in FGF23 cleavage in osteoblast- and osteocyte-like cell culture in our study, we performed mRNA isolation in all samples collected in the previous experiment.

At 14 days differentiation, *Fgf23* mRNA expression of cells was enhanced at all time points compared to vehicle treatment (Fig. 19A). *Furin* mRNA expression levels were decreased at 1 and 2 hours, but not at 6 hours following 100 ng PTH treatment. Interestingly, *Furin* mRNA expression was also decreased after 6 hours post-PTH treatment only with 10 ng PTH. *Fam20C* mRNA expression was upregulated whereas *Galnt3* was unchanged at 2 hours post-treatment with 100 ng PTH, pointing to elevated FGF23 cleavage at this treatment time point. Interestingly, reciprocal regulation of *Fam20C* and *Galnt3* mRNA expression was observed 6 hours post-PTH treatment suggesting a decrease in FGF23 cleavage at these time points (Fig. 19B).

At 21 days of differentiation *Fgf23* mRNA expression levels were significantly increased 2 hours after PTH treatment, both at 10 and 100 ng of PTH. *Furin*, *Fam20C* and *Galnt3* mRNA expression show no significant changes post-PTH treatment (Fig. 20B).

Summarized, these data provide significant evidence that PTH regulates FGF23 protein stability by modulating expression of the enzymes responsible for protein cleavage. Moreover, these data confirm observation of our previous experiments showing a direct effect of PTH on FGF23 expression and cleavage *in vivo*.

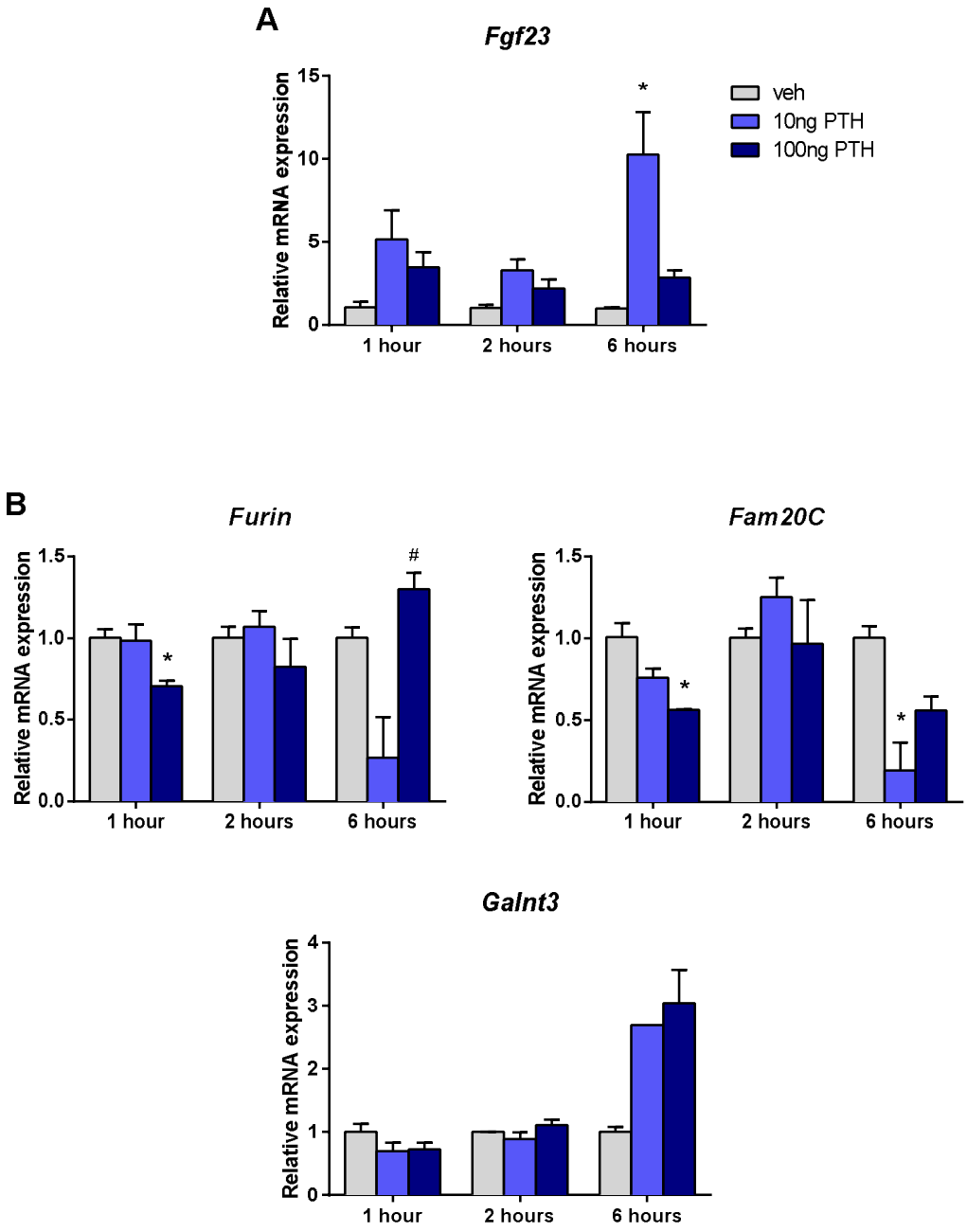


Figure 19: Relative *Fgf23* (A), *Furin*, *Fam20C* and *Galnt3* (B) mRNA expression in 14 days differentiated osteoblasts treated with vehicle (veh) or PTH (10 and 100 ng/ml) for 1, 2 or 6 hours

Data was standardized to *GAPDH* and represents mean ± SEM. Each group consists of n=3 samples. *p<0.05 vs vehicle; #p<0.05 vs 10 ng PTH

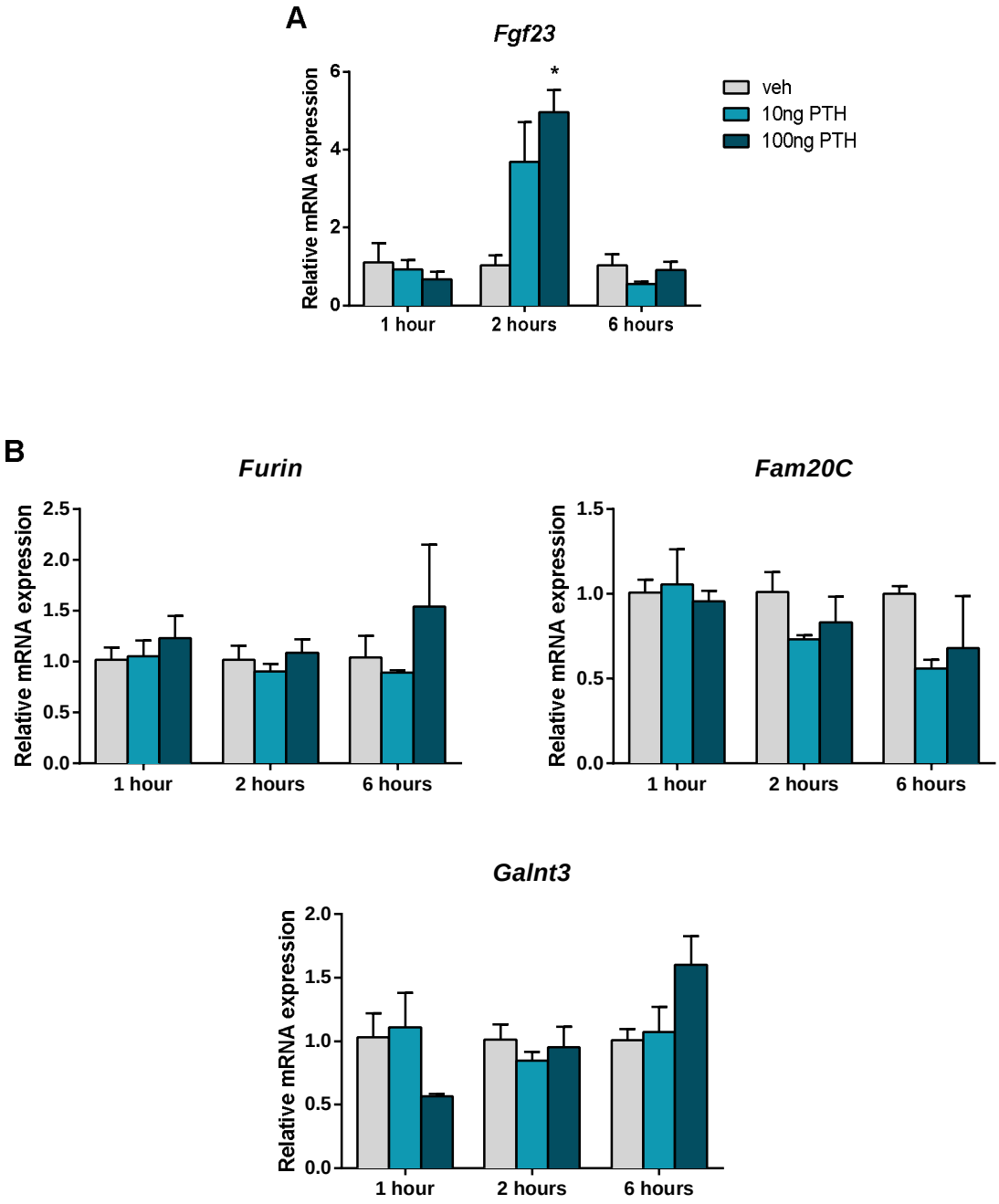


Figure 20: Relative *Fgf23* (A), *Furin*, *Fam20C* and *Galnt3* (B) mRNA expression in 21 days differentiated osteoblasts treated with vehicle (veh) or PTH (10 and 100 ng/ml) for 1, 2 or 6 hours

Data was standardized to *GAPDH* and represent mean ± SEM. Each group consists of n=3 samples. *p<0.05 vs vehicle

4. Discussion

FGF23 is one of the most important endocrine regulators of phosphate, calcium and vitamin D metabolism together with PTH. Controversial studies describe feedback regulation between FGF23 and PTH. The exact molecular mechanism for this interaction remains still unknown. We performed this study in order to investigate direct effects of FGF23 on PTH and vice versa within the feedback mechanisms between these two molecules.

Several studies suggest that FGF23 directly acts on PTH production and secretion (Ben-Dov et al., 2007; Krajisnik et al., 2007). In our study we used *Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$ compound mutant mice, held on rescue diet giving the possibilities to investigate FGF23 effects independently of VDR signaling. Mutant animals displayed significant increases in serum PTH, even higher in compound mutants indicating a direct inhibitory effect of FGF23 on PTH secretion, independent of vitamin D action. We confirmed this finding in isolated mouse parathyroid glands showing PTH expression upregulation in *Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$ mutant mice, and downregulation of PTH expression confirmed in WT mice, treated with recombinant FGF23 for 5 days. To further validate our results we utilized an *ex vivo* parathyroid organ culture system, which was demonstrated to be an optimal system for PTH expression monitoring *in vitro* (Ben-Dov et al., 2007). As expected, in all treated organs hypocalcemic stimulus led to elevated PTH secretion levels. Interestingly, combination of hypocalcemic stimulus with rFGF23 resulted in suppression of PTH secretion further confirming that FGF23 is involved in direct regulation of PTH secretion.

In agreement with our findings, earlier studies showed the direct action of FGF23 on the parathyroid decreasing PTH gene expression and secretion *in vivo* in rats and *in vitro* in rat parathyroid cultures through activating the mitogen-activated protein kinase (MAPK) pathway (Ben-Dov et al., 2007). Another study, using primary cultures of bovine parathyroid cells showed that FGF23 modulates PTH secretion, by mediating 1α-hydroxylase expression (Krajisnik et al., 2007). Moreover, studies in *Fgf23*$^{-/-}$ mice show decreased PTH levels (DeLuca et al., 2008), whereas overexpression of human FGF23 resulted in increase of PTH secretion (Shimada et al., 2004a).

Studies focusing on disorders with declining kidney function showed that parathyroid glands can get resistant to FGF23 action. The proposed mechanism underlying such FGF23 resistance is decreased abundance of FGFRs and Klotho in the parathyroid (Komaba et al., 2010; Krajisnik et al., 2010; Silver et al., 2012). In our study we were unable to detect any

changes in parathyroid *Fgfrs* and *Klotho* mRNA expression in *Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$ mutant mice. At the same time, we found decreased *Pthr1*, but increased *Casr* mRNA expression in parathyroid glands of *Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$ mutant mice, suggesting existence of regulatory feedback between FGF23 signaling and PTHR1 and CaSR expression. This notion is also reinforced by our gain-of-function experiment showing that treatment with rFGF23 resulted in decrease of parathyroid *Pth*, *Casr* and *Klotho* mRNA expression, but led to elevated *Pthr1* mRNA expression with no change in *Fgfr1* and *Fgfr4* expression. Moreover, presence of hypercalcemia and elevated circulating PTH in *Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$ mutant mice suggests that lack of FGF23 led to partial PTH resistance in these animals.

Several studies indicate Klotho as fundamental for the stimulation of PTH secretion during hypocalcemic conditions (Imura et al., 2007). Several other studies however, questioned the role of Klotho in regulation of PTH expression (Martuseviciene et al., 2011). In our study, in parathyroid organ culture of *Klotho*$^{-/-}$, VDR mutants and *Klotho*$^{-/-}$/VDR$^{\Delta/\Delta}$ compound mutant mice exposed to hypocalcemic conditions, we observed enhanced PTH secretion suggesting Klotho and vitamin D independent regulation of PTH secretion.

Studies indicated FGF23 acting on parathyroid glands by binding to its FGFR-Klotho complex to suppress PTH secretion (Ben-Dov et al., 2007; Krajisnik et al., 2007). In this study administration of recombinant FGF23 led to an increase in parathyroid Klotho expression which further resulted in decreased PTH level (Ben-Dov et al., 2007). The result of our study did not support this finding since 5-days rFGF23 treatment did not change parathyroid Klotho expression in WT mice. Moreover, Klotho expression was not regulated in *Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$ mutant mice.

Our finding is supported by a recent study (Olauson et al., 2013) showing a novel, Klotho-independent FGF23 signaling pathway in parathyroid glands that mediates suppression of PTH. Parathyroid-specific ablation of Klotho had no impact on FGF23-dependent PTH regulation in his study. This Klotho independent action of FGF23 in a Klotho-expressing target organ represents a paradigm shift in the conceptualization of FGF23 endocrine action.

Summarized, existing studies and our results strongly indicate that FGF23 has a direct inhibitory effect on PTH secretion in parathyroid glands. The exact intracellular mechanism and the role of parathyroid Klotho in FGF23 mediated PTH expression will be further revealed in future experiments.

The second focus of this study was to investigate whether PTH can regulate FGF23 production and secretion. FGF23 cleavage regulation is an important regulatory step in modulation of circulating intact FGF23 levels (Wolf and White, 2014). We further hypothesized that PTH can regulate not only the expression, but also cleavage processing of FGF23. To the best of our knowledge, no studies investigating PTH cleavage regulation of FGF23 were performed till now.

The existing data indicate that PTH infusion to WT mice and humans increased serum FGF23. Moreover, PTH increased expression of FGF23 in osteoblast-like UMR106 cells (Burnett-Bowie et al., 2009; Lavi-Moshayoff et al., 2010). Recent study suggests that this regulation occurs through PKA-mediated activation of Nurr1 resulting in increased FGF23 transcription (Meir et al., 2014). In other studies using the bone-derived cell line ROS 17/2.8 and organ tissue culture of rat calvariae, PTH treatment did not increase FGF23 expression levels (Liu, 2006; Saji et al., 2010).

The data of our study indicate, that single injections of PTH in wild type mice increases FGF23 levels over 3-fold, two hours post-injection. Besides, we demonstrated that this increase was associated with profound elevations in circulating C-terminal FGF23 levels, indicating enlarged cleavage of FGF23 post-PTH injection. Conversely, concomitant injections of PTH with furin inhibitor resulted in an increase of circulating intact FGF23 levels and decrease of C-terminal FGF23 levels. This suggests that PTH regulates FGF23 cleavage mainly through furin activation.

To prove if PTH is directly effecting processing of FGF23 in osteocytes *in vitro*, we used primary osteoblast/osteocyte cells, isolated from mouse calvariae. Indeed, we were able to detect increased FGF23 secretion in *in vivo* data in the osteocyte-like cells differentiated for 21 days and to a lower extend in osteoblast-like cells, differentiated for 14 days. Consistent with our *in vivo* data, the effect on the FGF23 production was most prominent at 2 hours post-PTH treatment, confirming rapid PTH effects on regulation of FGF23 cleavage.

Others have shown that the enzymes Furin, GalNac-T3 and Fam20C are responsible for posttranslational modifications of FGF23 (Bhattacharyya et al., 2012; Tagliabracci et al., 2014). Therefore, in our study we analyzed gene expression of *Furin*, *Galnt3* and *Fam20C* in osteoblast/osteocyte cell culture and correlated these data to the secreted intact and C-terminal FGF23 levels. The result of this analysis indicate that expression of FGF23 cleavage regulating molecules *Furin*, *Fam20C* and *Galnt3* is regulated by PTH treatment in osteoblast-like cells, signifying activation of FGF23 cleavage. Interestingly, no change in *Furin*, *Fam20C* and *Galnt3* expression in osteocyte-like cells was observed. This suggests cell-specific

regulation of FGF23 cleavage by PTH. It has been suggested however, that osteocytes are the main site of FGF23 production (Bonewald and Wacker, 2014). Additionally, a possible role of FGF23 cleavage in circulation was also proposed. Therefore it is plausible that increased circulating FGF23 post-PTH treatment is cleaved in the circulation. The fact that in our *in vitro* experiment *Furin*, *Fam20C* and *Galnt3* mRNA expression was unchanged in osteocyte-like cells whereas an increase in circulating FGF23 C-terminal is observed 2 hours post-PTH treatment *in vivo* also supports this notion. However, the mechanisms behind circulatory cleavage of FGF23 remain still elusive. Proposed mechanisms for PTH-driving regulation of FGF23 production are summarized in Figure 21.

Further experiments are required to reveal exact regulatory mechanisms behind PTH-mediated increase in FGF23 expression and cleavage in osteoblasts/osteocytes.

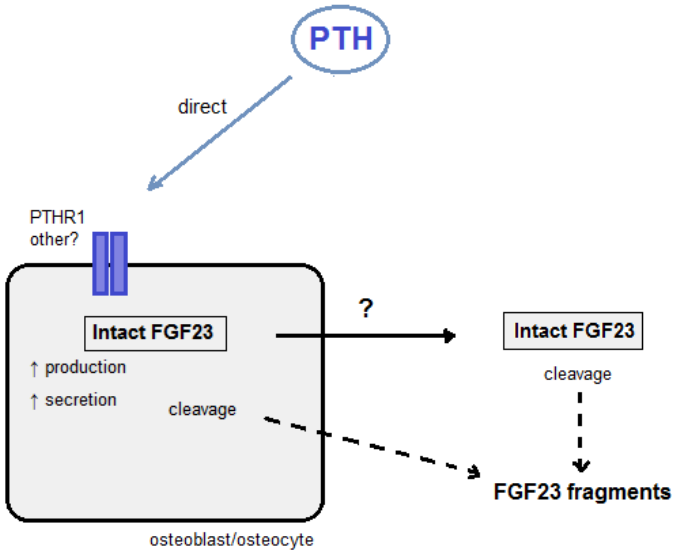


Figure 21: Proposed model of PTH action on FGF23 cleavage regulation

PTH directly acts on osteoblasts/osteocytes, the main site of FGF23 production. It binds to a receptor and activates downstream signaling through an unknown pathway. This leads to increased FGF23 production and secretion. Cleavage of FGF23 is activated and upregulated in the cell, resulting in N- and C-terminal FGF23 fragments production and secretion into the circulation. Intact FGF23 could also be secreted from the cell and cleaved in circulation.

In conclusion, our data show a for the first time clear regulatory feedback loop between FGF23 and PTH. Strong evidence from our *in vivo* and *ex vivo* experiments show: 1.) that FGF23 regulates PTH expression independently of vitamin D signaling; 2.) that PTH stimulates FGF23 expression and is involved in FGF23 cleavage regulation.

The recent data on the direct interaction between FGF23 and the parathyroid gland and PTH synthesis and secretion have important implications for understanding the pathophysiology of common mineral and bone disorders associated with impaired kidney function. Findings of the present study are of critical clinical significance and imply importance of correlation between circulating FGF23 and PTH levels in therapeutically strategy of secondary hyperparathyroidism and chronic kidney failure patients.

5. Summary

Parathyroid hormone (PTH) is a well-established regulator of calcium and phosphate homeostasis. PTH secretion increases in response to low blood calcium levels and normalize serum calcium by elevating renal calcium reabsorption as well as by increasing calcitriol production enhancing calcium intestinal absorption. Moreover, PTH stimulates osteoclast differentiation and activity, resulting in increased bone resorption and calcium efflux from the bone. Additionally, PTH increases urinary phosphate excretion by reducing phosphate uptake in the renal proximal tubules.

Fibroblast growth factor-23 (FGF23), a recently discovered bone-derived hormone, is produced in response to hyperphosphatemia and 1,25-dihydroxyvitamin D_3. FGF23 plays a major role in maintaining phosphate, calcium and sodium mineral homeostasis by regulating its renal excretion. FGF23 also reduces renal 1α-hydroxylase synthesis in the kidney and as a result circulating levels of biologically active vitamin D.

FGF23 is a 30-kDa protein which could be proteolytically cleaved and processed into smaller N-terminal and C-terminal fragments by the enzymes Furin, Fam20C and GalNac-T3.

FGF23 signals through FGF Receptors (FGFR) and a co-receptor Klotho. The Klotho-expressing tissues determine target organs of FGF23, including the parathyroid glands.

Accumulating evidence suggest a negative feedback loop between FGF23 and PTH expression regulation, where PTH stimulates FGF23 production and FGF23 in turn suppresses PTH synthesis, but the exact mechanisms are not well understood yet.

In the current study, we show that FGF23 has a direct inhibitory effect on PTH production as monitored in *Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$ compound mutants. We confirmed these results in mice treated with recombinant FGF23 and using *ex vivo* parathyroid gland organ culture. Further, using *in vivo* and *in vitro* studies we demonstrated that PTH acutely increases FGF23 expression. Our *in vivo* study has additionally uncovered that PTH regulates post-translational cleavage of FGF23 and expression of Furin, Fam20C and GalNac-T3 proteins. Further studies are required to verify molecular mechanisms behind.

6. Zusammenfassung

Parathormon (PTH) ist etabliert in der Regulation des Calcium- und Phosphathaushaltes. Bei niedrigem Calciumspiegel im Blut kommt es zur erhöhten Ausschüttung von PTH und zur Normalisierung durch erhöhte renale Calciumreabsorption, aber auch durch vermehrte Calcitriol Produktion, welche die intestinale Calciumabsorption vergrößert. Weiters stimuliert PTH die Differenzierung und Aktivität von Osteoklasten um die Resorption des Knochens und somit die Freisetzung von Calcium zu erhöhen. Die Phosphatausscheidung wird durch PTH vermehrt, indem die Phosphataufnahme in den proximalen Nierentubuli verringert wird.

Bei Fibroblast growth factor-23 (FGF23) handelt es sich um ein kürzlich entdecktes Hormon. Es wird bei Hyperphosphatämie und hohem 1,25-Dihydroxyvitamin D_3 aus dem Knochen freigesetzt. FGF23 spielt eine wichtige Rolle im Phosphat-, Calcium- und Natriumhaushalt durch Regulation der renalen Ausscheidung. In der Niere kommt es durch FGF23 zur verminderten Herstellung von 1α-Hydroxylase und zum Rückgang des biologisch aktiven Vitamin D in der Zirkulation.

FGF23 ist ein 30-kDa großes Protein. Es kann proteolytisch in N-terminale und C-terminale Fragmente gespalten werden. Laut Studien sind die Enzyme Furin, Fam20C und GalNac-T3 daran beteiligt. FGF23 bindet an FGF Rezeptoren (FGFR) und benötigt dafür den Ko-Rezeptor Klotho. Zielorgane für FGF23 werden durch die Expression von Klotho bestimmt, die Nebenschilddrüsen gehören dazu.

Publikationen haben auf eine Feedbackschleife zwischen FGF23 und PTH hingewiesen. PTH soll die FGF23 Produktion anregen und im Gegenzug vermindert FGF23 die PTH Synthese. Mechanismen dahinter sind jedoch noch nicht bekannt.

Mit dieser Studie konnte eine direkte hemmende Wirkung von FGF23 auf die PTH Produktion in *Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$ Mutanten gezeigt werden. Dies konnte in Mäusen, behandelt mit rekombinantem FGF23 und in Organkulturen der Nebenschilddrüse bestätigt werden.

Wir demonstrierten des Weiteren eine akute, erhöhte Expression von FGF23 durch PTH. In *in vivo* Experimenten konnte eine Regulation der Spaltung von FGF23 durch PTH gezeigt werden. Die Proteine Furin, Fam20C und GalNac-T3 zeigten eine Regulation, es benötigt weitere Studien um die molekularen Mechanismen dahinter zu verifizieren.

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8. List of Tables and Figures

9. Abbreviations

$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D3
ADAM	A Desintegrin and Metalloproteinase
BCP	1-Bromo-3-chloropropane
Ca^{2+}	Calcium
cAMP	cyclic adenosine monophosphate
CaSR	calcium-sensing receptor
Cl^-	chloride
DBP	vitamin D-binding protein
DCT	distal convoluted tubules
DMP	dentin matrix protein
ENPP	ecto-nucleotide pyrophosphatase/phosphodiesterase
Fam20C	family with sequence similarity 20
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GalNac-T3	O-glycosyl transferase N-acetylgalactosaminyltransferase 3
IP_3	Inositol triphosphate
KL	Klotho
MAPK	mitogen-activated protein kinase
Na^+	sodium
NaPi	sodium phosphate cotransporter
NCC	Na^+:Cl^- co-transporter
Nurr	nuclear receptor-associated protein
PCT	proximal convoluted tubules
PHEX	phosphate-regulating protein with homologies to endopeptidases on the X chromosome
PKA	Protein kinase A
PKC	Protein kinase C
PTC	proximal tubule cells

PTG	parathyroid glands
PTH	Parathyroid hormone
SPC	subtilisin-like proprotein convertase
TRPV	transient receptor potential vannilloid
VDR	Vitamin D receptor
VDRE	Vitamin D-responsive elements
WT	wild-type