Studies on the regulation of the phosphaturic hormone fibroblast growth factor 23 (FGF23)

Dissertation

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List of abbreviations

$1,25(OH)_2D_3$	1,25-Dihydroxy-Vitamin D3, Calcitriol, active vitamin D
ADHR	Autosomal dominant hypophosphatemic rickets/osteomalacia
AGEs	Advanced glycation end products
ANKH	Progressive ankylosis protein homolog
ARHR	Autosomal recessive hypophosphatemic rickets/osteomalacia
BCE	Bread crust extract
CaM	Calmodulin
CaMK	CaM-dependent kinases
CKD	Chronic kidney disease
CMD	Craniometaphyseal dysplasia
COX	Cytochrome c oxidase
CRAC	Ca^{2+} release-activated Ca^{2+} channel
CsA	Ciclosporin A
Cyp24a1	Cytochrome P450 24A1, 25-hydroxyvitamin D-24-hydroxylase
Cyp27b1	Cytochrome P450 27B1, 25-hydroxyvitamin D-1α-hydroxylase
Cyp7a1	Cytochrome P450 7A1; Cholesterol 7 alpha-hydroxylase
DAG	Diacylglycerol
DMP1	Dentin matrix protein-1
ENPP1	Ectonucleotide pyrophosphatase/phosphodiesterase 1
ER	Endoplasmic reticulum
FAM20C	Family with sequence similarity 20, member C
FGF	Fibroblast growth factor
FGFR	FGF receptors
FK-506	Tacrolimus
FOXO1	Forkhead box protein O1
FTC	Familial tumorous calcinosis
GNAS1	G protein alpha subunit S1
GSK3	Glycogen synthase kinase 3
HIF-1α	Hypoxia-inducible factor-1α
HOMA-IR	Homeostatic model assessment-insulin resistance
HPT	Hyperparathyroidism
IGF1	Insulin-like growth factor 1

IL	Interleukin
IP3	Inositol-1,4,5-trisphosphate
Klph	Klotho/lactase-phlorizin hydrolase-related protein
LPS	Bacterial lipopolysaccharides
LVH	Left ventricular hypertrophy
MAPK	Mitogen-activated protein kinase
MAS/FD	McCune-Albright syndrome/fibrous dysplasia
NaPi-2	Sodium phosphate co-transporters of type II
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nurr1	Nuclear receptor-associated protein1
PHEX	Phosphate regulating gene with homologies to endopeptidases on the X chromosome
Pi	Inorganic phosphate
PI3K	Phosphoinositid-3 kinase
PKB/Akt	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbolester 12-O-Tetradecanoylphorbol-13-acetat
PTH	Parathyroid hormone
RAGE	AGE receptor
r-BSA	Ribose-modified bovine serum albumin
Runx2	Runt-related transcription factor 2
SGK3	Serum and glucocorticoid kinase 3
SOCE	Store-operated Ca^{2+} entry
SPC	Furin/subtilisin-like proprotein convertase
STIM1	Stromal interaction molecule 1
STZ	Streptozotocin
TIO	Tumor-induced osteomalacia
TNFα	Tumor necrosis factor α
TRPV5	Transient receptor potential vanilloid type 5
VDR	Vitamin D receptor
VDRE	Vitamin D-responsive element
XLH	X-linked hypophosphatemic rickets/osteomalacia

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1 Introduction

1.1 The family of fibroblast growth factor 23

The fibroblast growth factor (FGF) superfamily affects an extremely broad range of biological processes such as development, organogenesis, and metabolism. This is feasible due to the large number of 22 members (FGF1-FGF23). FGF15 is the murine orthologue to human FGF19 and is therefore often summarized as FGF15/19 [1]. Most FGFs mediate their effect by binding to cell surface FGF receptors (FGFR). Four genes (FGFR1-4) encode for these receptor tyrosine kinases. Two isoforms (b and c) exist by alternative splicing of FGFR1-3 [2]. The 22 human members are divided into 7 subfamilies based on their phylogenetic origin and sequence homology [3]. FGFs of the FGF1, FGF4, FGF7, FGF8 and FGF9 subfamily act as paracrine/autocrine factors. A special feature of the FGF11 subfamily, also called FGF homologous factors, is that they do not bind to FGFRs and act as intracellular mediators [4], for example, by binding to intracellular domains of voltage-gated sodium channels and thus modulate subcellular transport [5]. The FGF19 subfamily, which hosts FGF19/15, 21, and 23, functions as circulating factors and is referred to as endocrine FGFs [3].

The endocrine FGFs are involved in physiological processes such as the regulation of bile acid homeostasis by FGF15/19 [6–8], the energy and lipid metabolism by FGF21 [9], and the mineral balance by FGF23 [10, 11], and are mainly expressed in the small intestine, in the liver and in the bones, respectively [12]. A unique feature of this subfamily is a divergent structure of the highly conserved heparin sulfate (HS)-binding domain [13]. Binding to heparin sulfate has two important roles. On the one hand, this component of the extracellular matrix prevents the diffusion of secreted FGFs, thereby increasing their local concentration and enabling the paracrine/autocrine effect [14]. On the other hand, HS is essential for the activation of FGF receptors, forming a 2:2:2 complex of heparan sulfate, FGF and FGFR [2]. The reduced affinity of the FGF19 subfamily to HS has as consequence that they are less bound to the cell surface, but also allows the diffusion into the bloodstream and the endocrine effect [13]. At the same time, however, it also represents a disadvantage for the receptor binding at the target site, which is compensated by the use of Klotho, the senescence-associated protein, as obligatory co-receptor [3, 15–17].

Three related Klotho proteins exist, α and βKlotho [18, 19] and Klotho/lactase-phlorizin hydrolase-related protein (Klph), also called lactase-like protein (Lctl) or γklotho [20]. These single-pass transmembrane proteins are characterized by a very short intracellular domain with unidentifiable functional unit and a large extracellular domain with two tandem repeats of

β-glucosidase-like domains, whereas γ-klotho has only one domain [18, 19]. Furthermore, these co-receptors exhibit a specific tissue expression and FGF/FGFR interaction [12]. αKlotho is expressed in the kidney and parathyroid gland [18], where it forms complexes with FGFR1c, 3c, and 4 and serves as a high affinity receptor for FGF23 [16]. βKlotho forms complexes with FGFR1-4 and supports the signaling of FGF15/19 and FGF21, respectively [12, 21, 17] and is expressed in liver and adipose tissue [19]. γKlotho forms complexes with FGFR1b, 1c, 2c and 4 and increases FGF19 activity and is expressed in the eyes, adipose tissue and kidney [12, 20]. The necessity of Klotho for the endocrine FGF effect is evident from the striking phenotypic similarities of knockout mice. Both *βKlotho*- [22] as well as *Fgfr4*- [23, 7] and *Fgf15*-deficient mice [7, 24] show increased synthesis and excretion of bile acids, which are associated with the activation of gene expression of Cholesterol 7 alpha-hydroxylase (cytochrome P450 7A1, *Cyp7a1*). Similarly, *Fgf23* null mice develop a phenotype associated with premature aging [11, 25], also observed in *αKlotho*-knockout mice [18].

While FGFRs show a very broad tissue distribution, the expression of the Klotho proteins is more limited [12]. Consequently, the sites of action for the endocrine FGFs are largely determined by the presence of Klotho proteins in combination with certain FGFR isoforms. However, this limitation of the site of action by expression of the essential co-receptor could also be less stringent, because a soluble form of αKlotho exists, which arises by alternative splicing [26, 27] or by cleavage of the large extracellular ectodomain via membrane-bound proteolytic enzymes [28]. Thus, it might be possible that FGFs can also act in quite different places by allowing the soluble Klotho to bind to the ubiquitously expressed FGFRs. However, further studies are still needed to clarify the exact effect of soluble Klotho.

1.2 The molecular basics and functions of FGF23

The *FGF23* gene codes for a protein of 251 amino acid [29, 10]. The detected protein size of 32 kDa of the recombinant expressed protein [10] does not correspond to the calculated molecular weight of 27.95 kDa (UniProt: Q9GZV9) and the bacterial FGF23 of 28 kDa [30], suggesting that FGF23 undergoes posttranslational modification that makes the protein heavier. In fact, enzymatic deglycosylation and mass analysis proved that FGF23 is glycosylated at several sites [30–32].

In addition to the 32 kDa glycoprotein, a 12 kDa fragment of FGF23 was detected via western blot [10, 33, 34]. Further research revealed that FGF23 is cleaved at the site of ^{179}Arg and ^{180}Ser, giving rise to an N- and C-terminal cleavage fragment, with only the C-terminal fragment being detected, due to the binding site of the used antibody [10, 33, 34]. De facto, the

furin/subtilisin-like proprotein convertase (SPC) consensus sequence is located in the region of ^{176}RXXR^{179} and is responsible for cleavage of FGF23 [33, 35]. SPCs are a family of serine proteases responsible for the processing of latent precursor proteins to their biologically active products by cleavage of the substrates at the R(X)n-R consensus site, as in the case of peptide hormones, growth factors and enzymes [36]. Further *in vivo* studies showed that only the 32 kDa full-length FGF23 has biological activity, while the two cleavage fragments alone have no effect [34]. Interestingly, the C-terminal fragment apparently competes for binding to the FGFR-αKlotho complex with intact FGF23, so that temporarily even the serum phosphate level in mice was increased, as in the case of FGF23 deficiency [29, 37]. In addition, several small compounds have been shown to interfere with the interaction between FGF23 and FGFR-αKlotho complex [38]. Thereby, these potentially competitive inhibitors could function as an effective therapeutic agent against FGF23 hyperfunction disorders, which, however, requires further investigation.

Interestingly, a missense mutation in the ^{176}RXXR^{179} motif (R176Q, R179W, and R179Q) results in increased levels of FGF23 and autosomal dominant hypophosphatemic rickets/osteomalacia (ADHR) [39]. The mutant FGF23 escapes proteolytic cleavage by SPCs, accumulates more frequently, and causes the symptoms associated with FGF23 hyperfunction disorders [33, 34, 40, 41]. Furthermore, the already mentioned glycosylation is also involved in the cleavage of FGF23 [31, 32]. FGF23 is protected from proteolytic cleavage by O-glycosylation around the SPC consensus sequence, and modification at T^{178} is crucial to preserve native full length FGF23 and complete glycosylated form [32]. Thus, FGF23 is subject to a post-translational regulatory mechanism involving competitive O-glycosylation and protease processing to produce intact FGF23.

FGF23 is mainly produced by osteoblasts and osteocytes in the bone [42, 43], and interacts with FGFR1c, 3c, and 4. Transmembrane αKlotho enhances the binding affinity of FGF23 [15, 16, 44]. Crystal structure analysis revealed that αKlotho acts as a scaffold protein, bringing FGF23 and FGFR into close proximity, and so increasing the stability of the ternary complex [45]. Following the receptor binding, the activation of several signaling pathways regulates the expression of various genes, which is the subject of current research.

FGF23 is the master regulator of phosphate and active vitamin D (1,25-dihydroxyvitamin D3, 1,25$(OH)_2D_3$) balance, acting predominantly in the kidney [10, 11], the major site of αKlotho expression [18]. Direct administration of recombinant FGF23 decreased serum phosphate in mice and the implantation of cells stably expressing FGF23 into nude mice resulted in hypophosphatemia, increased phosphate excretion (phosphaturia), bone softening

(osteomalacia), and reduced $1,25(OH)_2D_3$ levels in mice [10]. The cause for the altered phosphate balance was a reduced expression of sodium phosphate co-transporters of type II (NaPi-2a and -2c) in proximal renal tubules, which reduced renal phosphate absorption and increased excretion [46, 47]. The negative FGF23-mediated effect on $1,25(OH)_2D_3$ levels occurs by reducing the renal mRNA level of 25-hydroxyvitamin D-1α-hydroxylase (cytochrome P450 27B1, Cyp27b1), which is responsible for the production of $1,25(OH)_2D_3$. At the same time FGF23 increase 24-hydroxylase (cytochrome P450 24A1, Cyp24a1) mRNA, which in turn converts $1,25(OH)_2D_3$ into more hydrophilic metabolites with lower activity [48]. The effect of FGF23 on 24-hydroxylase is caused by a $1,25(OH)_2D_3$-dependent mechanism [49]. Furthermore, as a result of $1,25(OH)_2D_3$ reduction, there is a decrease in NaPi-2b expression in the small intestine [50], which suppresses the uptake of intestinal inorganic phosphate (Pi), and thus FGF23 has an additional negative effect on serum phosphate. By changing the expression of the key proteins of $1,25(OH)_2D_3$ and phosphate metabolism, as shown in Figure 1, FGF23 efficiently regulates mineral balance.

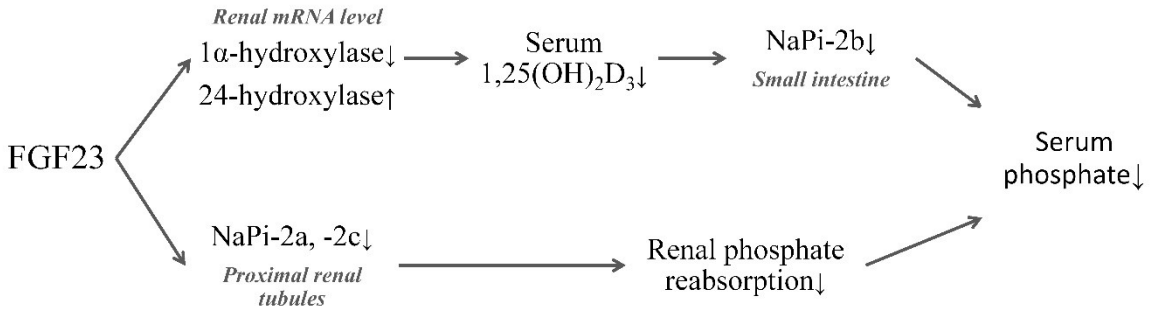

Figure 1: FGF23 as a master regulator of phosphate and $1,25(OH)_2D_3$ metabolism.
Overview is adapted from [51].

The regulation of FGF23 production is subject to feedback loops involving phosphate and $1,25(OH)_2D_3$. Their administration significantly increases the FGF23 level [48, 52–65], while the loss of vitamin D receptor (VDR) [63–67] or NaPi transporter [68–70] leads to a significant reduction in FGF23 levels.

The parathyroid gland, as another expression site of the essential co-receptor αKlotho, is an important target organ for FGF23 [71, 72]. The parathyroid hormone (PTH) has a phosphaturic effect similar to FGF23 by downregulating apical NaPi-2a/c expression in renal cells of the proximal tubules [73, 74]. But quite contrary to FGF23, PTH increases 1α-hydroxylase (*Cyp27b1*) expression and thus increases $1,25(OH)_2D_3$ levels [75]. The FGF23-mediated effect on parathyroid hormone (PTH) is less clear than in the case of Pi and

$1,25(OH)_2D_3$. Treatment with FGF23 lowers PTH synthesis and secretion [72, 76–78]. Clinical data and animal models of diseases with increased FGF23 production, on the other hand, were characterized by normal or elevated PTH levels and even by the development of hyperparathyroidism (HPT) [40, 79–84]. However, these conflicting results may be due to the fact that the currently used assays for measuring intact PTH cannot differentiate between biological inactive oxidized PTH and biologically fully active PTH. This could be particularly relevant in diseases with severe oxidative stress, where a large part of the PTH is oxidized and only a small part of the circulating PTH is bioactive [85]. Further, the detected stimulating effect of FGF23 on PTH may have been triggered by pathological changes in vitamin and mineral metabolism. Hasegawa *et al*. also postulated that the effects of $1,25(OH)_2D_3$ and calcium (Ca^{2+}) on PTH secretion is more dominant than FGF23 [80]. PTH is an important stimulator of $1,25(OH)_2D_3$ and Ca^{2+} providing negative feedback on PTH secretion [86–88]. Consequently, by decreasing $1,25(OH)_2D_3$ and/or Ca^{2+}, the inhibitory effect on PTH release would be reduced and its release increased.

Whether PTH and FGF23 are subject to a classical endocrine feedback loop is not entirely clear. In some studies, PTH increases FGF23 expression [89–94], presumably by induction of the nuclear receptor-associated protein1 (Nurr1) [95], and patients with manifested HPT showed elevated FGF23 serum levels [96–98]. While, in other studies, PTH has an inhibitory effect [67, 99] or no effect on FGF23 [59, 100]. Furthermore, mice lacking the PTH1 receptor in the proximal tubule are characterized by an elevated FGF23 level, suggesting that PTH inhibits FGF23 [101]. These contradictory results can be explained, at least in part, by the fact that PTH and FGF23 are independent regulators of phosphate, $1,25(OH)_2D_3$, and Ca^{2+}, which in turn affect the two in different ways. Besides, in pathophysiological circumstances, such as HPT, secondary changes occur, which in turn can affect FGF23 and thus do not reflect the physiological state. In the disease, impaired renal function is mainly responsible for the increase of FGF23 [102]. In the study by Yamashita *et al*., HPT patients had an overall significantly increased FGF23 level, but patients with normal renal function had FGF23 in the normal range [98].

Ca^{2+} is not only an important regulator of PTH [86–88], but also of FGF23. Intake of Ca^{2+} increases FGF23 [49, 58, 62, 103, 104], while its deficiency is associated with a reduction [104–106]. These Ca^{2+}-mediated effects proceed independently of PTH/$1,25(OH)_2D_3$ [49, 62, 106] and can be attributed, at least in part, to a direct regulation of *FGF23* gene transcription [49, 58, 103, 104]. Recently, it has also been shown that store-operated Ca^{2+} entry (SOCE) via essential pore subunit Orai1 of the Ca^{2+} release-activated Ca^{2+} channel (CRAC) [107]

significantly increased *Fgf23* expression in osteoblastic cells [108, 109]. The Orai1 dependency was also confirmed in *Orai1*$^{-/-}$ mice, in which the expression of *Fgf23* was greatly reduced [110]. SOCE is a process that serves the basal Ca^{2+} regulation of the cell. Activation of a receptor coupled to phospholipase C leads to the generation of inositol 1,4,5-trisphosphate (IP3), which in turn mediates the release of Ca^{2+} from the endoplasmic reticulum (ER), the main intracellular reservoir of Ca^{2+}, into the cytoplasm. The stromal interaction molecule 1 (STIM1) recognizes the Ca^{2+} decrease in ER, oligomerizes and interacts with Orai1, which is located in the cell surface membrane. This activates the Ca^{2+} influx into the cell to slowly replenish the Ca^{2+} levels in the ER [111–113].

Ca^{2+} itself is also regulated by FGF23 via increasing tubular Ca^{2+} resorption through transient receptor potential vanilloid type 5 (TRPV5) [114]. In healthy human, serum FGF23 correlated positively with serum Ca^{2+} and negatively with the Ca^{2+}/creatinine ratio in urine, suggesting that FGF23 is also a Ca^{2+} preserving hormone [115]. Further, $1,25(OH)_2D_3$ increases Ca^{2+} uptake in the small intestine in addition to phosphate transport [116]. Due to the inhibitory effect of FGF23 on $1,25(OH)_2D_3$, it can also inhibit Ca^{2+} absorption at the same time, probably contributing to Ca^{2+} balance and negative feedback loop of its own formation.

The interactions of all these factors are very complex and subject to numerous endocrinological feedback loops, as shown in Figure 2.

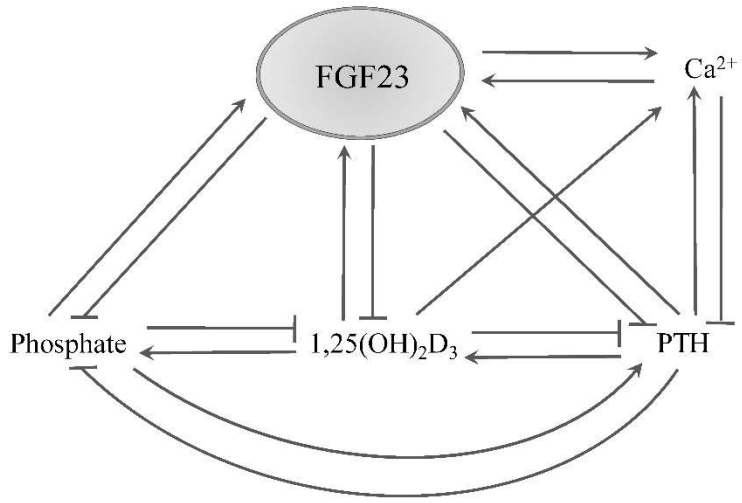

Figure 2: Endocrinological feedback loops regulating hormone and mineral homeostasis.
Overview is adapted from Bacchetta *et al.* [117] and **Paper 5**.

1.3 Clinical relevance of FGF23

Changes in FGF23 blood levels are associated with various diseases. Increased FGF23 levels are mainly observed in rare acquired or genetic disorders associated with decreased

phosphate absorption, hypophosphatemia, low or inadequate normal $1,25(OH)_2D_3$ levels, and skeletal mineralization defects (rickets or osteomalacia). As a result of various defects in FGF23 regulation, several types of FGF23-dependent hypophosphatemia are distinguished, which are summarized in Table 1 and considered more closely in **Paper 5**.

Table1: FGF23 hyperfunction disorders with hypophosphatemia.

Disorders	Cause/responsible gene
Autosomal dominant hypophosphatemic rickets/osteomalacia (ADHR)	Gain-of-function mutation of *FGF23*
Tumor-induced osteomalacia (TIO)	Overproduction of FGF23 in a causative tumor
X-linked hypophosphatemic rickets/osteomalacia (XLH)	Loss-of-function mutation of *PHEX*
Autosomal recessive hypophosphatemic rickets/osteomalacia 1 (ARHR1)	Loss-of-function mutation of *DMP1*
Autosomal recessive hypophosphatemic rickets/osteomalacia 2 (ARHR2)	Loss-of-function mutation of *ENPP1*
Autosomal recessive hypophosphatemic rickets/osteomalacia 3 (ARHR3)	Loss-of-function mutation of *FAM20C*
McCune-Albright syndrome/fibrous dysplasia (MAS/FD)	Gain-of-function mutation of *GNAS1*
Craniometaphyseal dysplasia (CMD)	Loss-of-function mutation of *ANKH*
Osteoglophonic dysplasia	Gain-of-function mutation of *FGFR1*
Translocation between chromosome 9 and 13 t(9,13)(q21.13:q13.1)	Enhanced plasma αKlotho

Adapted from Ito *et al*. [118] and **Paper 5**. PHEX: phosphate regulating gene with homologies to endopeptidases on the X chromosome; DMP1: dentin matrix protein-1; ENPP1: ectonucleotide pyrophosphatase/phosphodiesterase 1; FAM20C: family with sequence similarity 20, member C; GNAS: G protein alpha subunit S1; ANKH: progressive ankylosis protein homolog.

Chronic kidney disease (CKD) has a variety of causes including, kidney inflammation and infection, congenital disorders, high blood pressure or diabetes. However, it is always characterized by decreasing kidney performance, detectable by the decreasing glomerular filtration rate or functional nephron number [119–121]. Symptomatic of the advanced CKD stage is an up to 1000-fold increased FGF23 level, $1,25(OH)_2D_3$ deficiency, secondary hyperparathyroidism and hyperphosphatemia [119, 121]. Meanwhile, FGF23 progressively increases with gradual loss of renal function and is highest in end stage renal failure [82, 102, 122–134]. In addition to patient data, animal models of kidney failure also exhibit elevated FGF23 levels [135–141]. Hitherto investigations confirm the assumption of a fixed causal sequence in the CKD course (Figure 3), which can also be observed in patients in this order [125]. Due to the decreasing kidney capacity, the phosphate excretion per nephron must be

increased to maintain the phosphate balance [121], achieved by increasing the phosphaturic hormone FGF23. This results in a decrease in $1,25(OH)_2D_3$, which also serves as adaptive protection against further intestinal phosphate uptake [50], but also stimulates PTH secretion [80, 86]. Through these measures to compensate for increased phosphate load per nephron in the early stages of the disease, the serum phosphate level is kept in the norm, but also favors a downward spiral. The increased PTH additionally stimulates FGF23 [89–94], leading to a further decrease in $1,25(OH)_2D_3$ levels. Both increased FGF23 and decreased $1,25(OH)_2D_3$ inhibit the expression of αKlotho [142, 143], but also phosphate overload and kidney tissue damage may contribute to αKlotho reduction [144]. In fact, in humans and mice, a steady decline of αKlotho in kidney and parathyroid glands has been observed during CKD progression [126, 145–150]. This may be accompanied by resistance to FGF23 and compensatory additional release of the hormone, but also reduced phosphaturic effect, which also increases the vicious cycle of increased FGF23, decreased $1,25(OH)_2D_3$ and αKlotho, and increased PTH. Finally, in the end stage of kidney disease, persistent hyperphosphatemia occurs when the remaining nephrons are reduced to a level that the residual phosphate cannot be excreted via urine. With around 500 million people suffering from CKD worldwide [151], which is associated with significant public health burdens, CKD is the focus of current research. Thus, FGF23 is of high interest for the pathophysiological regulation of CKD due to its early occurrence in the course of the disease.

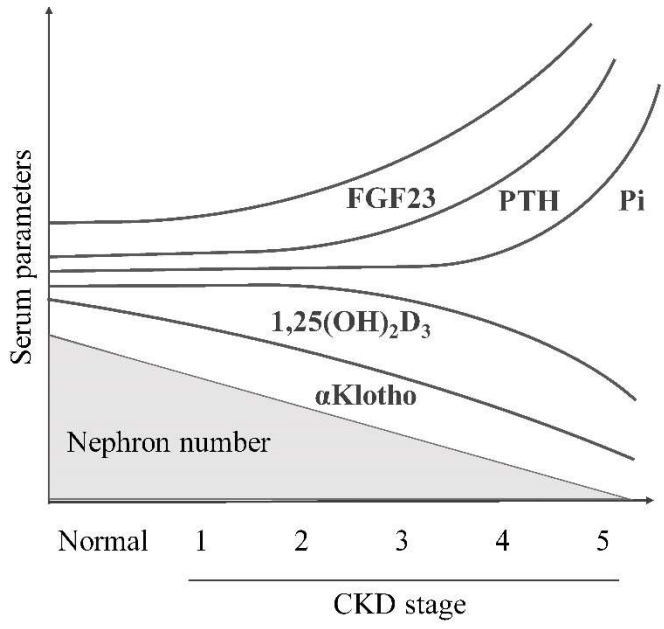

Figure 3: Changes in indicated serum parameters during chronic kidney disease (CKD) progression, resulting in a characteristic hormone profile in patients.
Figure is modified according to Hu *et al.* [3].

Loss of FGF23 activity is the cause of the rare hereditary disorder familial tumorous calcinosis (FTC), which is characterized by hyperphosphatemia, ectopic calcifications and increased $1,25(OH)_2D_3$ level [118, 152]. The cause is on the one hand a loss-of-function mutation in the gene *GALNT3* (N-acetylgalactosaminyltransferase 3), which O-glycosylates FGF23 on the T^{178} in the SPC consensus sequence. Due to its loss and concomitant glycosylation, FGF23 becomes more susceptible to proteolytic processing, with the result that only a small amount of intact hormone is secreted despite increased expression [31, 32, 153–155]. Also, various mutations in the *FGF23* gene, whether directly or by resulting conformational change, impair glycosylation [30], thereby promoting proteolytic cleavage or preventing secretion [156–162] because apparently mutant protein remains in the Golgi complex [163]. This demonstrates that posttranslational processing of the phosphaturic hormone is an important regulatory step.

Furthermore, FGF23 also has relevance in the aging process. *αKlotho*- [18] and *Fgf23*-deficient mice [11] offer similar phenotype with remarkably delayed growth and short lifespan. Age-related symptoms such as ectopic calcification, osteoporosis, pulmonary emphysema, skin atrophy and infertility have been shown to be triggered by the knock-out mediated increase in phosphate, $1,25(OH)_2D_3$ and Ca^{2+} [11, 18]. Rescue experiments suggest that phosphate excess is the leading cause for the age-related diseases, as a low-phosphate diets abolished many age-related symptoms in *Fgf23* null mice despite increased $1,25(OH)_2D_3$ and Ca^{2+} levels [164]. In addition, CKD can be regarded as an accelerated kidney aging [121, 165], in which, despite the high amount of FGF23, the phosphaturic effect remains inadequate and an excess of phosphate is existent.

Deregulation of the mineral balance is significantly associated with cardiac mortality, presumably due to hyperphosphatemia-related calcification and blockage of the arteries, as a strong predictor of cardiovascular disease [166–170]. As an important regulator of the phosphate balance, it is not surprising that the phosphaturic hormone FGF23 is also elevated in patients with cardiovascular disease [171–176] and is considered as an independent risk factor for cardiovascular mortality [177–182]. There is also a strong correlation between elevated FGF23 and left ventricular hypertrophy (LVH) [183–189]. It could be shown that FGF23 induces LVH via FGFR4-dependent but αKlotho-independent activation of the NFAT (nuclear factor of activated T-cells) signaling pathway [189, 190]. But also a direct LVH-mediated increase in FGF23 production, again by activation of NFAT, was observed [191].

The increased FGF23 levels in the various diseases, and especially the early onset of CKD, suggests that FGF23 may act as a biomarker of disease [192]. But it is still unclear

whether and to what extent FGF23 actively induces disease or is only symptomatic of it. This brings the pathophysiological role and molecular regulation of FGF23 production into the focus of current research. The identification of new regulators and signaling pathways would lead to a better understanding of the mode of action of this versatile factor and approaches to new therapies for FGF23-associated diseases.

The established regulators include, as noted above, phosphate, $1,25(OH)_2D_3$, parathyroid hormone, and Ca^{2+}, but newer regulators such as iron status, inflammatory responses, and metabolic processes are also of importance. Inflammation has been shown to be one of the main causes of FGF23 production [193] and in most of the diseases mentioned above, inflammatory processes contribute significantly to FGF23 production [194]. In fact, elevated FGF23 levels are associated with elevated inflammatory markers in patients with CKD [195–198] or other diseases [199–203]. *In vivo* and *in vitro* experiments also show that proinflammatory stimuli such as tumor necrosis factor α (TNFα), bacterial lipopolysaccharides (LPS), interleukin (IL)-1β/6, bacteria or their toxins upregulate FGF23 directly and dose-dependently [193, 204–211]. Numerous studies confirm the dependence of the inflammatory increase in *FGF23* transcription on the transcription factor complex NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) [109, 207–209], which is involved in a large number of proinflammatory cellular reactions [212, 213]. At the molecular level, the effect on FGF23 is mediated, at least in part, by NFκB-sensitive regulation of the Ca^{2+} channel Orai1 and its regulator STIM1 [214], as part of SOCE, which is also considered to be one of the major factors in the regulation of *Fgf23* gene expression [108, 109]. Figure 4 depicts how inflammatory mediators regulate FGF23 formation.

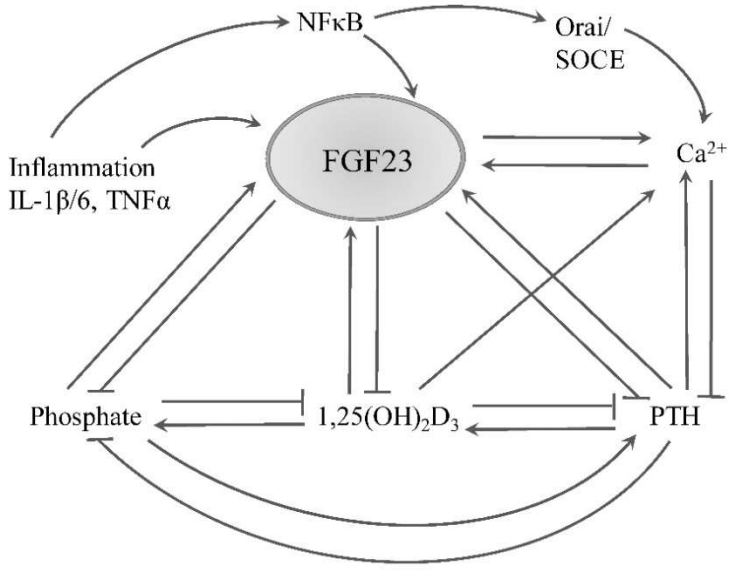

Figure 4: The most important regulators of FGF23 and their interaction.
Overview is adapted from Bacchetta *et al.* [117] and **Paper 5**.

2 Objective of this study

FGF23 mediates various physiological processes to maintain phosphate and $1,25(OH)_2D_3$ homeostasis, mainly by regulating renal and parathyroid function. Consequently, the disruption of these endocrine axes is of crucial importance for the pathophysiology of kidney and bone diseases. As a causative factor in the pathogenesis of rare hereditary forms of hypophosphatemic rickets and due to its early occurrence in chronic kidney disease, FGF23 is of high clinical relevance, also as a disease-relevant biomarker. However, it is still unclear whether changes in the FGF23 level are the cause or consequence of disease. For this reason, it is of great interest to identify the exact causes of the altered FGF23 production and to uncover the regulatory mechanisms and signaling pathways involved. This could also lead to the development of new therapeutic approaches for FGF23-associated diseases.

In the last 15 years of research, new mechanistic insight has been gained into the regulation of FGF23, but these still do not provide a clear picture and also rather show how complex the regulation is. This variety of known regulators was also examined in detail in **Paper 5**. Therefore, the aim of this work was to contribute to the clarification of the regulatory mechanism of FGF23 by deciphering new regulators and the underlying signal transduction pathways.

Many of the diseases already mentioned are associated with an increased inflammatory status [194] and it has been shown that inflammation is one of the most important regulators of FGF23 production [193]. Among other things, the triggered inflammatory reaction leads to the activation of SOCE and thus to an increase in FGF23 [108, 109]. Based on this, the focus of this work was on the investigation of intracellular processes, particularly those linked to inflammation that contribute to the regulation of FGF23. For this purpose, mainly *in vitro* experiments with UMR106 osteoblast-like cells, which express FGF23 endogenously, were performed in order to gain a precise insight into the signal transduction pathways involved in the regulation of FGF23 expression. The following questions were addressed within the scope of this work:

I) Which new regulators are relevant for FGF23 formation?

It is very important to understand how FGF23 formation is regulated and for this purpose the knowledge of new regulatory factors is of the highest interest. Since, among other things, inflammatory mechanisms are one of the most important triggers for FGF23 production [193], the influence of the involved mediators are of relevance.

II) What are the underlying mechanisms in the regulation of FGF23 expression?

After identifying new regulatory factors, it is of course of great interest to understand how they affect *FGF23* expression. For this purpose, the underlying intracellular mechanisms were investigated in cell culture experiments using specific inhibitors or activators of specific signaling pathways or genetic manipulation.

III) Which transcription factors are relevant for the regulation of *FGF23* transcription?

Following the clarification of the signaling pathways involved in the regulation of *FGF23* expression, the decisive question arose which transcription factors contribute exactly to the expression of *FGF23*.

3 Publications

3.1 Paper 1: Insulin suppresses the production of fibroblast growth factor 23 (FGF23)

Bär, L.; Feger, M.; Fajol, A.; Klotz, L.-O.; Zeng, S.; Lang, F.; Hocher, B.; Föller, M. Insulin suppresses the production of fibroblast growth factor 23 (FGF23). Proceedings of the National Academy of Sciences of the United States of America, 2018, 115, 5804–5809. DOI: 10.1073/pnas.1800160115.

Insulin suppresses the production of fibroblast growth factor 23 (FGF23)

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Edited by T. W. Mak, The Campbell Family Institute for Breast Cancer Research at Princess Margaret Cancer Centre, University Health Network, Toronto, ON, Canada, and approved April 23, 2018 (received for review January 4, 2018)

Fibroblast growth factor 23 (FGF23) is produced by bone cells and regulates renal phosphate and vitamin D metabolism, as well as causing left ventricular hypertrophy. FGF23 deficiency results in rapid aging, whereas high plasma FGF23 levels are found in several disorders, including kidney or cardiovascular diseases. Regulators of FGF23 production include parathyroid hormone (PTH), calcitriol, dietary phosphate, and inflammation. We report that insulin and insulin-like growth factor 1 (IGF1) are negative regulators of FGF23 production. In UMR106 osteoblast-like cells, insulin and IGF1 down-regulated FGF23 production by inhibiting the transcription factor forkhead box protein O1 (FOXO1) through phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB)/Akt signaling. Insulin deficiency caused a surge in the serum FGF23 concentration in mice, which was reversed by administration of insulin. In women, a highly significant negative correlation between FGF23 plasma concentration and increase in plasma insulin level following an oral glucose load was found. Our results provide strong evidence that insulin/IGF1-dependent PI3K/PKB/Akt/FOXO1 signaling is a powerful suppressor of FGF23 production in vitro as well as in mice and in humans.

PI3K | PKB/Akt | Klotho | phosphate

The proteohormone fibroblast growth factor 23 (FGF23) is synthesized by bone cells (1) and regulates phosphate and vitamin D metabolism (2). In the kidney, FGF23 inhibits phosphate reabsorption by stimulating the internalization of the phosphate transporter NaPiIIa and suppresses the formation of calcitriol or $1,25(OH)_2D_3$, the active form of vitamin D, by inhibiting 25-hydroxyvitamin D_3 1-α-hydroxylase (encoded by the Cyp27b1 gene) (1). In the parathyroid gland, FGF23 suppresses the formation of parathyroid hormone (PTH) (3). All these endocrine effects of FGF23 are mediated by a membrane receptor that is made up of fibroblast growth factor receptor 1 c-splicing form (FGFR1c) and membrane-anchored Klotho (1, 4). Apart from membrane Klotho, a soluble form exists (sKlotho) that itself exerts several endocrine effects. SKlotho can be found in blood, urine, and cerebrospinal fluid (5, 6). Interestingly, FGF23 further triggers hypertrophy of the left cardiac ventricle (7) through a cardiac receptor not depending on Klotho (8).

Klotho-null mice have a short life span with clear signs of neuronal, metabolic, muscle, skin, or cardiovascular aging that resembles human aging (9). In large part, the aging is due to dysregulated phosphate metabolism, which results in massive calcification in most tissues and organs (9). FGF23-deficient mice exhibit similar symptoms due to the joint action of Klotho and FGF23 in phosphate metabolism (2).

The plasma FGF23 concentration is elevated in several acute and chronic diseases, which makes FGF23 a candidate as a meaningful disease biomarker (10). The role of FGF23 is characterized best in chronic kidney disease (CKD), where a high FGF23 plasma concentration occurs very early even before the onset of appreciable hyperparathyroidism and hyperphosphatemia, which are typical sequelae of CKD (11). In CKD and coronary heart disease, FGF23 is a powerful predictor of mortality (12).

Known regulators of FGF23 production include PTH (13), $1,25(OH)_2D_3$ (14), iron status (15), dietary phosphate (16), and inflammation (15, 17). Polycystic kidneys are also a source of enhanced FGF23 production (18).

Insulin and insulin-like growth factor 1 (IGF1) exert their pleiotropic effects through phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB)/Akt signaling (19). Stimulation of insulin or IGF1 receptors leads to the activation of PI3K, which ultimately results in enhanced activity of PKB/Akt, a key mediator of insulin and IGF1 effects in the regulation of cell proliferation, survival, and metabolism (19). Downstream targets of PKB/Akt include glycogen synthase kinase 3 (GSK3) (19) and transcription factor forkhead box protein O1 (FOXO1) (20–22), which are both inhibited via phosphorylation. Transgenic mice expressing PI3K/PKB/Akt-resistant GSK3 (23) suffer from renal phosphate loss (phosphaturia) (24) and have an elevated serum concentration of FGF23 (25). Moreover, PKBβ/Akt2 and serum and glucocorticoid kinase 3 (SGK3), another downstream signaling element of PI3K, regulate the renal phosphate transporter NaPiIIa. Hence, both PKBβ/Akt2- and SGK3-deficient mice suffer from phosphaturia (26, 27).

The impact of insulin deficiency (type 1 diabetes) and resistance (type 2 diabetes) on FGF23 is complex (28). In most studies, diabetes was associated with higher serum levels of

Significance

Fibroblast growth factor 23 (FGF23) regulates phosphate and vitamin D metabolism and induces left heart hypertrophy. The importance of FGF23 is supported by the consequences of FGF23 deficiency: FGF23-deficient mice have a significantly reduced life span and recapitulate human age-associated diseases. Moreover, FGF23 has gained attention as a potential disease biomarker due to its positive correlation with disease activity, progression, and outcome in chronic kidney disease or cardiovascular disorders. It is, however, not entirely clear whether FGF23 merely indicates disease or actively contributes to disease progression. Therefore, it is important to explore the regulation of FGF23 production, which is similarly incompletely understood. Our paper identifies fundamental insulin/IGF1-dependent PI3K/Akt/FOXO1 signaling as a key suppressor of FGF23 formation.

Author contributions: F.L., B.H., and M. Föller designed research; L.B., M. Feger, A.F., and S.Z. performed research; L.-O.K. contributed new reagents/analytic tools; L.B. analyzed data; and L.B. and M. Föller wrote the paper.

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FGF23 (29–31), whereas other studies did not find an association (32, 33). Clearly, inflammation is a major trigger of FGF23 production (15), and most patients with type 2 diabetes, especially those with obesity, suffer from inflammatory conditions (34). Type 1 diabetes is similarly associated with inflammation, particularly at later disease stages (35). Therefore, the positive association of diabetes with FGF23 may be due, in large part, to inflammation. The role of insulin and PI3K/PKB/Akt signaling in the regulation of FGF23 production remained, however, hitherto elusive.

Here, we explored the significance of insulin and insulin-dependent signaling for the synthesis of FGF23.

Results

Insulin and IGF1 Down-Regulate Fgf23 Gene Expression by Activating PI3K/PKB/Akt Signaling. We treated UMR106 osteoblast-like cells with insulin and determined *Fgf23* transcripts by quantitative real-time RT-PCR (qRT-PCR). As demonstrated in Fig. 1*A*, insulin significantly lowered the abundance of *Fgf23* mRNA. The insulin effect was concentration-dependent (Fig. 1*B*). Since cellular effects of insulin are typically mediated by PI3K, we tested next whether PI3K is involved. Inhibition of PI3K with wortmannin up-regulated *Fgf23* gene expression (Fig. 1*C*). The coadministration of insulin overcame this effect to a large extent (Fig. 1*C*). The main downstream target of insulin-induced PI3K is PKB/Akt. Similar to wortmannin, PKB/Akt inhibition with MK-2206 increased *Fgf23* gene expression and also abrogated the inhibitory effect of insulin on *Fgf23* (Fig. 1*D*). IGF1 is another important inducer of PI3K/Akt signaling. As demonstrated in Fig. 2*A*, IGF1 indeed mimicked the insulin effect on *Fgf23* transcripts. Also, the IGF1 effect was significantly attenuated in the presence of MK-2206 (Fig. 2*A*). IGF1 was effective through the IGF1 receptor (IGF1R) as IGF1R antagonist BMS-754807 (Fig. 2*B*) and siRNA-mediated knockdown of the *Igf1r* gene (Fig. 2 *C* and *D*) significantly attenuated IGF1-induced inhibition of *Fgf23* gene expression.

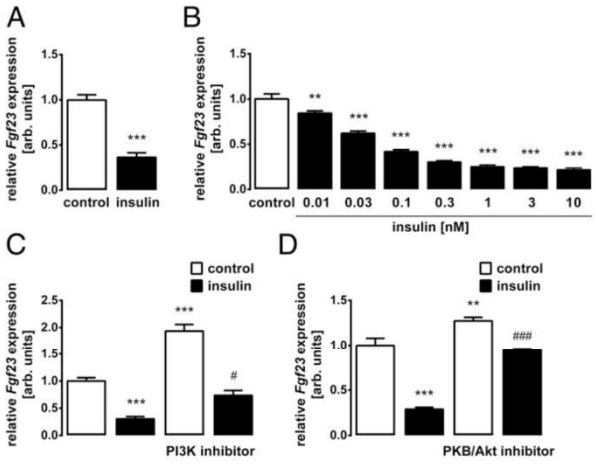

Fig. 1. Insulin down-regulates *Fgf23* expression through PI3K/PKB/Akt signaling. Bar diagrams represent the mRNA abundance of *Fgf23* measured by qRT-PCR in UMR106 cells incubated without or with insulin (10 nM; *A*, *C*, and *D*) or at the indicated insulin concentration (*B*) in the absence and presence of the PI3K inhibitor wortmannin (5 μM, *C*) or PKB/Akt inhibitor MK-2206 (1 μM, *D*). Gene expression was normalized to *Tbp* as a housekeeping gene, and the values are expressed as arithmetic means ± SEM (n = 5–8). $^{**}P <$ 0.01 and $^{***}P < 0.001$ indicate significant difference from vehicle (first bar). $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ indicate significant difference from an absence of wortmannin or MK-2206 (second bar vs. fourth bar). arb., arbitrary.

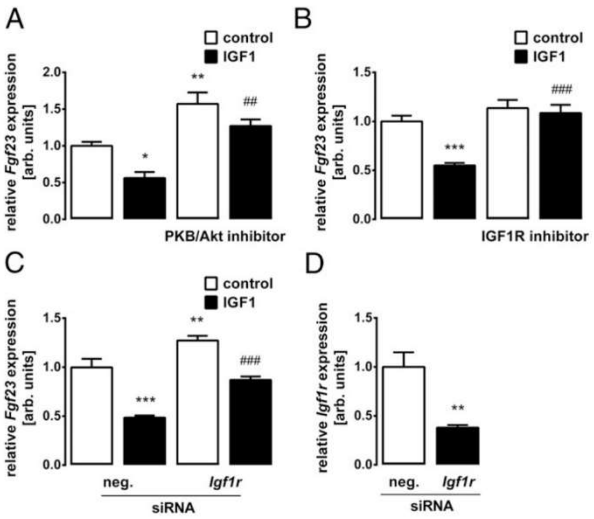

Fig. 2. IGF1 is a negative regulator of *Fgf23* transcription. *Fgf23* transcript levels in UMR106 cells incubated without or with IGF1 (0.5 μg/mL; *A*–*C*) in the absence and presence of the PKB/Akt inhibitor MK-2206 (1 μM, *A*), the IGF1R inhibitor BMS-754807 (1 μM, *B*), or treatment with *Igf1r*-specific siRNA and nonsense siRNA as a negative control (neg. siRNA, *C*) are shown. (*D*) *Igf1r* mRNA expression in UMR106 cells treated with nonsense siRNA or *Igfr1* siRNA. Gene expression was normalized to *Tbp* as a housekeeping gene, and the values are expressed as arithmetic means ± SEM (n = 6). $^{*}P < 0.05$, $^{**}P <$ 0.01, and $^{***}P < 0.001$ indicate significant difference from vehicle (first bar). $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ indicate significant difference from an absence of MK-2206, BMS-754807, or *Igf1r*-specific siRNA (second bar vs. fourth bar). arb., arbitrary.

The Insulin Effect on Fgf23 Is Mediated by the PKB/Akt-Regulated Transcription Factor FOXO1. Many metabolic effects of insulin-dependent PKB/Akt signaling are mediated by the inhibitory phosphorylation of FOXO1 (21). As illustrated by Western blotting in Fig. 3*A*, treatment of the cells with insulin induced the phosphorylation of FOXO1 at Ser256, a site phosphorylated by PKB/Akt (36), pointing to an inhibition of the transcriptional activity of FOXO1. Next, we studied whether transcriptional activity of FOXO1 is required for *Fgf23* gene transcription. Similar to treatment with insulin, the incubation of UMR106 cells with the FOXO1 inhibitor AS1842856 resulted in down-regulation of *Fgf23* gene expression (Fig. 3*B*). The knockdown of *Foxo1* gene expression using specific siRNA also lowered the abundance of *Fgf23* transcripts (Fig. 3*C*). The siRNA-mediated down-regulation of FOXO1 was confirmed by Western blotting (Fig. 3*D*). Conversely, the overexpression of FOXO1 up-regulated *Fgf23* gene expression (Fig. 3*E*). Efficient transfection with the respective plasmid encoding FOXO1, along with GFP, was confirmed by Western blotting as well as by fluorescence (Fig. 3*F*).

In Mice, Insulin Deficiency Elevates the FGF23 Serum Level, Which Is Again Normalized by Insulin Administration. Our in vitro studies suggest that insulin is a potent negative regulator of FGF23 production through PI3K/PKB/Akt/FOXO1 signaling. Next, we carried out in vivo experiments to assess the physiological relevance of insulin-dependent suppression of FGF23 production. To this end, we depleted wild-type mice of endogenous insulin production by means of streptozotocin (STZ), a substance with toxicity to insulin-producing β cells, thereby inducing acute insulin deficiency. As expected, we observed a surge in the blood glucose level upon treatment with STZ, which was prevented by daily insulin injections over a period of 10 d in another group of mice (Fig. 4*A*). Importantly, insulin deficiency in STZ-treated animals resulted in an

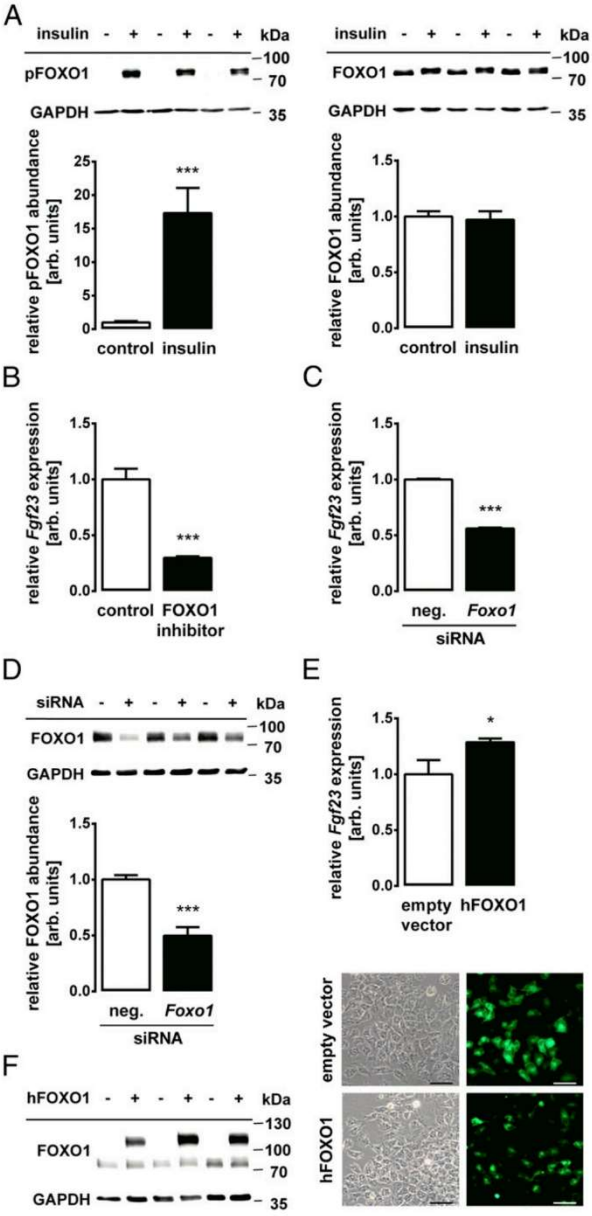

Fig. 3. Transcription factor FOXO1 regulates *Fgf23* expression. (*A*, *Upper*) Original Western blots of phospho-FOXO1 (pFOXO1) and GAPDH (*Left*) or FOXO1 and GAPDH (*Right*) abundance in UMR106 cells incubated with or without 10 nM insulin for 30 min. (*A*, *Lower*) Densitometric analysis (arithmetic means ± SEM, $n = 7$) of phospho-FOXO1 or FOXO1 protein abundance normalized to GAPDH. (*B*) Arithmetic means ± SEM ($n = 5$) of *Fgf23* mRNA abundance in UMR106 cells incubated with or without the FOXO1 inhibitor AS1842856 (50 nM). (*C*) Arithmetic means ± SEM ($n = 5$) of *Fgf23* mRNA abundance in UMR106 cells treated with *Foxo1*-specific or nonsense siRNA as a negative control (neg. siRNA). (*D*) Representative Western blot and densitometric analysis (arithmetic means ± SEM, $n = 7$) of FOXO1 protein abundance normalized to GAPDH in *Foxo1*-specific or nonsense siRNA-treated (neg. siRNA) cells. (*E*) Arithmetic means ± SEM ($n = 7$) of *Fgf23* mRNA in UMR106 cells transfected with a vector construct encoding human FOXO1-WT or with an empty vector pEGFP-C1 as a control. (*F*) Representative original Western blot (*Left*) demonstrating FOXO1 and the GFP-FOXO1 fusion protein abundance and representative microscopy image (*Right*) showing the GFP signal of the fusion protein or GFP alone in UMR106 cells following transfection with human FOXO1-WT or with an empty vector pEGFP-C1. (Scale bar: 50 μm.) $*P < 0.05$ and $***P < 0.001$ indicate significant difference from control-treated cells. arb., arbitrary.

increase in the serum FGF23 concentration that was significantly and almost completely prevented in STZ-treated mice injected daily with insulin (Fig. 4*B*). Hence, the inhibitory effect of insulin on FGF23 production is of high physiological relevance in vivo. Serum calcitriol (Fig. 4*C*), phosphate (Fig. 4*D*), calcium (Fig. 4*E*), and renal Klotho expression (Fig. 4*F*) were not affected by the treatment.

Plasma Insulin Is Negatively Correlated with the Plasma FGF23 Concentration in Humans. We finally investigated whether insulin-dependent suppression of FGF23 is also relevant in humans. In healthy volunteers, we analyzed baseline hormones as well as changes of hormones after an oral glucose load.

The baseline insulin concentration in the fasted subjects was inversely correlated with baseline FGF23 ($r = -0.282$, $P = 0.005$) as well as with homeostatic model assessment-estimated insulin resistance (HOMA-IR; $r = -0.293$, $P = 0.003$), a clinical index for insulin resistance, but not with proinsulin and HOMA-β, a parameter describing pancreatic β cell function and indicating insulin secretion (Table 1). These results are in line with an inhibitory effect of insulin on FGF23 secretion. The lack of correlation of baseline proinsulin and HOMA-β with baseline FGF23 suggests that a putative inhibitory effect of FGF23 on insulin secretion is a very unlikely explanation of our findings.

Baseline insulin increased from 12.2 ± 8.5 μU/mL to 113.1 ± 87.0 μU/mL 60 min after the oral glucose load of 75 g. The area under the curve (AUC) of the insulin time curve reflects insulin secretion 60 min after the glucose challenge, and the AUC of the FGF23 reflects the amount of secreted FGF23 within 60 min in response to the insulin challenge. We observed an inverse correlation ($r = -0.287$, $P = 0.004$) (Fig. 5 and Table 2), suggesting that insulin is a negative regulator of FGF23. In a further model, we correlated the AUC of insulin during the first 60 min after an oral glucose challenge with the AUC of FGF23 from 60 to 120 min after the glucose load. This model serves to test long-lasting effects of the insulin secretion in the first 60 min after glucose load. Again, we observed an inverse correlation ($r = -0.266$, $P = 0.008$) (Table 2). These results suggest that enhanced insulin secretion due to the oral glucose load suppressed FGF23 production in healthy volunteers with intact insulin signaling. We furthermore tested the correlation of insulin secretion (AUC of insulin during the first 60 min after glucose load) with the FGF23 concentrations at the time points of 60 min and 120 min. Again, an inverse relationship (Table 2) could be detected. Taken together, the analysis of baseline hormone concentrations (Table 1) and changes of hormones after the oral glucose load (Fig. 5 and Table 2) indicate that insulin is a potent negative regulator of FGF23 in humans as well.

Discussion

This study provides compelling evidence that insulin is a powerful and physiologically highly relevant suppressor of FGF23 synthesis in vitro as well as in mice and humans. According to our results, the insulin-induced inhibition of the transcription factor FOXO1 through PI3K/PKB/Akt signaling results in the down-regulation of *Fgf23* gene transcription.

Given the high prevalence of type 1 and type 2 diabetes, which are diseases associated with insulin deficiency or resistance, it may come as surprise that a direct insulin effect on FGF23 has not been described yet. In fact, several studies have addressed the impact of diabetes on plasma FGF23 and have found some associations (29–31), whereas others have not (32, 33). A plausible reason may be the fact that hyperinsulinemia, a state typical

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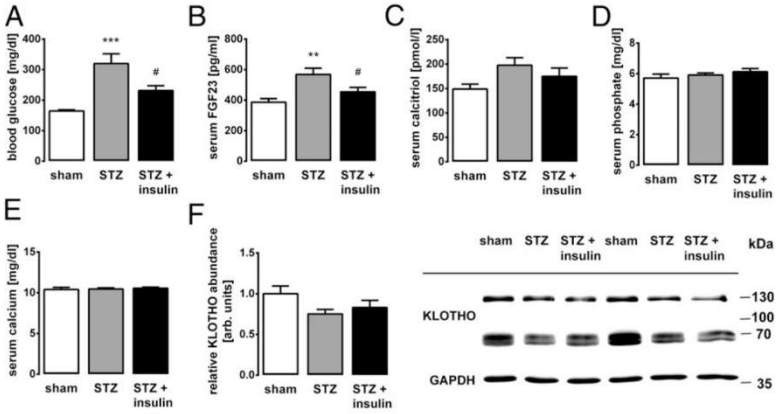

Fig. 4. Insulin deficiency results in a surge of the serum FGF23 level that is reversed by insulin. Arithmetic means ± SEM (n = 12 mice per group) of the blood glucose (*A*), serum C-terminal FGF23 (*B*), calcitriol (*C*), phosphate (*D*), and calcium (*E*) concentrations, as well as densitometric analysis of renal Klotho protein expression normalized to GAPDH (*F*, *Left*) and a representative Western blot (*F*, *Right*) in sham-treated (first bars) or STZ-treated mice receiving daily saline (second bars) or insulin (third bars) injections. arb., arbitrary. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant difference from sham-treated mice, and $^{\#}P < 0.05$ indicates significant difference between mice treated with STZ only and mice treated with STZ and insulin.

of many patients with type 2 diabetes, would be expected to be associated with low FGF23 levels according to our study. On the other hand, hyperinsulinemia and type 2 diabetes are clearly linked to inflammation (34) and kidney disease. Both conditions, alone or in combination, are associated with increased FGF23 production (15, 37). Hence, these effects may compensate for one other, resulting in the observed rather weak associations of diabetes with FGF23. In addition, confounding cofactors, such as inflammation, preexisting CKD, or preexisting coronary heart diseases, may have a further impact on FGF23 secretion in diabetes.

In contrast to the above-mentioned clinical studies analyzing patients with complex metabolic and/or cardiorenal diseases, and thus having many confounding factors with unpredictable effects on FGF23 secretion, our study of healthy pregnant women without renal impairment, manifest insulin resistance, and hyperinsulinemia (i.e., without known confounding factors) revealed a clear negative correlation of plasma insulin with FGF23. In patients with manifest type 2 diabetes, acute hyperinsulinemia was even reported to enhance FGF23 levels (38), suggesting that intact insulin signaling is required for the suppression of FGF23 secretion by insulin, a notion corroborated by the fact that insulin-resistant individuals have higher FGF23 serum levels (30).

The direct action of insulin on FGF23 was confirmed in mice. The abrogation of insulin secretion in STZ-treated mice resulted in the expected increase in serum FGF23 concentration in comparison to sham-treated animals. STZ-exposed mice additionally treated with daily insulin injections had normal FGF23 levels, suggesting that the STZ effect on insulin secretion, and not a possible side effect, was causative for the observed suppression of FGF23 serum levels. Similar effects were recently observed in STZ-exposed mice treated with insulin for a longer period (39). Insulin lowers the plasma phosphate concentration by inducing cellular phosphate uptake (40), an effect that may further down-regulate FGF23 secretion. However, neither STZ nor insulin treatment significantly changed the serum phosphate concentration or calcitriol, calcium, and renal Klotho expression. Suppression of FGF23 may be a physiological mechanism to prevent excessive hypophosphatemia due to cellular phosphate accumulation induced by insulin.

Our study also addressed the mechanism underlying insulin-induced inhibition of FGF23 formation. Inhibition of PI3K or PKB/Akt revealed the involvement of this signaling pathway. The high medical relevance of PI3K/PKB/Akt is based on the fact that in addition to insulin, many other growth factors are activators. Hence, PI3K/PKB/Akt signaling is utilized by many types of cancer to proliferate, survive, and migrate (41). In line with the relevance of the PI3K/PKB/Akt pathway for growth factor signaling, we found that IGF1 also attenuates FGF23 production involving IGF1R and PI3K/PKB/Akt signaling. Our study therefore suggests that all changes in PI3K/PKB/Akt activity, including possible future pharmacological interventions to combat cancer (41), may have an impact on FGF23.

We identified the transcription factor FOXO1 as the downstream mediator of the PKB/Akt effect on FGF23. Insulin action results in FOXO1 phosphorylation, thereby inhibiting this transcription factor. Pharmacological inhibition of FOXO1, as well as siRNA-mediated silencing, down-regulated *Fgf23* gene expression, whereas FOXO1 overexpression, which results in higher cellular levels of nonphosphorylated active FOXO1 (42), led to an enhancement of *Fgf23* expression. In general, FOXO1 is known to induce stress resistance in mammalian cells and has even been shown to contribute to longevity in worms and flies (43). In view of these antistress effects of FOXO1, it is interesting to speculate whether and how the FOXO1-dependent up-regulation of FGF23 fits into this concept: On the one hand, FGF23 deficiency is clearly associated with a short

Table 1. Correlation of baseline FGF23, insulin, proinsulin, HOMA-IR index, and HOMA-β index in fasted healthy pregnant women

	FGF23		Insulin		Proinsulin		HOMA-IR		HOMA-β	
Parameter	r_s	P	r_s	P	r_s	P	r_s	P	r_s	P
FGF23	1.000	—	−0.282**	0.005	0.035	0.735	−0.293**	0.003	−0.148	0.146
Insulin	−0.282**	0.005	1.000	—	0.324**	0.001	0.992**	0.000	0.799**	0.000
Proinsulin	0.035	0.735	0.324**	0.001	1.000	—	0.339**	0.001	0.208*	0.040
HOMA-IR	−0.293**	0.003	0.992**	0.000	0.339**	0.001	1.000	—	0.730**	0.000
HOMA-β	−0.148	0.146	0.799**	0.000	0.208*	0.040	0.730**	0.000	1.000	—

Spearman's rank correlation coefficient [Spearman's rho (r_s)] was determined for each group (n = 98). Correlation is significant at the $^{*}P < 0.05$ level and $^{**}P < 0.01$ level.

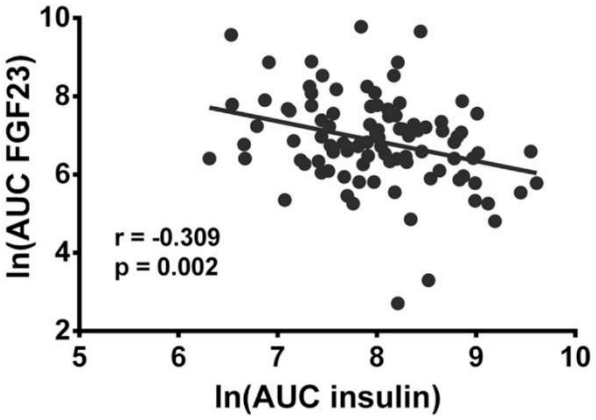

Fig. 5. Insulin and FGF23 secretion are negatively correlated in humans. A scatter plot representing the correlation of the AUC of the insulin time curve reflecting insulin secretion during the first 60 min after an oral glucose tolerance test in healthy volunteers and the AUC of the FGF23 time curve during the same time period reflecting the secreted FGF23 within 60 min is shown. Due to the right squid distribution of the raw data, a natural log transformation was performed. The Pearson correlation coefficient was determined (n = 98). ln, natural logarithm.

life span and age-associated diseases (2), but on the other hand, the up-regulation of FGF23 expression has not been associated with beneficial effects yet and is observed in many diseases (10).

Growth hormone (GH) exerts its growth-promoting effects in bone through IGF1. Bone growth requires a positive phosphate balance, and IGF1 indeed elevates serum phosphate concentration by stimulating renal phosphate reabsorption and calcitriol formation (44), which are effects expected to enhance FGF23 production. In line with this, a positive association of IGF1 with FGF23 was observed (45), and an elevation of FGF23 was found in patients with acromegaly (46) and in GH-deficient children treated with recombinant GH (47, 48). Therefore, IGF1 may have opposing effects on FGF23: A direct FGF23-lowering effect was demonstrated in our study, limiting the indirect FGF23-stimulating actions of IGF1, which may otherwise counteract its phosphate-retaining effect.

Taken together, we demonstrate that insulin- and IGF1-dependent PI3K/PKB/Akt/FOXO1 signaling is a powerful negative regulator of FGF23 production in vitro, in mice, and in humans.

Materials and Methods

The human study was approved by the Ethical Committee of the Medical Faculty of Humboldt University, Charité-Universitätsmedizin Berlin, Berlin, Germany. The animal study was approved by the Animal Care and Use Committee of Jinan University.

Glucose Tolerance Test in Healthy Pregnant Women. This study was performed in a subset of the Berlin Birth Cohort study (details are provided in refs. 49–51). Informed consent was obtained from the human subjects involved. Healthy women in the first trimester of pregnancy were fasted for at least 8 h, followed by an oral glucose load of 75 g and further blood collections after 60 and 120 min, respectively. Glucose, insulin, and proinsulin were measured by standard tests, and plasma FGF23 was measured using the Human FGF23 (Intact) ELISA Kit (Immutopics). The HOMA-IR index and HOMA-β index were calculated as described previously (52).

Animals and Experimental Design. Male DBA/2N mice (Charles River Laboratories) were subjected to five daily i.p. injections of 40 mg/kg of STZ (Sigma) or vehicle (sham). STZ-treated mice were then divided in two groups, with or without insulin treatment (n = 12 mice per group). The latter group received eight daily injections of 1 U/kg of Insulin Abasaglar (Eli Lilly) at 3:00 PM. From day 9 onward, mice received insulin injections every 12 h. On day 10, blood glucose was measured by a glucometer (Accu-Chek Aviva) 6 h after the last insulin injection. In anesthetized mice, retroorbital blood collection was performed. The following serum parameters were determined: C-terminal FGF23 and calcitriol with ELISA kits (Immutopics and IDS), inorganic phosphate by a photometric method using a Fluitest PHOS kit (Roche), and total calcium using a Fuji DRI CHEM NX500-Analyzer (Fujifilm).

Cell Culture. UMR106 rat osteosarcoma cells (American Type Culture Collection) were cultured as described elsewhere (53) and pretreated with 100 nM $1,25(OH)_2D_3$ (Tocris Bioscience) for 24 h since they do not have appreciable amounts of *Fgf23* mRNA per se (53, 54). Cells were incubated with 0.01–10 nM insulin or 0.5 μg/mL IGF1 (Sigma) in the absence or presence of 5 μM PI3K inhibitor wortmannin (Sigma), 1 μM PKB/Akt inhibitor MK-2206 (Selleckchem), 1 μM IGF1R inhibitor BMS-754807 (Sigma), or 50 nM FOXO1 inhibitor AS1842856 (Calbiochem) for a further 24 h.

Cell Transfection. Cells cultured in antibiotic-free complete medium for 24 h were treated with 25 nM siRNA: *Foxo1* (L-088495-02), *Igf1r* (L-091936-02), or nonsense (D-001810-10), and with 5 μL of DharmaFECT1 transfection reagent (T-2001) from Dharmacon for a further 48 h, with 100 nM $1,25(OH)_2D_3$ being present in the last 24 h. For FOXO1 overexpression, cells were transfected with a plasmid encoding EGFP-tagged human WT-FOXO1. The plasmid was generated by subcloning a fragment of FOXO1 expression plasmid 1306 pSG5L HA FKHR wt (kindly provided by W. Sellers, Dana–Farber Cancer Institute, Boston, MA, through Addgene no. 10693) into the SalI/SmaI site of pEGFP-C1 (Clontech). UMR106 cells cultured for 24 h without antibiotics were transfected with pEGFPC1-hFOXO1 or with the empty vector pEGFP-C1 as a negative control (6 μg of DNA) using FuGENE Transfection Reagent (Promega). Cells were incubated for 24 h without and for a further 24 h with 100 nM $1,25(OH)_2D_3$.

qRT-PCR. RNA was extracted using peqGOLD TriFast reagent (VWR), transcribed using a GoScript Reverse Transcription System (Promega), and subjected to PCR with GoTaq qPCR Master Mix (Promega) (95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 58 °C for 30 s, and 72 °C for 45 s) using the following primers: *Tbp* (ACTCCTGCCACACCAGCC, GGTCAAGTTTACAGCCAAGATTCA), *Fgf23* (TGGCCATGTAGACGGAACAC, GGCCCCTATTATCACTACGGAG), *Foxo1* (GGTGAAGAGTGTGCCCTACT, ATTTCCCACTCTTGCCTCCC), and *Igf1r* (TGCTCCAAAGACAAAATACC, CAAAGACTTTACGGTACTCAG). After normalization to *Tbp* expression, relative quantification of gene expression based on the double-delta Ct (threshold cycle) analysis was carried out.

Table 2. Correlation of insulin and FGF23 changes after an oral glucose test in fasted healthy pregnant women

	AUC insulin (0–60 min)		AUC FGF23 (0–60 min)		AUC FGF23 (60–120 min)		FGF23 (60 min)		FGF23 (120 min)	
Parameter	r_s	P	r_s	P	r_s	P	r_s	P	r_s	P
AUC insulin (0–60 min)	1.000	—	−0.287**	0.004	−0.266**	0.008	−0.200*	0.049	−0.275**	0.006
AUC FGF23 (0–60 min)	−0.287**	0.004	1.000	—	0.885**	0.000	0.917**	0.000	0.677**	0.000
AUC FGF23 (60–120 min)	−0.266**	0.008	0.885**	0.000	1.000	—	0.921**	0.000	0.868**	0.000
FGF23 (60 min)	−0.200*	0.049	0.917**	0.000	0.921**	0.000	1.000	—	0.669**	0.000
FGF23 (120 min)	−0.275**	0.006	0.677**	0.000	0.868**	0.000	0.669**	0.000	1.000	—

AUC represents the time curve of hormone release after glucose challenge between respective time points. In addition, a correlation with the FGF23 concentration at the appropriate times was performed. Spearman's rank correlation coefficient [Spearman's rho (r_s)] was determined for each group (n = 98). Correlation is significant at the *P < 0.05 level and **P < 0.01 level.

Western Blotting. To study FOXO1 phosphorylation, cells were cultured for 24 h in complete medium, followed by another 24 h in serum-free medium. Then, cells were treated with insulin for 30 min. Fifty micrograms of cell lysate or 30 μg of kidney lysate was subjected to a standard Western blot procedure using the following antibodies: anti-FOXO1 (C29H4, 2880), anti–phospho-FOXO1 (Ser256, 9461), and anti-GAPDH (D16H11, 5174S) (all from Cell Signaling Technology), as well as anti-KLOTHO (AF1819; R&D Systems). Secondary anti-rabbit IgG antibody (7074; Cell Signaling Technology) and anti-goat IgG antibody (HAF109; R&D Systems) conjugated with HRP were used. Visualization using ECL detection reagent (GE Healthcare) and densitometrical analysis were performed with a gel documentation system (Syngene G:BOX Chemi XX6; VWR) relative to GAPDH bands.

Statistics. Arithmetic means ± SEM were calculated, and *n* represents the number of independent experiments. Comparisons of two groups were made by an unpaired Student's *t* test, and for more than two groups, comparisons were calculated via one-way ANOVA, followed by Tukey's or Dunnett's multiple comparison tests, using GraphPad Prism. Data of the human study were analyzed with SPSS. Spearman's rank and Pearson correlations and a correlation matrix for FGF23 and other relevant variables were generated as described previously elsewhere (55). Differences were considered significant if $P < 0.05$.

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3.2 Paper 2: Advanced glycation end products stimulate gene expression of fibroblast growth factor 23

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RESEARCH ARTICLE

Advanced glycation end products stimulate gene expression of fibroblast growth factor 23

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Scope: Osteoblasts produce fibroblast growth factor 23 (FGF23), a hormone inhibiting renal phosphate reabsorption and the formation of biologically active vitamin D, calcitriol. FGF23-deficient mice age rapidly and develop age-associated diseases at least in part due to massive calcification. Elevated FGF23 serum levels are detected in patients suffering from acute and chronic renal, cardiovascular, inflammatory, and metabolic diseases. Advanced glycation end products (AGEs) are sugar-modified proteins, nucleic acid, and lipids which contribute to these disorders. Here, we studied the significance of AGEs for the generation of FGF23.

Methods and results: As AGE sources, bread crust extract (BCE) and ribose-modified bovine serum albumin (r-BSA) were used. UMR106 osteoblast-like cells were exposed to BCE and r-BSA, and Fgf23 transcripts were determined by qRT-PCR. UMR106 cells express the receptor for AGEs, RAGE. BCE and r-BSA were powerful stimulators of Fgf23 transcription. NFκB inhibitor wogonin and store-operated calcium entry (SOCE) antagonist 2-APB attenuated the r-BSA and BCE effects on FGF23 synthesis.

Conclusion: Sources of AGEs induce the transcription of Fgf23 in UMR cells. At least in part, the effect is mediated through up-regulation of NFκB and subsequent SOCE. AGE-induced FGF23 production may contribute to increased FGF23 serum levels observed in chronic disease.

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1 Introduction

Calcium and phosphate homeostasis are regulated by the classical hormones parathyroid hormone (PTH) and calcitriol, the active form of vitamin D [1]. In 1997, the renal protein Klotho was discovered. Klotho-deficient mice suffer from a very short life span, age extremely fast, and have many age-associated disorders typical of aging human beings [2]. Above all, Klotho-deficient mice were found to have calcification in most organs and tissues as well as abnormally high serum phosphate and calcitriol concentrations [2].

Further research revealed that renal Klotho is a transmembrane protein and the obligatory co-receptor for fibroblast growth factor 23 (FGF23), a hormone produced by bone cells (osteoblasts and osteocytes) [3, 4]. FGF23 along with its co-receptor Klotho inhibit renal phosphate reabsorption and 25-Hydroxyvitamin D3 1-alpha-hydroxylase, the key enzyme for calcitriol production in the kidney [5]. Not surprisingly, FGF23-deficient mice exhibit a phenotype very similar to Klotho-deficient mice including rapid aging and age-associated diseases [6]. The hyperphosphatemia seems to be highly relevant for their pronounced phenotype since feeding a low phosphate or low vitamin D diet normalizes the life span of both, Klotho- and FGF23-deficient mice [7, 8]. Conversely, overexpression of Klotho, but not of FGF23 increases the life span of mice by about 30% [9].

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Abbreviations: 2-APB, 2-Aminoethoxydiphenyl borate; **AGE**, advanced glycation end products; **arb**, arbitrary; **BCE**, bread crust extract; **CRAC**, Ca^{2+} release-activated Ca^{2+} channel; **dNTP**, deoxynucleoside triphosphate; **esRAGE**, endogenous soluble RAGE; **FCS**, fetal calf serum; **FGF23**, fibroblast growth factor 23; **NC**, negative control; **NFκB**, nuclear factor kappa-light-chain-enhancer of activated B cells; **PTH**, parathyroid hormone; **qRT-PCR**, quantitative RT-PCR; **RAGE**, receptor of advanced glycation end products; **r-BSA**, ribose-modified bovine serum albumin; **SOCE**, store-operated calcium entry; **Tbp**, TATA box-binding protein

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Recently, FGF23 has gained attention as it has become clear that FGF23 may be a valuable and highly sensitive biomarker for different acute and chronic disorders [10, 11]. Patients with renal (acute kidney injury, chronic kidney disease), cardiovascular (coronary heart disease, myocardial infarction, atrial fibrillation), inflammatory, and metabolic diseases were found to have high levels of FGF23 [10]. Inflammation implicated in most of the aforementioned diseases has been shown to be a major trigger of FGF23 formation [12]. In detail, activation of the transcription factor complex NFκB induces FGF23 formation through up-regulation of the Ca^{2+} release-activated Ca^{2+} (CRAC) channel Orai1 [13].

Advanced glycation end products (AGEs) are generated by the non-enzymatic reaction of sugars with proteins or lipids [14]. They are produced in the body and in the process of preparing food [14]. Aging, degenerative disorders, and in particular metabolic diseases including diabetes are associated with enhanced formation of AGEs [14]. AGEs are known to activate NFκB [15]. Interestingly, a high serum FGF23 negatively correlates with outcome in diabetic nephropathy, and positively correlates with the presence of the endogenous soluble receptor of advanced glycation end products (esRAGE) [16]. Bread crust extract (BCE) is rich in different AGEs and also activates NFκB [17].

Analyzing UMR106 osteoblast-like cells, we sought to test whether BCE and ribose-modified BSA as AGE sources are capable of inducing Fgf23 transcription. In addition, we studied the underlying mechanism.

2 Materials and methods

2.1 Generation of BCE

Bread crust was prepared as a mixture of rye flour, wheat flour, yeast, sourdough and NaCl as previously described [18]. Defatted brown and lyophilized bread crust powder was stored at -20°C. Water-soluble bread crust extract (250 mg bread crust powder in 1 ml PBS (GIBCO, Karlsruhe, Germany)) was prepared by a 4 min. sonication followed by 2 centrifugation steps at 8000 × *g* for 20 min. The supernatant was stored at −20°C until further use. For the cell culture application, the stock solution was concentrated via vacuum centrifuge. The extract was reduced to half, yielding a concentration of 500 mg/mL. Finally, 5.6 μL of the concentrated bread crust extract was added to the cells, which resulted in a corresponding concentration of 1.4 mg/mL. BCE is rich in several AGEs including Arg-pyrimidine, carboxymethyllysine, GA-pyridine [19], pronyl lysine [18], gliadin as well as AGE-modified gliadin [17].

2.2 Generation of ribose BSA

Bovine serum albumin solution (1 mM, Sigma) was glycated by incubation under sterile conditions with 0.5 M ribose in 50 mM PBS at 50°C for 8 weeks. The AGE-BSA solution (r-BSA) was then dialyzed against PBS. By immunoblotting we observed a strong carboxymethyllysine signal and a weak signal for carboxyethyllysine in BSA treated with ribose. Arg-pyrimidine and pentosidine modifications were not detected in r-BSA (data not shown).

2.3 Cell culture

Cell culture was performed as previously described [20]. Briefly, UMR106 rat osteosarcoma cells were cultured in DMEM high glucose medium supplemented with 10% FCS and 1% penicillin/streptomycin under standard culture conditions. Since UMR106 cells do not contain appreciable amounts of Fgf23 mRNA, the cells were pretreated with 100 nM $1,25(OH)_2D_3$ (TOCRIS, Bristol, UK) for 24 h to induce Fgf23 expression [21] and then in addition treated with or without 1.4 mg/mL BCE, 10 μM r-BSA or 10 μM BSA as a control, 25 μM SOCE inhibitor 2-APB (Sigma, Schnelldorf, Germany), 100 μM NFκB inhibitor wogonin (Sigma), or with vehicle only for another 24 h.

2.4 RT-PCR

Total RNA was isolated from the cells using Trifast reagent (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. After measuring the RNA concentration reverse transcription was performed using the GoScript™ Reverse Transcription System (Promega, Mannheim, Germany). Briefly, in a reaction volume of 5 μL, 1.2 μg of RNA was used as template mixed with an appropriate volume of RNAse-free water and incubated at 70°C for 5 min. 15 μL of the reverse transcription reaction mix were prepared with the following final concentrations of reaction buffer (1×), $MgCl_2$ (2.5 mM), dNTP mix (0.5 mM each), random primer (0.5 μg), reverse transcriptase (1 U/μL), and RNAse-free water, and added to the RNA. Reverse transcription was performed with conditions set at 25°C for 5 min, 42°C for 1 h, and 70°C for 15 min.

For analysis of RAGE expression in UMR106 cells PCR amplification was carried out using GoTaq Green Master Mix (Promega, Mannheim, Germany). The RT-PCR reaction mixture contained: 2 μl cDNA, 0.3 μM of the specific forward and reverse primers, 7.5 μl Green Master Mix, and sterile water up to 15 μL. Amplification conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 69°C for 30 s, 72°C for 1 min, and a final elongation step at 72°C for 5 min. The PCR products were separated on a 1.5 % agarose-gel and visualized by Midori Green (Biozym, Hessisch Oldendorf, Germany).

The following primers were used:

Rat RAGE (receptor for advance glycation endproducts):
forward (5′-3′): CCTGAGACGGGACTCTTCACGCT
reverse (5′-3′): AGCATGGATCATGTGGGCTCTG

2.5 Quantitative RT-PCR

For qRT-PCR analysis, the final volume of the reaction mixture was 20 μL and contained: 2 μL cDNA, 1 μM of each primer, 10 μL GoTaq qPCR Master Mix (Promega), and sterile water up to 20 μL. PCR conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 58°C for 30 s and 72°C for 45 s. Quantitative RT-PCR was performed on a Rotor-Gene Q (QIAGEN, Hilden, Germany).

The following primers were used:

Rat Tbp (TATA box-binding protein):
forward (5′-3′): ACTCCTGCCACACCAGCC
reverse (5′-3′): GGTCAAGTTTACAGCCAAGATTCA
Rat Fgf23
forward (5′-3′): TGGCCATGTAGACGGAACAC
reverse (5′-3′): GGCCCCTATTATCACTACGGAG

Calculated mRNA expression levels were normalized to the expression levels of Tbp of the same cDNA sample as internal reference. All reactions were performed in duplicates. Relative quantification of gene expression was accomplished using the ΔΔCt method.

2.6 Statistics

Data are provided as means ± SEM, n represents the number of independent experiments. All data were tested for significance using Student's paired t-test or one-way ANOVA multiple comparison test. Only results with $p < 0.05$ were considered statistically significant.

3 Results

In order to test whether FGF23 production is modified by bread crust extract, we incubated UMR106 osteoblast-like cells with or without BCE for 24 h. BCE treatment resulted in a significant increase in Fgf23 transcript levels as analyzed by qRT-PCR (Fig. 1), pointing to the capability of bread crust extract to induce the production of FGF23.

Bread crust extract is rich in AGEs. To study whether AGEs similarly induce FGF23 production, we exposed UMR106 cells to r-BSA, a typical AGE, in a new series of experiments. As illustrated in Fig. 2, similar to BCE, r-BSA potently increased Fgf23 transcripts. Hence, AGEs also stimulate FGF23 formation.

AGE-related effects are typically mediated by the receptor for advanced glycation end products (RAGE). Employing RT-PCR, we analyzed next whether UMR106 osteoblast-like cells express RAGE. As shown in Fig. 3, RAGE mRNA could readily be detected pointing to the expression of RAGE in UMR106 cells.

Bread crust extract and AGEs have already been shown to activate NFκB [15]. Moreover, activation of NFκB has been demonstrated to induce FGF23 production [13]. Therefore, we

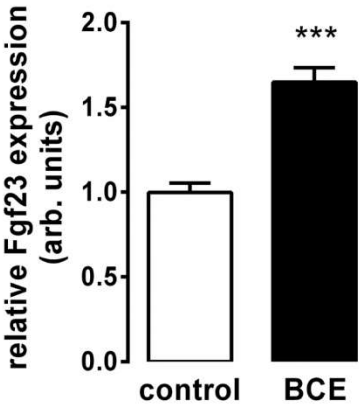

Figure 1. Bread crust extract (BCE) increases Fgf23 expression in UMR106 cells. UMR106 cells were incubated with 1.4 mg/mL BCE for 24 h. The mRNA abundance of Fgf23 was measured by quantitative real-time PCR. The gene expression was normalized to Tbp as housekeeping gene and the values are expressed as means ± SEM of 5 independent experiments. ***$p < 0.001$ indicates significant difference from control (paired t-test).

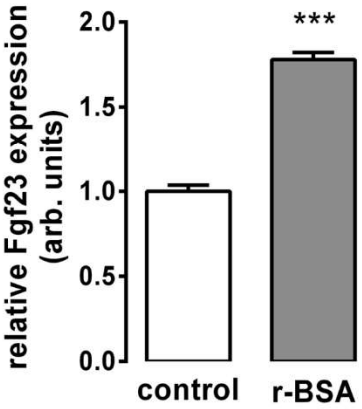

Figure 2. r-BSA enhances the expression of Fgf23 in UMR106 cells. The abundance of Fgf23 mRNA in r-BSA treated UMR-106 cells (10 μM; 24 h) was determined using quantitative real-time RT-PCR and the values were normalized to the expression of Tbp as housekeeping gene. Data are given as means ± SEM of 5 independent experiments. ***$p < 0.001$ indicates significant difference from control (paired t-test).

tested whether activation of NFκB is involved in the stimulating effect of bread crust extract and AGEs on Fgf23 transcription. To this end, UMR106 cells were treated with BCE and r-BSA in the absence and presence of NFκB inhibitor wogonin. Indeed, wogonin almost fully abrogated the effect of BCE (Fig. 4A) and r-BSA (Fig. 4B) on FGF23. Thus, activation of NFκB is required for AGE-induced Fgf23 transcription.

NFκB enhances FGF23 formation by up-regulating the expression of CRAC channel Orai1 which mediates SOCE [13]. In a last series of experiments, we therefore analyzed whether SOCE is also involved in the stimulating effect of AGE on

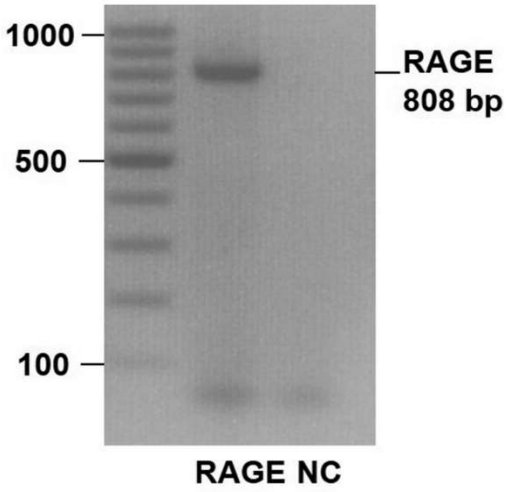

Figure 3. Expression of the receptor for advanced glycation end-products (RAGE) in UMR106 cells. RT-PCR of mRNA isolated from untreated UMR-106 cells was performed. A photograph of a 1.5 % agarose gel shows a sample with (left lane) and without a template as a negative control (right lane, NC). GeneRuler 1 kb DNA ladder was used as size marker. Expected PCR product size of RAGE is 808 bp.

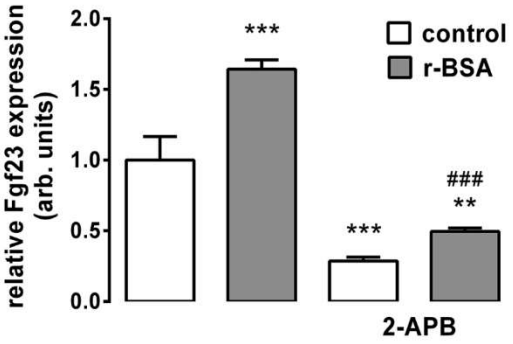

Figure 5. SOCE inhibitor 2-APB blunts the r-BSA-mediated increase in Fgf23 expression in UMR106 cells. Fgf23 mRNA abundance was determined by quantitative RT-PCR in UMR cells treated without (open bars) or with r-BSA (closed bars; 10 μM; 24 h) in the absence (left bars) or presence (right bars) of 25 μM SOCE inhibitor 2-APB.The values were normalized to Tbp expression and are given as means ± SEM of 5 independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significant difference from control (1st bar: absence of r-BSA and 2-APB), $^{###}p < 0.001$ indicates significant difference from absence of 2-APB (2nd versus 4th bar) (one-way ANOVA).

Fgf23 transcription. As illustrated in Fig. 5, Orai1 inhibitor 2-APB completely abrogated the AGE effect on FGF23. This result suggests that Ca^{2+} entry is also part of the cellular machinery leading to the transcription of Fgf23 upon treatment with AGEs.

4 Discussion

According to our study, AGE-rich bread crust extract was a powerful stimulator of Fgf23 transcription in UMR106 osteoblast-like cells. [22]. Moreover, r-BSA, also a source of AGEs, induced Fgf23 transcription. Thus, we conclude that the effect of BCE on FGF23 is, at least in part, due to the presence of AGEs in it. It must be kept in mind that the bioavailability of AGEs from food may be low as the intestinal absorption may be limited [25]. However, AGEs derived from endogenous sources could still be effective in inducing FGF23 production.

In this study, we determined Fgf23 transcript levels in UMR106 cells to estimate FGF23 formation as has been done by others before [22, 29, 30]. Altered Fgf23 mRNA abundance has explicitly been shown to correlate with FGF23 protein in UMR106 cells in the case of estrogens [32] and DMP1 [33].

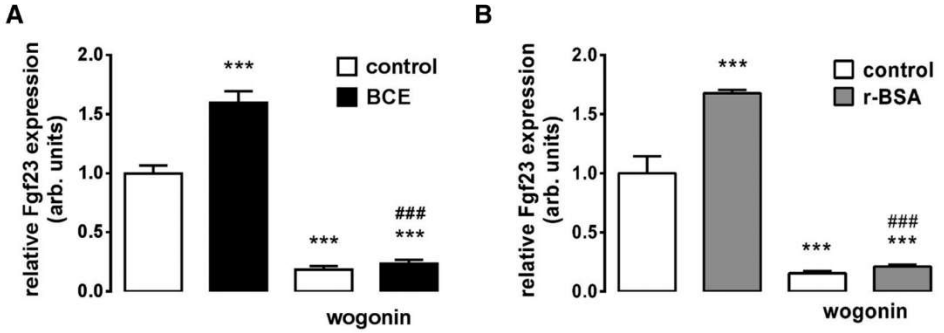

Figure 4. Effect of NF-κB inhibitor wogonin on the bread crust extract (BCE-) and r-BSA- induced Fgf23 transcript level increase. UMR-106 cells were treated without (open bars) or with (filled bars) BCE (A) or r-BSA (B) in the absence (left bars) and presence (right bars) of NF-κB inhibitor wogonin (100 μM; 24 h). Fgf23 mRNA abundance was determined by use of quantitative real-time RT-PCR and the values were normalized to Tbp expression. The values are given as means ± SEM of 5 independent experiments. $^{***}p < 0.001$ indicates significant difference from control (1st bar: absence of BCE, r-BSA, and wogonin), $^{###}p < 0.001$ indicates significant difference from absence of wogonin (2nd versus 4th bar) (one-way ANOVA).

Studying the underlying mechanism, we found that wogonin, a NFκB inhibitor, significantly attenuated the stimulating effect of BCE on FGF23-mRNA formation. NFκB is the pivotal transcription factor in inflammatory processes [23,24]. Previous studies have already established a clear role for inflammation in the synthesis of FGF23 [12, 26–28]. In detail, inflammatory cytokines have been shown to induce FGF23 production [31], and some acute and chronic inflammatory diseases are associated with an elevated serum FGF23 level [10]. Moreover, activation of NFκB itself enhances FGF23 generation [13]. On a molecular level, NFκB has been shown to up-regulate the expression of the CRAC channel Orai1. Orai1-mediated SOCE then triggers FGF23 synthesis [13]. Our results with wogonin suggest that also the effect of AGEs on FGF23 is, at least in part, mediated by activation of NFκB. However, our results do not exclude the possibility that ingredients of BCE other than AGEs also participate in the induction of NFκB. Orai1-inhibitor 2-APB also abrogated the stimulating effect of r-BSA on FGF23. NFκB may therefore be effective through up-regulation of Orai1 as has been shown before [13].

In line with a pivotal role of NFκB in cellular effects of bread crust extract, BCE has indeed been demonstrated to stimulate this transcription factor complex [17]. Also, cellular effects of AGEs depend on the induction of inflammatory processes [34, 35], in part by activating NFκB [36].

Some effects of AGEs are mediated by the receptor for advanced glycation end products (RAGE) [37]. Notably, our results document that UMR106 cells do express this receptor.

AGEs are particularly relevant in arteriosclerosis [38] and diabetes mellitus [39], conditions associated with a high cardiovascular risk. The serum FGF23 level is elevated in cardiovascular disease and strongly correlates with outcome [40,41]. It is intriguing to speculate that AGEs may contribute to enhanced FGF23 production in patients with those conditions. Further research is clearly warranted to establish a causal relationship.

In conclusion, AGEs enhance the expression of Fgf23 in UMR106 cells. The effect is at least in part mediated by stimulating NFκB activity and subsequent SOCE.

L.B. performed experiments, analyzed results, and wrote the manuscript, K.W., N.W., A.N.S., and A.S. provided material, M.F. designed the study and wrote the manuscript.

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The authors have declared no conflict of interest.

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3.3 Paper 3: Calcineurin inhibitors regulate fibroblast growth factor 23 (FGF23) synthesis

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ORIGINAL ARTICLE

Calcineurin inhibitors regulate fibroblast growth factor 23 (FGF23) synthesis

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Abstract Fibroblast growth factor 23 (FGF23) inhibits renal phosphate reabsorption and calcitriol formation, effects depending on Klotho as a co-receptor for FGF23. In addition, FGF23/Klotho strongly influences aging and the onset of age-associated diseases. The synthesis of FGF23 by bone cells is induced by store-operated Ca^{2+} entry (SOCE) through Orai1 in UMR106 osteoblast-like cells. Ca^{2+} entry activates the phosphatase calcineurin in many cell types which dephosphorylates nuclear factor of activated T cells (NFAT) thereby stimulating its transcriptional activity. Here, we explored whether calcineurin-NFAT signaling impacts on FGF23 production. Fgf23 transcripts were determined by qRT-PCR and FGF23 protein by ELISA. Calcineurin as well as NFAT expression were quantified by RT-PCR in UMR106 cells. UMR106 cells expressed calcineurin subunits Ppp3r1, Ppp3ca, Ppp3cb, and Ppp3cc as well as NFATc1, NFATc3, and NFATc4. Calcineurin inhibitors ciclosporin A (CsA) and tacrolimus (FK-506) decreased Fgf23 gene expression and FGF23 protein production. Moreover, calcineurin-NFAT interaction inhibitor INCA-6 reduced the abundance of Fgf23 transcripts as well as FGF23 protein. Calcineurin-NFAT signaling is a potent regulator of FGF23 formation.

Keywords Ciclosporin · Tacrolimus · Calcium · Phosphatase · Phosphate · Klotho

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Introduction

Fibroblast growth factor 23 (FGF23) is a member of the family of FGFs (Beenken and Mohammadi 2009). It is mainly produced by bone cells (osteoblasts and osteocytes) and acts in an endocrine fashion on other organs (Kuro-O and Moe 2016; Erben and Andrukhova 2016). Therefore, FGF23 is a classical hormone. In the kidney, FGF23 inhibits the reabsorption of phosphate, which is mainly accomplished by the Na^+-dependent phosphate transporters NapiIIa and NapiIIc, resulting in more urinary phosphate excretion (Kuro-O and Moe 2016; Erben and Andrukhova 2016). Moreover, FGF23 downregulates the expression of CYP27B1 or 25-hydroxyvitamin D3 1-alpha-hydroxylase, the renal key enzyme for the production of $1,25(OH)_2D_3$, active vitamin D (calcitriol). Hence, FGF23 lowers the serum concentration of $1,25(OH)_2D_3$ (Erben and Andrukhova 2016; Kuro-O and Moe 2016). Other target organs of FGF23 include the heart where FGF23 induces hypertrophy of the left ventricle (Faul 2016).

The renal (but not the cardiac) effects of FGF23 require the expression of transmembrane Klotho which serves as a co-receptor for FGF23 (Erben and Andrukhova 2016; Scholze et al. 2014). Apart from this function, soluble Klotho (sKlotho), which is a extracellular domain of transmembrane Klotho that can be cleaved off, also exerts endocrine effects including the regulation of NapiIIa, K^+ channel ROMK, and WNT signaling (Xu and Sun 2015).

Besides their role in controlling phosphate homeostasis, FGF23 and Klotho strongly influence aging. Mice deficient for FGF23 (Shimada et al. 2004) or Klotho (Kuro-o et al. 1997) suffer from a plethora of aging-associated disorders

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and age rapidly. Importantly, the premature aging of these mice is the consequence of the deranged phosphate metabolism as the mice are almost normal when maintained on a low phosphate or low vitamin D diet (Kuro-O and Moe 2016).

Several acute and chronic human diseases including acute renal failure, chronic kidney disease, polycystic kidney disease, atrial fibrillation, or sepsis result in an increase in the plasma FGF23 level (Schnedl et al. 2015; Leaf et al. 2016a; Munoz Mendoza et al. 2017; Leaf et al. 2016b; Di Giuseppe et al. 2015; Spichtig et al. 2014). The underlying causes are not completely understood. Known regulators of FGF23 production include inflammation (Sharaf El Din et al. 2017), dietary phosphate (Takeda et al. 2014), PTH (Knab et al. 2017), $1,25(OH)_2D_3$ (Nguyen-Yamamoto et al. 2017), and the iron status (Lewerin et al. 2017). The transcription factors binding to the FGF23 promoter are incompletely determined. Orphan nuclear receptor Nurr1 may play a role as it may be involved in the effect of PTH on FGF23, and the FGF23 promoter contains putative Nurr1 binding sites (Meir et al. 2014). $1,25(OH)_2D_3$ may induce FGF23 production through vitamin D response elements in the FGF23 promoter (Kolek et al. 2005). Also, a role of hypoxia-inducible factor 1α appears to be essential (Zhang et al. 2016b). Recently, store-operated Ca^{2+} entry (SOCE) through the Ca^{2+} release-activated Ca^{2+} channel (CRAC) Orai1 has been demonstrated to induce Fgf23 gene expression (Zhang et al. 2016a).

Calcineurin is a Ca^{2+}-dependent phosphatase expressed in many cell types including osteoblasts. It dephosphorylates nuclear factor of activated T cells (NFAT) proteins, transcription factors first implicated in T cell activation by controlling the expression of interleukin-2 (Winslow et al. 2006). However, calcineurin-NFAT signaling is also effective in osteoblasts (Winslow et al. 2006).

Calcineurin inhibitors such as ciclosporin A (CsA) or tacrolimus (FK-506) are widely used in the treatment of patients after organ transplantation or with severe autoimmune diseases as immunosuppressive drugs (Laurin et al. 2017; El-Gowelli and El-Mas 2015).

In view of the pivotal role of intracellular Ca^{2+} for the induction of FGF23 synthesis, we explored whether Ca^{2+}-dependent calcineurin-NFAT signaling is relevant for the production of FGF23. To this end, we carried out experiments in UMR106 osteoblast-like cells.

Materials and methods

Cell culture

UMR106 rat osteoblast-like cells were cultured as previously described (Bar et al. 2017). Briefly, cells were incubated in DMEM high-glucose medium supplemented with 10% FCS, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37 °C and 5% CO_2.

Cells were pretreated with 100 nM $1,25(OH)_2D_3$ (TOCRIS, Wiesbaden-Nordenstadt, Germany) for 24 h to upregulate Fgf23 expression (Saini et al. 2013) which is otherwise low. Then, cells were additionally treated with 5 μM ciclosporin A (CsA) (Sigma, Schnelldorf, Germany), 25 μM tacrolimus (FK-506) (TOCRIS) or 25 μM INCA-6 (TOCRIS), or with vehicle only for another 24 h.

RNA extraction

Total RNA was isolated from UMR106 cells by phenol-chloroform extraction using Trifast reagent (Peqlab, Erlangen, Germany). RNA concentration and purity were determined by spectrophotometric measurements at $\lambda = 260$ and 280 nm using NanoDrop® One (Thermo Scientific, Darmstadt, Germany).

RT-PCR

Complementary DNA (cDNA) was synthesized using random hexamers and the GoScript™ Reverse Transcription System (Promega, Mannheim, Germany). In detail, 1.2 μg of RNA was reverse transcribed in a reaction mixture of 15 μl containing $MgCl_2$ (2.5 mM), dNTP mix (0.5 mM each), random primer (0.5 μg), reverse transcriptase (1 U/μl), and RNase-free water. Reverse transcription was performed at 25 °C for 5 min, 42 °C for 1 h, and 70 °C for 15 min.

Calcineurin and NFAT expression in UMR106 cells were studied by RT-PCR using the GoTaq Green Master Mix (Promega) and the primers listed in Table 1. The reaction was carried out with 2 μl of the synthesized cDNA, 0.3 μM of specific forward and reverse primers, 7.5 μl Green Master Mix, and sterile water up to 15 μl. Reaction conditions were 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 69 °C for 30 s, 72 °C for 1 min, and a final elongation step at 72 °C for 5 min. PCR products were loaded on a 1.5% agarose gel and visualized by Midori Green (Biozym, Hessisch Oldendorf, Germany). GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific) was used as a size marker.

Quantitative RT-PCR

Relative expression levels of Fgf23 were determined by quantitative real-time RT-PCR using SYBR Green I technology and the Rotor-Gene Q (QIAGEN, Hilden, Germany) as a real-time PCR cycler detection system. PCR was performed using 2 μl cDNA, 1 μM of each gene-specific primer, 10 μl GoTaq qPCR Master Mix (Promega), and sterile water up to

Table 1 Sequences of primers used

Name	Sequence (5′-3′)	Product
Ppp3r1_forward	ACGACATGGATAAAGACGGC	151 bp
Ppp3r1_reverse	GATATTCTCCCGTCCCCGTC	
Ppp3r2_forward	CAGCTGGTGGACAAAAGCAT	203 bp
Ppp3r2_reverse	TCCCATGTACACAACCTCGA	
Ppp3ca_forward	ATAACGATGGGAAGCCTCGT	157 bp
Ppp3ca_reverse	CAAACTGTGACTGGGGCATC	
Ppp3cb_forward	CTTTTGACAGCTTGCCCCTT	150 bp
Ppp3cb_reverse	GCAAGTCACACATTGGTCCA	
Ppp3cc_forward	ACATTGTTTCTGCTTCGAGGG	157 bp
Ppp3cc_reverse	ACTGCTGGTTTAAGAGGGCA	
Nfatc1_forward	GAAGACTGTCTCCACCACCA	165 bp
Nfatc1_reverse	CCGATGTCTGTCTCCCCTTT	
Nfatc2_forward	CCAAGACGAGCTGGACTTTT	152 bp
Nfatc2_reverse	GAGGGTTGCATGGCTTGAG	
Nfatc3_forward	TGATGGCCTTGGATCTCAGT	241 bp
Nfatc3_reverse	CCCTCGGCTACCTTCAGTTT	
Nfatc4_forward	GAAAGAGATGGCTGGCATGG	163 bp
Nfatc4_reverse	CACCTCAATCCTCAGCTCCA	

20 μl. The PCR amplification reactions were carried out in 40 cycles of 95 °C for 10 s, 58 °C for 30 s, and 72 °C for 45 s. Calculated mRNA expression levels were normalized to the expression levels of Tbp (TATA box-binding protein) of the same cDNA sample. Relative quantification of gene expression was based on the ΔΔCt method.

The following primers were used:

Rat Tbp:
forward (5′-3′): ACTCCTGCCACACCAGCC
reverse (5′-3′): GGTCAAGTTTACAGCCAAGATTCA
Rat Fgf23
forward (5′-3′): TGGCCATGTAGACGGAACAC
reverse (5′-3′): GGCCCCTATTATCACTACGGAG
Rat NF-κB p65
forward (5′-3′): TTCCCTGAAGTGGAGCTAGGA
reverse (5′-3′): CAGTCGAGGAAGACACTGGA

Preparation of whole cell lysates

Cells were cultured and treated as described above. Then, cells were washed twice with ice-cold PBS and lysed in RIPA buffer (Cell Signaling Technology, Frankfurt, Germany) containing protease phosphatase inhibitor cocktail and EDTA (Thermo Fisher Scientific). Cell lysates were centrifuged at 12,000 g and 4 °C for 10 min. The supernatants were harvested as whole cell lysate. Finally, the protein concentration was determined using the Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific).

Immunoblotting

Proteins (45 μg) of the whole cell lysates were resuspended in Roti-Load 1 (Carl Roth; Karlsruhe, Germany) and an appropriate amount of water before denaturation at 95 °C for 5 min. Next, proteins were separated by SDS-PAGE, transferred to PVDF membranes (GE Healthcare-Amersham, Freiburg, Germany), and blocked with 5% nonfat milk with Tris-buffered saline containing 0.1% Tween 20 (TBST) at room temperature for 1 h. Finally, membranes were incubated with the following primary antibodies (Cell Signaling Technology): anti-NF-κB p65, anti-phospho-NF-κB p65 (Ser536), and GAPDH as loading control. After several washing steps with TBST, incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Cell Signaling Technology) for 1 h at room temperature was carried out. Membranes were washed again and the protein bands were detected by Syngene G:BOX Chemi XX6 (VWR, Dresden, Germany) using ECL reagent (GE Healthcare-Amersham). Densitometric analysis was performed with Syngene GeneTools relative to GAPDH.

FGF23 ELISA (C-term)

UMR106 osteoblast-like cell medium was collected and frozen at −80 °C. The medium was concentrated using Sartorius Vivaspin 6 Centrifugal Concentrators (Sartoritus, Göttingen, Germany). The concentration of C-terminal FGF23 was determined by an ELISA from Immutopics (San Clemente, CA, USA) according to the manufacturer's instructions.

Statistics

All data are given as arithmetic means ± SEM; *n* represents the number of independent experiments. Statistical comparisons were made by Student's *t* test. Differences were considered significant if $p < 0.05$.

Results

In this study, we used UMR106 osteoblast-like cells to study the regulation of FGF23 production. Our first experiments aimed to explore whether these cells have functional calcineurin-NFAT signaling. To this end, we analyzed the expression of calcineurin subunits and NFAT proteins by RT-PCR. As illustrated in Fig. 1a, mRNA specific for the regulatory calcineurin subunit Ppp3r1 and for the catalytic subunits Ppp3ca, Ppp3cb, and Ppp3cc could readily be detected in UMR106 cells. Moreover, RT-PCR revealed the expression of NFATc1, NFATc3, and NFATc4 in UMR106 cells (Fig. 1b).

As a next step, we tested whether calcineurin signaling is relevant for the production of FGF23 in UMR106 cells. We

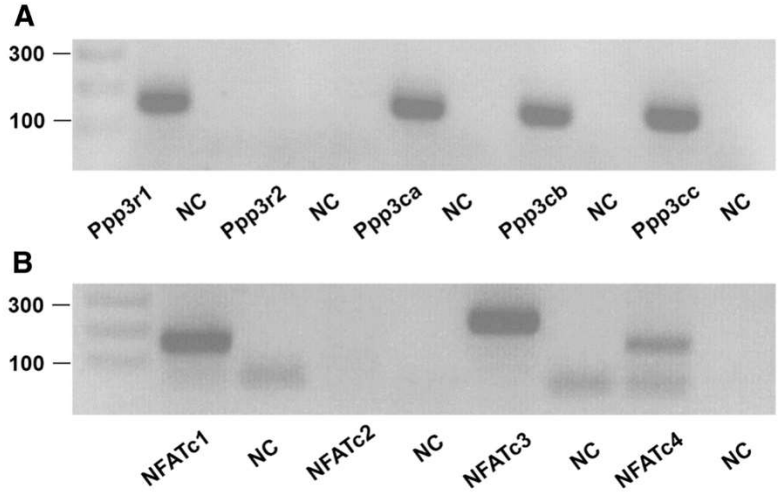

Fig. 1 Calcineurin and NFAT mRNA in UMR106 cells. **a** Calcineurin or protein phosphatase 3 (*Ppp3*) consists of a regulatory (Ppp3r1 or Ppp3r2) and catalytic subunit (Ppp3ca, Ppp3cb, and Ppp3cc). The gel photograph shows PCR products specific for the respective mRNAs. **b** PCR products specific for NFATc1-4 mRNAs are shown on the gel photograph. No bands are observed without template (*NC*)

incubated the cells with and without calcineurin inhibitor ciclosporin A (CsA) and determined relative Fgf23 transcript levels by qRT-PCR. Incubation with CsA significantly reduced the abundance of Fgf23 transcripts, pointing to a stimulating effect of calcineurin activity on FGF23 production (Fig. 2a). Reduced Fgf23 gene expression upon incubation with CsA translated into a lower FGF23 protein concentration in the supernatant of UMR106 cells (Fig. 2b).

We carried out further experiments to analyze whether tacrolimus (FK-506), another calcineurin inhibitor widely used in clinical practice, similarly interfered with Fgf23 transcription in UMR106 cells. According to Fig. 3a, incubation with FK-506 also decreased the gene expression of Fgf23. Accordingly, FGF23 protein production was reduced by FK-506 (Fig. 3b).

Calcineurin dephosphorylates NFAT thereby allowing this protein to enter the nucleus and act as a transcription factor. We investigated whether the interaction of calcineurin with NFAT is required for the effect of calcineurin on Fgf23 transcription. To this end, we incubated UMR106 cells with and without calcineurin-NFAT interaction inhibitor INCA-6. As shown in Fig. 4a, the presence of INCA-6 significantly lowered Fgf23 transcript levels suggesting that the effect of calcineurin on FG23 was mediated by NFAT. As expected, the FGF23 concentration was similarly decreased by INCA-6 (Fig. 4b).

CsA and FK-506 have been demonstrated to influence transcription factor complex NF-κB. Moreover, NF-κB signaling has a strong impact on FGF23 formation. We therefore tested whether treatment with CsA or FK-506 changed NF-κB expression and activity in UMR106 cells. As illustrated in Fig.

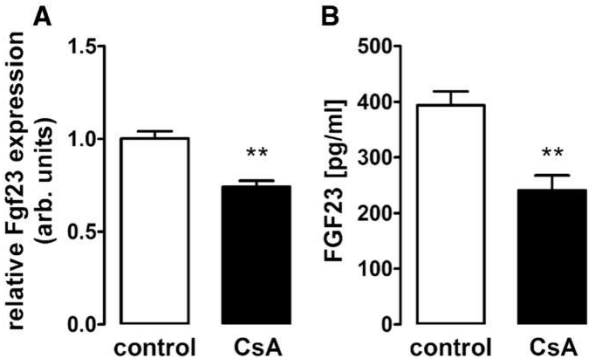

Fig. 2 Calcineurin inhibitor tacrolimus A (*CsA*) inhibits FGF23 production in UMR106 cells. UMR106 cells were treated without (*open bars*) and with 5 μM CsA (*filled bars*) for 24 h. **a** Arithmetic means ± SEM ($n = 4$) of relative Fgf23 mRNA abundance in UMR106 cells. **b** Arithmetic means ± SEM ($n = 5$) of FGF23 protein concentration in the cell culture supernatant. $^{**}p < 0.01$ indicates significant difference from control

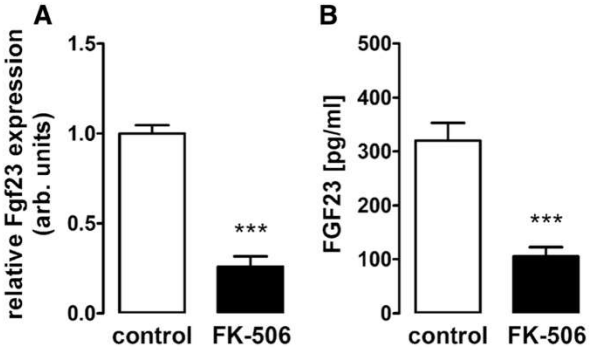

Fig. 3 Calcineurin inhibitor tacrolimus (*FK-506*) inhibits FGF23 production in UMR106 cells. UMR106 cells were treated without (*open bars*) and with 25 μM FK-506 (*filled bars*) for 24 h. **a** Arithmetic means ± SEM ($n = 3$) of relative Fgf23 mRNA abundance in UMR106 cells. **b** Arithmetic means ± SEM ($n = 5$) of FGF23 protein concentration in the cell culture supernatant. $^{***}p < 0.001$ indicates significant difference from control

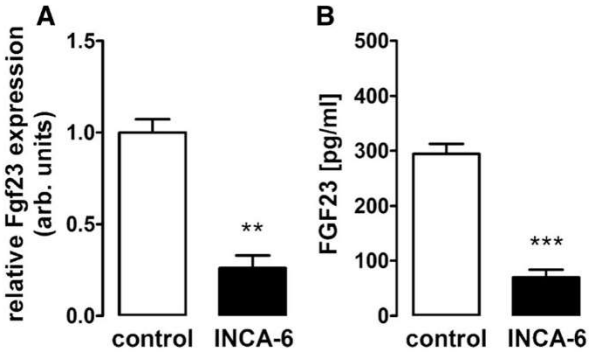

Fig. 4 A calcineurin-NFAT interaction is required for Fgf23 expression in UMR106 cells. UMR106 cells were treated without (*open bars*) and with 25 μM calcineurin-NFAT interaction inhibitor INCA-6 (*filled bars*) for 24 h. **a** Arithmetic means ± SEM (n = 3) of relative Fgf23 mRNA abundance in UMR106 cells. **b** Arithmetic means ± SEM (n = 5) of FGF23 protein concentration in the cell culture supernatant. $^{**}p < 0.01$, $^{***}p < 0.001$ indicate significant difference from control

5a, b, expression of the p65 subunit of NF-κB was not affected by CsA or FK-506. Moreover, phosphorylation of the p65 subunit of NF-κB was neither influenced by treatment with CsA (Fig. 5c, e) nor with FK-506 (Fig. 5d, f).

Discussion

We have demonstrated that calcineurin-NFAT signaling is present in UMR106 osteoblast-like cells and is relevant for the generation of FGF23. Moreover, our results suggest that calcineurin inhibitors decrease the gene expression of Fgf23, which translated into a reduced FGF23 protein concentration in the cell culture supernatant.

Calcineurin inhibitors such as ciclosporin A and tacrolimus are widely used to prevent transplant rejection (van Gelder et al. 2017). Following kidney transplantation, a dramatic decrease of the plasma FGF23 concentration has already been found (Economidou et al. 2009). Importantly, most patients in this study were treated with calcineurin inhibitors (Economidou et al. 2009). Therefore, it appears to be likely that the decreased FGF23 production in patients after kidney transplantation is not only the consequence of better renal function but also due to the inhibitory effect of calcineurin inhibitors on FGF23 formation.

Ciclosporin A and tacrolimus are also used in the treatment of patients with severe autoimmune diseases (Laurin et al. 2017). These conditions are associated with enhanced inflammation, which calcineurin inhibitors attenuate due to their immunosuppressive properties. Importantly, inflammation has also been shown to induce the synthesis of FGF23, and patients with inflammatory diseases have higher plasma FGF23 concentrations (David et al. 2017). Conversely, a high FGF23 level as observed in CKD patients may be immunosuppressive as it impairs neutrophil recruitment (Rossaint et al. 2016), and immune cells are also a source of FGF23 production (Masuda et al. 2015). It is intriguing to speculate that lower FGF23 levels in patients with autoimmune disease

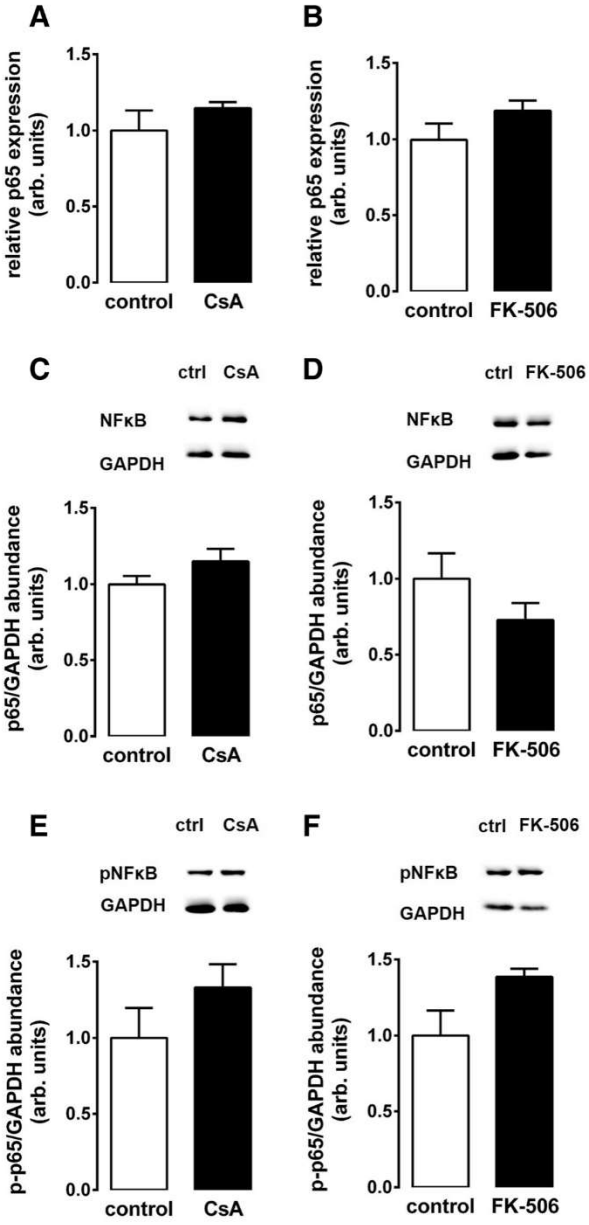

Fig. 5 Impact of calcineurin inhibitors ciclosporin A (*CsA*) and tacrolimus (*FK-506*) on expression and activation of NF-κB in UMR106 cells. UMR106 cells were treated without (*open bars*) and with (*filled bars*) 5 μM CsA (**a**, **c**, **e**) or 25 μM FK-506 (**b**, **d**, **f**) for 24 h. **a**, **b** Arithmetic means ± SEM (n = 3) of relative p65 mRNA abundance in UMR106 cells. **c**, **d** A representative western blot and arithmetic means ± SEM (n = 5) of the densitometric analysis of p65 protein expression are given. **e**, **f** A representative western blot and arithmetic means ± SEM (n = 5) of the densitometric analysis of p-p65 are shown

treated with calcineurin inhibitors are not only due to decreased inflammation but also because of the direct inhibitory effect of these drugs on FGF23 production. Definitely, further research is warranted to address this question.

A recent paper revealed that the treatment of mice with ciclosporin or tacrolimus results in an increase in the serum $1,25(OH)_2D_3$ concentration (Lee et al. 2011). FGF23 inhibits the renal formation of $1,25(OH)_2D_3$. Hence, it is likely that the inhibitory effect of calcineurin inhibitors on FGF23 uncovered in the present study is, at least in part, also responsible for the enhancement of $1,25(OH)_2D_3$ formation upon treatment with ciclosporin or tacrolimus as observed by Lee et al. (Lee et al. 2011).

In T lymphocytes, Orai1-mediated Ca^{2+} entry is part of the cellular machinery resulting in calcineurin activation, NFAT dephosphorylation, induction of transcriptional activity of NFAT, and ultimately in IL-2-stimulated T cell activation (Srikanth and Gwack 2013). In UMR106 cells, Ca^{2+} entry through Orai1 has been demonstrated to induce Fgf23 transcription (Zhang et al. 2016a). Our present study suggests that Ca^{2+} may trigger calcineurin activation in UMR106 cells as it does in T lymphocytes. Moreover, according to our experiments with calcineurin-NFAT interaction inhibitor INCA-6, the calcineurin-dependent activation of NFAT is also involved in the effect of calcineurin on Fgf23 transcription. A future study should address the signaling downstream of NFAT governing the transcriptional control of Fgf23 expression.

According to our RT-PCR analysis, NFAT1c is expressed in UMR106 osteoblast-like cells. The differentiation of osteoclasts depends on NFAT1c which has therefore a pivotal role in bone homeostasis (Asagiri et al. 2005).

Transcription factor complex NF-κB has already been demonstrated to control the formation of FGF23 (Zhang et al. 2016a) and to be inhibited by CsA and FK-506 (Du et al. 2009). However, we did not find evidence that the inhibitory effect of CsA and FK-506 on FGF23 formation was mediated by NF-κB as neither expression nor NF-κB activity were affected.

Our study did not address the complex signaling downstream of CsA and FK-506 mediating the suppressive effect on FGF23 formation. Since CsA and FK-506 affect a variety of further transcription factors and signaling molecules (Hogan et al. 2003; Serfling et al. 2006), their role in the production of FGF23 must be addressed in a future study.

Parathyroid hormone (PTH) may be oxidized at methionine residues resulting in loss of its activity (Hocher et al. 2013; Hocher and Yin 2017). FGF23 also comprises several methionine residues which may similarly be subject to oxidation with consequences for its activity. Clearly, further research must address this issue.

In conclusion, calcineurin-NFAT signaling is a powerful regulator of Fgf23 gene expression. Hence, calcineurin inhibitors CsA and tacrolimus inhibit FGF23 production.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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3.4 Paper 4: PKC regulates the production of fibroblast growth factor 23 (FGF23)

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PLOS ONE

RESEARCH ARTICLE

PKC regulates the production of fibroblast growth factor 23 (FGF23)

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Abstract

Serine/threonine protein kinase C (PKC) is activated by diacylglycerol that is released from membrane lipids by phospholipase C in response to activation of G protein-coupled receptors or receptor tyrosine kinases. PKC isoforms are particularly relevant for proliferation and differentiation of cells including osteoblasts. Osteoblasts/osteocytes produce fibroblast growth factor 23 (FGF23), a hormone regulating renal phosphate and vitamin D handling. PKC activates NFκB, a transcription factor complex controlling FGF23 expression. Here, we analyzed the impact of PKC on FGF23 synthesis. *Fgf23* expression was analyzed by qRT-PCR in UMR106 osteoblast-like cells and in IDG-SW3 osteocytes, and FGF23 protein was measured by ELISA. Phorbol ester 12-O-tetradecanoylphorbol-13-acetate (PMA), a PKC activator, up-regulated FGF23 production. In contrast, PKC inhibitors calphostin C, Gö6976, sotrastaurin and ruboxistaurin suppressed FGF23 formation. NFκB inhibitor withaferin A abolished the stimulatory effect of PMA on *Fgf23*. PKC is a powerful regulator of FGF23 synthesis, an effect which is at least partly mediated by NFκB.

OPEN ACCESS

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Introduction

Protein kinase C (PKC) isoforms are related serine/threonine kinases probably expressed in all cell types. Classically, PKC activity is induced upon stimulation of various G_q protein-coupled receptors and growth factor receptor tyrosine kinases [1]. Three classes of PKC isoforms can be distinguished: classical PKC (cPKC) isoforms are activated by both, diacylglycerol (DAG) and an increase in the intracellular Ca^{2+} concentration whereas novel PKC (nPKC) isoforms require only DAG, and atypical PKC (aPKC) isoforms are induced by other mechanism [2]. The classical activation is dependent on phospholipase Cβ or Cγ-mediated breakdown of membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) yielding inositol 1,4,5-trisphosphate (IP_3) and DAG. IP_3 binds the IP_3 receptor releasing Ca^{2+} from the endoplasmic reticulum (ER), while membrane-bound DAG activates PKC [1].

PKC is crucial for most cellular responses including the regulation of gene expression, cell migration, proliferation, differentiation, and apoptosis [3]. Moreover, PKC is implicated in the pathophysiology of frequent disorders such as heart failure, diabetes, Alzheimer and Parkinson disease, as well as inflammatory and immune disorders [3]. PKC is particularly relevant for

various malignancies, owing to its tumor and metastasis-promoting properties [3]. Plant-derived phorbol esters are potent carcinogens that are effective through stimulating PKC activity [3].

Inflammation [4,5], renal and cardiovascular disease [6–8] are major triggers of the production of fibroblast growth factor 23 (FGF23), a proteohormone mainly produced in the bone [9] and implicated in the regulation of phosphate reabsorption and $1,25(OH)_2D_3$ (active vitamin D [10]) formation in the kidney [11]. The FGF23-mediated inhibition of renal phosphate transporter NapiIIa and CYP27B1, the key enzyme for the generation of $1,25(OH)_2D_3$, is dependent on Klotho, a transmembrane protein [9]. The induction of left ventricular hypertrophy is, however, solely mediated by FGF23 without the involvement of Klotho [8,12,13], whereas vitamin D partly overcomes the FGF23 effect on cardiac hypertrophy [14]. FGF23 also impacts on neutrophil recruitment [15], erythropoiesis [16,17], or hepatic cytokine secretion [18] in a paracrine manner.

Klotho or FGF23 deficiency results in rapid aging, a very short life span, and multiple age-associated diseases affecting most organs and tissues [11,19]. This dramatic phenotype of Klotho or FGF23 null mice is almost completely rescued by a phosphate- or vitamin D-deficient diet [20,21], pointing to the predominant role of calcification in the pathophysiology of Klotho or FGF23 deficiency [22]. Apart from its endocrine and paracrine effects that are still incompletely understood, FGF23 is a putative disease biomarker [6,23]. The plasma level of FGF23 correlates well with progression of chronic kidney disease (CKD) and is a very sensitive marker that is elevated even before onset of hyperphosphatemia or hyperparathyroidism [24,25]. Furthermore, FGF23 levels are elevated in acute kidney injury [26,27]. Whether and to which extent FGF23 not only indicates disease but actively contributes to disease progression, as shown for the heart, remains unclear.

The identification of molecular regulators of FGF23 production is of high interest and relevance. Known regulators include PTH [28], $1,25(OH)_2D_3$ [29], phosphate [30,31], inflammatory cytokines and factors such as tumor necrosis factor α (TNFα) [32,33], interleukin (IL)-1/6 [4,33–35], NFκB [33,36], transforming growth factor (TGF)β [37], AMP-dependent protein kinase (AMPK) [38] or insulin-dependent PI3 kinase signaling [39].

The present study explored the contribution of PKC signaling to the production of FGF23 in bone cells.

Materials and methods

Cell culture

Cell culture and experiments with UMR106 rat osteoblast-like cells (purchased from ATCC, Manassas, VA, USA) were conducted as described before [39]. Briefly, cells were cultured in DMEM high-glucose medium containing 10% FBS and penicillin-streptomycin at 37˚C and 5% CO_2.

IDG-SW3 bone cells (purchased from Kerafast, Boston, MA, USA) were cultured as described earlier [40]. Briefly, non-differentiated cells were kept at 33˚C in AlphaMEM medium (with L-glutamine and deoxyribonucleosides) containing 10% FBS, penicillin-streptomycin and interferon-gamma (INF-γ; 50 U/ml). For differentiation, cells were plated on collagen-coated dishes at 37˚C in medium with 50 μg/ml ascorbic acid and 4 mM β-glycerophosphate but without INF-γ. All reagents were from ThermoFisher unless indicated.

IDG-SW3 osteocytes were used after 35 days of differentiation, and UMR106 cells were pretreated with 100 nM $1,25(OH)_2D_3$ (Tocris, Wiesbaden-Nordenstadt, Germany) for 24h before the experiment. Cells were then incubated with activator phorbol ester 12-O-tetradecanoylphorbol-13-acetate (PMA; Sigma, Schnelldorf, Germany; 0.1 μM; 6 h) with or without 1 μM

PKC inhibitors calphostin C (Tocris), Gö6976 (Tocris), sotrastaurin (Selleckchem, München, Germany), ruboxistaurin (Selleckchem), or NFκB inhibitor withaferin A (Tocris; 0.5 μM), or with vehicle only for another 24 h.

Expression analysis

Total RNA was extracted using peqGOLD TriFast reagent (VWR, Dresden, Germany). Complementary DNA (cDNA) was synthesized from 1.2 μg RNA using random primers and the GoScript™ Reverse Transcription System (Promega, Mannheim, Germany) at 25˚C for 5 min, 42˚C for 1 h, and 70˚C for 15 min.

The expression profile of PKC isoforms in UMR106 cells was studied by RT-PCR using the GoTaq Green Master Mix (Promega) and the primers listed in Table 1. For PCR, 2 μl synthesized cDNA were used. Settings were: 95˚C for 3 min, followed by 35 cycles of 95˚C for 30 s, 60˚C for 30s, 72˚C for 45s. PCR products were loaded on a 1.5% agarose-gel and visualized by Midori Green (Biozym, Hessisch Oldendorf, Germany), and a 100 bp DNA ladder (Jena Bioscience, Jena, Germany) was used as a size marker.

qRT-PCR

Relative expression levels of *Fgf23* and other relevant genes were determined by qRT-PCR using 2 μl synthesized cDNA (for primers see Table 2), and GoTaq qPCR Master Mix (Promega) on a Rotor-Gene Q (Qiagen, Hilden, Germany). PCR conditions were 95˚C for 3 min, followed by 35 cycles of 95˚C for 10 s, 58˚C for 30 s and 72˚C for 45 s. After normalization to *Tbp* (TATA box-binding protein) expression, relative quantification of gene expression was carried out based on the double-delta Ct (threshold cycle) method.

FGF23 ELISA (C-Term)

UMR106 osteoblast-like cell culture supernatant was collected and frozen at -80˚C. The medium was concentrated using Sartorius Vivaspin 6 Centrifugal Concentrators (Sartoritus,

Table 1. Primer sequences used for expression analysis.

name	sequence (5'-3')	product
*Pkcα*_forward	CTGAACCCTCAGTGGAATGAGT	326 bp
*Pkcα*_reverse	GGCTGCTTCCTGTCTTCTGAA	
*Pkcβ*_forward	AACGGCTTGTCAGATCCCTA	250 bp
*Pkcβ*_reverse	CCACTCCGGCTTTCTGTAGT	
*Pkcγ*_forward	AGTCCCACGGACTCCAAGAG	397 bp
*Pkcγ*_reverse	CGGCATAGAATGCTGCGTG	
*Pkcδ*_forward	TGTGAAGACTGCGGCATGAA	265 bp
*Pkcδ*_reverse	AGGTGAAGTTCTCAAGGCGG	
*Pkcε*_forward	CGAGGACGACTTGTTTGAATCC	389 bp
*Pkcε*_reverse	CAGTTTCTCAGGGCATCAGGTC	
*Pkcζ*_forward	GTGGACCCCACGACAACTT	207 bp
*Pkcζ*_reverse	GATGCTTGGGAAAACGTGGA	
*Pkcη*_forward	CCATGAAGATGCCACAGGGATC	249 bp
*Pkcη*_reverse	TCATCGATCGGAGTTAAAACAGG	
*Pkcθ*_forward	TGCCGACAATGTAATGCAGC	219 bp
*Pkcθ*_reverse	ACACTTGAGACCTTGCCTCG	
*Pkcι*_forward	CTCCTGATCCACGTGTTCCC	321 bp
*Pkcι*_reverse	GGATGACTGGTCCATTGGCA	

https://doi.org/10.1371/journal.pone.0211309.t001

Table 2. Primer sequences used for qRT-PCR.

name	sequence (5'-3')
*Tbp*_forward	ACTCCTGCCACACCAGCC
*Tbp*_reverse	GGTCAAGTTTACAGCCAAGATTCA
*Fgf23*_forward	TGGCCATGTAGACGGAACAC
*Fgf23*_reverse	GGCCCCTATTATCACTACGGAG
*Il6*_forward	CAGAGTCATTCAGAGCAATAC
*Il6*_reverse	CTTTCAAGATGAGTTGGATGG
*Tnfα*_forward	CTCACACTCAGATCATCTTC
*Tnfα*_reverse	GAGAACCTGGGAGTAGATAAG
*Nfatc1*_forward	GAAGACTGTCTCCACCACCA
*Nfatc1*_reverse	CCGATGTCTGTCTCCCCTTT
*Nfatc3*_forward	TGATGGCCTTGGATCTCAGT
*Nfatc3*_reverse	CCCTCGGCTACCTTCAGTTT
*Nfatc4*_forward	GAAAGAGATGGCTGGCATGG
*Nfatc4*_reverse	CACCTCAATCCTCAGCTCCA
*Hif1α*_forward	GAAAGGATTACTGAGTTGATGG
*Hif1α*_reverse	CAGACATATCCACCTCTTTTTG
*Phex*_forward	ATGGCTGGATAAGCAATAAC
*Phex*_reverse	GCTTTTTCAATCGCTTTCTC
*Dmp1*_forward	ACTGTTATCCTCCTTACGTTC
*Dmp1*_reverse	GGTCTATACTGGCTTCTGTC

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Göttingen, Germany). The concentration of C-terminal FGF23 was determined by an ELISA (Immutopics, San Clemente, CA, USA) according to the manufacturer's instructions.

Statistics

Arithmetic means ± SEM were calculated, and *n* represents the number of independent experiments. Comparisons of two groups were made by unpaired Student's t test, and for more than two groups, comparisons were calculated via one-way ANOVA, followed by Tukey's or Dunnett's multiple comparison tests, using GraphPad Prism. Differences were considered significant if $p < 0.05$.

Results

The relevance of PKC activity for the synthesis of FGF23 was studied in UMR106 osteoblast-like cells and IDG-SW3 osteocytes. First, the expression of *Pkc* isoforms was explored by RT-PCR. As demonstrated in Fig 1, mRNA specific for *Pkcα*, *Pkcδ*, *Pkcε*, *Pkcη*, *Pkcθ*, and *Pkcι* could readily be detected. The bands indicating the abundance of *Pkcβ*, *Pkcγ*, *Pkcζ* mRNA in UMR106 cells were weaker albeit detectable.

Phorbol ester 12-O-tetradecanoylphorbol-13-acetate (PMA) is a potent activator of PKC [3]. We treated UMR106 cells with and without PMA and determined *Fgf23* transcripts by qRT-PCR. PMA treatment significantly up-regulated the abundance of *Fgf23* mRNA (Fig 2A). As a next step, we explored whether PMA-stimulated *Fgf23* gene expression translates into enhanced FGF23 production. To this end, we determined FGF23 protein in the supernatant of UMR106 cells. As shown in Fig 2B, PMA indeed stimulated FGF23 synthesis. Similar to osteoblasts, PKC activation with PMA enhanced *Fgf23* gene expression in IDG-SW3 osteocytes (Fig 2C). These results suggest that PKC activity drives *Fgf23* gene expression in osteoblasts and osteocytes.

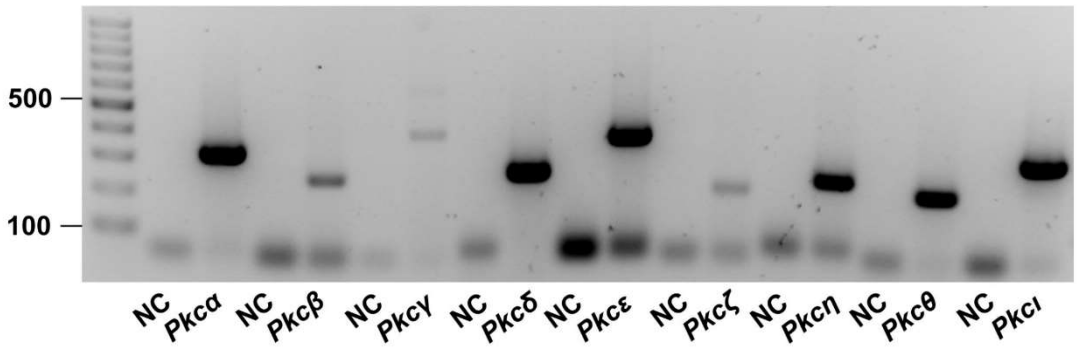

Fig 1. Expression of *Pkc* isoforms in UMR106 osteoblast-like cells. Original agarose gel photo showing *Pkcα*, *-β*, *-γ*, *-δ*, *-ε*, *-ζ*, *-η*, *-θ or -ι* specific cDNA in UMR106 cells. NC: non-template control.

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Our next series of experiments tested whether inhibition of PKC interferes with FGF23 expression. To this end, UMR106 cells were exposed to PKC inhibitors. As demonstrated in Fig 3, PKC inhibitor calphostin C (Fig 3A) and also PKCα/β inhibitor Gö6976 (Fig 3B) significantly and dose-dependently down-regulated *Fgf23* gene expression in UMR106 cells. PKCα/β inhibitor Gö6976 also lowered the FGF23 protein concentration in the cell culture supernatant (Fig 3C). Thus, PKC is a stimulator of FGF23 expression.

We investigated whether PMA-stimulated *Fgf23* gene expression is indeed dependent on PKC activity using UMR106 and IDG-SW3 cells. As demonstrated in Fig 4, the PMA effect on *Fgf23* gene expression was completely abrogated by PKC inhibitor Gö6976 in UMR106 osteoblast-like cells (Fig 4A) and in IDG-SWR3 osteocytes (Fig 4B), and also by PKC inhibitors sotrastaurin (Fig 4C) and ruboxistaurin (Fig 4D) in UMR106 cells.

Finally, we sought to identify the mechanism of PKC-dependent FGF23 regulation. Since PKC is an activator of NFκB [41], a known regulator of FGF23, we treated UMR106 cells with and without NFκB inhibitor withaferin A in the absence and presence of PMA and determined

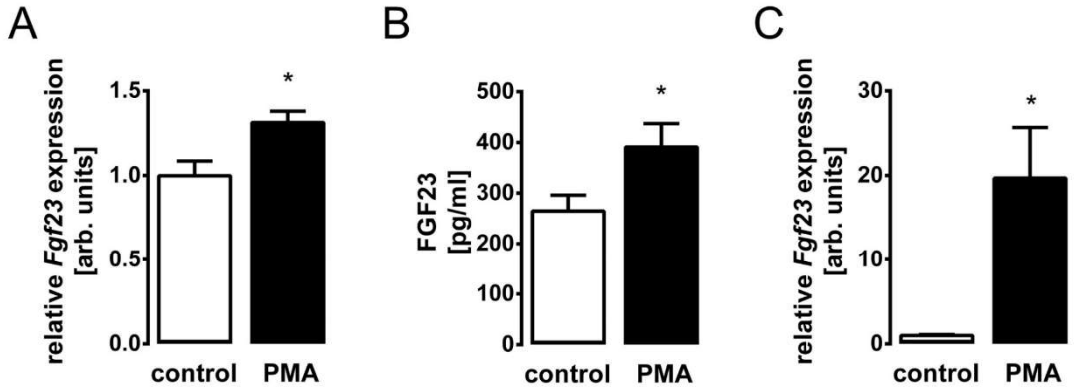

Fig 2. PKC activator PMA induces FGF23 production in UMR106 osteoblast-like cells and in IDG-SW3 osteocytes. Arithmetic means ± SEM (n = 6) of relative *Fgf23* mRNA abundance normalized to *Tbp* in UMR106 osteoblast-like cells (**A**) or IDG-SW3 osteocytes (**C**), and FGF23 concentration in the cell culture supernatant of UMR106 cells (**B**) incubated without (white bars) or with (black bars) 0.1 μM PKC activator PMA. * $p < 0.05$ indicates significant difference. arb., arbitrary.

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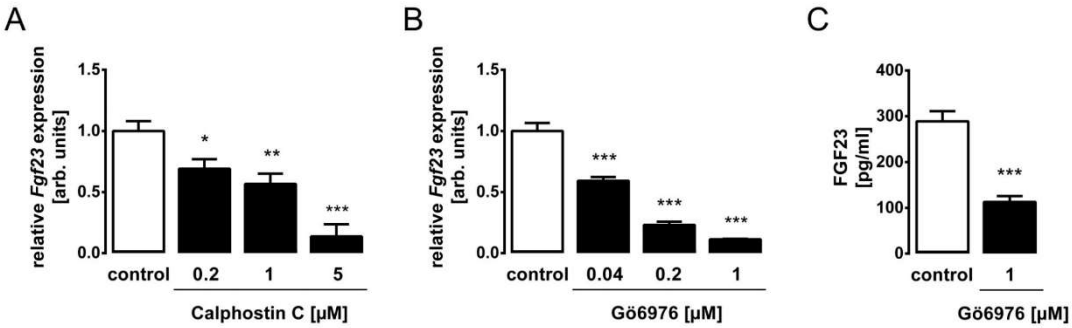

Fig 3. PKC inhibitors Calphostin C and Gö6976 decrease FGF23 expression levels in UMR106 osteoblast cells. UMR106 cells were treated without and with PKC inhibitors Calphostin C (**A**) or Gö6976 (**B, C**) at the indicated concentrations. Arithmetic means ± SEM (n = 6) of the relative *Fgf23* mRNA abundance in UMR106 cells (**A, B**). Gene expression was normalized to *Tbp* as housekeeping gene. Arithmetic means ± SEM (n = 6) of FGF23 protein concentration in the cell culture supernatant (**C**). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ indicate significant difference. arb., arbitrary.

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Fgf23 gene expression. As demonstrated in Fig 5A, the PMA effect was indeed abrogated by withaferin A. Hence, PKC was, at least in part, effective through NFκB activity. The pro-inflammatory cytokines TNFα [32,33] and IL-6 [33,35] are also regulators of FGF23 synthesis. We therefore checked whether PKC activity impacts on their expression. According to Fig 5B and 5C PKC activation resulted in enhanced gene expression of *Tnfα* and *Il-6* in UMR106 cells.

Further transcription factors involved in FGF23 production include NFAT [42,43] and HIF-1α [44–46]. Therefore, we tested whether PKC induces the expression of these transcription factors. As a result, incubation of UMR106 cells with 0.1 μM PMA did not significantly affect *Nfatc1* mRNA (control: 1.0 ± 0.02; PMA: 1.03 ± 0.02; n = 6), *Nfatc4* mRNA (control: 1.0 ± 0.03; PMA: 1.02 ± 0.06; n = 6) or *HIF-1α* mRNA (control: 1.0 ± 0.02; PMA: 1.0 ± 0.05; n = 6). The abundance of *Nfat3* mRNA was significantly down-regulated upon incubation with PMA (control: 1.0 ± 0.02; PMA: 0.79 ± 0.03; $p < 0.001$; n = 6).

The peptidase PHEX (phosphate regulating gene with homologies to endopeptidases on the X chromosome) and matrix protein DMP1 (dentin matrix protein-1) are further important regulators of FGF23 [47,48]. PKC activation did not affect *Phex* mRNA (control: 1.0 ± 0.02; PMA: 1.14 ± 0.04; n = 6) but significantly up-regulated *Dmp1* mRNA (control: 1.0 ± 0.02; PMA: 255.76 ± 12.82; $p < 0.001$; n = 6).

Discussion

Our study discloses PKC as a novel regulator of FGF23 production. We provide experimental evidence that activation of PKC induces and inhibition of PKC suppresses *Fgf23* gene expression: Four different PKC inhibitors were similarly capable of completely blocking PMA-induced *Fgf23* expression. Moreover, altered *Fgf23* gene expression translated into protein secretion as demonstrated by FGF23 protein measurements in the cell culture supernatant. These results unequivocally demonstrate the powerful role of PKC in regulating FGF23 production.

FGF23 is mainly produced by osteoblasts/osteocytes in the bone [9]. The differentiation of osteoblasts is driven by transcription factor MSX2 [49]. PKC enhances the proliferation of osteoblasts [50]. In contrast, PKC inhibits the differentiation of osteoblasts by targeting MSX2

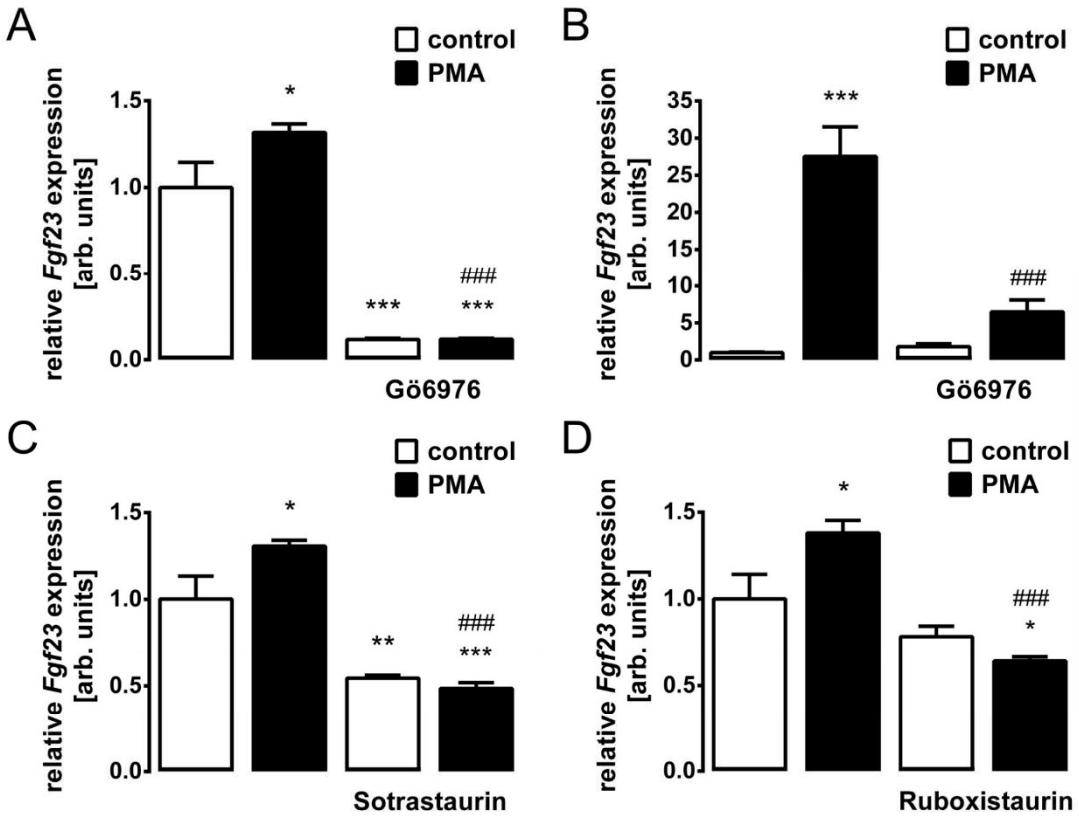

Fig 4. PKC inhibition abrogates the PMA-induced increase in *Fgf23* gene expression in UMR106 osteoblast-like cells and in IDG-SW3 osteocytes. Relative *Fgf23* transcript levels in UMR106 cells (**A,C,D**) or in IDG-SW3 cells (**B**) incubated without or with PMA (0.1 μM, **A-D**) in the absence and presence of PKCα/β inhibitor Gö6976 (1 μM, **A,B**), pan PKC inhibitor Sotrastaurin (1 μM, **C**) or PKCβ inhibitor Ruboxistaurin (1 μM, **D**). Gene expression was normalized to *Tbp* as a housekeeping gene, and the values are expressed as arithmetic means ± SEM (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ indicate significant difference from vehicle (first bar). $^{\#\#\#}p < 0.001$ indicates significant difference from the absence of PKC inhibitor (second bar vs. fourth bar). arb., arbitrary.

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[51] whereas FGF23 induces MSX2. [52]. Transcriptional activity of Runx2, also implicated in osteoblast differentiation, is enhanced by both PKC [53] and FGF23 [54].

We could show that four different pharmacological PKC inhibitors potently suppressed *Fgf23* gene expression. Enhanced PKC activity contributes to the pathophysiology of several disorders, and pharmacological PKC inhibition has therefore been suggested as a therapeutic approach in multiple diseases including cancer, sequelae of diabetes, cardiovascular diseases, or inflammatory disorders such as psoriasis [55]. Some of these diseases, particularly renal and cardiovascular diseases, are associated with elevated FGF23 plasma levels [6], and FGF23 not only indicates disease, but actively contributes at least to left heart hypertrophy [12,13]. It therefore appears to be possible that the therapeutic benefit of PKC inhibition in some of these diseases is at least in part also due its FGF23-lowering properties.

Two different cell lines representing both osteoblasts and mature osteocytes were used in our study to decipher the effect of PKC on FGF23: PKC activation with PMA enhanced

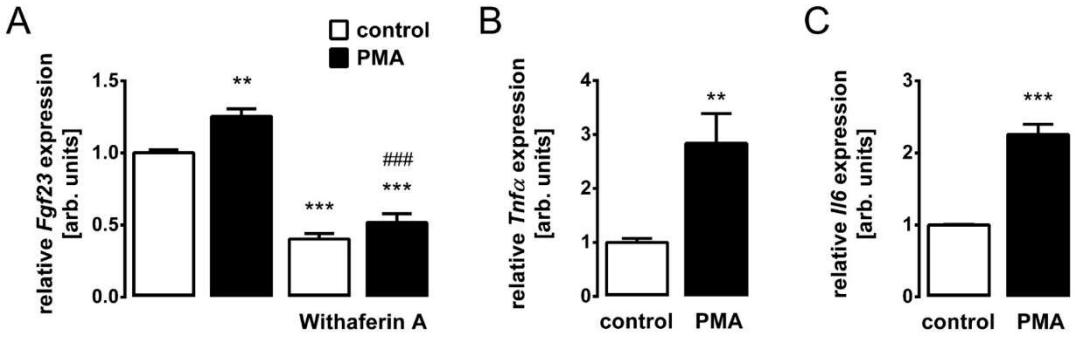

Fig 5. The PKC effect on *Fgf23* gene expression is dependent on inflammation. Relative *Fgf23* transcript levels in UMR106 cells incubated without or with PMA (0.1 μM) in the absence and presence of NFκB inhibitor Withaferin A (0.5 μM) (**A**). Relative *Tnfα* (**B**) and *Il6* (**C**) transcript levels in UMR106 cells incubated without or with PMA (0.1 μM). Gene expression was normalized to *Tbp* as a housekeeping gene, and the values are expressed as arithmetic means ± SEM (n = 6). $^{**}p < 0.01$, and $^{***}p < 0.001$ indicate significant difference from vehicle (first bar). $^{###}p < 0.001$ indicates significant difference from the absence of withaferin A (second bar vs. fourth bar). arb., arbitrary.

https://doi.org/10.1371/journal.pone.0211309.g005

and PKC inhibition with different PKC inhibitors suppressed *Fgf23* gene expression in both UMR106 osteoblast-like cells and IDG-SW3 osteocytes. This result suggests PKC-dependent regulation of FGF23 formation as a universal mechanism for the control of this hormone.

In an attempt to identify the underlying mechanism, we found that PKC-mediated up-regulation of *Fgf*23 expression is sensitive to NFκB inhibition. NFκB, as a p65/p50 dimer, is inactivated by binding inhibitory κB (IκB). The inhibitory κB kinase (IKK) phosphorylated IκB, leading to its ubiquitination and degradation, paralleled by the release and activation of NFκB [56]. Therefore, the activation of the IKK complex plays a crucial role in the induction of NFκB, and PKC activates NFκB through this kinase [41]. Clearly, inflammation is a major trigger of FGF23 production [4,36]. In detail, NFκB up-regulates Ca^{2+} release-activated Ca^{2+} (CRAC) channel Orai1/STIM1 accomplishing store-operated Ca^{2+} entry (SOCE) and inducing the transcription of the *Fgf*23 gene [36]. According to our experiment with NFκB inhibitor withaferin A, the PKC effect on FGF23 was, at least partly, dependent on NFκB pointing to the decisive role of this pro-inflammatory transcription factor complex in the regulation of FGF23. Moreover, PKC activation induced the expression of pro-inflammatory cytokines TNFα and IL-6 which are themselves stimulators of FGF23 production [33]. Therefore, the enhanced formation of these cytokines may contribute to the PKC effect on FGF23. NFAT isoforms and HIF1α, further regulators of FGF23, were not affected by PKC activity in UMR106 cells.

We observed a PKC-mediated up-regulation of FGF23 regulator DMP1 in UMR106 cells which would be expected to lower FGF23 levels. Possibly, the PKC effect on FGF23 involving NFκB overcomes the expected down-regulation of FGF23 due to PKC-mediated DMP1 induction. Alternatively, enhanced PKC-stimulated FGF23 formation is limited by counter regulatory DMP1 induction. Clearly, further studies are warranted to address this question. In addition, since our study was carried out *in vitro* only, its *in vivo* relevance will also have to be verified separately.

In conclusion, our study identified PKC as a novel regulator of FGF23 production in both osteoblast and osteocytes, being at least partially effective via NFκB signaling. Therefore, the therapeutic modulation of PKC activity in chronic disease may impact on the plasma FGF23 concentration.

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Supervision: Michael Föller.

Writing – original draft: Ludmilla Bär, Michael Föller.

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3.5 Paper 5: Regulation of fibroblast growth factor 23 (FGF23) in health and disease.

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Regulation of fibroblast growth factor 23 (FGF23) in health and disease

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Abbreviations

$1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; ADHR/ARHR, autosomal dominant/recessive hypophosphatemic rickets; ADPKD, autosomal dominant polycystic kidney disease; AGEs, advanced glycation end products; AKI, acute kidney injury; AMPK, AMP-activated protein kinase; ANKH, progressive ankylosis protein homolog; ASARM, acidic serine-aspartate rich MEPE-associated motif; BMP1, bone morphogenetic protein 1; CKD, chronic kidney disease; CRAC, calcium release-activated calcium channel; DMP1, dentin matrix protein-1; ENPP1, ectonucleotide pyrophosphatase/phosphodiesterase 1; EPO, erythropoietin; FAM20C, family with sequence similarity 20, member C; FGF, fibroblast growth factor; FGFR, FGF receptor; FTC, familial tumoral calcinosis; GALNT3, N-acetylgalactosaminyltransferase 3; GNAS1, G protein alpha subunit S1; GSK3, glycogen synthase kinase 3; HIF-1α, hypoxia-inducible factor 1-α; IGF1, insulin-like growth factor 1; JMS, Jansen's metaphyseal chondrodysplasia; LPS, lipopolysaccharides; LVH, left ventricular hypertrophy; MAS/FD, McCune-Albright syndrome/Fibrous dysplasia; NaPi, sodium-phosphate cotransporters; NCC, NaCl cotransporter; NFAT, nuclear factor of activated T-cells; OSR1, oxidative stress responsive kinase 1; PAI-1, plasminogen activator inhibitor-1; PC2, proprotein convertase 2; PHEX, phosphate regulating gene with homologies to endopeptidases on the X chromosome; PTH, parathyroid hormone; ROS, reactive oxygen species; SOCE, store-operated Ca^{2+} entry; SPAK, Ste20-related proline-alanine-rich kinase; SPC, furin/subtilisin-like proprotein convertase; TGFβ, transforming growth factor β; TIO, tumor-induced osteomalacia; VDR, vitamin D receptor; WNK, with no lysine/K kinase; XLH, X-linked hypophosphatemia.

Keywords: FGF23, phosphate, inflammation, Ca^{2+}, Klotho

Abstract

Fibroblast growth factor 23 (FGF23) is mainly produced in the bone and, upon secretion, forms a complex with an FGF receptor and co-receptor αKlotho. FGF23 can exert several endocrine functions, such as inhibit renal phosphate reabsorption and 1,25-dihydroxyvitamin D3 production. Moreover, it has paracrine activities on several cell types, including neutrophils and hepatocytes. Klotho and Fgf23 deficiencies result in pathologies otherwise encountered in age-associated diseases, mainly as a result of hyperphosphatemia-dependent calcification. FGF23 levels are also perturbed in the plasma of patients with several disorders, including kidney or cardiovascular diseases. Here, we review mechanisms controlling FGF23 production and discuss how FGF23 regulation is perturbed in disease.

Introduction

Phosphate is implicated in a wide variety of cellular functions including signal transduction, energy exchange, membrane biology, and the organization of the endoskeleton. In humans, approximately 85% of total phosphorus is found in bones and teeth [1, 2], the rest being part of nucleic acids, cell membrane phospholipids, energy-rich molecules (e.g. ATP), phosphoproteins, and as inorganic phosphate (Pi) in the serum. The serum phosphate level is kept within the physiological range through the coordination of intestinal absorption, bone deposition and renal excretion [1–3]. Hyperphosphatemia (i.e. elevated serum phosphate concentration) has been associated with cardiovascular disease and cardiac mortality, presumably due to associated vascular calcification through increased deposition of poor soluble calcium-phosphate in cardiovascular structures [4–8]. Therefore, the serum phosphate concentration must be tightly controlled. A circulating factor with the potential to regulate phosphate levels was first identified in studies with bone tumor patients that exhibited rickets and increased renal phosphate excretion was described in 1959 [9]. Later, Harrison postulated that a certain bone tumor releases a phosphaturic hormone responsible for rickets [10]. In the 1990s, studies with hypophosphatemic (Hyp)-mice reported the presence of a circulating factor that, upon transfer, could cause renal phosphate loss and hypophosphatemia, as well as suppress the synthesis of 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) in wild type mice [11]. Later, this factor was identified as fibroblast growth factor 23 (FGF23); gain of function mutations at ^{176}Arg and ^{179}Arg of FGF23 were associated with autosomal dominant hypophosphatemic rickets (ADHR) [12]. Shortly thereafter, FGF23 was also disclosed as the cause of tumor-induced osteomalacia (TIO), thereby confirming its role in phosphate metabolism [13–16].

The endocrine subfamily of fibroblast growth factors (FGF)

FGF23 is a 32 kDa glycoprotein and belongs to the superfamily of fibroblast growth factors (FGF). The 22 members of the FGF superfamily are involved in a plethora of biological processes, including development, organogenesis, and metabolism, and have been phylogenetically subdivided into seven subfamilies [17, 18]. FGF23 is part of the endocrine subfamily, along with FGF19 and FGF21 [17, 18]. All three are characterized by a divergent structure of the highly conserved heparin (HS)-binding domain, which is characteristic for prototypical paracrine FGFs and necessary for membrane receptor binding. Reduced HS-binding affinity allows for the release of FGF23 into the circulation [19]. FGF23, FGF19 and FGF21 can all bind FGF receptors (FGFR) with high affinity upon conjunction with the respective transmembrane Klotho protein (α, β, or γKlotho) [18–20].

FGF23 mainly binds to FGFR1c, FGFR3c and FGFR4, and αKlotho increases the binding affinity [21–23]. αKlotho acts as a scaffold protein bringing FGFRs and FGF23 together to form a 1:1:1 complex [24]. Whereas FGFRs are abundantly expressed throughout the body, tissue expression of αKlotho is tightly regulated [25], and therefore determines the effect of FGF23 on target organs. Apart from transmembrane αKlotho a soluble form exists generated by alternative splicing [26, 27] or cleavage of Klotho by membrane-bound proteolytic enzymes [28, 29], which could expand the target organs of FGF23.

FGF23 can be proteolytically cleaved between Arg^{179} and Ser^{180}, and cleavage releases inactive N- and C-terminal fragments [13]. Full length, intact FGF23 (iFGF23) is biologically active. C-terminal FGF23 (cFGF23) in excess inhibits FGF23-effects [30]. The mutation

causative for ADHR is within the consensus sequence ^{176}RXXR^{179} recognized by furin/subtilisin-like proprotein convertase (SPC) [12, 31] and prevents FGF23 cleavage, resulting in enhanced full-length FGF23 without change of its expression [32, 33]. Hence, cleavage significantly contributes to FGF23 biology.

FGF23: a phosphaturic hormone

FGF23 is mainly produced in the bone by osteoblasts and osteocytes [34, 35] and has predominantly endocrine, but also paracrine effects. Recent evidence showed that locally secreted FGF23 may act as a regulator of bone mineralization in osteocytes in an autocrine/paracrine manner [36, 37] and also the local secretion of FGF23 in the heart has putative paracrine functions [38]. As a hormone, it mainly acts in the kidney, owing to the strong expression of αKlotho in this organ [25], but may also have an effect in the parathyroid gland, also expressing αKlotho [39].

The regulation of renal phosphate excretion and $1,25(OH)_2D_3$ formation is among the best studied endocrine effects of FGF23: Administration of recombinant FGF23 to mice leads to a decrease in serum phosphate level. In addition, the implantation of FGF23-producing cells into nude mice causes hypophosphatemia, phosphaturia, osteomalacia and a decrease of the $1,25(OH)_2D_3$ concentration [13]. Similarly, overexpression of FGF23 in transgenic mice results in ADHR-like symptoms and reduced surface expression of type II sodium-phosphate cotransporter NaPi-2a in the epithelial cells of the renal proximal tubules accounting for enhanced urinary Pi excretion [40].

Moreover, FGF23 suppresses the synthesis and induces the degradation of $1,25(OH)_2D_3$. In detail, FGF23 decreases the renal mRNA level of 25-hydroxyvitamin D-1α-hydroxylase (*Cyp27b1)*, the key enzyme for the production of active $1,25(OH)_2D_3$ from 25(OH)D. In addition, FGF23 induces the expression of 24-hydroxylase (*Cyp24a1*), which inactivate $1,25(OH)_2D_3$ [41]. Via its suppressive effect on $1,25(OH)_2D_3$ formation, FGF23 also impacts on expression of NaPi-2b in the small intestine, thereby leading to decreased intestinal Pi absorption and further lowering the serum phosphate level [42].

Due to appreciable αKlotho expression, the parathyroid gland is another important target organ for FGF23 [25, 39]. Parathyroid hormone (PTH) is an important regulator of renal phosphate absorption as, similarly to FGF23, it can down regulate apical NaPi-2a/c expression in renal epithelial cells of the proximal tubule [43, 44]. Moreover, PTH is a powerful regulator of $1,25(OH)_2D_3$, as it induces gene expression of *CYP27B1* [45]. FGF23 has been shown to inhibit PTH synthesis and secretion directly through the MAPK pathway both in animals and *in vitro* [39, 46]. However, in particular chronic kidney disease (CKD) is widely known to be associated with elevated serum PTH [33, 47–50]. One aspect explaining this discrepancy could be that FGF23 can regulate PTH predominantly under physiological conditions and less in diseases for hitherto unknown reasons. Moreover, common tests do not discriminate between non-oxidized and oxidized PTH. The latter may be of reduced biological activity. Therefore, it could be that total PTH is elevated in CKD with most of it being oxidized with limited activity as this disease is linked to a reactive oxygen species (ROS) burden. [51]. In addition, reduced Klotho expression in CKD may account for the failure of increased FGF23 levels to prevent hyperparathyroidism [52, 53]. Moreover, the impact of calcium and $1,25(OH)_2D_3$ on PTH

secretion dominates over that of FGF23, and therefore, at least in CKD, the FGF23 effect on PTH is of minor relevance [49].

FGF23 regulation: insights from rare disorders and renal disease

FGF23 biology is disturbed in a number of pathological conditions that are either triggered by defects in the FGF23 pathway or involve FGF23 dysregulation as a secondary phenomenon. The disturbed endocrine FGF23 effect on the phosphate and vitamin D balance plays a major role in these processes. Table 1 summarizes these diseases and clinical conditions associated with altered FGF23 levels. Figure 1 graphically illustrates the effect of these and other mediators, which will be discussed further below.

FGF23 hyperfunction disorders with hypophosphatemia

Much can be learned about the molecular regulation of FGF23 production and secretion from rare forms of rickets including: autosomal dominant/recessive hypophosphatemic rickets (ARHR), X-linked hypophosphatemia, McCune-Albright syndrome (MAS)/Fibrous dysplasia (FD) and tumor-induced osteomalacia [54].

An activating mutation in the gene encoding the *G protein alpha subunit S1* (*GNAS1*, $G_s\alpha$), accounts for MAS/FD. As a consequence, cAMP/protein kinase A signaling is permanently active [55–57]. In addition, FD is characterized by permanent stimulation of FGF23 production due to unlimited cAMP/protein kinase A signaling [34, 58]. The exact mechanism of cAMP-regulated FGF23 production is unclear. In FD, FGF23 cleavage is altered. Hence, elevated cFGF23 and decreased intact FGF23 levels are observed which may be due to less glycosylation and enhanced furin-dependent cleavage [58].

XLH is linked to a loss-of-function mutation of the membrane-bound metalloendopeptidase *PHEX* (phosphate regulating gene with homologies to endopeptidases on the X chromosome) [50, 15, 59, 60]. Although it is a peptidase expressed in bone, PHEX does not cleave FGF23 [61]. Instead, PHEX regulates *Fgf23* gene expression [59, 60, 62] through FGF receptor signaling, a finding corroborated by the fact that FGFR inhibitor SU5402 prevents the increase in Fgf23 mRNA expression in bone marrow stromal cells from HYP mice [63].

The lack of PHEX interaction partner dentin matrix protein-1 (DMP1) with a common acidic serine-aspartate rich MEPE-associated motif (ASARM) [64] causes ARHR1 and stimulates FGF23 production [65–68]. The high FGF23 concentration of *Dmp1*-deficient mice but not of Phex-deficient (Hyp) mice is corrected by the overexpression of Dmp1 ($Dmp1^{Tg}$) [67]. Hyp/$Dmp1^{Tg}$ mice exhibit a reduced bone expression of *Fgf23*. Nevertheless, the serum FGF23 levels and the ratio of full-length/degraded FGF23 are elevated. Thus, PHEX and DMP1 need to interact to lower total plasma FGF23 [67]. Moreover, DMP1 inhibits *Fgf23* gene expression in cell culture studies [69, 70]. Possibly, DMP1 binds to PHEX through ASARM and to cell surface αvβ3-integrin thus decreasing FGF23 [71]. DMP1-dependent FGF23 regulation may involve focal adhesion kinase and ultimately ERK and JNK signaling activation [72, 73], since *Fgf23* gene expression is also dependent on actin-regulating Rac1/PAK1 signaling and the re-organization of the actin cytoskeleton [74]. Proprotein convertase 2 (PC2) cleaves FGF23, and pharmacologically inhibited PC2 activity results in decreased FGF23 degradation and increased *Fgf23* mRNA and protein. Mechanistically, decreased PC2 activity leads to a reduced abundance of active bone morphogenetic protein 1 (BMP1) subsequently

reducing the cleavage of DMP1. This causes an increase in *Fgf23* mRNA [70]. However, the exact role of the different convertases in FGF23 cleavage in osteocytes is still unclear, as in vitro PC1/3, PC5/6 and furin are able to cleave FGF23 in addition to PC2 [75]. The transport of FGF23 prevents PC2-mediated cleavage in vivo. Rather, PC5/6 seems to mediate the processing of FGF23 [75].

ARHR2 with high FGF23 levels results from inactivating mutations of ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*) [76–79], a transmembrane glycoprotein producing interstitial pyrophosphate (PPi), which is generated by chondrocytes and osteoblasts, regulates gene expression and inhibits calcium-phosphate deposition and therefore calcification [80, 81]. An *ANKH* (progressive ankylosis protein homolog) mutation causing reduced PPi transport is also associated with enhanced FGF23 synthesis [82]. Obviously, inactivation of either genes leads reduced PPi tissue levels, resulting in enhanced calcification due to increased calcium-phosphate deposition, and in addition to phosphate depletion in bone and increased FGF23 expression. Also, mutations of the *FAM20C* (Family with sequence similarity 20, member C) were identified as a cause of hypophosphatemia in ARHR3 [83]. Mice deficient for *Fam20c* exhibit FGF23-induced rickets and downregulation of DMP1. Hence, FAM20C may downregulate FGF23 expression through DMP1 [84]. The FAM20C protein directly phosphorylates FGF23 at Ser180 in the SPC consensus sequence preventing its GALNT3-mediated O-glycosylation and instead promoting its inactivation [85]. In contrast, PC5/6 and furin cleave FGF23 irrespective of its phosphorylation status. This points to glycosylation but not phosphorylation prevening FGF23 cleavage [75].

An activating mutation of the *FGFR1* gene is the underlying cause of osteoglophonic dysplasia, another form of FGF23-induced autosomal-dominant hypophosphatemia [86]. Actually, FGF receptor activation enhances FGF23 production [63, 87–93] whereas its inhibition lowers FGF23 levels [63, 91–94] suggesting a positive feedback mechanism. The essential co-receptor Klotho is also expressed in bone [90, 95], and FGF23 treatment increased its own expression in the presence of αKlotho [90]. A rare disease caused by a translocation between chromosome 9 and 13 (t(9,13)(q21.13:q13.1)) results in enhanced plasma αKlotho and FGF23 levels as well as hypophosphatemia [96], suggesting that αKlotho drives FGF23 expression. Alternatively, ectopic αKlotho has no organ specificity thus explaining more intense bone-FGFR/αKlotho expression rather than directly stimulating FGF23 expression [97]. As a matter of fact, *Klotho* null mice have a high FGF23 serum level which argues against a direct stimulatory effect of αKlotho on FGF23 [98–101], but suggests enhanced FGF23 production to compensate for FGF23 resistance in Klotho deficiency

FGF23-related disorders with hyperphosphatemia

Autosomal recessive familial tumoral calcinosis (FTC) is characterized by hyperphosphatemia, calcifications, as well as enhanced $1,25(OH)_2D_3$ and total FGF23 serum concentrations contrasting hardly detectable uncleaved intact FGF23 [54, 102–104]. The disease is due to a loss-of-function mutation in the genes *GALNT3* or *FGF23*. GALNT3 is an enzyme that O-glycosylates FGF23 at T^{178} in the SPC consensus sequence. GALNT3 deficiency and subsequent lack of glycosylation favors proteolysis of FGF23. As a consequence, less intact protein is secreted despite increased expression [103–107]. Mutations of the *FGF23* gene affecting proteolysis and resulting in less secretion of the intact hormone

have been described [108–112]. The same holds true for O-glycosylation [113]. Missing glycosylation as a direct consequence of a mutation or due to mutation-induced changes in the protein conformation may result in failure to secrete the protein or in accelerated clearance upon secretion [113–115]. According to case reports a missense mutation in *αKlotho* [116] and FGF23 autoantibodies [117] can also be the cause of FTC-associated symptoms.

FGF23 hyperfunction disorders with hyperphosphatemia and extra-osseous sources of FGF23

Patients with advanced stage of chronic kidney disease (CKD) have high FGF23, PTH and phosphate concentrations whereas $1,25(OH)_2D_3$ is low. With disease progression, FGF23 continues to increase [48, 118–127]. The same holds true for rodent disease models [90, 128–132]. FGF23 is a very sensitive biomarker for impaired kidney function [133]. Impaired renal function in CKD or acute kidney injury (AKI) is a major stimulus of FGF23 production [134–139]. In animal models of AKI, FGF23 rises within hours and before onset of hyperphosphatemia [139–141].

Further renal conditions including fibrosis [142], albuminuria [143] and autosomal dominant polycystic kidney disease (ADPKD) [144–146] are associated with elevated FGF23 levels. Also, impaired liver function [147] and lung disease [148–150] stimulates FGF23 production. Patients with heart failure [151, 152] or atherosclerosis [153–156] have higher FGF23 levels. Left ventricular hypertrophy (LVH) promotes FGF23 production through activation of the nuclear factor of activated T-cells (NFAT) pathway [157]. A strong correlation between increased FGF23 and LVH has been observed [99, 158–161]. Finally, high FGF23 is an independent risk factor for mortality, cardiovascular disease, and CKD progression [162–167].

Apart from bone [34, 35], FGF23 expression is detectable in thymus, brain, spleen, heart to name a few [13, 61]. Thus, it appears to be possible that the expression of FGF23 in these and other tissues contributes to enhanced FGF23 formation in some of the above-mentioned diseases. In AKI the elevated FGF23 plasma level [140] does not seem to be solely due to enhanced expression in bone. Instead, 5- to 15-fold upregulated *Fgf23* mRNA can be found in thymus, spleen and heart [168]. $1,25(OH)_2D_3$ and inflammation have been postulated to trigger FGF23 synthesis in AKI [168]. Moreover, the spleen is also a source of *Fgf23* during inflammation [169].

Renal FGF23 expression occurs in CKD [146, 170], but has been suggested to be irrelevant for the elevated plasma FGF23 concentration typical of this condition [170]. Local FGF23 production regulating fibroblasts in a paracrine manner occurs in injured kidneys [171].

Left heart hypertrophy drives myocardial FGF23 synthesis [157]. Conversely, FGF23 induces left heart hypertrophy [99] through FGFR4-dependent NFAT signaling in a paracrine manner [99, 172, 38]. The renin-angiotensin-aldosterone system activates FGF23 expression in cardiac myocytes [98, 157, 173].

Homeostatic regulation of FGF23

Regulation of FGF23 by phosphate and vitamin D metabolism

Primarily, bone-derived FGF23 is regulated by feedback loops involving the phosphate status, $1,25(OH)_2D_3$ and PTH, all determinants of the serum phosphate concentration and key regulators of FGF23. Dietary phosphate or the administration of $1,25(OH)_2D_3$, rapidly increases

the serum FGF23 level in rodents and humans [98, 175–186] (Fig. 1). Extracellular phosphate and $1,25(OH)_2D_3$ also induce *Galnt3* expression, which could be expected to counteract FGF23 degradation, as well as *Dmp1*, *Phex* and *Enpp1* genes, negative regulators of FGF23. Hence, phosphate and $1,25(OH)_2D_3$ may not only be involved in the translational, but also posttranslational regulation of FGF23 forming a feedback loop [181]. Therefore, local factors regulating FGF23 underlie an interdependence with the systemic regulators phosphate and 1,25(OH)2D3. FGF23 lowers both, the serum 1,25(OH)2D3 and phosphate concentration thereby completing the negative feedback loop [13, 41, 40] (Fig. 1).

The impact of PTH on FGF23 is somewhat controversial. According to some studies including cell culture experiments PTH induces FGF23 expression [95, 187–191], presumably by induction of the orphan nuclear receptor Nurr1 [192]. In line with this, high FGF23 is observed in patients with hyperparathyroidism [193–195] or with Jansen's metaphyseal chondrodysplasia (JMS; a rare autosomal dominant disorder caused by activating mutations of the PTH/PTHrP receptor) [196] and McCune-Albright syndrome (MAS)/Fibrous dysplasia (FD) (activating PTH signaling downstream of the receptor) [34, 58]. Others found an FGF23-lowering effect [69, 197] or no effect of PTH on FGF23 [182, 198]. Mice deficient for the PTH1 receptor in the proximal tubule are characterized by an elevated FGF23 level suggesting that PTH inhibits FGF23 [199]. The conflicting results may, at least in part, be explained by the fact that both PTH and FGF23 independently regulate $1,25(OH)_2D_3$, serum phosphate, and calcium which are all also independent regulators of PTH and FGF23. Moreover, the impact of PTH on FGF23 in pathological conditions such as hyperparathyroidism may not reflect the effect under physiological conditions since the disease causes secondary changes that may again impact on FGF23. It appears to be possible that the decline of renal function rather than hyperparathyroidism accounts for the surge in FGF23 in CKD [195, 118].

Dietary Ca^{2+} stimulates FGF23 production [98, 179, 200–202], and a Ca^{2+} deficit lowers FGF23 [202–204]. Ca^{2+}-dependent regulation of FGF23 seems to be independent of PTH/vitamin D [200, 179] and possibly be due to a direct effect on *Fgf23* gene expression [98, 200–202] via calcium channels [179, 202]. Ca^{2+} signaling is a major driver of FGF23 production: store-operated Ca^{2+} entry (SOCE) via calcium release-activated calcium channel (CRAC) Orai1 enhances gene expression of *Fgf23* [205–207]. *Fgf23* gene expression may furthermore be dependent on calcineurin/NFAT signaling activated by intracellular Ca^{2+} [208]. NFAT signaling also participates in enhanced FGF23 production due to cardiac hypertrophy [157].

Iron, hypoxia, and erythropoietin

Hypoxia, anemia, and iron deficiency are frequent sequelae in CKD [209]. They were all identified as stimuli of FGF23 production [128, 210–218]. Iron deficiency not only increases FGF23 transcription but also its cleavage [210, 216–218]. Interestingly, iron supplementation has been shown to increase [219–223] or decrease FGF23 [224–227], possibly depending on whether the supplementation stronger affects FGF23 transcription or cleavage.

Iron deficiency is often paralleled by hypoxia which may be another stimulus of FGF23 production through hypoxia-inducible factor 1-α (HIF-1α) [214, 228]. Moreover, HIF-1α may also be involved in FGF23 cleavage by inducing furin expression [229].

Erythropoietin (EPO) has also been shown to induce FGF23 [139, 230–233]. In two of the studies, EPO rather increased the level of C-terminal than intact FGF23 [230, 232], suggesting that EPO is involved in posttranslational regulation of FGF23. The other two studies found elevations of intact FGF23 in response to EPO, too.

Metal ions regulate FGF23 homeostasis

The magnesium (Mg^{2+}) status may regulate FGF23 as hypomagnesemia is associated with an increase in serum FGF23 [234–236]. Moreover, an inverse relationship between elevated FGF23 in CKD and Mg^{2+} is established [237, 238], and Mg^{2+} suppresses PTH and induces $1,25(OH)_2D_3$ [239] which also affect FGF23.

Cadmium (Cd^{2+}) intoxication causes phosphaturia and Itai-Itai disease, which is linked to renal failure and bone demineralization [240–242]. The administration of Cd^{2+} increases the plasma FGF23 concentration without changing *Fgf23* expression but releasing GALNT3 [243, 244].

Lithium ions stimulate FGF23 production [88, 205, 245].

WNK-OSR1/SPAK-NCC signaling also contributes to the regulation of FGF23 production [246–248]. WNK (with no lysine/K kinase) is a serine/threonine kinase involved in the regulation of blood pressure and salt balance by regulating renal tubular NaCl cotransporter (NCC) through oxidative stress-responsive kinase 1 (OSR1) and Ste20-related proline-alanine-rich kinase (SPAK) [249]. Enhanced FGF23 production is found in transgenic mice expressing WNK-resistant OSR1 [246], WNK-resistant SPAK [247], or deficient for NCC [248]. These results suggest that extracellular volume is a major regulator of FGF23 production.

Cytokines regulate FGF23 production

Inflammation is a common cause of many of diseases associated with elevated FGF23 levels. Indeed, enhanced inflammation in CKD [250–253] or in some other diseases [254–257] have been independently associated with an increase in FGF23 levels. These results suggest that inflammation could be a trigger of FGF23 production. Indeed, pro-inflammatory cytokines and other molecules including tumor necrosis factor α (TNFα), interleukin (IL)-1β/6, bacterial lipopolysaccharides (LPS), other bacterial components or their toxins and advanced glycation end products (AGEs) upregulate *Fgf23* gene expression directly and dose-dependently [210, 258–266] (Fig. 1). Acute or chronic inflammation induced by injection of IL1β injection or bacterial components into mice induces a surge in *Fgf23* gene expression in the bone and elevates the level of C-terminal FGF23 through HIF1α without appreciably influencing intact FGF23 [210].

Again, an interplay between the systemic regulator inflammation and local factors regulating FGF23 can be observed: Pro-inflammatory cytokines downregulate mRNA of *PHEX*, *DMP1,* and *ENPP1* and up-regulate *GALNT3*, thereby preventing FGF23 cleavage [264]. Transcription factor complex NFκB, which is implicated in a wide variety of pro-inflammatory cellular responses, induces *Fgf23* gene expression [206, 263–266]. NFκB up-regulates CRAC channel Orai1, and as detailed above, Orai1-mediated SOCE is a major driver of *Fgf23* gene expression mineral balance [205, 206, 266] (Fig. 1).

Chronic iron deficiency induces FGF23 in a way similar to chronic inflammation [210, 267]. IL-6 also enhances *Fgf23* promoter activity through signal transducer and activator of transcription 3 (STAT3) resulting in more *Fgf23* gene expression [260].

Transforming growth factor β (TGFβ) stimulates *Fgf23* gene expression by up-regulating SOCE [268]. Increased production of TGFβ during chronic inflammatory cystic fibrosis stimulates FGF23 signaling via upregulation of FGFR1 leading to interleukin IL-8 secretion [150]. In addition, an inhibitory effect of TGFβ on *FGF23* promoter activity may also be relevant [88].

FGF23 promotes the secretion of inflammatory cytokines in the liver by FGFR4-mediated activation of calcineurin/NFAT signaling [269] and stimulates TNFα secretion in macrophages [263, 265]. Therefore, a positive feedback loop may exist where FGF23 promotes the secretion of pro-inflammatory cytokines which induce more FGF23 secretion.

Recently, a direct effect of angiotensin II and aldosterone on FGF23 expression in cardiac myocytes has been demonstrated, which may contribute to myocardial fibrosis in CKD [173].

Metabolic regulators of FGF23 production

Insulin is a direct negative regulator of FGF23 synthesis: By activating PI3K/PKB/Akt/FOXO1 signaling, insulin suppresses the production of FGF23 [270]. Similarly, insulin-like growth factor 1 (IGF1) inhibits *FGF23* gene expression through this pathway [270].

The association between FGF23 and insulin deficiency (type 1 diabetes) and resistance (type 2 diabetes) is, however, complex. In many cross-sectional studies, FGF23 is positively correlated with obesity, diabetes or HOMA-IR, a parameter indicating insulin resistance [254, 257, 271–278]. According to other studies, FGF23 is inversely associated with insulin resistance [279–281].

Epinephrine, the neurotransmitter of the sympathetic nervous system, that also serves to mobilize energy reserves, promotes *Fgf23* mRNA production [282]. Thus, stress induces FGF23 production. In transgenic mice expressing a mutant form of glycogen synthase kinase 3 (GSK3) that cannot be inhibited by PI3K/PKB/Akt signaling any more, enhanced sympathetic activity accounts for stimulated FGF23 production [283].

AMP-activated protein kinase (AMPK), a serine/threonine kinase serving as a cellular energy sensor generally helps cells survive low energy states by activating energy-providing and inhibiting energy-consuming pathways [284]. AMPK inhibits FGF23 production by down-regulating SOCE [285].

A diet rich in high fats elevates the serum FGF23 level in rodents [261, 286, 287], at least in part through enhanced inflammation [261].

Adipokines, adipose tissue-derived hormones, like leptin [288, 289] and plasminogen activator inhibitor-1 (PAI-1) [100, 141] are positive regulators of FGF23. PAI-1 may enhance FGF23 levels by inhibiting furin-like proteases [290]. In line with this, plasminogen activators (PAs) directly cleave FGF23 [141].

Table 2 summarizes regulators of FGF23, the interaction of which is also shown in Figure 1. The mode of action of the FGF23 regulators is depicted in Table 3.

Conclusion

The regulation of FGF23 production is complex and far from being fully understood. Key regulators of bone-derived FGF23 are the phosphate status and its determinants including vitamin D hormone and FGF23 as well as inflammation. In addition, to these systemic regulators, local factors such as PHEX or DMP1 control the production and post-translational processing of FGF23 in bone. The precise interplay between these systemic and local factors, the exact regulation of FGF23 gene expression, the identification of all relevant transcription factors, and the mechanisms underlying post-translational modification (e.g. cleavage) are, however, still enigmatic. It appears to be particularly urgent to address the following open questions in the future: How are the post-translational modifications of FGF23 regulated? To which extent does FGF23 contribute to disease rather than merely indicating it? What is the different regulation of FGF23 production in health and disease? Why are some conditions associated with markedly increased C-terminal FGF23 without affecting intact FGF23? What is the hierarchy of FGF23 regulation with regard to local (e.g. PHEX, DMP1) vs. systemic (e.g. inflammation, iron status) factors? Future research focusing on these and further open questions are warranted to bring forward the important field of FGF23 biology.

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Table 1. Clinical conditions impacting on FGF23.

	Regulators	References
FGF23 ↑	***Primary causes***	
	Gain-of-function mutation of *FGF23* (ADHR)	[12, 31, 32]
	Overproduction of FGF23 in causative tumors (TIO)	[13–16]
	Gain-of-function mutation of *GNAS1* (MAS/FD)	[34, 58]
	Loss-of-function mutation of *PHEX* (XLH)	[50, 15, 59–63]
	Loss-of-function mutation/inactivation of *DMP1* (ARHR1)	[65–68]
	Loss-of-function mutation of *ENPP1*/*FAM20C* (ARHR2/3)	[76, 77], [83, 84]
	Loss-of-function mutation of *ANKH*	[82]
	Gain-of-function mutation/activation of *FGFR1*	[63, 87–93]
	Secondary causes	
	Inactivation of PC2	[70]
	Overproduction of Klotho	[96]
	CKD, AKI	[48, 90, 118–132], [134–141]
	Renal fibrosis, albuminuria, ADPKD	[142], [143], [144–146]
	Liver and lung diseases	[147], [148–150]
	Heart failure, atherosclerosis	[151, 152], [153–156]
	LVH	[157], [99, 158–161, 174]
FGF23 ↓	***Primary causes***	
	Loss-of-function mutation of *GALNT3*/*FGF23* (FTC)	[103–107], [108–115]
	Administration of DMP1	[69, 70]
	Inactivation of FGFR1	[63, 91–94]
	Secondary causes	
	Cytoskeleton reorganization, proprotein convertase 2 (PC2)	[74], [70]

Table 2. Summary of molecular regulators of FGF23.

	Regulators	References
FGF23 ↑	$1,25(OH)_2D_3$	[181–186]
	Phosphate	[98, 175–182, 186]
	PTH	[95, 187–192]
	Hyperparathyroidism, JMS, MAS/FD	[193–195], [196], [34, 58]
	Calcium	[98, 179, 200–202]
	SOCE	[205–207]
	Inflammatory markers, pro-inflammatory stimuli	[250–257], [210, 258–266]
	NFκB signaling	[206, 263–266]
	Transforming growth factor β (TGFβ)	[150, 268]
	Angiotensin II and aldosterone	[173]
	Hypoxia, anemia and iron deficiency	[128, 210–218]
	Iron supplementation	[219–223]
	Hypoxia (HIF-1α)	[214, 228]
	Erythropoietin	[139, 230–233]
	Magnesium deficiency, cadmium	[234–238], [243, 244]
	Lithium	[88, 205, 245]
	WNK-OSR1/SPAK-NCC signaling	[246–248]
	Obesity, diabetes or HOMA-IR	[254, 257, 271–278]
	Epinephrine, PI3K/PKB/Akt-resistant GSK3	[282], [283]
	Loss-of-function mutation/inactivation of AMPK	[285]
	High fat diet	[261, 286, 287]
	Leptin, PAI-1	[288, 289], [100, 141]
FGF23 ↓	Insulin/IGF1	[270]
	PTH	[69, 197]
	Hypocalcemia	[202–204]
	Transforming growth factor β (TGFβ)	[88]
	Iron supplementation	[224–227]
	Activation of AMPK	[285]

Table 3. Mode of action of FGF23 regulators.

	Regulators	References
FGF23 ↑	***Transcriptional regulation***	
	Loss-of-function mutation of *PHEX*	[59–63]
	Inactivation or loss of DMP1	[66, 68]
	Decreased PC2 activity	[70]
	Gain-of-function mutation/activation of *FGFR1*	[63, 87–93]
	CKD, AKI, LVH	[90, 132], [139, 140], [157]
	$1,25(OH)_2D_3$, phosphate	[181–184], [98, 179–182]
	PTH	[95, 189–192]
	Calcium, SOCE	[98, 200–202], [205–207]
	Proinflammatory stimuli	[210, 260–266]
	NFκB	[206, 263–266]
	TGFβ	[150, 268]
	Angiotensin II and aldosterone	[173]
	Hypoxia, anemia and iron deficiency, HIF-1α	[210, 214, 217, 218], [228]
	Erythropoietin	[139, 230–233]
	Lithium	[88, 205]
	Enhanced sympathetic activity	[282]
	Leptin	[288, 289]
	Post-translational processing	
	Increased cAMP activity in MAS/FD	[58]
	Acute or chronic inflammation	[210]
	Low serum iron	[210, 216–218]
	EPO	[230, 232]
	Cadmium	[243, 244]
	PAI-1	[141]
FGF23 ↓	***Transcriptional regulation***	
	Dmp1 transgene or recombinant DMP1	[67], [69, 70]
	Inhibition of FGFR	[63, 91–94]
	TGFβ	[88]
	Insulin/IGF1, AMPK	[270], [285]
	Post-translational processing	
	PC2	[70]
	FAM20C	[85]
	Loss-of-function mutation of *GALNT3*/*FGF23* in FTC	[104–107], [109–115]

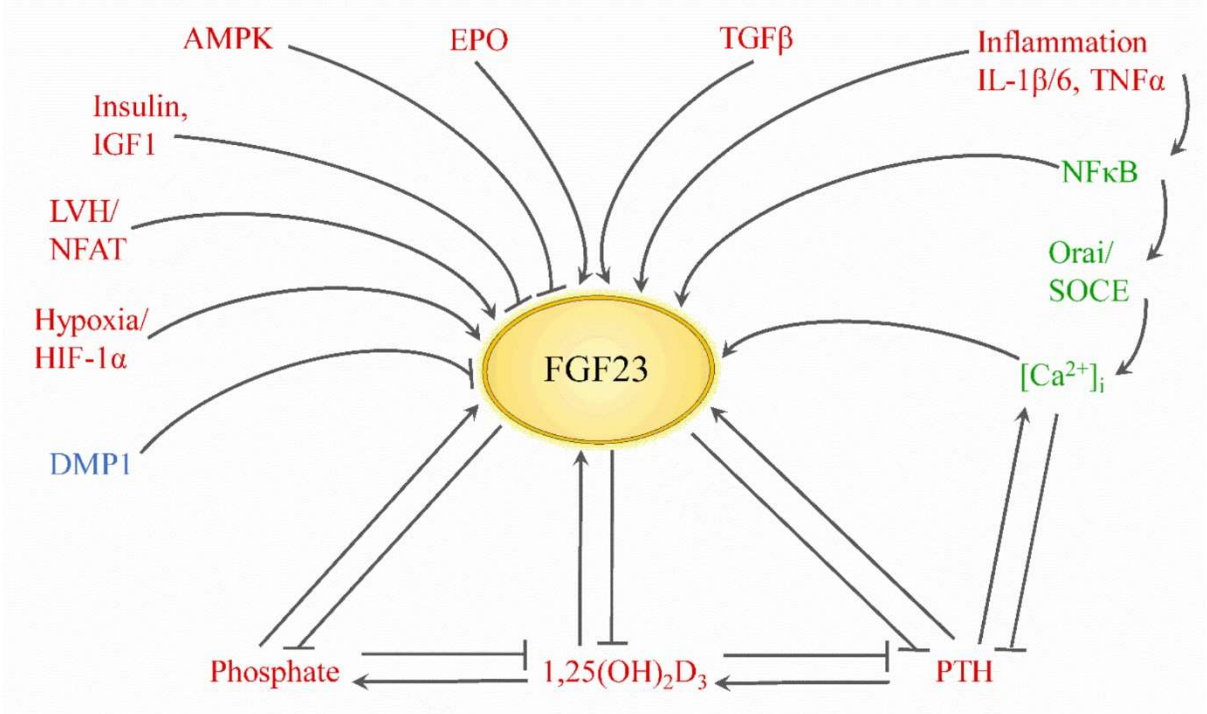

Fig. 1. Graphical illustration of some key regulators of FGF23. Systemic regulators are in red, intracellular mediators in green and local regulators in blue. Phosphate, 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$), parathyroid hormone (PTH), and Ca^{2+} upregulate FGF23. Intracellular Ca^{2+} also plays a decisive role in inflammation-induced activation of store operated calcium entry (SOCE) and stimulation of FGF23. Transforming growth factor β (TGFβ) and erythropoietin (EPO) are positive regulator of FGF23, whereas insulin and AMP-activated protein kinase AMPk are negative regulators. Left ventricular hypertrophy (LVH) and hypoxia induce FGF23 synthesis via transcription factor NFAT (nuclear factor of activated T-cells) and HIF-1α (hypoxia-inducible factor 1-α), respectively. Dentin matrix protein-1 (DMP1) is a negative local regulator of FGF23. $[Ca^{2+}]_i$: intracellular calcium.

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4 Discussion

4.1 Insulin as main regulatory hormone of glucose metabolism regulates FGF23

Inflammation is one of the most important regulators of FGF23 [193] and an association of FGF23 with metabolic disorders has also been uncovered [199, 200]. Based on these findings, the question arose whether mediators, which play a decisive role in diseases such as diabetes and metabolic syndrome, can also contribute to the regulation of the phosphaturic hormone. Besides, current research mainly associates type 2 diabetes [215], but also type 1 diabetes as an autoimmune disease [216], with chronic inflammatory diseases. Although the two types are generally regarded as different disease processes, they share the common fate of insufficient maintenance of glucose-induced insulin release due to dysfunction and/or progressive apoptotic decrease of pancreatic β cells, which ultimately leads to persistent hyperglycemia [216–220]. Thereby, an increased IL-1β production is observed in macrophages and/or β cells [221–223], leading to a decrease in insulin secretion and survival of β cells [224–228], partly due to NFκB-mediated apoptosis [222, 229].

Further, dietary nutrient intake can also serve as signal to activate the immune system by triggering mild inflammation [223, 230]. This short-term hyperglycemia exposure releases only small amounts of IL-1β, which even have a positive effect on insulin secretion, proliferation and survival of β cells [223, 224, 231]. But the initial physiological activation of the immune system is chronically activated in metabolic stress by overfeeding and promotes the onset of metabolic diseases [231]. Thus, local inflammation in tissues such as muscles, adipose tissue, liver, β cells, [232] and an increased amount of circulating inflammatory factors [215] are observed in type 2 diabetes and metabolic syndrome, in which overnutrition and hyperglycemia play a key role.

The chronic, systematic inflammation caused by diabetes [216] can of course also have an influence on other parts of the body, such as the bone and the phosphaturic hormone FGF23 expressed there, whose trigger is inflammation [193]. Indeed, there are some signs of inflammation-mediated stimulation of bone resorption and apoptosis of osteoblastic cells due to diabetes in the context of periodontal disease [233–237]. Furthermore, accumulated evidence confirms a rather complex association between FGF23 and diabetes. In most cross-sectional studies, FGF23 was positively correlated with obesity, diabetes or insulin resistance [199, 200, 238–245], while other studies showed an inverse association with insulin resistance [246–248].

A clinical link between diabetes and kidney disease, the main site of action of FGF23 [10, 11], has also been demonstrated. Diabetic nephropathy is an example of the involvement of immune-mediated inflammatory processes in the pathophysiology of diabetes and its complications [249]. Diabetes is also the most common cause of CKD and is associated with worse clinical events, such as earlier onset and greater severity of mineral metabolism disorders [238, 250]. Interestingly, insulin-resistant CKD patients show higher FGF23 levels while phosphate levels remain normal, suggesting a possible association between insulin resistance and phosphate homeostasis in CKD [239]. The decoding of the interaction between insulin effects and FGF23 may be important for understanding phosphate metabolism in relation to type 2 diabetes. In addition, subjects with type 2 diabetes in family history who did not develop hyperglycemia showed increased HOMA-IR (homeostatic model assessment-insulin resistance), insulin and FGF23 serum levels [251]. This finding suggests that disturbed insulin action, even without a pathological effect on blood glucose levels, may already have an effect on FGF23. Thus, an increased concentration of FGF23 may also be an indication of a decrease in metabolic function.

The inflammation-relevant aspect of this work raised the question whether chronic inflammatory diseases such as diabetes and metabolic syndrome play a decisive role in the regulation of the phosphaturic hormone. Insulin as the most important anabolic hormone is significantly involved in these two diseases, but to date there is little insight into the underlying mechanism of insulin-induced regulation of FGF23. For this reason, insulin was the focus of **Paper 1**. The investigations showed that insulin is an effective and physiologically highly relevant suppressor of FGF23 synthesis *in vitro* as well as in mice and humans. In UMR106 osteoblast-like cells, insulin and insulin-like growth factor 1 (IGF1) down regulated FGF23 production by inhibition of the transcription factor forkhead box protein O1 (FOXO1) via the phosphoinositid-3 kinase (PI3K)/protein kinase B (PKB)/Akt signaling pathway. In wild type mice, the induction of acute insulin deficiency by streptozotocin (STZ)-mediated destruction of insulin-producing β cells resulted in an increase in serum FGF23 levels, accompanied by an increase in blood glucose levels, confirming insulin deficiency. Treatment with insulin in this diabetic state led to a significant and almost complete decrease in FGF23 and blood glucose levels. However, neither STZ treatment nor insulin treatment significantly altered serum phosphate concentration, indicating a direct effect of insulin on FGF23. Similar effects have recently been observed in hypoinsulinemic mice treated with insulin for longer periods of time [376]. In addition, **Paper 1** revealed a highly significant negative correlation between FGF23

plasma concentration and the increase in plasma insulin levels in healthy pregnant women after oral glucose loading.

The clearly negative regulation of FGF23 by insulin contrasts with the above clinical studies in which hyperinsulinemia, a state typical of many patients with type 2 diabetes and metabolic syndrome, was associated with elevated FGF23 [199, 200, 238–245]. It should be kept in mind that most studies examined patients with complex metabolic and/or cardiorenal diseases. Therefore, many interfering factors may have unpredictable effects on FGF23 secretion. In addition, it is also widely known that most patients with type 2 diabetes, particularly those with obesity, suffer from inflammatory [215, 216] and also kidney disease [238, 250], conditions associated with increased FGF23 production [119, 193]. Therefore, it is very likely that the positive association of diabetes with FGF23 is largely due to inflammation and deregulated phosphate balance. Holecki *et al.* also determined that in older persons the increased FGF23 level is not associated with obesity or insulin resistance, but with inflammation [201].

The experiments of **Paper 1** in a non-pathological environment and the mechanistic insight show a clearly negative insulin-mediated effect on FGF23 formation, suggesting that an intact insulin signaling pathway is required for direct suppression of FGF23 secretion. For instance, it would be possible that in patients with manifest insulin resistance the FGF23 levels is increased [199, 200, 239] only due to a lack of negative insulin effects. In fact, the obesity-induced expression of NFκB target genes, such as *TNFα*, *IL-6* and *IL-1β*, results in a mild but chronic inflammation that can promote the development of insulin resistance [252], which not only affects adipose tissue but also has systemic influence on liver, vascularity, kidney, skeletal and cardiac muscle [215]. Confirming this, pioglitazone, an insulin sensitizer for the treatment of type 2 diabetes, reduced the expression of FGF23 [253].

The homeostasis of phosphate appears to be related to glucose metabolism and insulin, although the role of FGF23 in this relationship is still unclear [239]. Insulin is directly involved in phosphate homeostasis. On the one hand, it has an anti-phosphaturic effect by promoting phosphate reabsorption directly by stimulating NaPi-2 cotransporter, thereby increasing plasma phosphate [254]. Nevertheless, phosphate levels decrease both after acute insulin infusion *in vivo* and after a nutritional intake as a result of an insulin-induced increase in cellular phosphate uptake [255–257]. Insulin-mediated suppression of FGF23 may be a physiological mechanism to prevent excessive hypophosphatemia due to cellular phosphate accumulation. Insulin resistance may also limit cellular phosphate uptake, increasing plasma phosphate and thus stimulating FGF23 expression in addition to the lack of insulin-mediated negative effects,

such that phosphate level is kept within the norm, as observed in the study by Garland *et al.* [239]. For a better understanding of the relationship between insulin, FGF23 and phosphate balance, further investigations are required. However, the results of **Paper 1** clearly show that insulin is a potent suppressor of FGF23 regulation.

Stimulation of the insulin receptor leads to activation of PI3K, leading to phosphorylation and increased activity of PKB/Akt, an important mediator of insulin effects in the regulation of cell proliferation, survival and metabolism [258]. PKB/Akt mediates phosphorylation of glycogen synthase kinase 3 (GSK3), which inactivates it and thus also a large, functionally diverse set of direct downstream targets [258].

Paper 1 shows that the PI3K/PKB/Akt signaling pathway is directly involved in the regulation of FGF23 through the targeted use of inhibitors. In other studies, the involvement of this signaling pathway has already been shown indirectly. Transgenic mice expressing PI3K/PKB/Akt-resistant GSK3 [259] suffer from phosphaturia [260] and exhibit an elevated FGF23 serum level [261]. This would suggest that PI3K/PKB/Akt-sensitive GSK3 inhibition may be involved in the down-regulation of FGF23. In addition, PKBβ/Akt2 and serum and glucocorticoid kinase 3 (SGK3), another down streaming signaling element of PI3K, regulate the renal phosphate transporter NaPi-2a. Hence, both PKBβ/Akt2- and SGK3-deficient mice suffer from phosphaturia [262, 263].

The high medical relevance of PI3K/PKB/Akt is based on the fact that, in addition to insulin, many other growth factors affect its activity and that this signaling pathway is used by many types of cancer to proliferate, survive and migrate [264]. The involvement of this pathway in FGF23 regulation revealed in **Paper 1** suggests that any changes in PI3K/PKB/Akt activity, including possible future pharmacological interventions to combat cancer [264], may also have effects on FGF23 and thus on $1,25(OH)_2D_3$ and phosphate homeostasis.

4.2 Advanced glycation end products (AGEs) are strong stimulators of FGF23

Advanced glycation end products (AGEs) are a heterogeneous group of proteins, lipids and nucleic acids resulting from an irreversible non-enzymatic reaction with carbohydrates called glycation. It is distinguished into exogenous glycation, which is favored by strong heating of food with sugar, and endogenous glycation describes the body's reaction with sugar [265]. The endogenous AGEs formation is mainly favored by hyperglycemia, but also by oxidative stress and chronic inflammation, where accumulation over time turns out to be problematic [266]. Research indicates that AGEs are involved in the development of various

chronic inflammatory diseases such as type 2 diabetes [267], vascular and cardiovascular diseases [268], osteoporosis [269] an arthritis [270]. The cause is considered to be on the one hand that the normal function of numerous mediators such as amino acids, lipids, enzymes, hormones or growth factors is restricted by glycation-related changes in molecular conformation, enzymatic activity and receptor recognition, and on the other hand that AGEs themselves influence important metabolic processes by binding to the AGE receptor (RAGE) [271, 272]. Binding to RAGE on monocytes and macrophages induces via PI3K or mitogen-activated protein kinase (MAPK) signaling pathway NFκB and leads to oxidative stress and systemic inflammation, resulting in increased release of reactive oxygen species, as well as IL-1β/6 and TNFα [273–275]. These inflammatory factors increase insulin resistance [252], which in turn increases hyperglycemia and induces a dangerous vicious circle. The activation of NFκB also increases the cell expression of RAGE [276], which further enhances the inflammatory effect of AGEs.

Although hyperglycemia is considered to be one of the most important triggers in the pathogenesis of diabetic complications such as neuropathy, cardiomyopathy, nephropathy and retinopathy, the mechanisms involved remain unclear and AGEs are increasingly being ascribed a decisive role in their development [267, 272]. For example, treatment with Pimagedine, an AGE formation inhibitor, provided initial clinical evidence of AGE involvement in the pathogenesis of diabetic complications by slowing their progression [277]. Furthermore, patients with diabetes and end-stage renal disease exhibited a significantly higher AGEs level compared to healthy volunteers [278–283] and the accumulation of AGEs and RAGE in diabetic kidneys was strongly increased [284]. The increased abundance of AGEs in β cells is another reason for their dysfunction [285], as AGEs inhibit cytochrome c oxidase (COX) and ATP production in β cells, resulting in an impaired glucose-stimulated insulin secretion [286].

A direct diabetes-promoting effect of AGEs has been demonstrated in mice where AGEs administration has been associated with an accumulation of triglycerides and premature development of insulin resistance [287]. In contrast, in animal models for diabetes, a low AGE diet led to a decrease in the AGE concentration in serum, an improvement in insulin sensitivity [288, 289], as well as a significant decrease in AGE and RAGE expression, and macrophages in tissue [290]. In addition to a strong increase in weight, a diet rich in fat and AGEs led to the release of various inflammatory cytokines from fat tissue in mice [291]. Blockade of AGEs, on the other hand, reduces overexpression of these cytokines in diabetic mice [237]. This clarifies that AGEs can act as key modulators of endogenous metabolism and the increased release of cytokines and insulin resistance in diabetes is partly due to the formation of AGEs.

This clear link between AGEs and chronic inflammatory diseases such as diabetes suggests an interaction with FGF23, which is regulated by inflammation [194] and insulin (**Paper 1**). **Paper 2** therefore examined the influence of AGEs on FGF23 production. Since AGEs mediate their effect via RAGE [272], in **Paper 2** its expression was first demonstrated in UMR106 osteoblast-like cells and provided the basis for the AGE effect. Two types of AGEs also activating NFκB [292–294] were used. Ribose-modified bovine serum albumin (r-BSA) as classic AGE and bread crust extract (BCE) rich in various AGEs such as carboxymethyllysine [292], argenine and glycolaldehyde pyridine [293], gliadin [294], pronyllysine [295]. UMR106 cells were incubated with BCE and r-BSA and subsequently the *Fgf23* transcripts were quantified. It became clear that BCE and r-BSA are strong stimulators of *Fgf23* transcription, an effect at least partly mediated by the upregulation of NFκB and subsequent SOCE activation, since the use of NFκB inhibitor wogonin and SOCE antagonist 2-APB significantly reduced the r-BSA- and BCE-mediated effect on *Fgf23* mRNA synthesis. As a result of the findings of **Paper 2**, AGEs mediate the upregulation of FGF23 by activating the NFκB-dependent inflammatory reaction. However, these observations do not exclude the participation of BCE components other than AGEs that may be involved in the induction of FGF23.

In the treatment of CKD with phosphate binder Sevelamer it has already been observed that serum FGF23, Ca^{2+} and phosphate are reduced [296–298]. FGF23 seems to be reduced by Sevelamer-mediated suppression of AGEs accumulation [299] due to reversible AGEs binding in the intestine [300]. In addition, the markers for inflammation and oxidative stress associated with AGEs were down-regulated in patients with diabetes and early renal disease [300]. This effect of Sevelamer on inflammatory factors and FGF23 confirms the AGEs-mediated inflammation-dependent upregulation of the phosphaturic hormone in **Paper 2** and consequently AGEs could influence the progression of early diabetic CKD. Furthermore, AGEs-induced insulin resistance [287] may be associated with a reduced negative effect of insulin on FGF23 (**Paper 1**) and resulting in an increase in diabetes and metabolic diseases. In fact, AGEs have been shown to directly interfere with insulin signaling in granulosa cells by inhibiting insulin-mediated PI3K/PKB/Akt phosphorylation and thus signal transduction, and also by preventing membrane translocation of the GLUT-4 glucose transporter [301].

Insulin resistance and inflammation are typical of type 2 diabetes and metabolic syndrome associated with high FGF23 levels [199, 200, 238–245]. Furthermore, elevated FGF23 levels are observed in patients suffering from acute and chronic renal, cardiovascular, inflammatory and metabolic diseases [192], conditions associated with high vascular calcification and

cardiovascular morbidity. Several studies have revealed an association between elevated FGF23 levels and cardiovascular disease [171–176] and cardiovascular mortality [177–182]. AGEs also significantly contribute to these disorders [302–305]. It can therefore be speculated that accumulations of AGEs may contribute to elevated FGF23 serum levels in chronic inflammatory diseases and thus aggravate the course of the disease due to the disturbed phosphate balance. However, further research is required to establish a causal relationship.

4.3 Calmodulin as mediator of the Ca^{2+} signaling is involved in the regulation of FGF23

Current research demonstrates that FGF23 has many physiological and pathophysiological effects on the body. Accordingly, its expression is also influenced by various regulators and signaling pathways, as already considered in detail in **Paper 5**. One of the most important signaling pathways for the regulation of FGF23 is the NFκB signaling pathway [109, 207–209]. In addition to activating the expression of numerous inflammatory factors [212, 213], NFκB is also involved in controlling the intracellular Ca^{2+} concentration by upregulating the expression of the Ca^{2+} channel Orai1 and its activator STIM1 [109, 214], as well as store operated Ca^{2+} entry [108, 109]. Alterations of the cytosolic Ca^{2+} level is significantly involved in the regulation of numerous functions such as cell proliferation, migration and cell death, playing an important physiological role [306, 307]. It is also well known that Ca^{2+} upregulates *Fgf23* gene expression [49, 58, 103, 104]. The upregulation of the *Fgf23* mRNA is achieved at least in part via NFκB activated SOCE-dependent Ca^{2+} influx [108, 109]. Research on the underlying mechanism of **Paper 2** showed that the NFκB-inhibitor wogonin and the SOCE antagonist 2-APB significantly attenuated the stimulatory effect of r-BSA and BCE on *Fgf23* mRNA synthesis, suggesting that the Ca^{2+} influx is also part of the cellular machinery that contributes to *Fgf23* transcription induced by AGE treatment. However, the exact intracellular mechanism of Ca^{2+}-mediated regulation of FGF23 remains unclear.

Ca^{2+} as second messenger has a variety of different effects and affects many important intracellular processes. It can either directly influence the activity of target molecules, as in the case of protein kinase C (PKC), or modulate the activity of phosphodiesterases, adenylyl cyclases, CaM-dependent kinases (CaMK) and phosphatase calcineurin by binding to Ca^{2+}/calmodulin (CaM) [308]. Calcineurin, an important factor in the immune response, activates by dephosphorylation the transcription factor NFAT, which after translation into the cell nucleus stimulates the transcription of characteristic genes, such as interleukins, thereby initiating and enhancing the immune response of the activated T lymphocytes [309, 310]. In

addition to this initial discovery of T cell activation, calcineurin-NFAT signaling is also effective in osteoblasts regulating bone mass [311]. Several immunosuppressive agents like Ciclosporin A (CsA) and Tacrolimus (FK-506) mediate their effect by inhibiting calcineurin [309].

Paper 3 therefore investigated whether the Ca^{2+}-dependent calcineurin-NFAT signaling pathway is involved in the regulation of FGF23 production. To this end, it was first investigated whether UMR106 osteoblast-like cells have functional calcineurin-NFAT signaling. The expression of calcineurin subunits and NFAT proteins by RT-PCR were successfully demonstrated. The calcineurin inhibitors CsA and FK-506 decreased FGF23 gene expression and protein production, suggesting a stimulatory effect of calcineurin activity on FGF23 synthesis. Incubation with the calcineurin-NFAT interaction inhibitor INCA-6 also resulted in a significantly reduced amount of FGF23, suggesting that the effect of calcineurin on FGF23 is mediated by NFAT.

CsA and FK-506 have been shown to inhibit the transcription factor complex NFκB [312]. Since NFκB upregulates the formation of FGF23 [109], it is possible that the negative effect of calcineurin inhibitors on FGF23 formation could be mediated by the inhibition of NFκB. However, **Paper 3** did not provide evidence for the CsA- or FK-506-dependent regulation of NFκB expression nor activity. Thus, the calcineurin-NFAT signaling pathway is a strong regulator of FGF23 formation.

The Orai1-mediated Ca^{2+} influx with the resulting stimulation of the calcineurin-NFAT complex is part of the cellular machinery to activate T lymphocytes [310]. In UMR106 cells it was also shown that Ca^{2+} entry via Orai1 induces transcription of *Fgf23* [108, 109] and **Paper 3** proves that the calcineurin-NFAT signaling pathway can influence FGF23 expression. As in T lymphocytes, the NFκB/SOCE-related Ca^{2+} influx could have a down streaming effect on the calcineurin-NFAT signaling pathway and subsequently on the transcriptional regulation of *Fgf23*.

The involvement of calcineurin-NFAT signaling in FGF23 regulation can also be assumed on the basis of *in vivo* experimental data. Calcineurin inhibitors are frequently used to prevent transplant rejection [313]. After kidney transplantation, a drastic decrease in FGF23 levels was observed and, interestingly, the majority of patients in this study received treatment with calcineurin inhibitors [314]. This leaves room for speculation that reduced FGF23 production in patients after kidney transplantation is not only due to improved renal function, but also to the direct inhibitory effect of calcineurin inhibitors on FGF23 formation. Furthermore, the treatment of mice with CsA or FK-506 led to an increase in $1,25(OH)_2D_3$

serum concentration [315]. Since FGF23 acts as an inhibitor of $1,25(OH)_2D_3$ formation in the kidney [48], it is possible that the inhibitory effect of calcineurin inhibitors on FGF23 revealed in **Paper 3** is at least partly responsible for the $1,25(OH)_2D_3$ increase after treatment with CsA or FK-506 in the study of Lee *et al*. [315]. However, both observations require further investigation to confirm causal relationships between calcineurin inhibitors and FGF23.

Further investigations for the direct NFAT-dependent transcriptional control of *Fgf23* expression are still required. In addition, CsA and FK-506 influence a variety of other transcription factors and signaling molecules [316, 317] that may also play a role in the formation of FGF23. In summary, it can be said that calcineurin-NFAT signaling is relevant for the formation of FGF23, due to the inhibitory effect of calcineurin inhibitors on FGF23 expression.

4.4 Protein kinase C (PKC) regulates the FGF23 production

As mentioned above, Ca^{2+} can also mediate its effect directly via the activation of PKC. The large family of serine/threonine kinases is typically activated by stimulation of various G_q protein-coupled receptors and growth factor receptor tyrosine kinases by phospholipase C (PLC) mediated induction of the release of IP3 and diacylglycerol (DAG). IP3 binds to the receptor in the ER, releasing Ca^{2+} from the internal reservoir, which together with the membrane-bound DAG activates PKC and catalysis the phosphorylation of many target proteins [318]. Three classes of PKC isoforms are distinguished, based on their second messenger requirements: classical PKCs (cPKC) are activated by DAG and Ca^{2+}, while novel PKCs (nPKC) are induced only by DAG and atypical PKCs (aPKC) by other mechanisms [318].

PKCs are attributed to numerous cellular functions, including regulation of gene expression, cell migration, proliferation, differentiation and apoptosis [319]. In addition, PKC is involved in the pathophysiology of common diseases such as heart failure, diabetes, Alzheimer's and Parkinson's disease as well as inflammatory and immune diseases. PKC has been implicated in a range of cancer diseases due to its tumor and metastasis-promoting properties [319]. PKC is also relevant for the increased proliferation and decreased differentiation of osteoblasts [320, 321]. This would also allow PKC to participate in the regulation of the osteogenic hormone FGF23. A potential link between PKC and FGF23 also becomes apparent, as both enhance the transcriptional activity of Runt-related transcription factor 2 (*Runx2*), a key transcription factor in osteoblast differentiation [322, 323].

Due to the issue of how exactly the NFκB-mediated Ca^{2+}-dependent regulation of FGF23 proceeds [108, 109], the contribution of the PKC signal to the production of FGF23 was investigated in **Paper 4**. First, the expression of PKC isoforms in bone cells was successfully demonstrated. PKC is activated by the tumor promoter phorbolester 12-O-Tetradecanoylphorbol-13-acetat (PMA) [319], which has proven to be an effective stimulator of FGF23 mRNA and protein expression in **Paper 4**. In contrast, PKC inhibitors suppressed FGF23 synthesis. To investigate the underlying mechanism of PKC-dependent FGF23 regulation, the NFκB-Inhibitor withaferin A was used, which almost completely extinguished the stimulatory effect of PMA on *Fgf23* transcription. This makes PKC a strong regulator of FGF23 synthesis, which acts at least partially through NFκB. In addition, PKC activation induced the mRNA expression of the proinflammatory cytokines *TNFα* and *IL-6*.

The results of **Paper 4** suggest that FGF23 production is promoted via PKC activity. The involvement of the NFκB signaling pathway in this PKC-dependent regulation of FGF23 is plausibly conditioned by the fact that PKC contributes to the activation of NFκB [324]. This and the increased formation of cytokines, which in turn activate NFκB [212, 213] and are themselves stimulators of FGF23 production [204], are likely to potentiate the PKC effect on FGF23. It is also possible that the NFκB/SOCE-induced Ca^{2+} influx further activates PKC. In addition, PCK could also activate other signal cascades, which in turn may affect FGF23. For the signaling downstream of PKC governing the transcriptional control of *Fgf23* expression further investigations are necessary.

4.5 New insights into the transcriptional regulation of FGF23

FGF23 production is modulated by numerous regulators and signaling pathways, but the exact regulation at the transcriptional level is still not well understood. However, some transcription factors have already been identified. By using inhibitors of transcription or translation, actinomycin D or cycloheximide, it could be shown that $1,25(OH)_2D_3$ regulates FGF23 at the transcriptional level, probably via the VDR and presumably depending on the synthesis of an intermediate transfactors [57]. However, to date no functional vitamin D-responsive element (VDRE) has been identified in the *FGF23* gene, which may be related to the fact that $1,25(OH)_2D_3$ perhaps requires a composite responsive element in the FGF23 promoter [325]. The study by Meir *et al.* demonstrated *in vivo* and *in vitro* experiments that PTH mediates its stimulatory effect on *Fgf23* transcription via the orphan nuclear receptor Nurr1. A putative Nurr1 binding site was identified in the FGF23 promoter and Nurr1 also appears to play a role in the CKD environment [95].

Hypoxia-inducible factor-1α (HIF-1α), a transcription factor whose nuclear import and activity is regulated by iron status [326], is a direct transcriptional activator of FGF23 confirmed by chromatin immunoprecipitation assays [327]. Clinical observations and studies with mouse models for ADHR have already indicated the involvement of HIF-1α in the FGF23 regulation [328, 329]. The work of David *et al.* demonstrates that IL-1β stimuli directly increased FGF23 serum level and additionally the activity of HIF-1α [193]. Indeed, NFκB is a critical transcriptional activator of HIF-1α [330] and HIF-1α stabilization in turn lead to activation of NFκB [331]. These data suggest that HIF-1α activation may be a key mechanism for inflammation-mediated FGF23 production and providing a link to iron metabolism.

In **Paper 3**, the calcineurin-NFAT interaction inhibitor INCA-6 was used, which inhibits the calcineurin-related dephosphorylation of the phosphorylated nuclear localization signal of NFAT and thus the translocation into the cell nucleus, and the transcriptional regulation of target genes [309]. The amount of *Fgf23* mRNA was significantly reduced (**Paper 3**), indicating that NFAT is an important transcriptional regulator of FGF23. Recently it has also been detected that the promoter region of the *Fgf23* gene contains two putative NFAT-binding sites, where the proximal NFAT binding site is responsible for the transcriptional regulation of *Fgf23* [191]. Thus, NFAT proves to be a direct transcription factor of FGF23 and confirms the assumptions of **Paper 3**. Further evidence of the importance of NFAT on FGF23 regulation is that the increase in FGF23 serum levels initiated by cardiac hypertrophy is mediated by the calcineurin/NFAT pathway [191]. Furthermore, Orai1 deficiency associated with decreased *Fgf23* transcription [108, 109] (**Paper 2**) leads to a reduced nuclear localization and transcription activity of NFAT [110].

The **Paper 1** addressed the mechanism underlying insulin-induced inhibition of FGF23 formation. The transcription factor FOXO1 as the downstream mediator of the PI3k/PKB/Akt effect on FGF23 was identified, as reduction of active FOXO1 was accompanied by significant down-regulation of *Fgf23* transcription and conversely, the overexpression of FOXO1 up-regulated *Fgf23* gene expression.

Through insulin signaling PKB/Akt phosphorylates FOXO1, leading to its exclusion from the cell nucleus and phosphorylated FOXO1 is then ubiquitinated and degraded by the proteasome [332, 333]. FOXO1 gained prominence as it contributes to the longevity of worms and flies by inducing stress resistance [334]. But also in mammalian cells FOXO1 reduces stress and induces cell cycle arrest, DNA repair, and apoptosis, so that tumorigenicity can be reduced and the life span extended [335, 334]. Since FOXO1 is a central downstream target of the insulin/IGF1 pathway, it is not surprising that insulin/IFG1 also matter for longevity. Mice

deficient for receptor for either insulin or IGF1 had a prolonged life span and increased resistance to oxidative stress stimuli [336, 337]. In view of this life-shortening effect of insulin, there is room for speculation that the insulin-induced FGF23 decrease (**Paper 1**) contributes to aging, since FGF23 deficiency is also clearly associated with a short lifespan and age-associated diseases [11]. But also, a complete lack of the insulin signaling in turn leads to pathological phenomena such as diabetes and increased FGF23 levels, so that probably the key to longevity is only a moderate inactivation of the insulin signaling pathway. It is also interesting to investigate how the life-prolonging and anti-stress effects of FOXO1 and the FOXO1-dependent upregulation of *Fgf23* fit into this concept, because upregulation of FGF23 expression has not yet been associated with beneficial effects and instead it is observed in many diseases [192]. Therefore, further research is necessary with regard to insulin/FOXO1-dependent regulation of the phosphaturic hormone, also with a view to stress resistance and longevity. It must also be clarified whether FOXO1 is a direct transcription factor of FGF23, or whether other mediators and signaling pathways are still involved.

4.6 Conclusion

The aim of this work was to reveal new regulatory mechanisms of FGF23 regulation, in particular those associated with inflammation. To this end, factors that could be significantly involved in chronic inflammatory diseases such as diabetes and metabolic syndrome were investigated in more detail with regard to their influence on FGF23 formation. Subsequently, the underlying mechanisms of the relevant mediators were uncovered in a variety of ways.

The studies carried out and published as part of this work were able to answer most of the questions completely and comprehensively:

I) Which new regulators are relevant for FGF23 formation?

Insulin was detected as a potent negative regulator of FGF23 formation. *Fgf23* expression in UMR106 osteoblast-like cells was inhibited by treatment with insulin. In animal experiments, insulin deficiency led to an increase in serum FGF23 levels, which was reversed by insulin administration. In addition, plasma insulin levels in humans were negatively associated with plasma FGF23 levels. Since insulin plays a role in diabetes and metabolic syndrome in particular, and a connection with FGF23 has also been observed in these diseases, the involvement of insulin in FGF23 regulation is of high clinical relevance.

Furthermore, AGEs with an influence on inflammatory diseases have been identified as new positive regulators of FGF23. AGEs-mediated increase in FGF23 expression was

controlled by NFκB- and SOCE-inhibitors. Thus, a clear connection to inflammations could be established and also confirms the previous knowledge about the inflammation-relevant regulation of FGF23. In addition, several studies have confirmed the involvement of Ca^{2+} as an important regulator of FGF23.

II) What are the underlying mechanisms in the regulation of FGF23 expression?

Inflammation, as one of the main regulators of FGF23, also proved to be an important mediator of FGF23 expression in these studies, due to the use of NFκB-inhibitors or inflammation-relevant effectors such as AGEs. This clearly shows that chronic inflammatory diseases such as diabetes and metabolic syndrome can play a decisive role in the regulation of the phosphaturic hormone.

Moreover, the involvement of the Ca^{2+} signaling pathway was successfully demonstrated in this work. Calcineurin/NFAT- and PKC-signaling as transmitters of the Ca^{2+} signal could influence the expression of FGF23.

In connection with insulin and the use of specific inhibitors and genetic manipulation, it was shown that the underlying intracellular mechanism for the inhibition of FGF23 is based on the PI3K/PKB/Akt-induced inactivating phosphorylation of FOXO1 and thus the absence of induction of the gene expression. Since the PI3K/PKB/Akt signaling pathway exerts an extremely diverse influence in the cell, it is possible that other mediators may also influence FGF23 expression.

III) Which transcription factors are relevant for the regulation of *FGF23* transcription?

FOXO1 could be detected as a new transcription factor of the FGF23 regulation. It still has to be examined whether FOXO1 binds directly in the *FGF23* gene region or is responsible for the recruitment of other direct effectors. It is also interesting to speculate on how far FOXO participation in the FGF23 regulation also has an influence on stress resistance and longevity.

NFAT has also proven to be an important transcription factor in the regulation of FGF23.

5 Summary

Fibroblast growth factor 23 (FGF23) is an important regulator of the active vitamin D ($1,25(OH)_2D_3$) and phosphate balance. The regulation of this homeostasis is very finely and diversely regulated, and deregulation is often accompanied by severe diseases. Hyperphosphatemia is a common cause of vascular calcifications and occlusions associated with increased risk of myocardial infarction and mortality. Increased FGF23 is also considered as an independent risk factor for cardiovascular mortality. Thus, the knowledge gained about the function and regulatory mechanisms of FGF23 with regard to various chronic inflammatory, kidney and bone diseases as well as metabolic processes represents a highly relevant topic for human health from a pharmacological point of view.

Numerous studies demonstrate that the regulation of FGF23 production is highly complex and far from being fully understood. Many regulators have been described, but the precise regulation of *FGF23* gene expression, the identification of all relevant transcription factors and the mechanisms underlying posttranslational modification (e.g. cleavage) are still enigmatic. Therefore, the aim of this work was to identify new regulators of FGF23 and their underlying mechanisms in order to contribute to a better understanding of this diverse and clinically relevant factor.

Many diseases associated with elevated FGF23 levels, such as chronic kidney disease (CDK) or diabetes, are accompanied by inflammatory processes. Inflammatory reaction leading to activation of store-operated Ca^{2+} entry (SOCE), among other things, is one of the most important triggers of FGF23 increase. Therefore, the focus of this work was on investigation of inflammation-relevant processes that can influence the regulation of FGF23. The understanding of the systemic impact of inflammation has enormous implications for the design of novel therapies to reduce morbidity and mortality of patients by preventing the associated chronic inflammatory diseases.

It could be shown that insulin is a potent negative regulator of FGF23 and that regulation occurred via the phosphoinositid-3 kinase/protein kinase B/Akt/forkhead box protein O1 (PI3K/PKB/Akt/FOXO1) signaling pathway. The majority of previous studies are characterized by hyperinsulinemia in diabetes or metabolic syndrome with increased FGF23 release. However, rather this seems to reflect the pathophysiological state, while in this work in the non-pathological environment and the mechanistic findings clearly show a negative effect. It must also be kept in mind that diabetes and metabolic syndrome are strongly associated with inflammatory reactions that are known to upregulate FGF23. Due to the high clinical

relevance of insulin or PI3K/PKB/Akt-Signaling, pharmacological interventions for disease control can have an influence on FGF23 and thus also on the phosphate and $1,25(OH)_2D_3$ balance.

The inflammation-related upregulation of the phosphaturic hormone FGF23 could also be demonstrated in the context of this work through the use of advanced glycation end products (AGEs), which are associated with chronic inflammatory diseases such as diabetes. AGEs mediate their effect at least in part through stimulation of NFκB (nuclear factor of activated T-cells) and subsequent SOCE activation. Thus, AGEs-induced FGF23 production could contribute to an increased FGF23 serum level in chronic diseases and so influence the course of the disease due to the disturbed phosphate balance.

For the development of new therapeutic approaches for FGF23-associated diseases, an understanding of the underlying regulatory mechanisms is of great importance. Ca^{2+} is a well-known regulator of FGF23, which partially acts on *Fgf23* transcription via NFκB-dependent SOCE activation. Ca^{2+} also proved to be an important intracellular regulator in this work. It was shown that the calcineurin/NFAT (nuclear factor of activated T-cells) and protein kinase C (PKC) signaling pathways are involved in the Ca^{2+}-dependent regulation of FGF23. NFAT proves to be an important transcription factor that has already been associated with left ventricular hypertrophy (LVH)-induced FGF23 increase and thus illustrates the importance of calcineurin/NFAT for FGF23 and heart failure.

6 Zusammenfassung

Fibroblasten-Wachstumsfaktor 23 (FGF23) ist ein wichtiger Regulator des aktiven Vitamin D ($1,25(OH)_2D_3$)- und Phosphathaushaltes. Die Regulation dieser Homöostase ist sehr fein und vielfältig geregelt, und eine Deregulation geht oftmals mit schweren Erkrankungen einher. Hyperphosphatämie ist dabei häufige Ursache für Gefäßverkalkungen und -verstopfungen, die mit erhöhtem Herzinfarktrisiko und Sterblichkeit assoziiert sind. Erhöhtes FGF23 gilt ebenfalls als unabhängiger Risikofaktor für die kardiovaskuläre Mortalität. Damit stellt der Erkenntnisgewinn um die Funktion und Regulationsmechanismen von FGF23 hinsichtlich verschiedener chronischer Entzündungs-, Nieren- und Knochenerkrankungen sowie Stoffwechselprozesse aus pharmakologischer Sicht eine hochaktuelle Thematik für die Gesundheit des Menschen dar.

Zahlreiche Studien belegen, dass die Regulierung der FGF23-Produktion höchst komplex und noch lange nicht vollständig verstanden ist. Es wurden viele Regulatoren beschrieben, jedoch sind die genauen Regulationen der *FGF23*-Genexpression, die Identifizierung aller relevanten Transkriptionsfaktoren und die Mechanismen, die der posttranslationalen Modifikation (z. B. Spaltung) zugrunde liegen, immer noch rätselhaft. Daher war Ziel dieser Arbeit, neue Regulatoren von FGF23 und deren zugrundeliegenden Mechanismen zu identifizieren, um so zu einem besseren Verständnis über diesen vielfältigen und an zahlreichen klinisch relevanten Aspekten beteiligten Faktor beizutragen.

Zahlreiche Erkrankungen, die mit erhöhten FGF23-Werten assoziiert sind, wie chronisches Nierenversagen (*chronic kidney disease*, CKD) oder Diabetes werden von Entzündungsprozessen begleitet. Entzündungsreaktion, die unter anderem zur Aktivierung von speichergesteuerten Ca^{2+}-Einstrom (*store-operated* Ca^{2+} *entry*, SOCE) führt, ist einer der wichtigsten Auslöser der FGF23-Steigerung. Daher lag der Fokus dieser Arbeit auf Untersuchungen von entzündungsrelevanten Vorgängen, die die Regulation von FGF23 beeinflussen können. Das Verständnis der systemischen Auswirkung von Entzündungen hat enorme Auswirkungen auf das Design neuartiger Therapien, um Morbidität und Mortalität von Kranken durch Prävention der damit verbundenen chronischen entzündlichen Erkrankungen zu reduzieren.

Es konnte eindrucksvoll gezeigt werden, dass Insulin ein potenter negativer Regulator von FGF23 ist und die Regulation über den Phosphoinositid-3-Kinase/Proteinkinase B/Akt/ *forkhead box protein O1* (PI3K/PKB/Akt/FOXO1)-Signalweg verläuft. Der überwiegende Teil bisheriger Untersuchungen brachten eine Hyperinsulinämie in Zusammenhang mit Diabetes

oder metabolischen Syndrom mit einer gesteigerten FGF23-Freisetzung in Verbindung. Jedoch scheint dies eher den pathophysiologischen Zustand widerzuspiegeln, während in dieser Arbeit im nicht-pathologischen Umfeld die mechanistischen Erkenntnisse deutlich eine negative Wirkung aufzeigen. Dabei muss auch bedacht werden, dass Diabetes und metabolisches Syndrom stark mit Entzündungsreaktionen verbunden sind, welche bekanntermaßen FGF23 heraufregulieren. Durch die hohe klinische Relevanz des Insulin- bzw. PI3K/PKB/Akt-Signalweges können pharmakologische Maßnahmen zur Krankheitsbekämpfung Einfluss auf FGF23 und damit auch auf den $1,25(OH)_2D_3$- und Phosphathaushalt haben.

Die entzündungsbedingte Heraufregulation des phosphaturischen Hormons FGF23 konnte im Rahmen dieser Arbeit ebenfalls demonstriert werden durch Einsatz von AGEs (*advanced glycation end products*), welche in Verbindung mit chronischen Entzündungserkrankungen wie Diabetes gebracht werden konnten. Dabei vermitteln AGEs ihren Effekt zu mindestens zum Teil durch Stimulation von NFκB (*nuclear factor kappa-light-chain-enhancer of activated B cells*) und anschließender SOCE-Aktivierung. Damit könnte diese AGEs-bedingte FGF23-Produktion zu einem erhöhten FGF23-Serumspiegel bei chronischen Erkrankungen beitragen und damit den Krankheitsverlauf aufgrund des gestörten Phosphathaushaltes beeinflussen.

Zur Entwicklung neuer Therapieansätze für FGF23-assoziierten Krankheiten ist das Verständnis der zugrundeliegenden regulatorischen Mechanismen von großer Bedeutung. Ca^{2+} ist ein bekannter Regulator von FGF23, der zum Teil über die NFκB-abhängige SOCE-Aktivierung auf die *Fgf23* Transkription wirkt. Auch im Rahmen dieser Arbeit erwies sich Ca^{2+} als ein wichtiger intrazellulärer Regulator. Dabei konnte gezeigt werden, dass an der Ca^{2+}-abhängigen Regulation von FGF23 der Calcineurin/NFAT (*nuclear factor of activated T-cells*)- und Proteinkinase C (PKC)-Signalweg beteiligt sind. NFAT erweist sich damit als ein wichtiger Transkriptionsfaktor, der bereits in Zusammenhang mit linksventrikulärer Hypertrophie (LVH)-induzierter FGF23 Steigerung gebracht wurde und verdeutlicht damit die Bedeutung des Calcineurin/NFAT für FGF23 und Herzinsuffizienz.

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Eidesstattliche Erklärung

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

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