Studies on novel regulators of the fibroblast growth factor 23 (FGF23) synthesis in bone cells: The impact of p38MAPK, PPARα, and myostatin

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List of abbreviations

$1,25(OH)_2D_3$	1,25-Dihydroxy-Vitamin D_3, Calcitriol, active vitamin D
$[Ca^{2+}]_c$	Cytoplasmic calcium
ADHR	Autosomal dominant hypophosphataemic rickets/osteomalacia
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANKH	Progressive ankylosis protein homolog
ARHR	Autosomal recessive hypophosphataemic rickets/osteomalacia
ASARM	Acidic serine-aspartate rich MEPE-associated motif
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
CKD	Chronic kidney disease
Cl^-	Chloride
CRAC channel	Ca^{2+} release-activated Ca^{2+} channel
CRP	C-reactive protein
CYP24A1	25-hydroxyvitamin D-24-hydroxylase
CYP27B1	25-hydroxyvitamin D-1α-hydroxylase
DMP1	Dentin matrix protein 1
ENPP1	Ectonucleotide pyrophosphatase/phosphodiesterase 1
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1/2
FAM20C	Family with sequence similarity 20, member C
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor

FK-506	Tacrolimus
FOXO	Forkhead box protein O
FTC	Familial tumoral calcinosis
GALNT3	UDP-*N*-acetyl-α-D-galactosamine-polypeptide *N*-acetylgalactosaminyltransferase 3
GFR	Glomerular filtration rate
HB	Heparan sulfate-binding site
HDL	High-density lipoprotein
HS	Heparan sulfate
IP_3	Inositol-1,4,5-triphosphate
IP_3R	Inositol-1,4,5-triphosphate receptor
IL	Interleukin
K^+	Potassium
kDa	Kilodalton
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
mTOR	Mammalian target of rapamycin
MAPK	Mitogen-activated protein kinase
MEPE	Matrix extracellular phosphoglycoprotein
mRNA	Messenger RNA
Na^+	Sodium
NaPi-II	Na^+-phosphate co-transporter type II
NCC	Na^+-Cl^- co-transporter
NCX	Na^+/ Ca^{2+} exchangers
NFκB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells

NFAT	Nuclear factor of activated T-cells
OPN	Osteopontin
PHEX	Phosphate regulating endopeptidase homolog X-linked
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLCγ1	Phospholipase Cγ1
Pi	Inorganic phosphate
PPARα	Peroxisome proliferator-activated receptor alpha
PPi	Inorganic pyrophosphate
PTH	Parathyroid hormone
RA	Rheumatoid arthritis
SERCA	Sarco-endoplasmic Ca^{2+}-ATPase
SGK-1	Serum/glucocorticoid-regulated kinase-1
siRNA	Small interfering RNA
SOCE	Store-operated Ca^{2+} entry
SPC	Subtilisin-like proprotein convertase
STIM1	Stromal interaction molecule 1
TGF-β	Transforming growth factor-β
TIO	Tumor-induced osteomalacia
TNAP	Tissue-nonspecific alkaline phosphatase
TNF-α	Tumor necrosis factor alpha
TRPV5	Transient receptor potential vanilloid 5
VDR	Vitamin D receptor
XLH	X-linked hypophosphataemia

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1 Introduction

1.1 The fibroblast growth factor (FGF) superfamily and its receptors

The fibroblast growth factor superfamily consists of 22 members in human and mice numbered from FGF1 to FGF23 with a molecular weight ranging from 17-34 kilodalton (kDa), which share a 13-71% amino-acid identity on a ~ 120-amino-acid residue core sequence [1, 2]. It seems that human *FGF19* and mouse *Fgf15* are orthologues, without *FGF15* in humans and *Fgf19* in mice [3]. The FGFs can be phylogenetic classified into seven subfamilies [4]. Within these seven subfamilies the FGFs can be categorized into three groups as shown in Figure 1, according to their mode of actions: intracellular, paracrine and endocrine FGFs [3].

The secreted FGFs bind to FGF receptors (FGFRs) on the cell-surface. *FGFR1-4* form the human *FGFR* gene family [5]. FGFR1-4 are transmembrane receptors, with three extracellular immunoglobulin-like domains I, II and III and an intracellular tyrosine kinase domain. Immunoglobulin-like domain II and III as well as the linker region between, are essential for the ligand binding specificity [4, 5]. For immunoglobulin-like domain III two splicing alternatives (b and c) exist, which are encoded by the genes *FGFR1*, *FGFR2* and *FGFR3*. Therefore, seven FGFR proteins exist (FGFRs 1b, 1c, 2b, 2c, 3b, 3c, 4) [5]. Ligand binding results in dimerization and activation of FGFR, which causes intracellular substrate phosphorylation of phospholipase Cγ1 (PLCγ1), subsequent release of intracellular calcium, activation of FGFR substrate 2α and induction of the Ras mitogen-activated protein kinase (RAS-MAPK) and phosphoinositide 3-kinase (PI3K)-AKT signaling pathway [2, 5]. FGFs and FGFRs are localized in different tissues and are included in the regulation of developmental processes, metabolism, tissue maintenance, repair and organogenesis [1–5].

The group of intracellular FGFs (iFGF) comprises the FGF11 subfamily (FGF13/14/12/11) [5, 6]. The mechanism of action of iFGFs differs from the paracrine and endocrine FGF subfamilies, as iFGFs are not secreted and do not have an N-terminal signal peptide to bind to an FGFR. Therefore, iFGFs show no significant interactions with the existing FGFR1-4 in a physiological relevant manner [5–7]. IFGFs prominently occur in the nervous system and act by binding to the cytoplasmatic C-terminal region of voltage-gated sodium channels, thereby regulating the initiation and the propagation of action potentials [8–10].

The FGF1 (FGF1/2), FGF4 (FGF4/5/6), FGF7 (FGF3/7/10/22), FGF9 (FGF9/16/20) and FGF8 (FGF8/17/18) gene subfamilies represent the group of secreted paracrine or rather canonic FGFs [4, 5]. In contrast to endocrine FGFs, paracrine FGF subfamilies show high

heparan sulfate (HS)- binding (HSB) affinity [11]. The binding to HS determines the transport between cells and is required to bind to FGFR, forming the FGF-FGFR signaling complex [12]. HS proteoglycans are bound on the cell-surface or the extracellular matrix and modulate the distribution and the signaling cascade of secreted molecules [13]. Because of their high affinity for binding to the HSB site, secreted paracrine FGFs can diffuse only a small radius around the secreting tissue before complexing with FGFR via binding to HS [14]. That causes the paracrine mechanism of action of this subfamily, for example, the regulation of the modulation of cardiac development by FGF2 [15], hair growth by FGF5 [16], FGF7-related delay of wound healing [17], the sex determination by FGF9 [18] or the role of FGF8 during development of the midbrain [19].

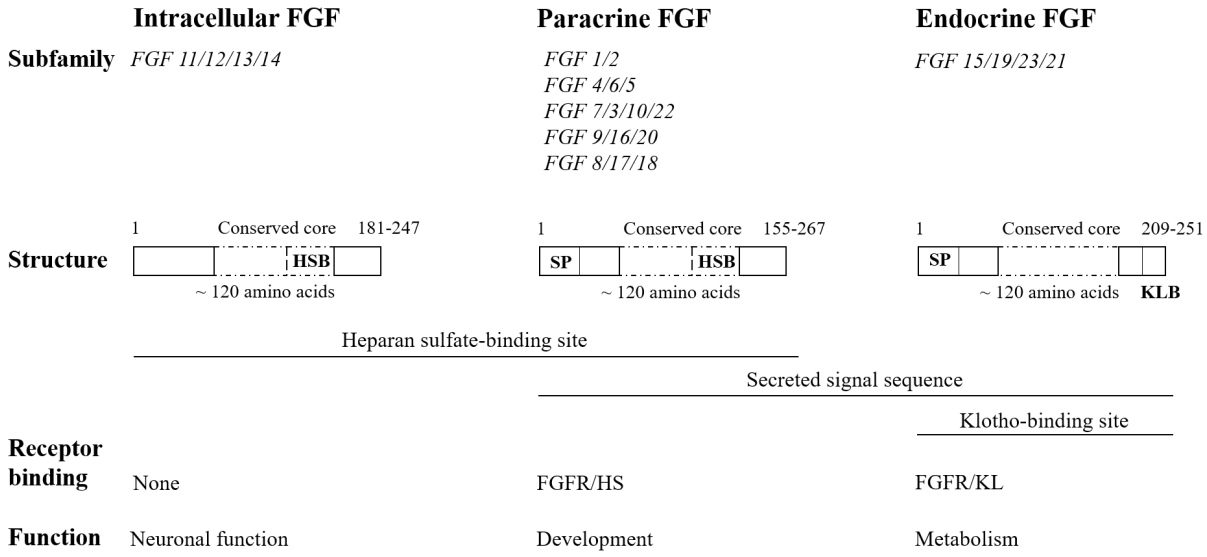

Figure 1: The *FGF* gene family.
The *FGF* gene family is divided into three groups according to their mechanisms of action, comprising one to five of the seven phylogenetic subfamilies. The schematic drawing shows the common core structure of the FGFs, as well as the heparan sulfate (HS)-binding (HSB) site, the Klotho-binding site (KLB), and the signal peptide (SP), which represents the cleavable secreted signal sequence. For more details see text. Figure is modified according to [3, 5].

The endocrine FGF subfamily comprises the orthologues FGF15/19 and the other hormone-like proteins FGF21 and FGF23 [20]. In contrast to intracellular and paracrine FGFs, the endocrine subfamily shows altered HSB topology mainly in the β10-β12 region. The resulting low affinity for binding to HS prevents endocrine FGFs from being trapped in the extracellular matrix and therefore allows them to diffuse freely and enter the bloodstream to exert their function as hormones on distant tissues [21]. In addition, the low binding affinity to

related FGFRs limits direct interaction. These two aspects suggest that hormone-like FGFs require a different co-factor to bind to FGFRs to exert their effect on the target tissue [22]. The required co-factor for the receptor binding of the endocrine FGFs is Klotho [21, 23]. Klotho is a type 1 single-pass transmembrane glycoprotein and located in the plasma membrane and the Golgi apparatus. The Klotho gene encodes a three-part protein family, consisting of α-Klotho and the two paralogs β- and γ-Klotho [24]. β-Klotho is localized in the cell membrane and mainly expressed in the liver and adipose tissue, where it forms a complex with FGFR1c and FGFR4, mediating the metabolic functionality of FGF19 and FGF21 [25–27]. FGF19 controls bile acid synthesis via suppression of Cyp7a1 expression, through binding on the FGFR4-β-Klotho-complex [25]. FGF21 is expressed mainly in the liver, where it is involved as downstream target of PPARα in metabolic adaptation to fasting, but also in the adipose tissue, where it modulates lipolysis [28–30]. γ-Klotho is encoded by the *Klph* gene and mainly expressed in the eyes but also in the kidney, adipose tissues, and skin [31, 32]. It is found that γ-Klotho interacts with FGFR1b, 1c, 2c, and 4 and promotes activation of FGF signaling by FGF19 in HEK293 cells [32]. α-Klotho is the co-factor of the endocrine subfamily member FGF23 [23, 33, 34]. FGF23 and its physiological role is described in detail below.

1.2 Basic molecular aspects and effects of FGF23

The endocrine FGF family member FGF23 was first identified in the year 2000 in the ventrolateral thalamic nucleus of the mouse brain [35]. In the same year, the physiological importance of FGF23 became apparent as the clinical picture of autosomal dominant hypophosphataemic rickets (ADHR) and its pathology could be attributed to a point mutation in the *FGF23* gene [36].

FGF23 is most similar to FGF21 and the gene is localized on chromosome 12p13 in humans [35]. The 32 kDa glycoprotein FGF23 comprises 251 amino acids. The 251 amino acids are distributed among a hydrophobic 24 amino acid signal sequence; the NH_2-terminal conserved FGF core region containing 154 amino acids that allows binding to FGFR; the COOH-terminal region containing 73 amino acids and therein the Klotho-binding site required for formation of the FGF23-α-Klotho-FGFR complex [35, 37–39]. FGF23 circulates in the bloodstream in three different forms: full length ^{25}FGF23^{251} form, the shorter variant ^{25}FGF23^{179}, lacking the COOH-terminal 73 amino acid fragment, and the cleaved C-terminal fragment itself [38, 39]. The N-terminal and C-terminal fragments results from the cleavage of the mature form by the subtilisin-like proprotein convertase (SPC) furin at the ^{176}RXXR^{179} site [40]. Since

the Klotho-binding site is located in the C-terminal region, only full-length FGF23 is biologically active [38, 41]. Interestingly, the C-terminal fragment appears to compete with the full-length variant for binding at the FGFR-Klotho complex, as a result of which injection of C-terminal FGF23 in rodents results in a reduction in phosphate excretion [42, 43]. A mechanism that protects FGF23 from cleavage and increases the circulating levels is the O-glycosylation of Thr^{178} by UDP-*N*-acetyl-α-D-galactosamine-polypeptide *N*-acetylgalactosaminyltransferase 3 (GALNT3), overlapping the furin-cleavage site, resulting in the secretion of biologically active intact full-length FGF23 [40, 44, 45]. In contrast, phosphorylation of Ser^{180} close to the SPC ^{176}RXXR^{179} motif by family with sequence similarity 20, member C (FAM20C), leads to the absence of O-glycosylation and consequently favors the increased cleavage of FGF23 [40]. Thus, the interplay between phosphorylation and O-glycosylation influences the relative amounts of intact and C-terminal FGF23 and provides a crucial physiological interface [46]. *Fgf23* is expressed in multiple tissues like brain, heart, kidney, thymus, spleen, liver, and the small intestine [47]. However, the most important cellular source of *FGF23* expression are osteoblasts and osteocytes in bone [47, 48].

The classical signal transfer of FGF23 to the target tissue requires the formation of the trimeric complex of FGF23-α-Klotho-FGFR, as in the kidney [46]. α-Klotho binds to FGFR2 with lower affinity, which is why FGF23 mainly complexes with the other subtypes of FGFR-1, -3, and -4, and binding with the c-isoform of FGFR1 induces the largest signal [23, 33, 38, 46, 49, 50]. The proximity and stability of the FGF23-FGFR linkage is achieved through α-Klotho, as the scaffold protein in this ternary complex, resulting in a 20-fold increased binding affinity of FGF23 to its receptor [34, 49].

A *Klotho* gene mutation in mice causes an age-like phenotype, including a shortened lifespan, arteriosclerosis, osteoporosis, skin atrophy, infertility, and emphysema [51]. The protein comprises a N-terminal short signal sequence, the large ectodomain, containing two internal repeats termed KL1 and KL2, mediating α-Klotho activity and function, the transmembrane domain, and a short intracellular domain [27, 51, 52]. Three forms can be distinguished: full-length transmembrane α-Klotho (135kDa), the 130 kDa shed soluble form, and the shorter truncated secreted variant of Klotho (65 kDa) [51, 53, 54]. Soluble Klotho is formed by proteolytic cleavage, termed α-cut, of full-length transmembrane α-Klotho on the cell surface by α-secretases A disintegrin and metalloproteinase domain-containing proteins 10 and 17 and β-APP cleaving enzyme 1 (BACE1), consisting of the KL1 and KL2 domains [27, 55, 56]. The β-cut of α-Klotho by A disintegrin and metalloproteinase domain-containing proteins 10 and 17 generates the two 65 kDa fragments KL1 and KL2 [55]. Therefore, after

shedding, soluble Klotho enters the blood, urine or cerebrospinal fluid as KL1 or KL2 only or both KL1 and KL2 and unfolds its biological function in peripheral tissues and organs [27, 54, 57, 58]. α-Klotho is mainly expressed in the kidney but also in the brain such as the cerebellum, cerebral cortex, spinal cord, and in other tissues with existing but lower expression like thyroid gland, aorta, urinary bladder, ovary, skeletal muscle, pancreas, prostate gland, testis or the adrenal gland [51, 59].

The functional importance of α-Klotho for FGF23 is shown in the overlapping phenotype of *kl*$^{-/-}$, *Fgf23*$^{-/-}$, and *Fgf23*$^{-/-}$/*kl*$^{-/-}$ double-knockout mice. The injection of bioactive FGF23 into mice results in a decrease of serum phosphate in wild-type and in *Fgf23*$^{-/-}$ mice, but not in *Fgf23*$^{-/-}$/*kl*$^{-/-}$ double-knockout mice, demonstrating the requirement of α-Klotho for the FGF23 pathway. All three phenotypes are characterized by premature ageing, shortened lifespan, skin atrophy, infertility, arteriosclerosis, and ectopic calcifications relative to wild-type mice [60]. *Fgf23*$^{-/-}$ mice show increased serum phosphate levels and significantly elevated 1,25$(OH)_2D_3$ (active vitamin D) concentrations [61]. A lack of the co-receptor α-Klotho causes a similar abnormal 1,25$(OH)_2D_3$ plasma level and mineral ion metabolism in mice [62]. There is good evidence showing that the similar phenotypes of *kl*$^{-/-}$ and *Fgf23*$^{-/-}$ knockout mice are caused by the unregulated production of 1,25$(OH)_2D_3$ and the resulting chronic hyperphosphataemia and hypercalcaemia [63–66]. Ablation of 1,25$(OH)_2D_3$ signaling in mice lacking a functional vitamin D receptor (VDR) is shown to completely prevent the premature aging phenotype in *kl*$^{-/-}$ and *Fgf23*$^{-/-}$ mice, indicating the importance of FGF23 as an essential regulator in 1,25$(OH)_2D_3$ and phosphate homeostasis [67–69].

Classical effects

The following mechanisms are illustrated in Figure 2. The hormone FGF23 is the master counter-regulator of 1,25$(OH)_2D_3$ synthesis [61]. The enzyme 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) regulates the last step in the activation of 1,25$(OH)_2D_3$ and its renal gene expression is lowered by FGF23 [70, 71]. Concomitantly, FGF23 increases 1,25$(OH)_2D_3$ -24-hydroxylase (*Cyp24a1*) expression, whose gene product promotes 1,25$(OH)_2D_3$ degradation, which entails a lowering of the circulating 1,25$(OH)_2D_3$ levels [61, 70, 71].

The phosphaturic effects of FGF23 has long been known [72]. The renal phosphate co-transport is maintained through Na^+-phosphate co-transporter type IIa and IIc (NaPi-IIa and NaPi-IIc), localized in the apical surface of renal proximal tubular epithelial cells [73]. NaPi-IIa is the most relevant regulator in the perpetuation of the serum and urinary phosphate balance [73]. Pharmacological inhibition of NaPi-IIa causes reduced plasma phosphate due to the dose-

dependent increase of urinary phosphate in mice [74]. FGF23 is able to decrease the expression of NaPi-IIa and NaPi-IIc in the proximal tubule in the kidney due to its binding on FGFR1 and FGFR4 and the resulting extracellular signal-regulated kinase (ERK 1/2) activation [70, 71, 75, 76]. Moreover, Andrukhova *et al.* demonstrate the internalization and degradation of NaPi-IIa from the membrane due to the phosphorylation of its scaffolding protein Na^+/H^+ exchange regulatory cofactor 1 (NHERF-1), through the activation of ERK1/2 and the serum/glucocorticoid-regulated kinase-1 (SGK-1) in a α-Klotho-dependent fashion [77]. The reduction of NaPi-IIa and NaPi-IIc results in a reduced phosphate resorption in the proximal tubular and causes an increased urinary phosphate excretion [70]. Overexpressing of *Fgf23* in transgenic mice causes reduced serum phosphate levels and significant urinary phosphate wasting due to a notable reduction of the NaPi-IIa expression [78, 79]. In contrast, *Fgf23* null mice are characterized by soft tissue calcifications and hyperphosphataemia caused by enhanced NaPi-IIa expression and resulting renal phosphate resorption [65, 80].

The brush-border membrane localized isoform NaPi-IIb is important for intestinal phosphate absorption and its absorption rate and mRNA expression is upregulated by $1,25(OH)_2D_3$ [81]. As described above, FGF23 can decrease the production of $1,25(OH)_2D_3$. Therefore, it can be hypothesized that FGF23 indirectly reduces intestinal phosphate uptake, by inhibition of vitamin D activation.

Besides the inhibition of phosphate resorption in the proximal renal tubule, FGF23 demonstrates also activity in the distal renal tubule [82]. Thus, activation of the ERK1/2, SGK-1, and with-no-lysine kinase 4 (WNK4) cascade through the formation of the FGF23-FGFR1-α-Klotho complex is shown to lead to increased membrane abundance of the transient receptor potential vanilloid 5 (TRPV5) calcium channel and, consequently, increased renal Ca^{2+} reabsorption [82]. This is supported by the fact, that TRPV expression in $Fgf23^{-/-}$/$VDR^{\Delta/\Delta}$ mice is decreased [82]. Furthermore, the same research group reported that FGF23 is capable, using the same signaling cascade as described above, to induces the membrane abundance of the Na^+-Cl^- co-transporter (NCC), followed by hypertension, volume expansion, and heart hypertrophy as a consequence of the induced increased renal Na^+ resorption [83].

Another organ of FGF23 activity is the parathyroid gland, which expresses FGFR1-3 as well as α-Klotho and is the original source for parathyroid hormone (PTH) [84]. The phosphaturic hormone PTH is the major stimulator of the synthesis of active vitamin D and the major regulator of maintaining serum Ca^{2+} levels [85]. Moreover, PTH is a regulator of phosphate excretion, also like FGF23, through the phosphorylation of NHERF-1 and the resulting degradation of NaPi-IIa, but unlike FGF23, in a protein kinase C (PKC)- and protein

kinase A (PKA)-dependent manner [86, 87]. Different studies demonstrate the FGF23-dependent decrease of the expression and the secretion of PTH *in-vivo* and *in-vitro* [88–91], marking a parathyroid-bone-axis in mineral homeostasis. However, this research viewpoint has not been conclusively clarified, as further study results give rise to controversy. Thus, Galitzer and coworkers show a PTH lowering by FGF23. However, they observe in a progressing disease status of chronic kidney disease (CKD) in a rat model, that the *PTH* mRNA increased, while *FGFR1* as well as α-*Klotho* expression sank, whereas the observed FGF23-dependent lowering of PTH was missing [90]. In addition, a recent study demonstrates a long-term stimulation of parathyroid cell proliferation and PTH secretion via FGF23 treatment *in-vitro* [84]. An secondary hyperparathyroidism can be observed in patients with CKD, triggered by elevated FGF23 levels, suggesting a missing suppression of PTH secretion by FGF23 [92]. In mice, overexpressing FGF23, PTH is significantly increased and flanked by hypertrophic parathyroid glands, likely resulting from persistent stimulation of PTH secretion as a consequence of increased FGF23 [79, 93]. These conflicting results may be potentially explained locally or systemically as follows: One aspect is probably the observed FGF23 signaling resistance in parathyroid glands in rats with CKD as well as in patients with secondary hyperparathyroidism, on the basis of the reduction of *FGFR1* and α-*Klotho* expression [90, 94]. Another perspective is the decrease in $1,25(OH)_2D_3$ levels, caused by elevated FGF23 levels in CKD and the resulting hypocalcemia, which leads to progressive PTH stimulation and secondary hyperparathyroidism [92]. Thus, FGF23 neutralization by antibody treatment is shown to normalize $1,25(OH)_2D_3$ levels by increasing *Cyp27b1* and decreasing *Cyp24a1* expressions [92]. This results in raised serum Ca^{2+} and consequently, decreased serum PTH in rats with early CKD [92]. In summary, it appears that FGF23 and PTH may form a regulatory loop, with the aspect that the described FGF23-PTH correlations are owed by impairments of other locally, systemic, and pathological factors [38].

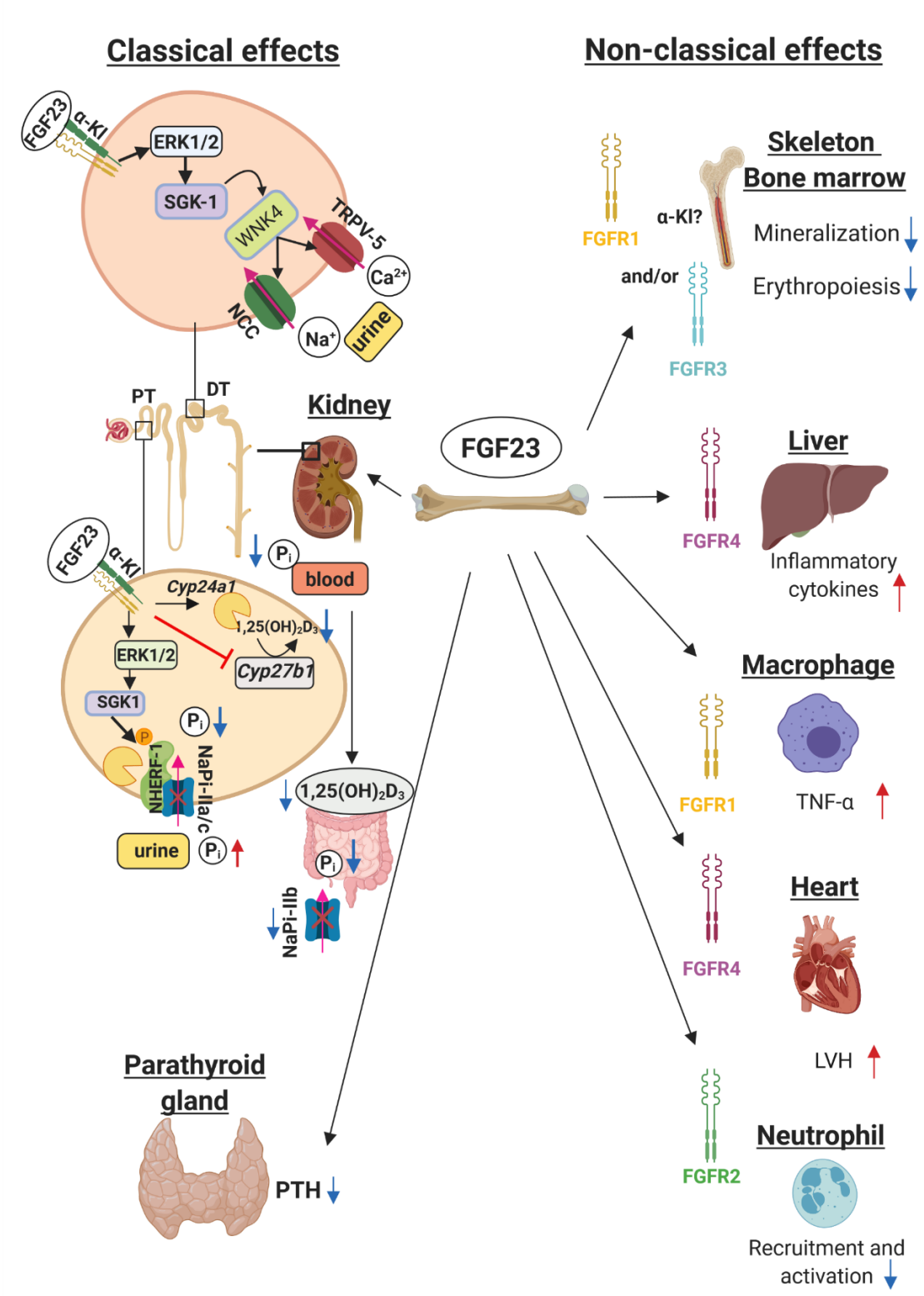

Figure 2: Overview of the classical and non-classical effects of FGF23.
Figure according to [50, 95]. For more details see text. Illustration was created with biorender.com
Fibroblast growth factor 23 (FGF23); Fibroblast growth factor receptor (FGFR); α-Klotho (α-Kl); Extracellular signal-regulated kinase 1/2 (ERK1/2); Serum/glucocorticoid-regulated kinase-1 (SGK-1); With-no-lysine kinase 4 (WNK4); Na^+-Cl^- co-transporter (NCC); Transient receptor potential vanilloid 5 (TRPV5) Ca^{2+} channel; Sodium (Na^+); Calcium (Ca^{2+}); Proximal tubule (PT); Distal tubule (DT); Inorganic phosphate (P_i); $1,25(OH)_2D_3$ (active vitamin D); 25-hydroxyvitamin D-24-hydroxylase (Cyp24a1); 25-hydroxyvitamin D-1α-hydroxylase (Cyp27b1); Na+-phosphate co-transporter type II (NaPi-II); Na+/H+ exchange regulatory cofactor 1 (NHERF-1); Parathyroid hormone (PTH); Tumor necrosis factor alpha (TNF-α); Left ventricular hypertrophy (LVH)

Non-classical effects

The action of FGF23 in the kidney and parathyroid gland and its controlled mechanisms in phosphate, $1,25(OH)_2D_3$ and PTH homeostasis are well described and are among the classical functions of this hormone. In addition, ongoing research has identified further extra-renal target organs and regulatory mechanisms of FGF23, which constitutes non-classical functions [50] and will be summarized in the following and pictured in Figure 2.

Progressing CKD is marked by increasing FGF23 levels [96], which is associated with occurring atherosclerotic and heart failure events [97], as well as left ventricular hypertrophy [98, 99]. At first, it was unclear whether left ventricular hypertrophy was a direct or indirect mechanism, until it was shown that FGF23 directly affects the cardiomyocytes by FGFR4 dependent PLCγ/calcineurin/NFAT activation, α-Klotho independently [100, 101]. Furthermore, it is demonstrated that FGF23 can intensify diastolic dysfunction due to myocardial fibrosis via upregulating β-catenin and transforming growth factor-β (TGF-β) [102].

FGF23 has also been shown to play a physiological role in the suppression of bone mineralization, which is independent of phosphate homeostasis [103, 104]. Thus, Shalhoub *et al.* show that FGF23 inhibits mineralization in mouse osteoblast-like cells due to suppression of the tissue-nonspecific alkaline phosphatase (TNAP) via FGFR1, and that this effect is enhanced by soluble Klotho [105]. TNAP breaks the inhibitory effect of inorganic pyrophosphate (PPi) on mineralization by splitting it to promote mineralization [106]. In contrast, Murali *et al.* report an autocrine or paracrine action of FGF23, in which the hormone regulates the osteoblast mineralization locally due to binding on FGFR3 in a α-Klotho-independent fashion, suppressing TNAP, whereby regulating indirectly osteopontin (*OPN*) transcription [107]. The cellular mechanisms of FGF23 to inhibit mineralization via FGFR1 and/or FGFR3 requires further clarification and whether this is α-Klotho-dependent or -independent.

Moreover, an increased erythropoiesis in *Fgf-23*$^{-/-}$ mice is shown, an effect which is reversed by treatment with FGF23 protein [108]. The inhibition of FGF23 signaling abolishes renal anemia and iron deficiency, reinforces erythropoiesis and encourages the commitment of hematopoietic stem cells towards the erythroid linage, marking the bone marrow as another place of FGF23 action [109].

Given the fact, that the liver is one of the organs with the highest expression of FGFR4, but α-*Klotho* is not expressed [32, 110] and FGF23 is expressed but not secreted [111], the question arises to what extent FGF23 affects this organ. Singh *et al.* show that FGF23 is capable

to induce the expression of inflammatory cytokines in primary mouse hepatocytes, such as C-reactive protein (CRP) or interleukin-6 (IL-6), via the PLCγ/calcineurin/NFAT signaling in an FGFR4-dependent manner [110]. Tumor necrosis factor alpha (TNF-α) is also upregulated by FGF23 in hepatocytes [111]. These data show the involvement of FGF23 in inflammatory processes and their stimulation by increased expression of corresponding cytokines.

In addition, the involvement of FGF23 in inflammatory processes described above is also relevant for immune response, as shown by the marked increase of FGF23 in the serum of mice after immunological stimuli and the correlation of FGF23 levels with infection-related hospitalization [112, 113]. Different studies demonstrate the expression of FGF23 in cultured macrophages, the FGF23-dependent upregulation in the macrophages cell number as well as the TNF-α expression via FGFR1 in these cells [112, 114]. This shows a paracrine function of FGF23 in the innate immune system, which is counter-regulatory to the effects of $1,25(OH)_2D_3$ in the immune response [114]. Moreover, Rossaint *et al.* demonstrate an impaired neutrophil recruitment and host defense during CKD by FGF23-mediated inhibition of leukocyte activation, adhesion, and migration via FGFR2 [115].

1.3 FGF23-related disorders and clinical implications

The role of FGF23 in maintaining phosphate and $1,25(OH)_2D_3$ homeostatic balance was described above. Therefore, a disturbed FGF23 regulation and production as a consequence of hereditary or non-hereditary disorders causes systemic hypo- and hyperphosphataemic states and an impaired $1,25(OH)_2D_3$ production. These FGF23-related disorders are described below.

Hypophosphataemic FGF23-related disorders

Table 1: Hypophosphataemic FGF23-related disorders.

Disorder	Cause/ responsible gene
Autosomal dominant hypophosphataemic rickets/osteomalacia (ADHR)	Gain-of-function mutation of *FGF23*
X-linked hypophosphataemic rickets/osteomalacia (XLH)	Loss-of-function mutation of *PHEX*
Autosomal recessive hypophosphataemic rickets/osteomalacia 1 (ARHR1)	Loss-of-function mutation of *DMP1*
Autosomal recessive hypophosphataemic rickets/osteomalacia 2 (ARHR2)	Loss-of-function mutation *ENPP1*
Autosomal recessive hypophosphataemic rickets/osteomalacia 3 (ARHR3)	Loss-of-function mutation *FAM20C*
McCune-Albright syndrome/fibrous dysplasia (MAS/FD)	Gain-of-function mutation of *GNAS1*
Craniometaphyseal dysplasia (CMD)	Loss-of-function mutation *ANKH*
Osteoglophonic dysplasia (OD)	Gain-of-function mutation *FGFR1*
Tumor-induced osteomalacia (TIO)	Overproduction of FGF23 in a causative tumor
t(9,13)(q21.13:q13.1)	Chromosomal translocation adjacent to *Klotho*

Table 1 is adapted from [39]. Fibroblast growth factor 23 (FGF23); Phosphate regulating endopeptidase homolog X-linked (PHEX); Dentin matrix protein-1 (DMP1); Ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1); Family with sequence similarity 20, member C (FAM20C); G protein alpha subunit S1 (GNAS1); Progressive ankylosis protein homolog (ANKH); Fibroblast growth factor receptor 1 (FGFR1)

Table 1 summarizes different disorders characterized by high FGF23 levels which cause a systemic hypophosphataemic state as a consequence of a disrupted molecular regulation and production. At this, Autosomal dominant hypophosphataemic rickets/osteomalacia, X-linked hypophosphataemic rickets/osteomalacia, Autosomal recessive hypophosphataemic rickets/osteomalacia 1-3, craniometaphyseal dysplasia, and osteoglophonic dysplasia are diseases, which are based on hereditary germline mutations causing FGF23-related rickets/osteomalacia [39].

Autosomal dominant hypophosphataemic rickets/ osteomalacia is caused by a missense mutation in the *FGF23* gene adjacent to the cleavage site in Arg (179) and Ser (180), which leads to a resistance to protein cleavage of FGF23, thus maintaining its biological activity and causing hypophosphataemia, rickets, osteomalacia, lower extremity deformities, and stunted growth in affected patients [36, 41, 116].

X-linked hypophosphataemic rickets/osteomalacia, the most common form of familial hypophosphataeamic rickets, is generated by an inactivation mutation of the human *phosphate regulating endopeptidase homolog X-linked* (*PHEX*) gene [117]. *Hyp* mice, the murine XLH model, show high Fgf23 levels, reduced phosphate and calcium, and decreased $1,25(OH)_2D_3$ levels in the serum, as well as mineralization defects [118, 119]. Moreover, patients suffering from XLH show, like patients with ADHR, an increased phosphate wasting and a resulting hypophosphataemia, rickets, leg deformities, and short stature [120].

Autosomal recessive hypophosphataemic rickets/osteomalacia (ARHR) are divided into three subclasses. ARHR1 is caused by a loss-of-function mutation in the *dentin matrix protein-1 (DMP1*) gene, mainly expressed in bone cells and encoding a non-collagenous bone matrix protein, which results in hypophosphataemic rickets/osteomalacia, defective osteocyte maturation, and excessive FGF23 production [121–123]. The inactivation mutation in the *ectonucleotide pyrophosphatase/phosphodiesterase 1* (*ENPP1*) gene, encoding a protein involved in the regulation of PPi and inhibition of calcification, generates ARHR2 and causes hypophosphataemic rickets resulting from elevated FGF23 levels [124, 125]. Moreover, the progressive ankylosis protein (ANK) is another regulator of cellular PPi levels [126]. A mutation in the human *progressive ankylosis protein homolog* (*ANKH*) gene causes the rare genetic disorder craniometaphyseal dysplasia, which is characterized by hyperostosis of craniofacial bones and widened metaphysis in long bones [127–129]. *Ank* loss-of-function impairs PPi transport and induces *Fgf23* expression in a murine craniometaphyseal dysplasia model [129]. ARHR3 results from a loss-of-function mutation in the *FAM20C* gene [130], causing high FGF23 levels, because of the reduced cleavage due to the lack of phosphorylation near the cleavage site [40]. Other symptoms are tooth decay, osteosclerosis rather than rickets, and ectopic calcifications [130]. In addition, a conditional *Fam20c* knockout in mice leads to hypophosphataemic rickets, affected mineralization and differentiation of bone cells and increased Fgf23 production [131].

McCune-Albright syndrome/fibrous dysplasia (MAS/FD) is characterized by an activating mutation of the human *G protein alpha subunit S1* gene, which causes increased cyclic adenosine monophosphate (cAMP) production [132]. Moreover, the high cAMP levels

in MAS/FD are involved in the observed elevated FGF23 production and altered cleavage due to decreased ppGALNT3 and increased furin activity in this disease [133, 134].

Osteoglophonic dysplasia is caused by a gain-of-function mutation in the *FGFR1* gene [135]. Activation of FGFR induces FGF23 synthesis [136]. This possibly leads to the observed phosphate wasting and disturbed vitamin D metabolism in OD, as well as non-ossifying bone lesions, a depressed nasal bridge, and rhizomelic dwarfism [135].

A chromosome translocation t(9,13)(q21.13:q13.1) adjacent to the *Klotho* gene causes a rare disease characterized by high α-Klotho and FGF23 levels and hypophosphataemic rickets [137].

Tumor-induced osteomalacia (TIO) is a rare acquired paraneoplastic disease characterized by benign mesenchymal tumors producing high levels of FGF23 and causing renal phosphate wasting, reduced $1,25(OH)_2D_3$, and osteomalacia [37, 72, 138–140].

Hyperphosphataemic FGF23-related disorders

The mirror image of FGF23-induced hypophosphataemic rickets/osteomalacia is found in the disorders of familial tumoral calcinosis (FTC), characterized by an impairment or loss of FGF23 function and resulting hyperphosphataemia, normal to high $1,25(OH)_2D_3$, and massive ectopic calcifications in the skin and subcutaneous tissues [39, 141]. One disorder in FTC is a loss-of-function mutation in the *FGF23* gene, inducing FGF23 proteolysis and absent or extremely reduced secretion of intact full-length FGF23 [142–144]. Another mutation, causing FTC, affects the *GALNT3* gene, leading to FTC symptoms and high C-terminal but absent intact FGF23 levels, pointing out the necessity of GALNT3-dependent O-glycosylation of FGF23 for its secretion and functionality [145, 146]. As described above, α-Klotho is an essential co-factor for FGF23 signaling. An observed missense mutation in the *KL* gene leads to attenuated α-Klotho production, resulting in impaired FGF23 bioactivity and causes FTC [147].

Chronic kidney disease (CKD) as disorder with hyperphosphataemia and FGF23 hyperfunction

CKD arises through irreversible alterations of kidney function and structure caused by different disease pathways, such as diabetes [148–150], hypertension [148, 150], inflammation [151] or congenital disorders [152], establishing a chronic reduction of the kidney function [153]. CKD is defined by a decline of the glomerular filtration rate (GFR) <60 mL/min per 1. 73 m^2 or marker of kidney damage, or both, for at least 3 months duration [153]. With a worldwide prevalence of 9.1% and 697.5 million recorded cases of all-stage CKD, as well as 1.2 million

caused deaths in 2017, this disease shows a high global clinical relevance [154]. Patients with CKD show elevated levels of FGF23, which increases with decreasing GFR [96, 155–157]. This was also observed in rats with early-stage kidney disease [92]. Due to the impairment of kidney performance and the reduction of nephrons and their function, more phosphate per nephron must be excreted to maintain phosphate balance. [158]. This compensation is achieved by an increased production of FGF23 at the beginning and during the progression of the disease, which initially keeps the serum phosphate levels in normal range (Fig. 3) [96, 155, 156].

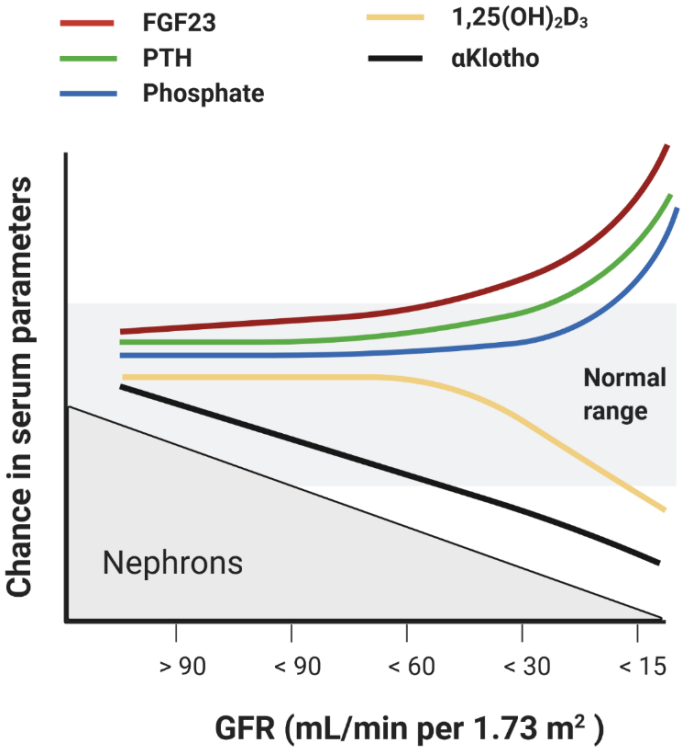

Figure 3: Changes of serum parameters of FGF23, PTH, phosphate, $1,25(OH)_2D_3$ and α-Klotho during progression of chronic kidney disease.
Figure according to [159–161]. For more details see text. Illustration was created with biorender.com.
Glomerular filtration rate (GFR); Fibroblast growth factor 23 (FGF23); Parathyroid hormone (PTH); $1,25(OH)_2D_3$ (active vitamin D)

However, in the further course of CKD this compensation increasingly disappears and consequently results in hyperphosphataemia [155]. This enhancement of FGF23 production results in decreased $1,25(OH)_2D_3$ that is followed by increased PTH levels and produces secondary hyperparathyroidism (Fig. 3) [92, 155]. The increased PTH levels in turn increase FGF23 production [162, 163], which leads to a further reduction of $1,25(OH)_2D_3$. In contrast,

a recent study shows an increasing plasma concentration of PTH and FGF23 with decreasing GFR in children with CKD and kidney transplant patients, but the increased PTH was predominantly oxidized and biologically less active PTH [164]. This provides insight into the complexity of the FGF23 and PTH relationship in the context of renal loss of function and points to other influencing factors, such as the associated increase in oxidative stress in these diseases [164]. The increasing oxidation of PTH and the associated loss of function, while the progressive increase of FGF23 remains constant, indicates that other factors besides secondary hyperparathyroidism must have a decisive influence [164]. Moreover, a decline of α-Klotho expression has been observed in CKD [94, 165], possibly due to elevated FGF23 and low $1,25(OH)_2D_3$ levels, as both suppress α-Klotho expression [66, 166]. In addition, high Pi intake decreases α-Klotho expression in murine models [167, 168]. The reduction of α-Klotho in the kidney could lead to resistance to FGF23 signaling due to deteriorated FGFR binding, which decreases the phosphaturic effect [158], thus reinforcing the described vicious circle of increased FGF23, decreased $1,25(OH)_2D_3$ and α-Klotho, high PTH and hyperphosphataemia (Fig. 3). Considering the high number of CKD diseases and deaths worldwide, research on FGF23 and its regulation and role in the pathophysiology of CKD is of great interest.

1.4 Regulatory mechanisms of FGF23 production

The multitude of mechanisms controlled by FGF23, its relevance to phosphate homeostasis, and its involvement in hereditary and non-hereditary pathologies has initiated numerous research projects that addresses the underlying mechanisms of production of this bone-derived hormone [50, 169, 170]. Several studies in recent years have provided insights into the system of local and systemic regulation of FGF23 transcription and formation (pictured in Fig. 4), as well as post-translational modifications and processing, with numerous mechanisms identified but the complex network not yet fully elucidated [171].

Bone-localized regulators of FGF23

As described above, several human diseases based on mutated genes and the impaired bioactivity of their protein products point to the existence of various bone-localized factors and their influence on the FGF23 regulation.

The *PHEX* gene is expressed in osteoblasts and osteocytes, encoding a 100-105 kDa glycoprotein. Inactivating mutations in its sequence caused the dominant disorder X-linked hypophosphataemia (XLH), which is characterized by elevated FGF23 levels [117, 118, 172–174]. It is shown that the peptidase PHEX does not cleave Fgf23, but suppresses its expression, which is conveyed due to FGFR-signaling [175–177]. Possibly, PHEX interacts thereby with DMP1 by binding to the acidic serine-aspartate rich MEPE-associated motif (ASARM) of DMP1 and αvβ3-integrin, which results in decreased *Fgf23* expression [177–179]. Moreover, binding interaction possibly results in increased FGF23 protein degradation due to the chaperone 7B2 coactivation of proprotein convertase 2. In contrast, PHEX mutations resulted in reduced 7B2•proprotein convertase 2 activity and decreased FGF23 cleavage, which is further exacerbated through reduced DMP1 cleavage and consequent increased *Fgf23* mRNA expression [179, 180]. The displacement of DMP1-PHEX interaction results also in increased FGF23 production [179]. Inactivating mutations in *DMP1* are responsible for autosomal recessive hypophosphataemic rickets (ARHR) as a consequence of elevated FGF23 levels, emphasizing the regulatory function of that protein [181]. DMP1, which is localized in bone and involved in mineralization processes, inhibits *Fgf23* expression *in-vitro* [182, 183] and B6-DMP1^{Tg} mice exhibited reduced Fgf23 serum levels [183]. Taken together, FGF23 is suppressed by PHEX and DMP-1 under physiological conditions; therefore the *loss-of-function* mutation in XLH and ARHR results in increased FGF23 expression and protein stability due to reduced cleavage [179].

In addition, impairment of local mineralization processes in bone and the supply and abundance of PPi can regulate FGF23 production. Thus, ENPP1 generates PPi and its knockdown results in increased Fgf23 plasma levels and bone-derived mRNA expression in *Enpp1*$^{-/-}$ mice [184, 185]. Moreover, a loss-of-function mutation of *ENPP1* causes the human disease ARHR2, with high FGF23 levels and hypophosphataemic rickets/osteomalacia [124]. In addition, the protein ANK channels intracellular PPi into the extracellular matrix and its inactivation in a murine model results in decreased mineral deposition, reduced *Phex* expression, and greatly increased *Fgf23* mRNA expression [129].

Systemic Regulation

Serum phosphate and $1,25(OH)_2D_3$ levels are closely controlled in a physiological range by FGF23. Therefore, the amount of phosphate and $1,25(OH)_2D_3$ are important regulators of FGF23 production. Several *in-vitro* and *in-vivo* studies describe phosphate- and $1,25(OH)_2D_3$-dependent FGF23 stimulation and demonstrate the existing feedback loops between FGF23, phosphate and $1,25(OH)_2D_3$ [48, 186–193] as pictured in Fig. 4. $1,25(OH)_2D_3$ can affect FGF23 directly through a vitamin D- response element (VDRE) in its promotor region and indirectly through increased intestinal phosphate intake through NaPi-IIb [81, 191].

In addition, dietary phosphate intake also affects systemic regulation of FGF23. Thus, a phosphate-rich diet in humans has been shown to increase serum phosphate and urinary phosphate excretion, accompanied by increased intact FGF23 levels, inversely correlated $1,25(OH)_2D_3$, and declined PTH [194]. Consistent with this, another study initially observes a decrease in serum FGF23 levels in healthy men following a low phosphate diet, which increased again with subsequent phosphate restoration and supplementation, accompanied with increased urinary phosphate excretion and negative correlated serum $1,25(OH)_2D_3$ [195]. In addition, an increase in serum FGF23 levels is also observed in sham-operated rats on a high phosphate diet compared with normal phosphate diet [196]. In this context, it is of interest whether phosphate can directly influence FGF23 synthesis. Vidal *et al.* recently show that high phosphate stimulates *Fgf23* mRNA expression in UMR106 osteoblast-like cells in the presence of high glucose medium [197]. In addition, phosphate treatment enhances *Fgf23* promotor activity and increases *Fgf23* expression in the presence of $1,25(OH)_2D_3$ in UMR106 cells, but also by stimulating of NAPH-mediated production of reactive oxygen species and the MEK-ERK pathway [198]. Interestingly, there is evidence that bone cells are able to sense high Pi levels by ligand-independent activation of FGFR1c and post-translationally affect FGF23 regulation [187]. Thereby, the increased FGF23 serum levels do not result from increased expression, but

from reduced cleavage rates due to protective O-glycosylation as a consequence of increased *Galnt3* expression [187]. $1,25(OH)_2D_3$ also increases *Galnt3* expression and possibly counteracts FGF23 degradation [186].

Consumption of a calcium- and phosphate-rich diet increases FGF23 serum levels in human subjects [194]. However, infusion-induced short-term changes in serum Ca^{2+} have no effect on FGF23 in healthy subjects or dialysis patients [199], whereas additional oral calcium carbonate administration for 8 weeks in $1,25(OH)_2D_3$-treated patients with CKD is associated with increased FGF23 levels [200]. Consistent with this, an increase of dietary Ca^{2+} results in increased serum Ca^{2+} levels, which are associated with higher Fgf23 levels in rats [201]. Furthermore, both dietary Ca^{2+} supplementation and continuous intravenous Ca^{2+} infusion increase both the circulatory Fgf23 level and bone-localized *Fgf23* mRNA expression in mice and rats [202, 203]. Consequently, a reduction in circulatory FGF23 levels was observed under hypocalcaemic conditions [201].

The feedback loop of ↑ PTH → ↑ FGF23 → ↓ PTH, as already partly described in 1.2.1, is still controversial. Different studies demonstrate a PTH- and PTH receptor-dependent induction of *FGF23* mRNA and FGF23 serum levels *in-vitro* and *in-vivo* via both the orphan nuclear receptor, nuclear receptor-associated protein 1 (Nurr1), and the Wnt signaling pathway [162–164, 204–207]. Furthermore, this is evidenced by increased levels of FGF23 in hyperparathyroidism, which is reversed due to parathyroidectomy in primary and secondary hyperparathyroidism and renal dysfunction [162, 163, 208, 209]. Interestingly, a recent study has been shown that only non-oxidized PTH has a stimulatory effect on *Fgf23* expression *in-vitro*, whereas oxidation in PTH at methionine-8, -18 or in combination weakens or even eliminates this effect [164]. In contrast, there are studies exist showing a lowering [210] or no FGF23 stimulation by PTH [211, 212]. The dissent probably results from the independent regulation of $1,25(OH)_2D_3$, Ca^{2+}, and phosphate by PTH and FGF23 and that these factors in turn are independent regulators of these two hormones, as illustrated in Fig. 4. In addition, the effect of PTH on FGF23 under pathological conditions, such as CKD, is not equivalent to the effects under physiological conditions because the disease may affect FGF23 secondarily. Therefore, it may be possible that the decline in renal function under these conditions has a more significant effect on FGF23 levels than hyperparathyroidism [213].

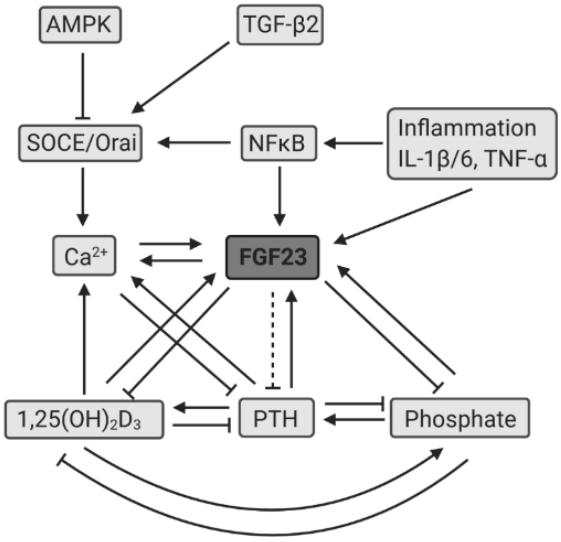

Figure 4: Systemic and local regulatory mechanisms of FGF23 production and their interaction.
Figure according to [213]. For more details see text. Illustration was created with biorender.com
The dotted line indicates conflicting data regarding the effect of FGF23 on PTH particularly in clinical conditions of CKD and hyperparathyroidism, as described in section 1.2. AMP-activated protein kinase (AMPK); Transforming growth factor-β2 (TGF-β2); Interleukin (IL); Tumor necrosis factor alpha (TNF-α); Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NFκB); Calcium (Ca^{2+}); Store-operated Ca^{2+} entry (SOCE); Fibroblast growth factor 23 (FGF23); Parathyroid hormone (PTH); $1,25(OH)_2D_3$ (active vitamin D)

Local regulatory mechanisms

Ca^{2+} and store-operated Ca^{2+} entry (SOCE)- dependent regulation

There is evidence, indicating a direct Ca^{2+}-stimulating effect on *Fgf23* mRNA expression in bone [202, 203], partially due to the regulation of the *Fgf23* promoter activity by calcium [214]. Furthermore, Zhang *et al.* demonstrate that the NFκB-sensitive Ca^{2+} channel Orai-1 accomplishes SOCE and, as a consequence, increases the cytosolic Ca^{2+} activity, which causes the upregulation of *Fgf23* transcripts [215]. Moreover, the regulatory role of Orai-1 for FGF23 production was also confirmed in *Orai1*$^{-/-}$ mice, showing a decreased *Fgf23* expression [216]. The importance of SOCE for FGF23 production is exemplified through different studies [215, 217–220]. SOCE is an important mechanism for the maintenance of intracellular Ca^{2+} balance as well as Ca^{2+}-dependent cellular signaling [221]. The initiation starts due to the activation of a receptor coupled with PLCγ and subsequent generation of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol. IP_3 binds to and opens IP_3 receptor (IP_3R) channels on the endoplasmic reticulum (ER) membrane, which causes an ER Ca^{2+} efflux into the cytoplasm [222]. The depletion of ER Ca^{2+} stores lead to dissociation of Ca^{2+} from ER-luminal EF-hand domains of stromal interaction molecule 1 (STIM1) and STIM2, which initiates its conformational change

leading to oligomerization and subsequent translocation to the ER-plasma membrane [223, 224]. The activated STIM oligomers recruits, binds to, and activates Ca^{2+} release-activated Ca^{2+} (CRAC) channels formed by ORAI-1 proteins, located in the cell membrane [222, 223]. The formation of these activated and opened CRAC channels causes an extracellular Ca^{2+} influx into the cell [223], which is termed as SOCE, because of its dependency on the ER Ca^{2+} concentration. The increase of cytoplasmic [Ca^{2+}] ($[Ca^{2+}]_c$) replenishes the ER Ca^{2+} stores via Sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA)2b Ca^{2+} pump, which translocate cytoplasmic Ca^{2+} into the lumen of ER [225], but also modulates calmodulin, which activates the serine/threonine phosphatase calcineurin resulting in the activation of the transcription factor nuclear factor of activated T-cells (NFAT) [222, 226, 227]. The calcineurin/NFAT signaling is besides Ca^{2+}-sensitive NFκB mediated induction of *Fgf23* expression [215] another modulator of intracellular Ca^{2+} -dependent FGF23 regulation [183, 228, 229].

NFκB and pro-inflammatory stimuli

NFκB is an important regulator of FGF23 as it mediates Ca^{2+}-dependent FGF23 induction as well as pro-inflammatory stimuli, which also induce the production of FGF23 (Fig. 4). Thus, inflammatory stimuli have been shown to promote FGF23 expression in a NFκB activity-dependent manner, and also to prevent cleavage by increasing *Galnt3* expression [230]. Moreover, cytokines like TNF-α [230–233], IL-1β and IL-6 [230, 233–235] as well as bacterial lipopolysaccharides (LPS) [230, 233, 236] are potent regulators to elevate FGF23 production, indicating the relevance of inflammation for FGF23 levels [237]. The fact of induction of inflammatory cytokines by FGF23 was pointed out above [110]. Thus, pro-inflammatory stimuli lead to an increase in FGF23 levels, which in turn promote the formation of cytokines in macrophages, liver, and other tissues and its own synthesis [238].

Recently discovered regulators of FGF23

Ongoing research in the network of systemic and local regulation of FGF23 production has identified a number of additional regulators and mechanisms [171]. Prominent among these are TGF-β2, via TGF-β receptor and receptor-related kinases induced SOCE [218], metabolic regulators, such as insulin [239] or AMP-activated protein kinase (AMPK) mediated decline of Orai-1 membrane insertion and subsequent reduced SOCE [220], leptin [240] or high fat diet [232] and also iron deficiency [241], anemia [241, 242], hypoxia [237, 243], erythropoietin [244], the renin-angiotensin-aldosterone system [245], and various metal ions [217, 246, 247].

2 Objective of this thesis

Two decades after its discovery as a causative factor of phosphate wasting in rare hereditary diseases with hypophosphataemic rickets [36, 78], the realization developed that FGF23 is the master regulator of the maintenance of phosphate and $1,25(OH)_2D_3$ homeostasis [61, 70]. The disruption of this physiological homeostasis results from and in the pathophysiological development of bone and kidney diseases [248, 249]. Thus, FGF23 not only plays a role in inherited disorders with disturbed bone mineralization [250], but is also significantly involved in the pathogenesis of chronic kidney disease as well as concomitant comorbidities [161, 251]. Therefore, FGF23 is discussed both as a potential biomarker for altered mineral metabolism and cardiovascular disease and as clinical target [50]. Nevertheless, the exact regulation of FGF23 production and whether this is a cause or consequence of the pathologies is not understood in detail. Therefore, it is important to understand the underlying local and systemic regulation of the synthesis of this hormone. The present work attempts to contribute to this and to fill existing gaps in the previously known regulatory network. Thus, knowledge of the local regulation of FGF23 formation in bone will be expanded by identifying and studying novel regulators and the underlying signaling mechanisms in osteoblasts and osteocytes. This is also important with regard to the development of therapeutic approaches for the aforementioned FGF23-related pathologies.

Paper 1

As outlined in the introduction section 1.4, FGF23 formation is controlled by a complex regulatory network. Likewise, the importance of inflammation as well as various inflammatory regulators for FGF23 formation was presented. At the cellular level, the transcription factor NFκB possesses an important role in the mediation of intracellular signaling mechanisms of FGF23 expression. Thus, NFκB is involved in FGF23 formation by both Ca^{2+}-sensitive [215, 219] and pro-inflammatory stimuli [230]. Interestingly, NFκB transcriptional activity is also induced by the p38MAPK [252–255], a serine/threonine kinase activated among others by inflammation [256, 257] and stress-stimuli [258]. In this context, the first study presented in this thesis addresses the following question:

I) What is the role of p38MAPK in the regulation of FGF23 synthesis and is the transcription factor NFκB involved?

To this end, in rat UMR106 osteoblast-like cells endogenously expressing FGF23 [190], the expression of p38MAPK isoforms was first examined. In subsequent experiments, p38MAPK-specific inhibitors and an activator were used in UMR106 cells and mouse IDG-SW3 osteocytes to test a possible influence of p38MAPK on FGF23 gene and protein expression. To understand the underlying signaling and to find out a possible role of NFκB in it, further experiments were performed with specific inhibitors of NFκB in the presence of the p38MAPK activator in UMR106 cells.

Paper 2

The influence of signaling pathways on the formation of FGF23, which are energy-dependent and involved in cellular energy homeostasis, are the subject of several studies in recent years [197, 232, 239]. Of particular note is the influence of FGF23 synthesis by AMPK [220], as sensor of cellular energy deprivation [259]. Peroxisome proliferator-activated receptor (PPAR)-α is a transcription factor, which regulates the expression of genes involved in cellular fatty acid uptake, β-oxidation, and triglyceride catabolism [260]. Thus, PPARα has an important function in cellular energy homeostasis through transcriptional control of target genes for mitochondrial and peroxisomal energy supply [261]. Moreover, energy availability is shown to influence formation of FGF23 *in-vivo* and *in-vitro* [197]. Cellular energy supply by restoring ATP levels upon energy deficiency is subject to the activity of AMPK [259], which is also activated by an PPARα agonist [262, 263]. Therefore, we explored the following question in Paper 2:

II) What is the impact of PPARα on FGF23 formation and is AMPK involved in the underlying signaling?

Since UMR106 osteoblast-like cells endogenously express FGF23, first it was tested if *Pparα* expression after treatment with its agonist fenofibrate was also detectable in these cells. Next, experiments were carried out in UMR106 cells using PPARα agonists, an antagonist or siRNA mediated *Pparα*-specific gene silencing to test a possible impact of PPARα on FGF23 gene and protein expression. The next series of experiments addressed the question whether the PPARα agonist fenofibrate modulates FGF23 production via AMPK activity and AMPK-mediated SOCE, as described elsewhere [220]. Therefore, AMPK phosphorylation after treatment with fenofibrate was determined and SOCE was quantified using Ca^{2+}-dependent fluorescence measurements in Fura-2-loaded UMR106 cells.

Paper 3

TGF-β is a cytokine that acts in a large variety of cells and biological mechanisms involving cell proliferation, differentiation, apoptosis, immune response, and autophagy, and is the primary factor driving fibrosis in most forms of CKD [264, 265]. In addition, TGF-β2 is found to act via TGF-β receptor I (TGF-βRI) to control the formation of Fgf23 via upregulating SOCE in UMR106 cells [218]. Myostatin, a potent negative regulator of skeletal muscle growth [266], is a TGF-β family member [264] and acts in a TGF-β-like signaling pathway via binding on activin receptor type 2B (ACVR2B) and complex formation with TGF-βRI [267, 268]. In addition, myostatin also acts via p38MAPK [269], a novel regulator of FGF23 synthesis in UMR106 and IDG-SW3 cells identified in **Paper 1** of this thesis. Therefore, Paper 3 addressed the following question:

III) What is the relevance of myostatin in the regulation of FGF23?

To this end, the expression of the myostatin receptors *Acvr2a* and *Acvr2b* was first tested in UMR106, IDG-SW3 and MC3T3-E1 cells. Next, these cells were treated with recombinant myostatin to determine whether it affects *Fgf23* mRNA expression and protein synthesis. Subsequent experiments were conducted to investigate the underlying regulatory mechanisms. Therefore, UMR106 cells were treated with either TGF-βRI, p38MAPK, NFκB, or SOCE inhibitor in the presence of recombinant myostatin.

3 Experimental Studies

3.1 Paper 1: p38MAPK controls fibroblast growth factor 23 (FGF23) synthesis in UMR106-osteoblast-like cells and in IDG-SW3 osteocytes

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ORIGINAL ARTICLE

p38MAPK controls fibroblast growth factor 23 (FGF23) synthesis in UMR106-osteoblast-like cells and in IDG-SW3 osteocytes

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Abstract

Background p38 mitogen-activated protein kinase (p38MAPK) is a serine/threonine kinase activated by cellular stress stimuli including radiation, osmotic shock, and inflammation and influencing apoptosis, cell proliferation, and autophagy. Moreover, p38MAPK induces transcriptional activity of the transcription factor complex NFκB mediating multiple pro-inflammatory cellular responses. Fibroblast growth factor 23 (FGF23) is produced by bone cells, and regulates renal phosphate and vitamin D metabolism as a hormone. FGF23 expression is enhanced by NFκB. Here, we analyzed the relevance of p38MAPK activity for the production of FGF23.

Methods *Fgf23* expression was analyzed by qRT-PCR and FGF23 protein by ELISA in UMR106 osteoblast-like cells and in IDG-SW3 osteocytes.

Results Inhibition of p38MAPK with SB203580 or SB202190 significantly down-regulated *Fgf23* expression and FGF23 protein expression. Conversely, p38MAPK activator anisomycin increased the abundance of *Fgf23* mRNA. NFκB inhibitors wogonin and withaferin A abrogated the stimulatory effect of anisomycin on *Fgf23* gene expression.

Conclusion p38MAPK induces FGF23 formation, an effect at least in part dependent on NFκB activity.

Keywords Anisomycin · NFκB · Calcium · Phosphate · Klotho

Introduction

p38 mitogen-activated protein kinase (p38MAPK) is a member of the MAPK family of serine/threonine kinases activated by cellular stress stimuli [1, 2] including radiation [3], osmotic shock [4], and inflammation [5]. Four isoforms of this stress kinase have been identified (p38-α, -β, -γ, and -δ) [6, 7] influencing apoptosis [8–10], cell proliferation [11–13], and autophagy [14]. Moreover, p38MAPK induces activity of the transcription factor complex NFκB mediating multiple pro-inflammatory cellular responses [15–17].

The NFκB-dependent up-regulation of Orai1 [18], a Ca^{2+} release-activated Ca^{2+} (CRAC) channel [19] involved in various cellular processes, with subsequent store-operated Ca^{2+} entry (SOCE) [20] induces the formation of fibroblast growth factor 23 (FGF23) [21–28]. FGF23 is a hormone produced by bone cells (osteoblasts/osteocytes) that induces renal phosphate excretion and inhibits renal formation of $1,25(OH)_2D_3$, active vitamin D. These endocrine effects of FGF23 require transmembrane αKlotho as a co-receptor [21, 29–32]. In mice, FGF23 or αKlotho deficiency results in rapid aging and various age-associated diseases due to hyperphosphatemia-induced massive calcification in organs and tissues [31–34]. FGF23 also induces left heart hypertrophy without a contribution of αKlotho [35, 36]. Further paracrine effects of FGF23 include the regulation of neutrophil recruitment [37, 38]. Cleavage of transmembrane αKlotho yields soluble Klotho (sKL) which can be found in blood, CSF, and urine and exerts FGF23-independent endocrine effects [29, 35, 39].

The FGF23 serum level is elevated in frequent human disorders including chronic kidney (CKD) and cardiovascular disease [35, 40–42]. In CKD, the FGF23 serum concentration goes up before hyperphosphatemia or a marked decline of glomerular filtration rate (GFR) can be observed [43–46], pointing to FGF23 being a powerful disease biomarker [47].

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Other factors regulating the production of FGF23 include $1,25(OH)_2D_3$ [30, 48], parathyroid hormone (PTH) [49–51], dietary phosphorus intake [52, 53], inflammation (in part through NFκB-mediated Orai1 up-regulation [22, 24]) [54–58], the iron status [59, 60], insulin [61], and AMP-dependent protein kinase (AMPK) [22].

In view of the p38MAPK-dependent regulation of NFκB, we explored whether this kinase is also a regulator of FGF23 synthesis.

Materials and methods

Cell culture

Culture of and experiments with UMR106 rat osteoblast-like cells were conducted as described elsewhere [21].

Cells were pretreated with 100 nM $1,25(OH)_2D_3$ (Tocris, Bristol, UK) for 24 h followed by incubation with p38MAPK inhibitors SB202190 or SB203580 (both from Tocris, 10 µM, 24 h) or with activator anisomycin (Tocris, 1 µM, 6 h) or with vehicle only. Where indicated, NFκB inhibitors withaferin A (500 nM; Tocris) or wogonin (100 µM; Sigma) were added for 24 h.

IDG-SW3 mouse osteocytes were also cultured as described [62]: 0.15×10^6 cells were plated on rat tail type I collagen-coated 12-well plates in αMEM medium, containing 10% FBS, 1% penicillin–streptomycin and 50 U/ml IFN-γ, and incubated at 33 °C. After adherence, 50 µg/ml ascorbic acid and 4 mM β-glycerophosphate replaced IFN-γ in the medium (all reagents from ThermoFisher Scientific). After 21 days of differentiation at 37 °C and 5% CO_2, IDG-SW3 cells were incubated with 20µM SB202190 for 24 h or with vehicle only. Three wells were pooled to one sample of treatment or vehicle.

Expression analysis

Total RNA was extracted with Tri-Fast (Peqlab, Erlangen, Germany). cDNA synthesis using 1.2µg RNA, random primers, and the GoScript™ Reverse Transcription System (Promega, Mannheim, Germany; 25 °C for 5 min, 42 °C for 1 h, and 70 °C for 15 min) was performed.

RT-PCR with 2µl cDNA (95 °C for 3 min, 35 cycles of 95 °C for 10 s, 58 °C (*p38MAPK-α*) or 60 °C (*p38MAPK-β/-γ/-δ*) for 30 s, and 72 °C for 30 s) was carried out. PCR products were loaded on a 2.4% agarose gel and visualized by Midori Green.

Primers used:

p38MAPK-α
F: CCGAGCGATACCAGAACCT
R: CTTCACTGCCACACGATGTC
p38MAPK-β
F: CAGAAGGTGGCTGTGAAGAAG
R: ACTTCGCTGAAATCCTCGAT
p38MAPK-γ
F: GACTTGAAACCTGGAAACCTG
R: GTGTAACGCATCCAATTCAAGA
p38MAPK-δ
F: GATATCTGGTCTGTTGGCTGC
R: CGCCTTGTCTTTCAGCTTCT

qRT-PCR

Total RNA (1.2 µg) was reverse-transcribed with the GoScript™ Reverse Transcription System (Promega). Relative *Fgf23* expression was determined by qRT-PCR using the Rotor-Gene Q (Qiagen, Hilden, Germany) and the GoTaq qPCR Master Mix (Promega). qPCR settings were 95 °C for 3 min, 35 cycles of 95 °C for 10 s, 60 °C for 30 s, 72 °C for 30 s (UMR106 cells) and 95 °C for 3 min, 40 cycles of 95 °C for 10 s, 58 °C for 30 s (*Fgf23*) and 60 °C for 30 s (*Tbp*), 72 °C for 30 s (IDG-SW3 cells). Calculated *Fgf23* mRNA expression levels were normalized to the expression levels of *Tbp* (TATA box-binding protein) of the same cDNA sample.

Primers used:

Rat Tbp
F: ACTCCTGCCACACCAGCC
R: GGTCAAGTTTACAGCCAAGATTCA
Rat Fgf23
F: TGGCCATGTAGACGGAACAC
R: GGCCCCTATTATCACTACGGAG
Mouse Tbp
F: CCAGACCCCACAACTCTTCC
R: CAGTTGTCCGTGGCTCTCTT
Mouse Fgf23
F: TCGAAGGTTCCTTTGTATGGA
R: AGTGATGCTTCTGCGACAAGT

Measurement of FGF23 protein

UMR106 cells were treated without or with 10µM SB202190 for 24 h. The cell culture supernatant was collected and stored at − 80 °C. Next, it was concentrated using Sartorius Vivaspin 6 Centrifugal Concentrators (Sartorius, Göttingen, Germany). C-terminal FGF23 was determined by an ELISA Kit (Immuntopics, Athens, USA) according to the manufacturer's protocol.

Statistics

All data are given as arithmetic mean ± SEM, *n* represents the number of independent experiments. Statistical comparisons were made by Student's *t* test or one-way ANOVA. Differences were considered as significant if $P < 0.05$.

Results

UMR106 osteoblast-like cells were used to study the impact of p38MAPK activity on FGF23 production. We first investigated the expression of different p38MAPK isoforms in this cell line. RT-PCR was carried out to identify isoform-specific transcripts. As illustrated in Fig. 1, all four p38MAPK isoforms, i.e., p38-α, p38-β, p38-γ, and p38-δ, could readily be detected in UMR106 cells.

Next, we assessed the contribution of p38MAPK activity to the production of FGF23. To this end, we exposed UMR106 osteoblast-like cells to p38MAPK inhibitor SB203580 and determined *Fgf23* mRNA by qRT-PCR as a measure of *Fgf23* gene expression. SB203580 (10 µM, 24 h) significantly reduced *Fgf23* transcripts compared to control (Fig. 2a). Similarly, another p38MAPK inhibitor, SB202190, also lowered the abundance of *Fgf23* mRNA in UMR106 cells (Fig. 2b). Moreover, SB202190 (20 µM, 24 h) down-regulated *Fgf23* mRNA in IDG-SW3 osteocytes (Fig. 2c) Employing ELISA, we measured C-terminal FGF23 in the cell culture supernatant. p38MAPK inhibitor SB202190 also suppressed FGF23 secretion by UMR106 cells (Fig. 2d). These results suggest that p38MAPK activity induces *Fgf23* gene expression and protein synthesis in both UMR106 osteoblast-like cells and in IDG-SW3 osteocytes.

A new series of experiments explored the effect of pharmacological p38MAPK activation on FGF23. UMR106 cells were treated with p38MAPK activator anisomycin, and *Fgf23* transcripts were again quantified by qRT-PCR. As demonstrated in Fig. 3, a 6h incubation with 1µM

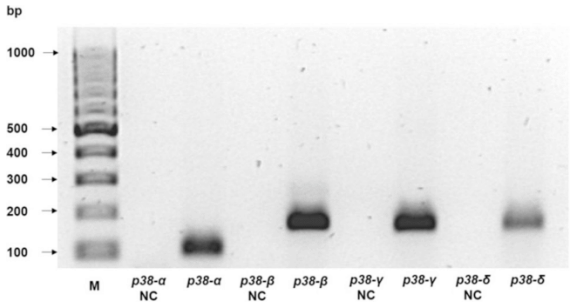

Fig. 1 Expression of p38MAPK isoforms in UMR106 rat osteoblast-like cells. Original agarose gel photo showing p38MAPK -α, -β, -γ, or -δ-specific cDNA in UMR106 cells. *NC* non-template control

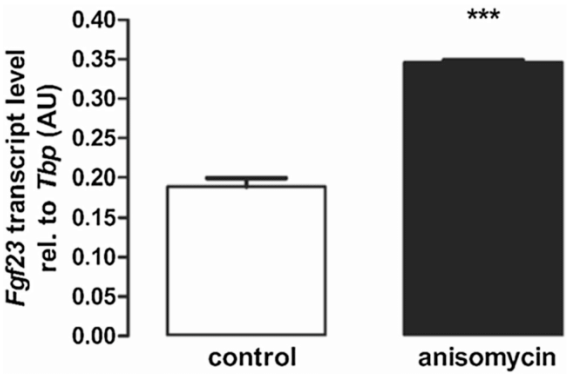

Fig. 3 p38MAPK activator anisomycin induces *Fgf23* gene expression in UMR106 cells. Arithmetic mean ± SEM ($n = 3$) of relative (rel.) *Fgf23* mRNA abundance in UMR106 cells incubated without (white bar) or with (black bar) p38MAPK activator anisomycin (1 µM, 6 h). ***$P < 0.001$ indicates significant differences. *AU* arbitrary units

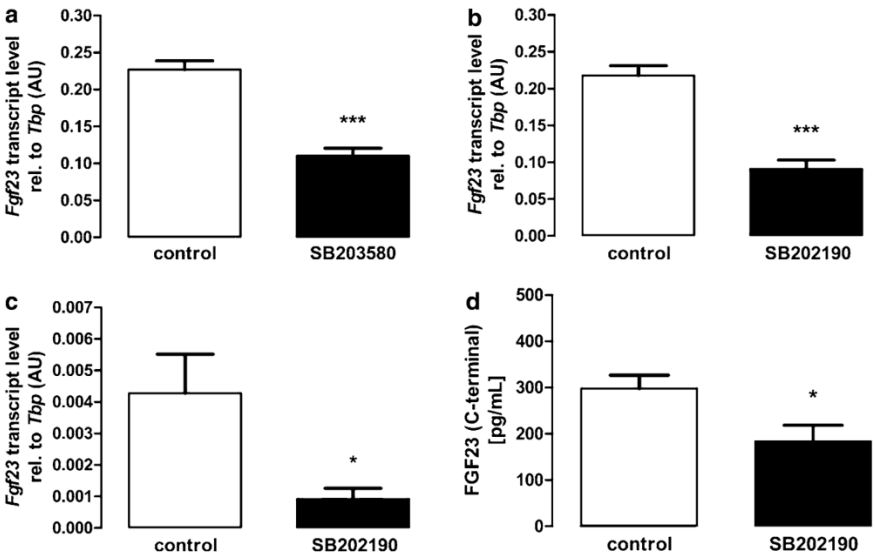

Fig. 2 p38MAPK inhibitors SB203580 or SB202190 suppress FGF23 production in UMR106 osteoblast-like cells and in IDG-SW3 osteocytes. Arithmetic mean ± SEM of relative (rel.) *Fgf23* mRNA abundance (**a**, **b**: $n = 7$, **c:** $n = 8$) or C-terminal FGF23 concentration in the cell culture supernatant (**d**: $n = 5$) of UMR106 osteoblast-like cells (**a**, **b**, **d**) or IDG-SW3 osteocytes (**c**) incubated without (white bars) or with (black bars) p38MAPK inhibitor SB203580 (**a**: 10 µM, 24 h) or SB202190 (**b**, **d**: 10 µM **c**: 20 µM, 24 h). ***$P < 0.001$; *$P < 0.05$ indicate significant differences. *AU* arbitrary units

anisomycin significantly induced *Fgf23* gene expression, a result again confirming a stimulatory effect of p38MAPK on FGF23 formation.

Since *Fgf23* gene expression is dependent on pro-inflammatory transcription factor NFκB, we tested whether the stimulating effect of p38MAPK on FGF23 is mediated by this transcription factor. To this end, we treated UMR106 cells with and without p38MAPK activator anisomycin in the presence and absence of NFκB inhibitors wogonin or withaferin A. As illustrated in Fig. 4, both wogonin (Fig. 4a) and withaferin A (Fig. 4b) abrogated the enhancement of *Fgf23* gene expression induced by anisomycin. These results suggest that the effect of p38MAPK on FGF23 is at least in part dependent on the induction of NFκB transcriptional activity.

Discussion

According to our study, p38MAPK is a powerful regulator of the production of FGF23: Pharmacological inhibition with two different p38MAPK inhibitors decreased whereas pharmacological p38MAPK activation elevated *Fgf23* transcripts in UMR106 osteoblast-like cells. Clearly, the p38MAPK effect also affects FGF23 protein synthesis as p38MAPK inhibition resulted in decreased FGF23 secretion into the supernatant of UMR106 osteoblast-like cells. Moreover, p38MAPK is similarly effective in IDG-SW3 osteocytes as the inhibitor also suppressed *Fgf23* gene expression in these cells. Our results suggest that p38MAPK activity up-regulates both gene expression of *Fgf23* and FGF23 protein production.

p38MAPK is a ubiquitously expressed serine/threonine kinase that belongs to the MAPK family of protein kinases up-regulated by different cellular stressors. Members of this kinase family translate extracellular signals (e.g., stress, cytokine stimuli) into intracellular responses [1, 2]. The main observation in our study, i.e., the stimulation of FGF23 production by p38MAPK in osteoblast-like cells, fits well into the established concept of p38MAPK being highly relevant for osteoblast differentiation as well as bone and skeleton formation [12, 63]. In line with this, deficiency of different p38 proteins resulted in skeletal defects and bone abnormalities [64]. p38MAPK regulates important osteogenic proteins including RUNX2 [64, 65] and controls migration of bone cells and is, therefore, particularly relevant after fractures [66–68].

p38MAPK is required for the differentiation of osteoclasts [69]. FGF23 inhibits osteoclastogenesis and increases the activity of osteoclasts [70]. It is intriguing to speculate that the p38MAPK-dependent regulation of FGF23 production may, therefore, also impact on osteoclast differentiation.

FGF23 formation in bone cells is stimulated by TGFβ [21]. Importantly, TGFβ activates p38MAPK in osteoblasts [71, 72], an effect that could contribute to TGFβ-mediated FGF23 production. Moreover, PTH [51, 73] and pro-inflammatory cytokines including TNFα [68, 74] and IL-1 [75, 76] are potent stimulators of p38MAPK and of FGF23 [50, 75, 77] synthesis in bone cells. Therefore, it is tempting to speculate that the signaling of different stimulators of FGF23 production converges on p38MAPK and that this kinase might, therefore, be an universal regulator of the formation of FGF23.

Our experiments further revealed that the p38MAPK effect on FGF23 was at least in part mediated by NFκB, a transcription factor complex implicated in a plethora of pro-inflammatory cellular responses [16]. p38MAPK is an established activator of NFκB [17], and NFκB has been demonstrated to up-regulate expression of CRAC channel Orai1 in UMR106 cells facilitating SOCE that triggers FGF23 production [18, 24, 25]. Interestingly, also TGFβ-dependent FGF23 production is dependent on Orai1-mediated SOCE

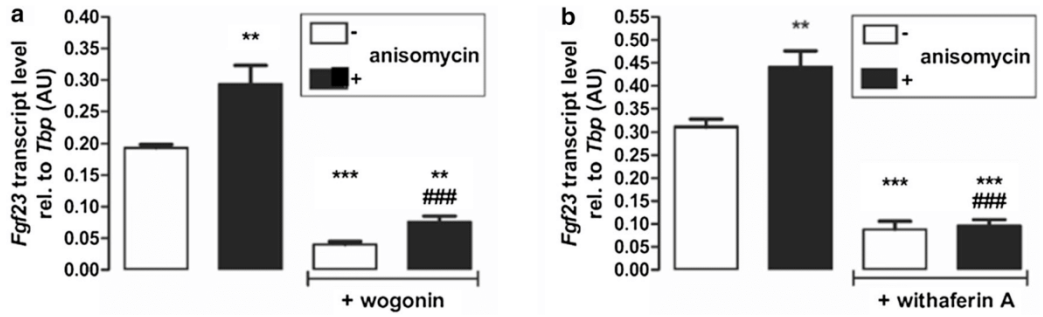

Fig. 4 NFκB inhibitors wogonin and withaferin A abrogate anisomycin-induced *Fgf23* gene expression in UMR106 cells. Arithmetic mean ± SEM of relative (rel.) *Fgf23* mRNA abundance in UMR106 cells incubated without (white bars) or with (black bars) p38MAPK activator anisomycin (1 μM, 6 h) in the presence or absence of NFκB inhibitor **a** wogonin ($n=4$; 100 μM, 24 h) or **b** withaferin A ($n=5$; 500 nM, 24 h). $^{**}P<0.01$, $^{***}P<0.001$ indicate significant differences from control. $^{\#\#\#}P<0.001$ indicates significant difference from the absence of NFκB inhibitor. *AU* arbitrary units

[21]. Therefore, it appears to be likely that p38MAPK/NFκB/Orai1 signaling is a major regulator of FGF23 synthesis in bone cells.

A wide range of pharmacological p38MAPK inhibitors have been developed and are suggested for the treatment of inflammatory diseases including multiple sclerosis, Alzheimer's disease, arthritis, asthma, and cancer [78–81]. In line with the stimulatory effect of NFκB on FGF23, inflammatory conditions are indeed associated with a high FGF23 plasma concentration [57, 58], and it appears to be possible that p38MAPK inhibition may not only be therapeutically beneficial in these disease, but also lower the abnormally high FGF23 plasma concentration typical of these conditions.

A clear limitation of our study is that it only includes in vitro data based on cell culture experiments. The in vivo relevance of p38MAPK for the production of FGF23 must be addressed in future investigations.

In conclusion, p38MAPK is a potent stimulator of *Fgf23* gene expression, at least in part by up-regulating NFκB.

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Compliance with ethical standards

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

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent is not required in this type of study.

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3.2 Paper 2: Peroxisome proliferator-activated receptor α (PPARα)-dependent regulation of fibroblast growth factor 23 (FGF23)

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SIGNALING AND CELL PHYSIOLOGY

Peroxisome proliferator–activated receptor α (PPARα)–dependent regulation of fibroblast growth factor 23 (FGF23)

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Abstract
Bone cells secrete fibroblast growth factor 23 (FGF23), a hormone that inhibits the synthesis of active vitamin D ($1,25(OH)_2D_3$) and induces phosphate excretion in the kidney. In addition, it exerts paracrine effects on other cells including hepatocytes, cardiomyocytes, and immune cells. The production of FGF23 is controlled by different factors including parathyroid hormone, $1,25(OH)_2D_3$, alimentary phosphate, insulin, inflammation, and AMP-dependent kinase (AMPK) regulation of store-operated Ca^{2+} entry (SOCE). Peroxisome proliferator–activated receptor α (PPARα) is a transcription factor with anti-inflammatory properties regulating lipid metabolism. Fibrates, PPARα agonists, are used in the treatment of dyslipidemia and activate AMPK. Here, we tested whether PPARα is a regulator of FGF23. *Fgf23* gene expression was analyzed in UMR106 rat osteoblast-like cells by qRT-PCR, AMPK phosphorylation by Western blotting, and SOCE assessed by fluorescence optics. PPARα agonists fenofibrate and WY-14643 suppressed, whereas PPARα antagonist GW6471 and siRNA-mediated knockdown of PPARα induced *Fgf23* gene expression. Fenofibrate induced AMPK activity in UMR106 cells and lowered SOCE. AMPK inhibitor compound C abrogated the PPARα effect on FGF23 in large part. Silencing of Orai-1 resulted in failure of PPARα to significantly influence *Fgf23* expression. Taken together, PPARα is a potent regulator of FGF23. PPARα agonists down-regulate FGF23 formation at least in part through AMPK-mediated suppression of SOCE.

Keywords Phosphate · $1,25(OH)_2D_3$ · Klotho · Inflammation

Introduction

Bone cells synthesize fibroblast growth factor 23 (FGF23), a hormone with renal effects and mediator with further paracrine actions on other cells [2, 6, 14, 29]. In the kidney, FGF23 inhibits the last reaction of the synthesis of active vitamin D, $1,25(OH)_2D_3$, which is catalyzed by 25-hydroxyvitamin D_3 1-alpha-hydroxylase [2]. Moreover, FGF23 reduces the reabsorption of phosphate that is accomplished by Na^+-dependent transporters in the proximal tubule [2, 11]. Extrarenal effects of FGF23 were found in the heart, in the liver, or in immune cells: FGF23 induces left heart hypertrophy [7], enhances the secretion of pro-inflammatory cytokines by hepatocytes [28], or regulates the recruitment of neutrophils [25].

The renal effects of FGF23 are dependent on transmembrane protein αKlotho [29]. The generation of mice deficient for either FGF23 or αKlotho revealed further significant effects of FGF23 and αKlotho that are dependent on deranged phosphate metabolism in large part: The animals age within weeks thereby exhibiting a broad spectrum of age-associated diseases affecting most organs and tissues and being typical of human aging [15, 27]. A low-phosphate or low-vitamin-D diet results in a normalization, pointing to a hitherto underestimated role of alimentary phosphate in the pathophysiology of aging-related diseases [16, 31].

FGF23 has been shown to be a reliable disease biomarker in various conditions including renal, metabolic, and cardiovascular disorders [24]. Particularly in chronic kidney disease (CKD), the FGF23 plasma level goes up early and predicts outcome [13].

The production of FGF23 is regulated by phosphate metabolism (alimentary phosphate) [33], $1,25(OH)_2D_3$ [23], parathyroid hormone (PTH) [18], inflammation [3, 9], iron

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metabolism [5], erythropoietin [4], and intracellular signaling cascades including AMP-dependent kinase (AMPK) signaling [10] and insulin-dependent phosphoinositide 3-kinase (PI3K) signaling [1]. The AMPK effect on FGF23 is dependent on store-operated Ca^{2+} entry (SOCE) that enhances *Fgf23* gene expression [10].

Peroxisome proliferator–activated receptors (PPAR) are a family of three transcription factors (PPARα, PPARβ/δ, and PPARγ) [17]. PPARα is expressed in many organs including the heart, liver, adipose tissue, skeletal muscle [17], and bone [32]. Upon ligand binding and forming a dimer with retinoid X receptor (RXR), PPARα binds to DNA in the nucleus and induces gene expression [17]. Target genes of PPARα affect cellular fatty acid uptake and oxidation [17]. Activation of PPARα by endogenous ligands including fatty acids and phospholipids results in enhanced fatty acid oxidation [17]. Hence, patients with hypertriglyceridemia are treated with fibrates, pharmacological PPARα agonists. PPARα agonists have been shown to induce AMPK activation [30].

Since AMPK is a powerful regulator of FGF23, this study explored in UMR106 rat osteoblast-like cells whether and how PPARα controls the production of FGF23.

Results

PPARα agonists suppress *Fgf23* gene expression

UMR106 osteoblast-like cells were used to investigate whether PPARα agonist fenofibrate, a drug widely used in the treatment of hypertriglyceridemia, influences FGF23. The cells were pretreated with $1,25(OH)_2D_3$ to induce *Fgf23* gene expression and then incubated without or with different concentrations of fenofibrate for 24 h, and *Ppara* gene expression was analyzed by qRT-PCR. As illustrated in Fig. 1a, fenofibrate significantly induced *Ppara* gene expression in a dose-dependent manner. Moreover, fenofibrate suppressed *Fgf23* gene expression in UMR106 cells (Fig. 1b). Also, WY-14643, another PPARα agonist, downregulated *Fgf23* gene expression in UMR106 cells within 24 h (Fig. 1c) in a dose-dependent manner. The suppressive effects of fenofibrate and WY-14643 on *Fgf23* expression translated into lower C-terminal FGF23 secretion into the cell culture supernatant (Fig. 1d, e). Intact FGF23 in the cell culture supernatant was not significantly different between control cells (2.5 ± 0.3 pg protein, $n = 10$) and cells exposed to 70 μM fenofibrate for 24 h (2.3 ± 0.4 pg protein, $n = 10$). In another series of experiment, intact FGF23 in the supernatant was significantly lower in UMR106 cells treated with 500 μM WY-14643 for 24 h (1.1 ± 0.3 pg protein, $n = 10$) than in control cells (1.6 ± 0.2 pg protein, $n = 10$, $p < 0.05$, Mann-Whitney *U* test). The ratio cFGF23-iFGF23/iFGF23 was not significantly different between control cells (12.3 ± 1.4, $n = 10$) and fenofibrate-treated cells (9.4 ± 1.4, $n = 10$) as well as between control cells (10.2 ± 1.1, $n = 10$) and WY-14643-treated cells (9.6 ± 2.9, $n = 10$).

In UMR106 cells pretreated with PTH to induce *Fgf23* expression, both fenofibrate and WY-14643 were similarly effective in lowering *Fgf23* transcripts (Fig. 2).

PPARα antagonist and silencing enhance Fgf23 gene expression

In view of the suppressive effect of PPARα agonists on FGF23 production, we studied the impact of PPARα antagonism. To this end, UMR106 cells were treated with PPARα antagonist GW6471 (25 μM). As shown in Fig. 3a, GW6471 significantly enhanced *Fgf23* gene expression in UMR106 cells within 6 h. To further characterize the role of PPARα for *Fgf23* gene expression, we employed the siRNA-mediated knockdown of *Ppara* in UMR106 cells. Similar to PPARα antagonism, silencing of the gene encoding PPARα resulted in an up-regulation of *Fgf23* transcripts (Fig. 3b).

The PPARα effect on FGF23 involves AMPK

AMPK has been demonstrated to be a powerful inhibitor of FGF23 production. Since PPARα agonists induce AMPK activity, we performed further experiments to assess whether the PPARα effect on FGF23 depends on AMPK. First, we employed Western blotting to study AMPK phosphorylation in UMR106 cells. As demonstrated in Fig. 4a, PPARα agonist fenofibrate increased the phosphorylation of AMPKα within 3 h, suggesting increased AMPK activity. Next, we explored whether the inhibitory effect of PPARα on *Fgf23* gene expression is dependent on AMPK. To this end, UMR106 cells were exposed to PPARα agonists fenofibrate or WY-14643 in the presence or absence of AMPK inhibitor compound C (1 μM, 24 h). PPARα agonists fenofibrate (Fig. 4b) and WY-14643 (Fig. 4c) failed to suppress *Fgf23* gene expression in the presence of compound C compared with control albeit the presence of PPARα agonist still resulted in lower *Fgf23* transcripts.

PPARα influences store-operated Ca^{2+} entry

The inhibitory effect of AMPK on *Fgf23* gene expression is in large parts dependent on the suppression of SOCE which facilitates FGF23 production. Our next series of experiments therefore focused on the impact of PPARα agonist fenofibrate on SOCE in UMR106 cells. Employing fluorescence optics, SOCE was estimated from the increase in Ca^{2+}-dependent fluorescence in Fura-2-loaded cells after the depletion of intracellular Ca^{2+} stores by adding thapsigargin and the addition of extracellular Ca^{2+}. As demonstrated in Fig. 5a, c,

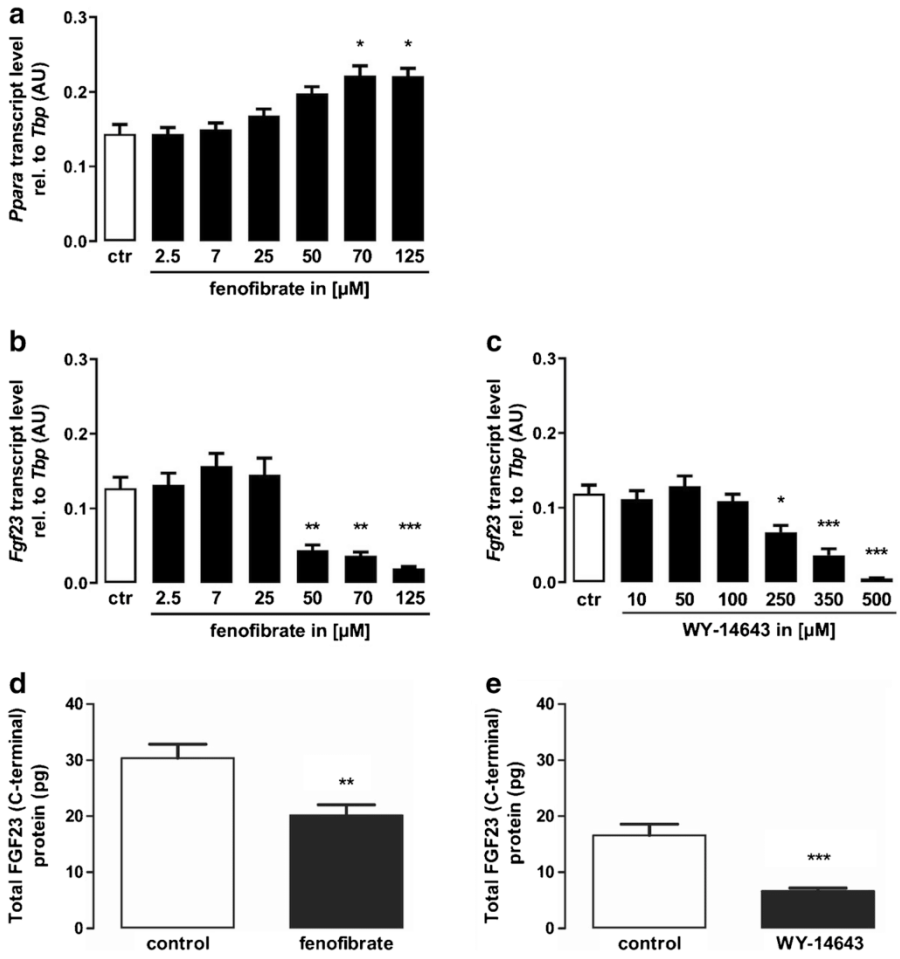

Fig. 1 PPARα agonists regulate *Fgf23* gene expression in UMR106 cells pretreated with $1,25(OH)_2D_3$. Arithmetic means ± SEM of relative (rel.) **a** *Ppara* or **b**, **c** *Fgf23* mRNA abundance or **d**, **e** total C-terminal FGF23 protein concentration in the cell culture supernatant concentrate of UMR106 osteoblast-like cells incubated without (white bars) or with (black bars) PPARα agonist fenofibrate (**a**, **b**, $n = 5$; **d** 70 μM, $n = 10$) or WY-14643 (**c** $n = 6$; **e** 500 μM, $n = 10$) for 24 h. The cells were pretreated with 100 nM $1,25(OH)_2D_3$ for 24 h to induce *Fgf23* expression. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ indicate significant difference from control. AU, arbitrary units (**a** Kruskal-Wallis; **b**, **c** one-way ANOVA; **d** unpaired Student's *t* test; **e** unpaired Student's *t* test with Welch's correction)

fenofibrate significantly reduced SOCE. Fenofibrate treatment also resulted in a significantly reduced depletion of intracellular Ca^{2+} stores following the addition of sarco-endoplasmic Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin (Fig. 5b).

The effect of PPARα on SOCE contributes to the suppression of FGF23 production

Our last series of experiments aimed to explore whether the regulation of SOCE by PPARα is needed to suppress FGF23

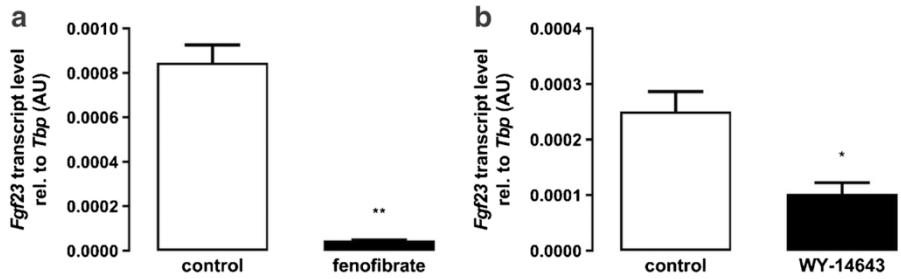

Fig. 2 PPARα agonists regulate *Fgf23* gene expression in UMR106 cells pretreated with PTH. **a** Arithmetic means ± SEM of rel. *Fgf23* mRNA abundance in UMR106 cells incubated without (white bars) or with (black bars) PPARα agonist fenofibrate (**a** 70 μM, 24 h, $n = 3$) or WY-14643 (**b** 500 μM, 24 h, $n = 3$). The cells were pretreated with 100 nM PTH for 24 h to induce *Fgf23* expression. $^*p < 0.05$ and $^{**}p < 0.01$ indicate significant difference. AU, arbitrary units (**a** unpaired Student's *t* test with Welch's correction; **b** unpaired Student's *t* test)

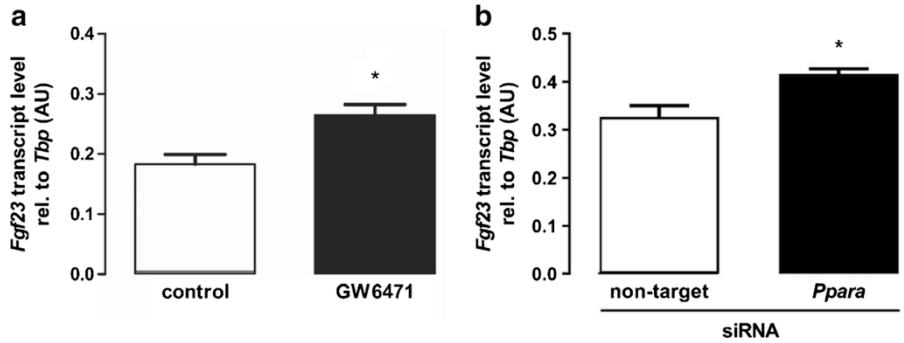

Fig. 3 PPARα antagonist GW6471 and siRNA-mediated knockdown of *Ppara* induce *Fgf23* gene expression in UMR106 cells. **a** Arithmetic means ± SEM of rel. *Fgf23* mRNA abundance in UMR106 cells incubated without (white bar) or with (black bar) PPARα inhibitor GW6471 (25 μM, 6 h, $n = 4$). **b** Arithmetic means ± SEM of rel. *Fgf23* mRNA abundance in UMR106 cells incubated with non-targeting (white bar) or *Ppara*-specific (black bar) siRNA (200 nM, 72 h, $n = 4$). $^{*}p < 0.05$ indicates significant difference. AU, arbitrary units (**a** unpaired Student's *t* test; **b** Mann-Whitney *U* test)

production. To this end, the effect of PPARα antagonist GW6471 was tested in UMR106 cells exposed to non-target siRNA or to cells treated with *Orai-1*-specific siRNA. Figure 6 reveals that GW6471 failed to significantly affect *Fgf23* gene expression in UMR106 cells treated with *Orai-1*-specific siRNA. This effect suggests that SOCE is, at least in part, needed for PPARα to regulate FGF23 production.

Discussion

Our study uncovers transcription factor PPARα as a potent suppressor of the formation of FGF23 in bone cells: Two different PPARα agonists attenuated whereas a PPARα antagonist and PPARα gene silencing enhanced *Fgf23* gene expression in UMR106 osteoblast-like cells. Interestingly,

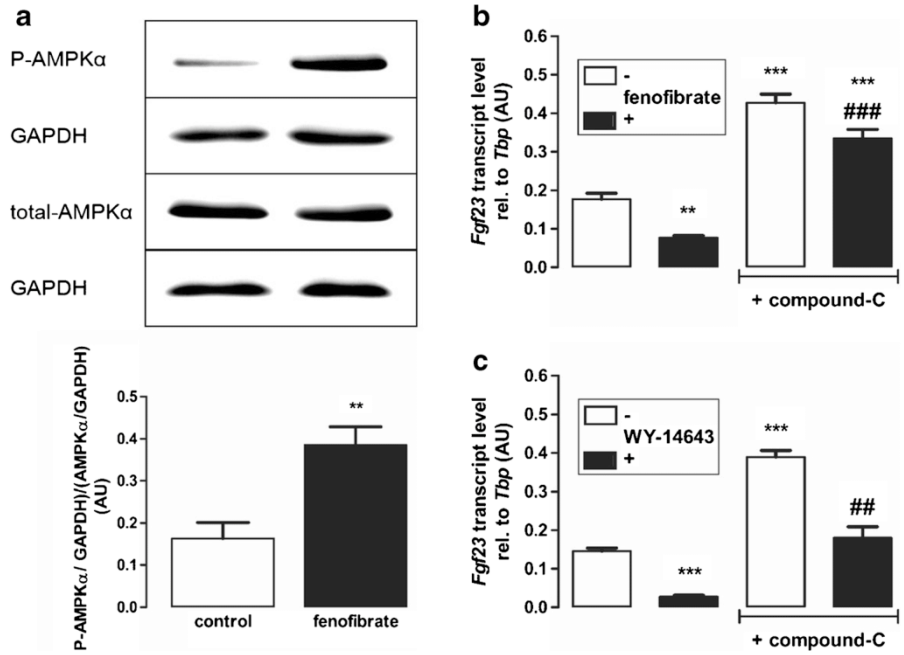

Fig. 4 The PPARα effect on FGF23 is at least in part dependent on AMPK. **a** Original Western blots (upper panel) demonstrating phospho-AMPKα, total AMPKα, and GAPDH protein abundance. The lower panel depicts the densitometric analysis (arithmetic means ± SEM, $n = 5$) of phospho-AMPKα and AMPKα abundance normalized to loading control GAPDH. UMR106 cells were treated without (white bar) or with (black bar) fenofibrate (70 μM, 3 h). **b**, **c** Arithmetic means ± SEM of rel. *Fgf23* mRNA abundance in UMR106 cells incubated without (white bars) or with (black bars) PPARα agonist fenofibrate (**b**, 70 μM, 24 h, $n = 5$) or WY-14643 (**c**, 500 μM, 24 h, $n = 9$) in the presence or absence of AMPK inhibitor compound C (1 μM, 24 h). $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significant difference from control. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ indicate significant difference from the absence of AMPK inhibitor (2nd vs 4th bar). AU, arbitrary units (**a** unpaired Student's *t* test; **b**, **c** one-way ANOVA)

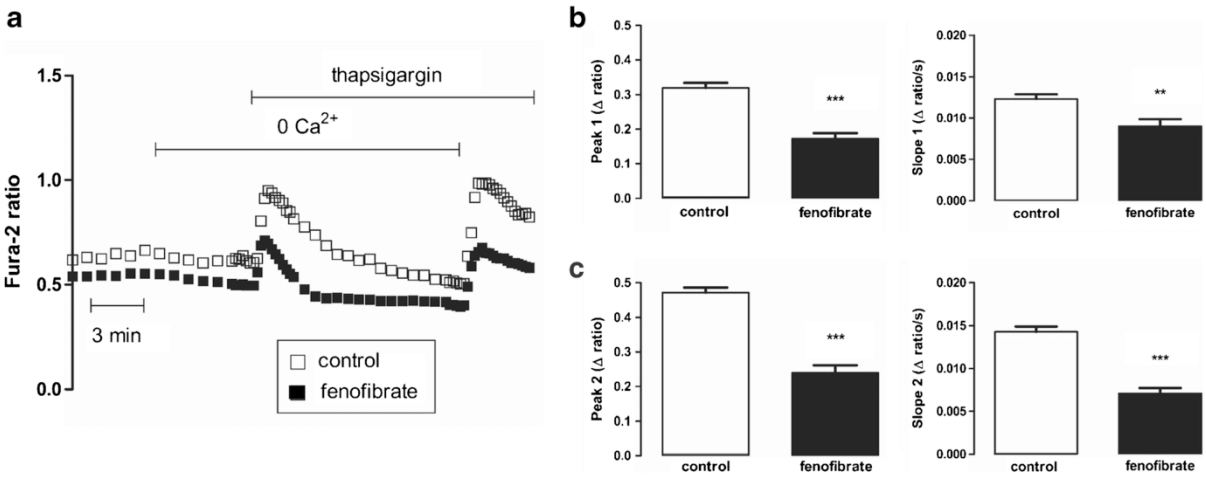

Fig. 5 Fenofibrate reduces store-operated Ca^{2+} entry (SOCE) in UMR106 cells. **a** Representative original tracings showing the ratio of fluorescence intensities emitted at 340 nm and 380 nm reflecting intracellular Ca^{2+}in UMR106 cells loaded with Fura-2. First, extracellular Ca^{2+} was eliminated, then 1 μM of the sarco-endoplasmic Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin was added, and finally, extracellular Ca^{2+} was readded to the cells treated without or with fenofibrate (70 μM, 24 h). **b** Arithmetic means ± SEM of the peak (left) and the slope (right) values of the $[Ca^{2+}]_i$ surge after the addition of thapsigargin. These values reflect Ca^{2+} release from intracellular Ca^{2+} stores ($n = 35–89$ cells measured on four different days). **c** Arithmetic means ± SEM of the peak (left) and the slope (right) values of the $[Ca^{2+}]_i$ surge after the final readdition of extracellular Ca^{2+}. These values reflect SOCE ($n = 35–89$ cells measured on four different days). $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significant differences. (**b**, **c** Mann-Whitney *U* test)

fenofibrate exhibited a sharp dose response in the concentration range of 25–50 μM.

Notably, the effect of PPARα agonists on *Fgf23* was observed in both UMR106 cells pretreated with $1,25(OH)_2D_3$ or PTH to induce *Fgf23* expression. Hence, it appears to be unlikely that the interference of vitamin D receptor (VDR) and PPARα signaling explains the effect of PPARα agonists on *Fgf23*. PPARα agonists suppressed C-terminal FGF23 in the cell culture supernatant whereas the effect on intact FGF23 appears to be weaker (WY-14643) or even lacks statistical significance (fenofibrate). This may hint at PPARα agonists affecting not only *Fgf23* gene expression and protein synthesis but also post-translational processing of FGF23 protein.

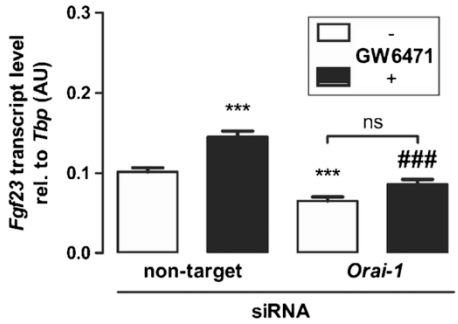

Fig. 6 PPARα antagonist GW6471 failed to significantly induce *Fgf23* expression in UMR106 cells after siRNA-mediated downregulation of *Orai-1*. Arithmetic means ± SEM of rel. *Fgf23* mRNA abundance in UMR106 cells incubated without (white bars) or with (black bars) PPARα antagonist GW6471 (25 μM, 6 h) in the presence or absence of non-target or *Orai-1*-specific siRNA (100 nM, 48 h, $n = 10$). $^{***}p < 0.001$ indicates significant differences from control. $^{###}p < 0.001$ indicates significant difference from the absence of *Orai-1*-specific siRNA (2nd vs 4th bar). AU, arbitrary units (one-way ANOVA)

Recently, cellular energy sensor AMPK has been identified as a powerful regulator of FGF23 production [10]. Since PPARα agonists have been demonstrated to induce AMPK [30], we sought to study whether AMPK is involved in the PPARα effect on FGF23. By Western blotting, we confirmed that PPARα agonist fenofibrate activates AMPK. Moreover, we found that PPARα agonists were less capable of suppressing *Fgf23* gene expression in the presence of AMPK inhibitor compound C. These results suggest that PPARα requires AMPK activity to mediate the downregulation of *Fgf23* gene expression.

Fgf23 gene expression has been shown to be triggered by SOCE [34]. In addition, the AMPK effect on FGF23 is dependent on SOCE [10]. Therefore, we employed fluorescence optics to determine whether PPARα agonist fenofibrate modifies SOCE in UMR106 cells. Our experiments revealed that fenofibrate downregulates SOCE. However, fenofibrate also led to an attenuated depletion of intracellular Ca^{2+} stores. Importantly, PPARα antagonist GW6471 was not capable of significantly influencing *Fgf23* expression in UMR106 treated with *Orai-1*-specific siRNA. These results hint at AMPK-regulated SOCE contributing to the PPARα effect on FGF23 but do not rule out the possibility that the blunting effect of PPARα on Ca^{2+} release from intracellular Ca^{2+} stores is also relevant.

PPARα exerts strong anti-inflammatory effects including the inhibition of NFκB signaling [12, 22]. Importantly, pro-inflammatory cytokines and NFκB signaling are also major stimuli of FGF23 production. In particular, NFκB has been demonstrated to induce FGF23 production through upregulation of SOCE [34]. Hence, the anti-inflammatory effects of PPARα are likely to contribute to the suppression of *Fgf23* gene production.

PPARα agonists, fibrates, are commonly used in the treatment of patients with dyslipidemia. They are particularly useful in hypertriglyceridemia. The drugs are supposed to prevent vascular disease and its sequelae, i.e., stroke or myocardial infarction. Interestingly, a positive association between dyslipidemia and plasma FGF23 levels has been established in patients on dialysis [21]. Moreover, the serum triglyceride levels are positively correlated with the plasma FGF23 concentration [19]. Recent research has established that FGF23 not only indicates disease as a biomarker but also actively drives pathophysiological processes, e.g., heart hypertrophy, a frequent and detrimental consequence of CKD [7]. The FGF23-lowering effect of PPARα agonists as suggested by our study may therefore have two implications: In line with FGF23 as a biomarker, the decrease of FGF23 by PPARα agonists may indicate a reduction of cardiovascular risk. Beyond this, it may also prevent FGF23-induced pathophysiological processes.

PPARα agonists reduced *Fgf23* gene expression in UMR106 cells. This effect is likely to have in vivo relevance: In line with a stimulating effect of FGF23 on left heart hypertrophy, an association of a higher FGF23 level with left heart hypertrophy has been found in children with CKD. In this study, the mean FGF23 level was 147 RU/ml in children with and 114 RU/ml in children without left heart hypertrophy [20]. The obviously clinically relevant difference in the FGF23 level of the two groups is well in the range of the effects of PPARα agonists revealed by our study.

Taken together, PPARα is a powerful regulator of *Fgf23* gene expression. The suppressive effect of PPARα on FGF23 is at least partially mediated by AMPK-dependent control of SOCE.

Methods

Cell culture and treatments

UMR106 rat osteoblast-like cells were cultured under standard conditions and pretreated with 100 nM $1,25(OH)_2D_3$ (Tocris, Bristol, UK) for 24 h followed by an incubation with PPARα agonists fenofibrate (2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoic acid isopropyl ester, 2.5–125 μM, 24 h, Sigma-Aldrich, Schnelldorf, Germany), WY-14643 (10–500 μM, 24 h, Tocris), or with antagonist GW6471 (25 μM, 6 h, Tocris). The pretreatment with $1,25(OH)_2D_3$ is needed to upregulate *Fgf23* gene expression which is otherwise low in UMR106 cells [26]. In other experiments, parathyroid hormone (100 nM PTH fragment 1–34, Sigma-Aldrich) was used to upregulate *Fgf23* gene expression. In some experiments, AMPK inhibitor compound C (1 μM, 24 h, Tocris) was added.

Silencing

For silencing of *Ppara* or *Orai-1*, 1.5×10^5 cells were seeded per well for 24 h in complete medium. UMR106 cells were transfected with 200 nM ON-TARGETplus Rat SMARTpool *Ppara* siRNA (L-080000-02-0005, Dharmacon, Lafayette, CO, USA), 100 nM ON-TARGETplus Rat SMARTpool *Orai-1* siRNA (L-081151-02-0010, Dharmacon), or non-targeting control siRNA (D-001810-10-20, Dharmacon) using 5 μl DharmaFECT 1 transfection reagent in an antibiotic-free complete medium. Twenty-four hours after *Orai-1* silencing and 48 h after *Ppara* silencing, 100 nM $1,25(OH)_2D_3$ were added and the cells incubated for another 24 h. Silencing efficiency was tested by qRT-PCR. In cells, treated with control siRNA, relative *Ppara* expression was 0.21 ± 0.01 arbitrary units ($n = 4$) and 0.09 ± 0.00 ($n = 4$) in cells treated with *Ppara*-specific siRNA ($p < 0.001$). In another series of experiments, relative *Orai-1* expression was 0.67 ± 0.01 a.u. ($n = 10$) in cells treated with control siRNA and 0.28 ± 0.01 a.u. ($n = 10$) in cells exposed to siRNA specific for *Orai-1* ($p < 0.01$).

Quantitative real-time PCR

Total RNA was isolated with Tri-Fast (Peqlab, Erlangen, Germany) and 1.2 μg hereof was used for cDNA synthesis with random primers and the GoScript™ Reverse Transcription System (Promega, Mannheim, Germany; 25 °C for 5 min, 42 °C for 1 h, and 70 °C for 15 min). qRT-PCR using a Rotor-Gene Q cycler (Qiagen, Hilden, Germany) and GoTaq qPCR Master Mix (Promega) was carried out to determine relative *Fgf23, Ppara* and *Orai-1* expression. The qPCR conditions were as follows: 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 60 °C for 30 s, 72 °C for 30 s. The calculated *Fgf23*, *Ppara*, and *Orai-1* mRNA transcript levels were normalized to the transcript levels of *Tbp* (TATA box-binding protein).

The primers used are as follows:

Rat Tbp

F: ACTCCTGCCACACCAGCC
R: GGTCAAGTTTACAGCCAAGATTCA

Rat Fgf23

F: TGGCCATGTAGACGGAACAC
R: GGCCCCTATTATCACTACGGAG

Rat Ppara

F: CTGCTATAATTTGCTGTGGAG
R: GAGTTTTGGGAAGAGAAAGG

Rat Orai-1

F: AGCCTCAACGAGCACTCGAT
R: CTGATCATGAGGGCGAACAGG

Western blotting

To examine AMPKα activity, 0.7×10^6 UMR106 cells were seeded in a 25-cm^2 cell culture flask and cultured for 45 h. After treatment with 70 μM fenofibrate for 3 h, the cells were lysed using 1× RIPA buffer (Cell Signaling Technology, Frankfurt, Germany) containing Halt™ protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Darmstadt, Germany). Next, 50 μg of the total protein lysate was subjected to a standard Western blot procedure using the following antibodies: AMPKα (#5831), phospho-AMPKα (Thr172, #2535), GAPDH (#5174), and secondary antibodies: anti-rabbit IgG (#7074) conjugated with HRP (all antibodies from Cell Signaling Technology). Protein bands were visualized by means of ECL detection reagent (GE Healthcare-Amersham, Amersham, UK) and Syngene G:BOX Chemi XX6 (VWR, Dresden, Germany) documentation system. Band intensities were referred to GAPDH and the relative phosphorylation of AMPKα was normalized to total AMPKα.

Ca^{2+} measurements

The measurements were carried out according to a procedure based on Fura-2 fluorescence as described [8]. To determine SOCE, extracellular Ca^{2+} was first removed. Next, Ca^{2+} was readded along with sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase inhibitor thapsigargin (1 μM; Tocris). Ca^{2+} entry was quantified by calculating the slope (delta ratio/s) and the peak (delta ratio) following readdition of Ca^{2+}. The cells were in Ringer solution containing the following in millimolar: 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.8 $CaCl_2$, 2 Na_2HPO_4, 32 N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid), 5 glucose, pH 7.4. Ca^{2+}-free Ringer contained the following in millimolar: 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 2 Na_2HPO_4, 32 N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid), 0.5 ethylene glycol-bis-(2-aminoethylether)-N,N,N′,N′-tetraacetic acid, 5 glucose, pH 7.4.

Measurement of FGF23 protein in the culture media

For the determination of FGF23 release into the cell culture medium, UMR106 cells were cultured as described above and treated with or without fenofibrate (70 μM) or WY-14643 (500 μM) for 24 h. After the treatment, the cell culture supernatant was collected and stored at − 80 °C for subsequent protein detection. The supernatant was concentrated using Sartorius Vivaspin 6 Centrifugal Concentrators (Sartorius, Göttingen, Germany). The amount of C-terminal and intact FGF23 was determined by ELISA (Mouse/Rat FGF-23 (C-Term) or Mouse/Rat FGF-23 (Intact), Immutopics, San Clemente, CA, USA) according to the manufacturer's protocol. The detected FGF23 is referred to the volume of the concentrate and is given as total protein amount (pg) per concentrate.

Statistics

The data are shown as arithmetic means ± SEM and *n* represents the number of independent experiments. Data were tested for normality and variance homogeneity. Statistical comparisons of two groups were made by unpaired Student's *t* test (if necessary with Welch's correction) or Mann-Whitney *U* test (for data not passing normality). More than two groups were tested for significance by one-way ANOVA followed by Bonferroni's multiple comparison test. For experiments with more than two groups and data not passing normality, Kruskal-Wallis followed by Dunn's multiple comparison test was used as indicated in the figure legends. Differences were considered significant if $p < 0.05$.

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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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3.3 Paper 3: Myostatin regulates the production of fibroblast growth factor 23 (FGF23) in UMR106 osteoblast–like cells

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SIGNALING AND CELL PHYSIOLOGY

Myostatin regulates the production of fibroblast growth factor 23 (FGF23) in UMR106 osteoblast–like cells

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Abstract
Myostatin is a signaling molecule produced by skeletal muscle cells (myokine) that inhibits muscle hypertrophy and has further paracrine and endocrine effects in other organs including bone. Myostatin binds to activin receptor type 2B which forms a complex with transforming growth factor-β type I receptor (TGF-βRI) and induces intracellular p38MAPK and NFκB signaling. Fibroblast growth factor 23 (FGF23) is a paracrine and endocrine mediator produced by bone cells and regulates phosphate and vitamin D metabolism in the kidney. P38MAPK and NFκB-dependent store-operated Ca^{2+} entry (SOCE) are positive regulators of FGF23 production. Here, we explored whether myostatin influences the synthesis of FGF23. *Fgf23* gene expression was determined by qRT-PCR and FGF23 protein by ELISA in UMR106 osteoblast–like cells. UMR106 cells expressed activin receptor type 2A and B. Myostatin upregulated *Fgf23* gene expression and protein production. The myostatin effect on *Fgf23* was significantly attenuated by TGF-βRI inhibitor SB431542, p38MAPK inhibitor SB202190, and NFκB inhibitor withaferin A. Moreover, SOCE inhibitor 2-APB blunted the myostatin effect on *Fgf23*. Taken together, myostatin is a stimulator of *Fgf23* expression in UMR106 cells, an effect at least partially mediated by downstream TGF-βRI/p38MAPK signaling as well as NFκB-dependent SOCE.

Keywords TGF-β · p38MAPK · Ca^{2+} · Vitamin D · Phosphate

Introduction

Myostatin is part of a group of signaling molecules produced by skeletal muscle cells (myocytes) that are known under the name "myokines" in analogy to "cytokines" [25]. It was discovered in 1997 as a member of the TGF-β superfamily and first named "growth differentiation factor 8 (GDF-8)" [33]. Myostatin counteracts muscle hypertrophy as mice deficient in myostatin exhibit 2–3 times more muscle mass than wild-type animals [33]. Also the Belgian Blue, cattle with a loss of function mutation in the gene encoding myostatin, is characterized by enormous muscle mass [16, 25]. Finally, mutations in the human gene encoding myostatin, which result in muscle hypertrophy, are similarly described [25, 41].

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In addition to myocytes, myostatin also affects bone metabolism including bone formation and osteoclastogenesis [25]. In osteocytes, myostatin upregulates sclerostin and dickkopf-related protein 1 (Dkk1) [25]. Thus, the myokine myostatin plays a role as regulatory molecule in the cross-talk between muscle and bone [25, 29].

Activin receptor type 2A and B (ACVR2A/B) are the membrane receptors for myostatin. Upon binding of myostatin, transcription factor activity of SMAD2/3 and forkhead box O (FOXO) is induced, ultimately resulting in the degradation of skeletal muscle proteins [17]. Further downstream effectors of myostatin also include p38MAPK [35].

Fibroblast growth factor 23 (FGF23) can be considered as an osteokine, a hormone mainly synthesized in the bone [25, 43]. Having classical endocrine effects, its main target organ is the kidney where it binds to a membrane receptor, which is dependent on transmembrane protein αKlotho [19], and inhibits the formation of calcitriol ($1,25(OH)_2D_3$, biologically active vitamin D_3) by suppressing 25-hydroxyvitamin D-1α-hydroxylase (encoded by *CYP27B1*), the renal key enzyme for $1,25(OH)_2D_3$ synthesis. Moreover, FGF23 fosters the internalization of membrane NaP_iIIa (*SLC34A1*),

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the major Na^+-dependent phosphate cotransporter in the proximal tubule of the kidney, thereby increasing the urinary excretion of phosphate [20, 39].

FGF23 may also be produced locally by other cells including hepatocytes [32] or cardiomyocytes thus exerting additional paracrine effects [28]. Thus, it was shown that FGF23 affects cardiac muscle and induces left ventricular hypertrophy [12], which underlines that this bone-derived molecule participates in the cross-talk between bone and muscle [25].

Moreover, the plasma concentration of FGF23 goes up in many acute and chronic diseases, notably renal and cardiovascular disorders [3, 21, 42]. Interestingly, myostatin is also upregulated in an early stage of chronic kidney diseases (CKD) [46]. Whether FGF23 is only a disease biomarker or actively drives pathophysiological processes is not entirely clear, yet [42].

The regulation of the production of FGF23 is subject to current research. Well-established regulators of FGF23 include $1,25(OH)_2D_3$ [31], dietary phosphate [44], parathyroid hormone [27], pro-inflammatory cytokines and pathways [8, 14], interleukin-6 (IL-6) [7], erythropoietin [5, 18], iron metabolism [6], transforming growth factor (TGF)-β (TGF-β) [13], peroxisome proliferator-activated receptor α [11], and intracellular signaling cascades including AMP-dependent protein kinase (AMPK) [15] and insulin/IGF-1-dependent phosphoinositide 3-kinase/Akt/FOXO signaling [2]. Remarkably, myostatin downstream signaling effector p38MAPK also controls *Fgf23* gene expression [10].

Some human diseases with enhanced myostatin plasma levels including dermatomyositis and CKD are characterized by enhanced FGF23 production [23, 46]. This and the well-established muscle and bone cross-talk [25] prompted us to investigate whether myostatin directly impacts on FGF23 formation and to uncover the underlying mechanism. A direct effect could be of high relevance given that pharmacological manipulation of myostatin in disease may be a future option [25].

Methods

Cell culture and treatments

UMR106 rat osteoblast-like cells (CRL-1661; ATCC, Manassas, VA, USA) were cultured under standard conditions [13]. Per se, basal *Fgf23* expression is low in UMR106 cells [40]. Therefore, they first have to be treated for 24 h with 100 nM $1,25(OH)_2D_3$ (Tocris, Bristol, UK), which strongly enhances *Fgf23* expression [40]. Next, cells were treated for further 24 h with recombinant myostatin protein (5–100 ng/mL, PeproTech, Rocky Hill, NJ, USA) in the presence or absence of TGF-β type I receptor (TGF-βRI) inhibitor SB431542 (10 µM, Sigma-Aldrich, Schnelldorf, Germany), p38MAPK inhibitor SB202190 (10 µM, Tocris), NFκB inhibitor withaferin A (500 nM, Tocris), or SOCE inhibitor 2-APB (150 µM, Sigma).

IDG-SW3 mouse osteocytes (CVCL_0P23; Kerafast, Boston, MA, USA) were plated on rat tail type I collagen–coated 12-well plates (150,000 cells per well) in α-Minimum Essential Medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 50 U/mL interferon (IFN)-γ (all reagents from Gibco, Life Technologies, Darmstadt, Germany). Cells were grown for 24 h at 33 °C and 5% CO_2. Next, differentiation was induced by replacing IFN-γ with 50 µg/ml ascorbic acid (Sigma-Aldrich) and 4 mM β-glycerophosphate (AppliChem, Darmstadt, Germany) and further incubation at 37 °C and 5% CO_2. The medium was changed every 2nd to 3rd day. At day 28, cells were incubated with 100 ng/mL recombinant myostatin or vehicle in duplicate for 8 h.

MC3T3-E1 Subclone 4 mouse pre-osteoblast cells (CRL-2593; ATCC) were cultured in α-MEM with 2 mM L-glutamine, nucleosides (Gibco, Life Technologies), 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. They were studied from passages 23 to 27. To this end, cells were seeded on rat tail type I collagen–coated 12-well plates (80,000 cells per well) for 24 h and incubated with 50 µg/mL ascorbic acid, and 4 mM β-glycerophosphate for 6 days. Then, 100 ng/mL myostatin or vehicle only was added in the presence of $1,25(OH)_2D_3$ (10 nM) 24 h before harvesting the cells.

Quantitative real-time polymerase chain reaction

Total RNA was isolated with TriFast reagent (Peqlab, Erlangen, Germany), and 1.2 µg was used along with random primers and the GoScript™ Reverse Transcription System (Promega, Mannheim, Germany) for cDNA synthesis (program: 25 °C for 5 min, 42 °C for 1 h, and 70 °C for 15 min). Quantitative real-time polymerase chain reaction (qRT-PCR) using the Rotor-Gene Q cycler (Qiagen, Hilden, Germany) and GoTaq qPCR Master Mix (Promega) was performed. The amplification conditions for analysis of *Fgf23* and *TATA box-binding protein* (*Tbp*) were 95 °C for 3 min, 40 cycles of 95 °C for 10 s, 57 °C for 30 s, 72 °C for 30 s (in UMR106 cells), and 95 °C for 3 min, 40 cycles of 95 °C for 10 s, 58 °C for 30 s (*Fgf23*), and 60 °C for 30 s (*Tbp*), 72 °C for 30 s (in IDG-SW3 and MC3T3-E1 cells). QRT-PCR conditions for analysis of *Acvr2a* and *Acvr2b* expression in UMR106, IDG-SW3, and MC3T3-E1 cells were 95 °C for 3 min, 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. The calculated *Fgf23*, *Acvr2a*, and *Acvr2b* mRNA transcript levels in UMR106, IDG-SW3, and MC3T3-E1 cells were normalized to the transcript levels of *Tbp*.

The *Acvr2a* and *Acvr2b* qRT-PCR products of the UMR106 cells were loaded on a 1.5% agarose gel and visualized by Midori Green.

The following primers (5′→3′ orientation) were used:

Rat Acvr2a:
F: CAATATCTCACAGGGACATC,
R: TTTGGAAGTTTATAGCACCC;
Rat Acvr2b:
F: AACATCATCACGTGGAAC,
R: AACATTCTTGCTTTTGAAGTC;
Rat Fgf23:
F: TAGAGCCTATTCAGACACTTC,
R: CATCAGGGCACTGTAGATAG;
Rat Tbp:
F: ACTCCTGCCACACCAGCC,
R: GGTCAAGTTTACAGCCAAGATTCA;
Mouse Acvr2a:
F: GGTCTCTTGGAATGAACTTTG,
R: TTACTTTTGATGTCCCTGTG;
Mouse Acvr2b:
F: ATTACCTCAAGGGGAACATC,
R: CATTCTTGCTTTTGAAGTCC;
Mouse Fgf23:
F: TCGAAGGTTCCTTTGTATGGA,
R: AGTGATGCTTCTGCGACAAGT;
Mouse Tbp:
F: CCAGACCCCACAACTCTTCC,
R: CAGTTGTCCGTGGCTCTCTT.

Enzyme-linked immunosorbent assay

UMR106 cells were cultured as described and treated with 100 ng/mL myostatin for 24 h. The cell culture supernatant was stored at − 80 °C. C-terminal FGF23 was determined by an ELISA kit (Mouse/Rat FGF-23 (C-Term), Immuntopics, San Clemente, CA, USA) according to the manufacturer's protocol. Intact FGF23 was determined in the supernatant after its concentration by means of Sartorius Vivaspin 6 Centrifugal Concentrators (Sartorius, Göttingen, Germany), with an ELISA kit (Mouse/Rat FGF-23 (Intact), Immuntopics).

Statistics

The data are shown as arithmetic means ± SEM, and *n* represents the number of independent experiments. Normal distribution was tested by Shapiro–Wilk normality test. Two groups were compared by unpaired Student's *t* test (if necessary with Welch's correction) or with Mann–Whitney-*U*-test for data not normally distributed. More than two groups were tested for significance by one-way ANOVA followed by Bonferroni's multiple comparisons test (if necessary with Welch's ANOVA followed by Dunnett's T3 multiple comparisons test). Differences were considered significant if $p < 0.05$.

Results

Expression of the activin type 2 receptors in UMR106 cells

The impact of myostatin on *Fgf23* expression was studied in UMR106 osteoblast–like cells. Employing RT-PCR, we first investigated the expression of myostatin receptors activin type 2A and B (*Acvr2a* and *Acvr2b*). As illustrated in Fig. 1, mRNA specific for receptor *Acvr2b* and to a markedly lesser extent for *Acvr2a* could be detected in UMR106 cells. Other cell lines used to study Fgf23 include IDG-SW3 and MC3T3-E1 cells. We used quantitative RT-PCR to compare *Acvr2a* and *Acvr2b* expression in all three cell lines. As a result, expression of *Acvr2a*

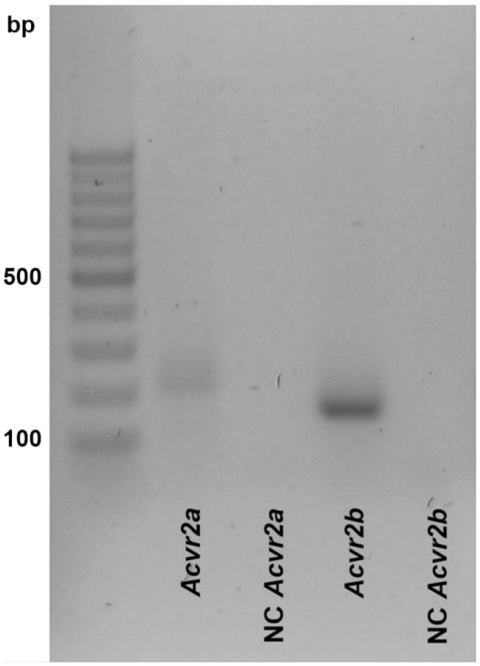

Fig. 1 Expression of activin type2 receptor isoforms in rat UMR106 osteoblast–like cells. Original agarose gel photo showing cDNA specific for activin type 2 receptor A (Acvr2a) and activin type 2 receptor B (Acvr2b) in UMR106 cells. NC, non-template control

relative to *Tbp* was 0.0008 ± 0.0000 in UMR106 cells, an expression level significantly ($p < 0.001$) lower than in IDG-SW3 cells (0.6014 ± 0.0615) and MC3T3-E1 cells (0.2768 ± 0.0162; for all $n = 7$). In contrast, expression of *Acvr2b* relative to *Tbp* was 0.5797 ± 0.0465 in UMR106 cells, an expression level significantly ($p < 0.001$) higher than in IDG-SW3 cells (0.0054 ± 0.0009) and MC3T3-E1 cells (0.0170 ± 0.0017; for all $n = 7$).

Myostatin induces Fgf23 expression in UMR106 cells

Next, we examined whether myostatin influences *Fgf23* expression. To this end, UMR106 cells were incubated without or with different concentrations of myostatin for 24 h and *Fgf23* gene expression was analyzed by quantitative RT-PCR. As shown in Fig. 2a, myostatin significantly increased *Fgf23* gene expression in a dose-dependent manner. Using ELISA, we tested whether the stimulatory effect of myostatin on *Fgf23* gene expression is translated into enhanced FGF23 protein secretion into the cell culture supernatant. Indeed, 24 h treatment with myostatin also upregulated C-terminal FGF23 (Fig. 2b) and intact FGF23 (Fig. 2c) production. In IDG-SW3 cells, an 8 h treatment with 100 ng/mL myostatin resulted in a relative *Fgf23* expression of 0.0006 ± 0.0004 ($n = 4$), a level not significantly different from that in control cells (0.0006 ± 0.0002; $n = 4$). Similarly, a 24 h treatment with 100 ng/mL myostatin led to a relative *Fgf23* expression of 0.0382 ± 0.0025 ($n = 7$) in MC3T3-E1 cells, a level not significantly different from that in control-treated cells (0.0401 ± 0.0042; $n = 7$).

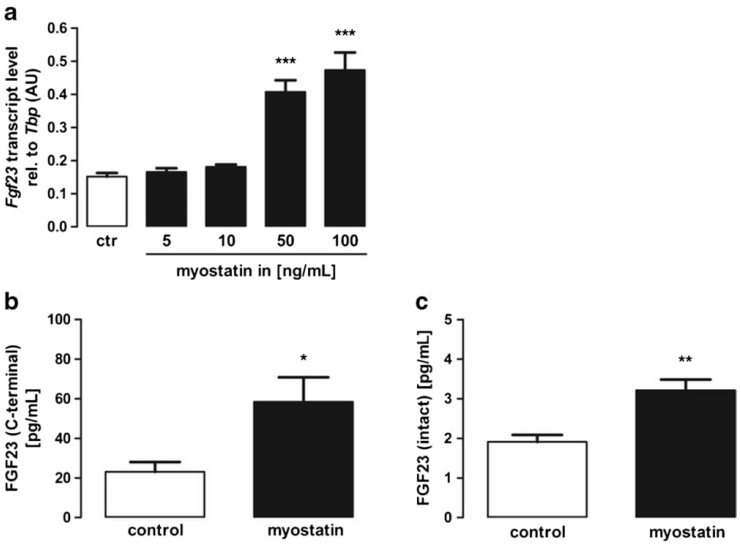

Fig. 2 Myostatin induces *Fgf23* expression in UMR106 cells, **a** Arithmetic means ± SEM of relative (rel.) *Fgf23* mRNA abundance or **b** C-terminal or **c** intact FGF23 protein concentration in the cell culture supernatant of UMR106 osteoblast–like cells incubated without (white bars) or with (black bars) myostatin (**a** indicated concentrations, $n = 7$; and **b**, **c** 100 ng/mL, $n = 10$) for 24 h. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ indicate significant difference from control. AU, arbitrary units; ctr, control (**a** one-way ANOVA; **b** unpaired Student's *t* test with Welch's correction; **c** Mann–Whitney-*U*-test)

Transforming growth factor-β type I receptor is necessary for myostatin-induced *Fgf23* expression

The next experiments explored the signaling of the myostatin effect on *Fgf23*. The binding of myostatin to ACVR2B induces complex formation with TGF-βRI [38]. In order to study whether this process is involved, we incubated UMR106 cells with 100 ng/mL myostatin in the presence or absence of TGF-βRI inhibitor SB431542 (10 μM) for 24 h. SB431542 treatment significantly abrogated myostatin-induced *Fgf23* expression in UMR106 cells (Fig. 3).

The myostatin effect on *Fgf23* is at least in part dependent on p38MAPK and NFκB-mediated store-operated Ca^{2+} entry (SOCE)

P38MAPK is a downstream target of myostatin [45] and also a potent regulator of FGF23 production [10]. To test whether p38MAPK contributes to the myostatin effect on *Fgf23* gene expression, UMR106 cells were incubated with or without 100 ng/mL myostatin in the presence or absence of 10 μM p38MAPK inhibitor SB202190 for 24 h. As demonstrated in Fig. 4a, SB202190 significantly blunted myostatin-mediated upregulation of *Fgf23* gene expression. Nevertheless, myostatin was capable of significantly enhancing *Fgf23* gene expression even in the presence of SB202190, pointing to the involvement of further effectors. Since pro-inflammatory transcription factor complex NFκB is a powerful regulator of FGF23 and myostatin induces NFκB activity [1], we performed further experiments to elucidate an involvement of NFκB.

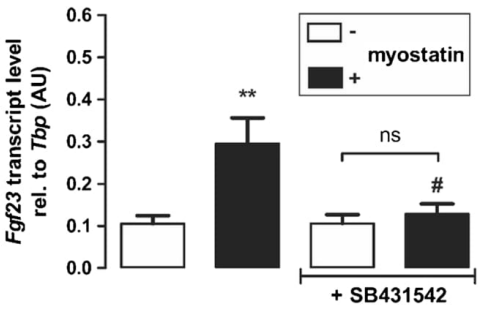

Fig. 3 Transforming growth factor-β type I receptor (TGF-βRI) is necessary for myostatin-induced *Fgf23* gene expression. Arithmetic means ± SEM of relative (rel.) *Fgf23* mRNA abundance in UMR106 osteoblast–like cells incubated without (white bars) or with (black bars) myostatin (100 ng/mL, 24 h, $n = 7$) in the presence or absence of TGF-βRI inhibitor SB431542 (10 µM, 24 h). $^{**}p < 0.01$ indicates significant difference from control. $\#p < 0.05$ indicates significant difference from the absence of SB431542 (2nd vs 4th bar). AU, arbitrary units (one-way ANOVA)

To this end, UMR106 cells were treated with and without 100 ng/mL myostatin in the presence or absence of NFκB inhibitor withaferin A (500 nM) for 24 h. As shown in Fig. 4b, myostatin did not significantly alter *Fgf23* gene expression in the presence of withaferin A.

NFκB enhances *Fgf23* gene expression by upregulating SOCE [47]. Hence, we sought to determine whether the myostatin effect is also dependent on SOCE. To this end, UMR106 cells were treated with or without myostatin in the presence or absence of SOCE inhibitor 2-APB (150 µM) for 24 h. No significant effect of myostatin on *Fgf23* gene expression was observed in UMR106 cells treated with 2-APB (Fig. 4c).

Discussion

This study provides evidence that myostatin, a signaling molecule produced by skeletal muscle cells, is a potent regulator of the production of FGF23, a hormone produced by bone cells. According to our results, myostatin upregulated *Fgf23* gene expression and secretion of FGF23 protein in UMR106 osteoblast–like cells. Both, production of C-terminal and intact FGF23 were enhanced upon treatment with myostatin, suggesting that the myokine indeed regulates biologically active FGF23.

Myostatin was discovered as a myokine with mainly paracrine effects in skeletal muscle, i.e., the inhibition of skeletal muscle growth [16]. Our study adds to the growing

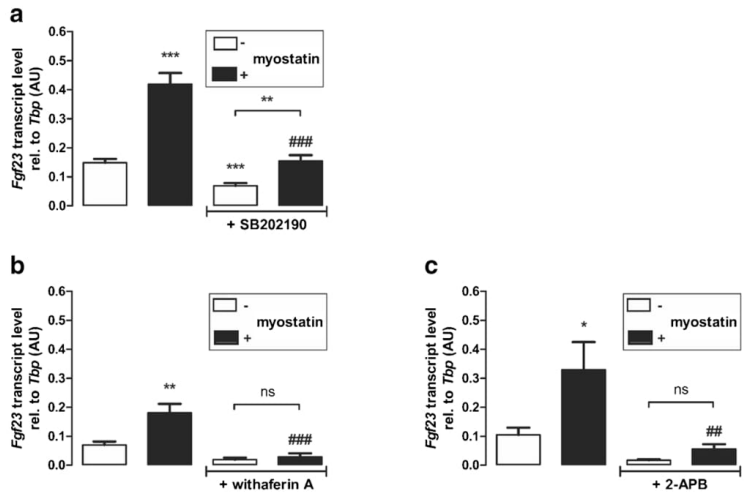

Fig. 4 The myostatin effect on Fgf23 is at least in part dependent on p38MAPK and on NFκB-mediated store-operated Ca^{2+} entry (SOCE). Arithmetic means ± SEM of relative (rel.) *Fgf23* mRNA abundance in UMR106 cells treated without (white bars) or with (black bars) myostatin (100 ng/mL, 24 h) in the presence or absence of p38MAPK inhibitor SB202190 (**a** 10 µM, 24 h, n = 16) or NFκB inhibitor withaferin A (**b** 500 nM, 24 h, n = 6), or SOCE inhibitor 2-APB (**c** 150 µM, 24 h, n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ indicate significant difference from control. $\#\#p < 0.01$ and $\#\#\#p < 0.001$ indicate significant difference from the absence of **a** SB202190; **b** withaferin A, or **c** 2-APB (2nd vs 4th bar). AU, arbitrary units (**a** Welch's ANOVA; **b**, **c** one-way ANOVA)

concept of a muscle-bone cross-talk and further supports the notion of myostatin having paracrine and endocrine effects [25]. In this respect, it shares similarity with FGF23 which is also characterized by both paracrine and endocrine effects in different tissues and cells [28]. Other factors involved in this cross-talk are, among others, irisin, receptor activator of NF-κB ligand (RANKL), osteocalcin, sclerostin, or TGF-β [25, 29]. The regulation of bone-derived FGF23 through muscle-derived myostatin according to our study again underlines the mutual influence of bone and muscle. Interestingly, also IL-6 and TGF-β, other factors involved in bone-muscle cross-talk, are potent regulators of FGF23 [7, 13]. Conversely, bone-derived FGF23 also acts on skeleton muscle, inducing muscle atrophy [24, 29]

Our experiments also addressed the cellular mechanisms through which myostatin exerts its stimulatory effect on FGF23. The main membrane receptor for myostatin, ACVR2B [25, 38], was strongly expressed in UMR106 cells, whereas the expression level of ACVR2A was markedly lower. Conversely, expression of ACVR2B was low and that of ACVR2A was high in IDG-SW3 and MC3T3-E1 cells. Interestingly, myostatin failed to significantly affect *Fgf23* expression in these cells, a result in line with ACVR2B being the major mediator of the myostatin effect on FGF23. Moreover, our results suggest that TGF-βRI is involved, as TGF-βRI inhibitor SB431542 significantly attenuated the myostatin effect on *Fgf23*. In line with a decisive role of TGF-βRI signaling for the production of FGF23, an earlier study identified TGF-β as a major trigger of FGF23 formation [13].

Downstream intracellular effectors of myostatin include p38MAPK [45]. Using p38MAPK inhibitor SB202190, we could demonstrate that also the myostatin effect on FGF23 is, at least in part, dependent on p38MAPK. This finding corroborates another study showing that p38MAPK signaling is a regulator of FGF23, in part through pro-inflammatory transcription factor complex NFκB [10]. Importantly, NFκB is a downstream target of myostatin [1] and itself is an important mediator of the stimulatory effect of inflammation and pro-inflammatory cytokines on FGF23 synthesis [22, 47]. NFκB is effective through inducing SOCE [47]. In line with this, both, NFκB and SOCE inhibition, significantly prevented myostatin from upregulating *Fgf23* gene expression. A summary of the putative signaling is presented in Fig. 5.

Myostatin not only prevents muscle hypertrophy but also an increase in bone mass as myostatin deficiency results in higher bone mass [9], bone mineral density and mineral content [34]. In line with this, myostatin inhibits osteoblast differentiation [37]. FGF23 induces a reduction of the plasma phosphate and $1,25(OH)_2D_3$ concentration. Both effects are expected to favor a reduction in bone mineralization and mass [3]. Hence, the stimulatory effect of myostatin on

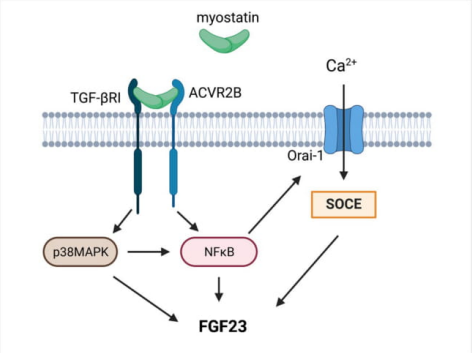

Fig. 5 Schematic illustration of myostatin-induced FGF23 production in UMR106 cells. Myostatin binding to ACVR2B and following partnering with TGF-βRI activates p38MAPK and NFκB. NFκB induces SOCE, resulting in induction of *Fgf23* gene expression. Created with BioRender.com. Activin type 2 receptor B (ACVR2B); fibroblast growth factor 23 (FGF23); nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB); p38 mitogen–activated protein kinase (p38MAPK); store-operated Ca^{2+} entry (SOCE); transforming growth factor-β type I receptor (TGF-βRI)

FGF23 fits well into the concept of myostatin limiting skeletal muscle and bone mass.

In dermatomyositis, an inflammatory condition, the plasma myostatin concentration is elevated [23]. Importantly, the same study also found higher FGF23 levels in patients with dermatomyositis [23]. Moreover, CKD, another disease associated with elevated FGF23 levels [26], is characterized by a higher myostatin plasma concentration [46] and enhanced myostatin expression in muscle of mice [48]. These results are in line with our major finding, i.e., myostatin-dependent stimulation of FGF23 production.

Pharmacological manipulation of myostatin has already been tested as a therapeutic approach [25]: Myostatin inhibition may theoretically be beneficial in diseases with muscle weakness such as Duchenne muscular dystrophy or rheumatoid arthritis and was already tested [25]. According to our study, myostatin inhibition could result in lower FGF23, which may indeed be beneficial with regard to the reduced bone mass typical of both Duchenne muscular atrophy [4] and rheumatoid arthritis [30].

Data on the in vivo relevance of our in vitro results are sparse thus far: No gross differences in serum Ca^{2+} or phosphate were reported in Holstein Friesian calves and Belgian Blue calves [36]. Clearly, further in vivo studies exploring FGF23 and phosphate metabolism in myostatin-deficient mice or in Belgian Blue cattle are needed to confirm the significance of our findings.

Taken together, this study found a direct stimulatory effect of myostatin on *Fgf23* gene expression and protein

production in UMR106 cells. Moreover, it uncovered that this effect is, at least in part, mediated by p38MAPK and NFκB. These findings may contribute to higher FGF23 levels in some diseases with enhanced myostatin production and may be relevant for future therapeutic approaches involving pharmacological manipulation of myostatin.

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Author contribution F. Ewendt and M. Föller designed the research; F. Ewendt analyzed the data; F. Ewendt and M. Feger performed the research; and M. Föller, F. Ewendt, and M. Feger wrote the paper.

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Data Availability. Data and material will be shared.

Code availability. Not applicable.

Declarations

Ethics approval Not applicable.

Consent to participate. Not applicable.

Consent for publication. Not applicable.

Competing interests The authors declare no competing interests.

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4 Discussion

4.1 p38MAPK controls FGF23 synthesis

Inflammation is a potent stimulator of FGF23 production [234, 237]. Various pro-inflammatory cytokines like TNF-α, IL-1β, IL-6 or also LPS are capable of inducing FGF23 expression [230, 231, 233, 235]. NFκB signaling is an important mediator of both pro-inflammatory stimuli and FGF23 production [112, 215, 230]. p38MAPK is another important regulator for the production of cytokines and is itself activated by pro-inflammatory stimuli [257]. Thus, p38MAPK stimulates the expression of TNF-α and IL-1β and these in turn activate p38MAPK [256, 257, 270–272]. Moreover, different studies have shown the capability of p38MAPK to activate NFκB signaling, resulting in increased TNF-α expression [252, 253, 255].

The results from **Paper 1** fit very well into this concept, which extends the participation of p38MAPK and subsequent NFκB signaling pathway in the mediation of inflammation to the regulation of FGF23. Thus, we demonstrated that in UMR106 and IDG-SW3 cells p38MAPK inhibitors SB202190 and SB203580 caused decreased *Fgf23* expression, which was further supported by the detected decreased secretion of C-terminal Fgf23 protein into the cell culture supernatant. In contrast, p38MAPK activator anisomycin stimulated the expression of *Fgf23* in UMR106 cells. These results suggest that activated p38MAPK induces gene expression and protein synthesis of Fgf23 in UMR106 and IDG-SW3 cells. Given the fact, that NFκB upregulates FGF23 [215], we tested whether p38MAPK is capable of regulating FGF23 via activation of the NFκB signaling. The inhibition of NFκB abrogated the induction of *Fgf23* expression by anisomycin, demonstrating that p38MAPK upregulates *Fgf23* expression at least in part through NFκB activity. Therefore, it is possible that p38MAPK is a part of the described upregulation of *Fgf23* transcripts by Zhang *et al.*, as a consequence of NFκB-sensitive Orai-1 upregulation and SOCE [215]. This notion is supported by the results in **Paper 3** showing attenuation of *Fgf23* expression after treatment with the TGF-β family member myostatin in UMR106 cells in the presence of either a p38MAPK, NFκB or SOCE inhibitor. Consistent with this, Yan *et al*. show that p38MAPK and NFκB activity are required for TGF-β-induced upregulation of SOCE in megakaryocytes [273]. Moreover, it is worth noting that TGF-β has been described to act in a non-canonical Smad-independent pathway via p38MAPK in bone [274, 275] and to activate p38MAPK in osteoblastic cells [276–278]. Interestingly, TGF-β itself upregulates *Fgf23* expression SOCE-dependently in UMR106 cells [218]. Thus, it could be postulated that this is mediated, at least in part, by the activation of p38MAPK. Sundivakkam *et al.* describe the p38β-MAPK isoform also as an important regulator of SOCE, but in contrast,

they show a suppression of SOCE as a consequence of p38β activation in human lung microvascular endothelial cells (HLMVECs) [279]. Moreover, in intestinal epithelial cells, SOCE itself was shown to activate p38MAPK, but this leads to suppression of NFκB activation, in contrast to the results described above [280].

Nevertheless, the induction of *Fgf23* expression and protein synthesis by p38MAPK that we found, is supported by the publication of Kido *et al.* The authors show a cadmium-dependent activation of p38MAPK and an increase of intact Fgf23 *in-vivo* and *in-vitro* [247]. The effect is achieved at the post-transcriptional level by lowering *Furin* and increasing *Galnt3* expression, resulting in a reduction of Fgf23 cleavage and subsequent rise of intact Fgf23 levels [247]. Further, the study of Kido *et al*. shows that the p38MAPK inhibitor SB203580 inhibits the expression of *Galnt3* [247]. TNF-α can activate p38MAPK in bone, as described above [256, 271]. TNF-α induces *Fgf23* transcription in IDG-SW3 cells via NFκB and TNF-α upregulates gene expression of *Galnt3* [230], similar to what was described in **Paper 1** and by Kido *et al.* regarding p38MAPK [247]. Since it was shown in **Paper 1** that p38MAPK also affected *Fgf23* gene expression in IDG-SW3 cells, it is conceivable that it is also involved in TNF-a mediated *Fgf23* regulation in these cells. Taken together, p38MAPK is not only an activator of Fgf23 protein synthesis and gene expression, mediated partly by NFκB activity as shown in **Paper 1**, but also causes an increased secretion of intact Fgf23 through a reduction of cleavage as a result of upregulated *Galnt3* expression [247].

Given the pivotal role of SOCE and intracellular Ca^{2+} signaling for *Fgf23* transcription, it is not surprising that the Ca^{2+}-dependent calcineurin/NFAT pathway is also involved in cellular regulation of *Fgf23* gene expression [228, 229]. Bär *et al*. report decreased *Fgf23* gene expression and protein synthesis in UMR106 cells after treatment with calcineurin inhibitor tacrolimus (FK-506) or calcineurin-NFAT-interaction inhibitor INCA-6 [228]. Interestingly, several studies show an inhibitory effect of tacrolimus on p38MAPK in different cell types [281–283] and further an abrogation of TNF-α- induced activation of p38MAPK and NFκB in myofibroblasts in the presence of FK-506 [284]. In addition, NFATc is activated by p38MAPK in T-cells [285]. Within those lines, it is theoretically possible to speculate that the observation of a tacrolimus-dependent reduction of *Fgf23* transcription in UMR106 cells [228] may also be, at least in part, due to inhibition of p38MAPK. However, Bär *et al*. do not observe any significant influence of NFκB by FK-506 in UMR106 cells [228].

FGF23 is not only synthesized as a consequence of p38MAPK activation, as described in **Paper 1**, but is also capable of activating p38MAPK itself [286, 287]. This reciprocal regulation may lead to a feed forward loop of mutual upregulation. Moreover, different

cytokines like TNF-α, IL-6 or LPS induce both p38MAPK activity and FGF23 transcription [230, 231, 235, 271, 288, 289]. The inhibition of p38MAPK activity seems to be useful to decrease cytokine production, as shown in a study by decreasing Il-6 secretion of MC3T3-E1 pre-osteoblastic cells after treatment with the p38MAPK inhibitor SB203580 [290]. Therefore, it is possible to speculate that the upregulation of FGF23 by these pro-inflammatory cytokines is, at least in part, mediated by activation of p38MAPK. This mechanism fits well into the concept of the vicious circle of inflammation and FGF23, already described by Faul *et al.*, marking p38MAPK as an important and universal modulator [238, 257].

The important role of the p38MAPK pathway in inflammatory diseases, like asthma, inflammatory bowel disease, systemic inflammation and rheumatoid arthritis is therefore not surprising [291]. Furthermore, the application of p38MAPK inhibitors attenuates the progression of inflammatory stimuli in these conditions [291]. In addition, also the usage of synthetic glucocorticoids, like dexamethasone, in such conditions is well established [292]. It is remarkable, that also dexamethasone has an inhibitory effect on p38MAPK [293, 294] and NFκB [295]. Interestingly, it is further observed that concomitant treatment with p38MAPK inhibitors and dexamethasone has additive anti-inflammatory effects by inhibiting TNF-α- or IL-1β-induced cytokine expression in human lung fibroblasts and epithelial cells from asthma patients [296, 297]. Given the fact, that inflammatory conditions are strongly correlated with increased FGF23, the effects of application of specific p38MAPK inhibitors alone or combined with others, like dexamethasone, may not only be beneficial in these diseases, but also potentially reduce the high levels of FGF23 under these conditions. This notion is supported by a recent publication showing a reduction of Fgf23 mRNA and protein production in UMR106 and MC3T3-E1 cells and in mice after treatment with dexamethasone [298].

Protein kinase C (PKC) is described to induce *Fgf23* gene transcription in UMR106 cells [299] and its activator Phorbol ester 12-O-tetradecanoylphorbol-13-acetate (PMA) induces Fgf23 mRNA and protein production *in-vivo* [300]. It is remarkable, that PKC induces *Fgf23* transcription in UMR106 cells, at least in part, NFκB-dependently [299], which gives rise to speculation about the possible involvement of p38MAPK. This is supported by several studies showing both activation of p38MAPK by PKC or its activator PMA and blunted effects in the presence of p38MAPK inhibitors [301–304]. In contrast, Gö6976 and calphostin C, both PKC inhibitors, are shown to reduce both *Fgf23* transcription [299] and activation of p38MAPK [305–308]. In addition, PMA treatment in UMR106 cells also induces *Tnf-α* and *Il-6* gene expression [299], which are both activator and downstream targets of p38MAPK as well [270, 271, 289, 290]. Therefore, p38MAPK may play a potential role in the PKC-mediated induction

of *Fgf23* transcription in response to NFκB activity and inflammation described by Bär *et al*. [299].

Several *in-vivo* and *in-vitro* studies highlight the importance of PTH as a systemic key regulator of FGF23 production [163, 164, 204–206]. PTH was shown to activate *Fgf23* transcription in UMR106 cells and cultured osteocytes via the cAMP/PKA/Nurr1 pathway [163, 204, 205, 309]. It is noteworthy, that PTH-regulated osteoblastic genes are centered on the p38MAPK and NFκB gene network [310]. In addition, studies find that treatment of osteoblasts with p38MAPK inhibitor SB203580 in the presence of PTH abolishes its effects [311, 312]. Furthermore, cAMP/PKA-dependent p38MAPK activation by PTH and induction of target gene expression is observed in osteoblastic cells [313, 314]. The importance of p38MAPK for PTH effect mediation is also evident *in-vivo*, as PTH-dependent increase of bone mass and formation is blunted in *p38α* conditional knockout mice [314]. Interestingly, cAMP/PKA-dependent activation of p38MAPK is also observed in MC3T3-E1 (FGF23-producing pre-osteoblasts [298]) cells in response to treatment with PTH [313]. It is reasonable to speculate that the observed PTH-dependent induction of *Fgf23* transcription in UMR106 cells [205] may also be mediated via the activation of the novel regulator p38MAPK described in **Paper 1** and subsequent NFκB activity. Thus, p38MAPK may play an important role in linking systemic and local regulation of FGF23 synthesis.

Furthermore, 17β-estradiol (E_2) and the estrogen receptor (ER) is shown to activate p38MAPK in MC3T3-E1 and MG63 osteoblastic cells [315, 316]. Of note, E_2 is also capable of inducing dose-dependently Fgf23 mRNA and protein expression in UMR106 cells as well as increasing Fgf23 serum levels and gene expression in bone of E_2-treated ovariectomized CKD rats [317]. The authors postulate a possible binding of nuclear ERs on corresponding estrogen response elements in the *FGF23* promoter, but a direct mechanism is not described [317]. It is therefore tempting to speculate, that the induction of E_2-dependent FGF23 synthesis in bone cells is also mediated at least in part via p38MAPK activation.

Moreover, it is reported that feeding high-fat diets (HFD) in rats enhances the formation of advanced glycation end products (AGEs) in serum and different tissues accompanied by the activation of p38MAPK in heart and liver [318]. The direct activation of p38MAPK by AGEs is also described in bone cells and cells of other tissues [319–322]. Interestingly, a recent publication observes an induction of *Fgf23* gene expression by AGEs in UMR106 cells, an effect that has been shown to depend on NFκB activity and SOCE-mediated cellular Ca^{2+} entry [219]. In this context, p38MAPK activity may take part in the AGEs-dependent activation of NFκB-mediated SOCE and subsequent *Fgf23* transcription.

In addition, AGEs- and LPS-induced increases in both *Sost* mRNA expression and its gene product sclerostin are detected in mouse osteocytic MLO-Y4 cells, which is blunted in the presence of p38MAPK inhibitor SB203580 [322, 323]. Furthermore, a reduction of *Sost* expression in *p38α* conditional knockout mice is demonstrated [314]. Interestingly, a recent study finds that sclerostin directly stimulates *Fgf23* mRNA expression and protein secretion in IDG-SW3 cells [324]. Inhibition of p38MAPK in IDG-SW3 cells, as shown in **Paper 1**, resulted in decreased *Fgf23* expression. In light of this, it is conceivable that the decrease in *Fgf23* expression could also be mediated by a reduction in sclerostin expression via p38MAPK inhibition in these cells.

In addition, it is recently described that a HFD induces Fgf23 production by TNF-α upregulation in mice, and TNF-α stimulates *Fgf23* transcription in a time- and dose-dependent manner in UMR106 cells [232]. TNF-α is active via NFκB [230] and, further, has been shown to induce p38MAPK activity [271]. Given the fact, that p38MAPK also acted, at least in part, via NFκB in UMR106 cells, as described in **Paper 1**, it could be speculated that the described TNF-α-dependent induction of *Fgf23* transcription in UMR106 [232] is also mediated via p38MAPK activity. This aspect is further strengthened by the fact that HFD itself is shown to induce p38MAPK activation [325, 326]. Thus, it is conceivable that the marked increase in serum Fgf23 in HFD-fed *Tnf-α* knockout mice compared with both wild-type and *Tnf-α* knockout mice on control diet [232] is partially mediated via activation of p38MAPK.

In this context, Abdelmegeed *et al.* also show interesting data regarding upregulation of TNF-α in HFD-fed mice [327]. Interestingly, peroxisome proliferator-activated receptor α (*Pparα*)-null mice used, show increased activation of p38MAPK in the liver. Using a standard diet, *Pparα*-null mice show a threefold increase in phosphorylation of p38MAPK compared with the wild type mice. However, the highest p38MAPK activity is observed in HFD-fed Pparα-null animals, whose level of phosphorylated p38MAPK is again 33% higher compared with standard diet-fed Pparα-null mice. This indicates a PPARα-feeding-interaction in the regulation of p38MAPK. The data suggest a potential inhibitory effect of PPARα on p38MAPK activity. Consistent with this, a PPARα agonist-mediated reduction of TNF-α-induced phosphorylation of p38MAPK is described [328]. Interestingly, PPARα was identified in this thesis in **Paper 2** as an independent and novel regulator of *Fgf23* expression in UMR106 cells, which is discussed in the subsequent section.

In conclusion, p38MAPK was identified as novel regulator of *Fgf23* gene expression in UMR106 and IDG-SW3 cells, acting, at least in part, via NFκB activity. The *in-vivo* role of p38MAPK for FGF23 formation must be addressed in future studies. The role of p38MAPK as

a potent regulator of FGF23 formation in bone cells is supported by its involvement in several cellular signaling pathways that are also involved in FGF23 production in a regulatory manner. This indicates the important position that p38MAPK occupies in the complex network of local and systemic influence on FGF23 synthesis in bone cells.

4.2 PPARα-dependent regulation of FGF23

PPARα is the major ligand-activated transcription factor for lipid and fasting metabolism, that binds as a heterodimer with the retinoid X receptor (RXR) PPAR response elements in the promoter region of target genes involved in β-oxidation, fatty acid transport, triglyceride catabolism, and the production of ketone bodies [260, 261]. Fibrates, which are PPARα agonists, have been shown to increase high-density lipoprotein (HDL) and decrease low-density lipoprotein (LDL) and triglyceride plasma levels, improving atherogenic lipoprotein profiles [329–331].

Paper 2, presented in this thesis, demonstrates a novel function of PPARα by suppressing FGF23 formation. Thus, in **Paper 2** the PPARα agonists fenofibrate and WY-14643 were shown to reduce *Fgf23* gene expression in UMR106 cells in a dose-dependent manner, whereas treatment with PPARα antagonist GW6471 or siRNA-mediated *Ppara* knockdown enhanced *Fgf23* expression in these cells. It should be noted that PPARα agonists regulated *Fgf23* expression in both PTH- and $1,25(OH)_2D_3$-pretreated UMR106 cells. Therefore, it is unlikely that the effects of PPARα and its agonists and antagonists on FGF23 resulted from a reciprocal influence of the PPARα pathway and the $1,25(OH)_2D_3$-dependent VDR pathway, as described elsewhere [332, 333].

Treatment with fenofibrate in UMR106 cells stimulated *Ppara* gene expression in a dose-dependent manner, as shown in **Paper 2**, an effect that is also observed in pre-osteoblastic MC3T3-E1 cells [334].

The next series of experiments in **Paper 2** addressed the effect of PPARα agonists on Fgf23 protein synthesis. The post-translational modifications of FGF23 protein by GALNT3-dependent O-glycosylation or FAM20C-mediated phosphorylation is decisive for its cleavage by furin/subtilisin-like proprotein convertase into N- and C-terminal fragments [40]. As shown in **Paper 2**, treatment of UMR106 cells with fenofibrate or WY-14643 resulted in significantly decreased C-terminal Fgf23 levels in the cell culture supernatant. In contrast, the effect on intact Fgf23 in the cell culture supernatant was weaker in the presence of WY-14643 or even lacked statistical significance in the presence of fenofibrate. This gives possible hints that PPARα

agonists not only regulate FGF23 gene transcription and protein production but also post-translational processing. This notion is supported by the fact that it was observed that fenofibrate and WY-14643 are able to induce *Furin* mRNA expression in human hepatocytes [335]. However, data from **Paper 2** demonstrated no significant difference of the ratio C-terminal FGF23 - intact FGF23/ intact FGF23 between control and fenofibrate- or WY-14643-treated UMR106 cells. Therefore, future studies have to address the possible underlying mechanisms of PPARα agonists-mediated regulation of proteins involved in the post-translational processing of FGF23.

The cellular energy sensor AMPK was recently identified as a potent regulator of FGF23 formation *in-vitro* and *in-vivo* by Glosse *et al*., downregulating the synthesis of this bone-derived hormone [220]. Because PPARα agonists are shown to activate AMPK in bone cells [336] and cells of other tissues [337–340], we addressed the question whether AMPK is involved in the PPARα action on FGF23. Interestingly, western blot experiments in **Paper 2** revealed an increase of phosphorylated AMPK protein abundance after treatment with fenofibrate in UMR106 cells. In line with this, treatment with bezafibrate, another PPARα agonist, is shown to induce phosphorylation of AMPK also in pre-osteoblastic MC3T3-E1 cells [336]. Notably, further experiments in **Paper 2** showed an abrogation of PPARα agonists-dependent down-regulation of *Fgf23* transcription in the presence of AMPK inhibitor compound-C in UMR106 cells. These results suggest that AMPK activity is involved in the PPARα-dependent reduction of *Fgf23* transcription. This notion is supported by the observations of several studies, which show a blunting of PPARα agonists-mediated effects in the presence of compound-C also in MC3T3-E1 cells [336] and cells of other tissues [262, 341, 342]. However, in **Paper 2**, compound-C was less effective in the presence of WY-14643 than with fenofibrate, demonstrating differential activation and involvement of AMPK by these two agonists, as described elsewhere [263, 340, 343]. Thus, compared with WY-14643, fenofibrate may also exert its effects independently of PPARα. This consideration is supported by the fact that fenofibrate also induces phosphorylation of AMPK in *Ppara* knockout mice [343].

SOCE is a potent stimulator of *Fgf23* gene transcription through which various regulators mediate their effects on FGF23 production [215, 217–219]. Of note, also AMPK effectively regulates FGF23 via SOCE [220]. In detail, AMPK is identified to suppress SOCE and reduces the insertion and cell membrane abundance of Ca^{2+} release-activated Ca^{2+} channel Orai-1 [220, 344, 345]. Therefore, experiments were performed using fluorescent optics to determine whether PPARα agonist fenofibrate also affects SOCE in UMR106 cells, and it was found to downregulate SOCE. Interestingly, in pulmonary arterial smooth muscle cells it is

observed that PPAR family member γ also inhibits SOCE [346]. Our experiments in **Paper 2** showed that the PPARα agonist fenofibrate mediated the observed reduction of *Fgf23* expression in large part via AMPK activity and downstream SOCE suppression. Consistent with the experiments in **Paper 2,** which showed an abrogation of the fenofibrate-induced decrease in *Fgf23* expression in the presence of AMPK inhibitor compound-C in UMR106 cells, Glosse *et al*. show an attenuation of the increase in *Fgf23* transcription by compound-C in UMR106 cells in the presence of both the SOCE inhibitors 2-APB and MRS 1845 and the Orai-1 inhibitor AnCoA4 [220].

The extent to which the influence of PPARα on the detected reduced depletion of intracellular Ca^{2+} stores is also relevant for FGF23 production remains to be elucidated. A possible explanation for the lower basal levels measured at baseline and subsequent reduced Ca^{2+} efflux after incubation with thapsigargin in fenofibrate-treated cells could be the influence of AMPK activation on other cellular Ca^{2+} homeostasis sites. The depletion of ER Ca^{2+} stores due to binding of IP_3 on IP_3R results in the initiation of the SOCE cascade [222, 227]. Interestingly, AMPK activation is shown to negatively regulate IP_3R and subsequent IP_3-induced Ca^{2+} release from ER stores [347]. The suppression of ER Ca^{2+} store depletion potentially inhibits the initiation of SOCE and further reduces the influx of additional Ca^{2+} during AMPK activation. Control of external Ca^{2+} influx could be used to reduce $[Ca^{2+}]_c$ accumulation, since AMPK activation also inhibits sarco-endoplasmic Ca^{2+}-ATPase (SERCA) [348], which otherwise keeps $[Ca^{2+}]_c$ low by translocating Ca^{2+} into the ER [225]. Furthermore, the calcium efflux via Na^+/ Ca^{2+} exchangers (NCX) is also determined by AMPK activation. Thus, bone marrow-derived dendritic cells (DC) from *Ampk*$^{+/+}$ mice show lower NCX activity than *Ampk* $^{-/-}$ DC [344]. In addition, AMPK activation leads to impairment of the Na^+/K^+ ATPase, causing an increase in cytosolic $[Na^+]$, possibly altering the electrochemical gradient of the NCX [349]. This would lead to an increased influx instead of efflux of cytosolic Ca^{2+}. AMPK-mediated inhibition of NCX and Na^+/K^+ ATPase likely serves to prevent further Ca^{2+} influx into energy-depleted cells [349]. The described regulated mechanisms in cellular Ca^{2+} homeostasis, as a consequence of AMPK activation, could lead to the maintenance of a low cellular $[Ca^{2+}]$ status. This low cellular $[Ca^{2+}]$ status of the cells could explain the detected low Ca^{2+} basal levels as well as the reduced depletion of intracellular Ca^{2+} stores in the UMR106 cells after 24 h of treatment with fenofibrate in **Paper 2**. In line with this, in vascular smooth muscles cells it is shown that fenofibrate treatment reduces intracellular Ca^{2+} levels [350]. Thus, the PPARα agonist-mediated reduction of *Fgf23* expression, in addition to the reduction of SOCE, could also be explained by observed low $[Ca^{2+}]_c$ levels after treatment with fenofibrate,

as a result of which Ca^{2+}-dependent signaling pathways are inhibited. In light of this, the FGF23-inducing and Ca^{2+}-dependent signaling pathway calcineurin-NFAT, which links SOCE, intracellular Ca^{2+}, and *Fgf23* gene expression, may be inhibited [228, 229, 351, 352]. Of note, fenofibrate and overexpression of PPARα blunts the endothelin-1 induced nuclear translocation of NFAT in cardiac myocytes [353]. Besides the discussed potential role of low intracellular Ca^{2+} levels and attenuated depletion of intracellular Ca^{2+} stores for FGF23 production, data published in **Paper 2** showed that the PPARα antagonist GW6471 failed to increases *Fgf23* expression after siRNA-mediated knockdown of *Orai-1* in UMR106 cells. These results indicate the involvement of AMPK-mediated reduced Orai-1 insertion and SOCE suppression in the PPARα effect on FGF23. Moreover, fenofibrate-mediated activation of AMPK could lead to inactivation of the AMPK downstream target mammalian target of rapamycin (mTOR) ([354]) and, via this, additionally downregulate FGF23 production. This links fenofibrate-mediated regulation to the influence of cellular energy homeostasis and energy-sensors on FGF23 production. Thus, fenofibrate is shown to suppress mTOR activity, partly involving AMPK activation, in osteoarthritic-chondrocytes [355] and various cancer cells [356–358]. Interestingly, Vidal *et al*. show that energy intake increases FGF23 production *in-vivo* and *in-vitro*, whereas co-treatment with mTOR inhibitors rapamycin and everolimus in the presence of high glucose blunts *Fgf23* gene transcription in UMR106 cells [197]. The notion that the energy-sensor mTOR is involved in the regulation of FGF23 synthesis is further supported by the observation of a trend towards decreased Fgf23 plasma levels in mice treated with rapamycin [359]. In addition, mTOR is shown to upregulate the expression of STIM1 and ORAI-1, leading to an enhancement of SOCE [360–362] and that treatment with rapamycin in UMR106 cells decreases *Stim1* expression in the presence of high glucose [197]. Taken together, it could be speculated that the observed suppression of *Fgf23* gene expression by PPARα agonists in **Paper 2** is mediated by both activation of AMPK with subsequent reduction of Orai-1 and SOCE, and possibly by suppression of mTOR activity and subsequent downregulation of Stim1 and SOCE. In addition, as described above, NFκB is a potent inducer of *Orai-1* transcription and SOCE-dependent upregulation of *Fgf23* gene transcription in UMR106 [215]. Of note, PPARα antagonist GW6471 failed to increase *Fgf23* gene transcription in the presence of siRNA-mediated knockdown of *Orai-1* in **Paper 2**. GW6471 is shown to abrogate the inhibitory effects of WY-14643 on LPS-induced NFκB activity [363]. This indicates a potential importance of PPARα activity for the NFκB signaling pathway. Interestingly, PPARα and its agonists are shown to inhibit NFκB signaling in different cell types [363–368]. Moreover, abrogation of the fenofibrate-induced decrease in AGEs-mediated

activation of NFκB activity is observed in SVEC4 cells in the presence of both siRNA-mediated *Ampk* knockdown and compound-C-mediated AMPK inhibition [369]. Consistent with this, the fenofibrate-induced decrease in TNF-α-mediated increase in NFκB activity is also abolished in SVEC4 cells in the presence of both siRNA-mediated *Ampk* knockdown and AMPK inhibition [370]. This provides evidence for another mechanism of fenofibrate-induced SOCE suppression, as shown in **Paper 2**, via inhibition of NFκB signaling and subsequent reduced *Fgf23* gene expression. It is conceivable that AMPK activated by fenofibrate, as shown in **Paper 2**, and the resulting direct inhibition of NFκB may also be involved. This consideration is supported by the description of a number of mechanisms of AMPK-mediated NFκB inhibition [371].

In addition to its role in the signaling cascade of SOCE, NFκB mediates pro-inflammatory stimuli and cytokine expression that is inhibited by PPARα and its agonists [372–375]. Thus, reduced secretion of TNF-α, IL-6, IL-1β, and CRP are observed in the presence of PPARα agonists, which is associated with inhibition of NFκB signaling [366, 372, 373, 376]. Moreover, in the presence of fenofibrate in C2C12 cells, a reduction in TNF-α-induced activation of p38MAPK and phosphorylation of IκB, the inhibitor of NFκB, is demonstrated [328]. P38MAPK was identified in **Paper 1** as novel regulator of *Fgf23* expression and the mentioned cytokines and NFκB activity are strong inducer of FGF23 production as well [230]. Pro-inflammatory stimuli and cytokine expression are potent stimulators of FGF23 formation, which in turn activates cytokine production in various tissues, resulting in a vicious cycle of systemic inflammation [238]. Therefore, anti-inflammatory effects of PPARα and its agonists may be involved in the observed reduction of *Fgf23* gene expression in **Paper 2**. Additionally, PPARα agonists could be useful as modulators in diseases with systemic inflammation, decreasing both pro-inflammatory stimuli and FGF23 production. This notion is supported by the fact that patients with osteoarthritis show higher FGF23 levels than controls [377] and FGF23 is correlated with inflammation in subjects with rheumatoid arthritis (RA), a disease with chronic inflammation [378]. Interestingly, in a pilot study in patients with RA treated with fenofibrate, a reduction in CRP and IL-6 levels is observed [374], both of which are associated with FGF23 levels [379]. In addition, fenofibrate reduces *Tnf-α* mRNA in the liver and myostatin mRNA and protein expression in muscle in rats with arthritis [380]. Remarkably, myostatin was identified as novel inducer of Fgf23 formation in UMR106 cells in **Paper 3** of this thesis. Moreover, fenofibrate reduces CRP and IL-6 plasma levels in subjects with nondiabetic insulin-resistant metabolic syndrome [381]. Interestingly, it is shown that patients with metabolic syndrome have also higher FGF23 levels compared to subjects without

metabolic syndrome [382]. Therefore, it is possible that fenofibrate can be used to decrease bone-derived FGF23 directly (as described in **Paper 2**) or indirectly by reducing pro-inflammatory signaling pathways and systemic inflammation in the aforementioned pathologies.

In addition, pro-inflammatory biomarkers, like TNF-α and IL-6, are positively associated with serum triglyceride levels, whereas the correlation with HDL is negative [383–386]. Further, a study in children shows a positive correlation of TNF-α and IL-6 with body mass index, waist circumference, and percentage of body fat and higher *TNF-α* and *IL-6* expression in obese children [383]. Interestingly, serum FGF23 is also shown to be positively associated with body mass index, body fat mass, waist circumference, and triglyceride level, whereas the association of serum FGF23 with HDL is negative in two independent cohorts of elderly subjects [382]. In contrast, a positive correlation of serum FGF23 and HDL is observed in obese children and adolescents [387], whereas no significant association of FGF23 with HDL, LDL, triglyceride and body mass index is found in a study of patients with CKD [388]. In addition to the described anti-inflammatory effects of fibrates, these PPARα agonists, are shown to be useful in the treatment of dyslipidemia by increasing HDL and decreasing LDL and triglyceride plasma levels [329–331]. Thus, it could be speculated, that the use of fibrates in dyslipidemia, obesity, and correlated inflammatory stimuli improves lipid profiles and directly or indirectly lowers serum FGF23. Moreover, dyslipidemia is positively associated with leptin levels [389, 390]. Of note, treatment with leptin increases both serum Fgf23 levels and bone-derived *Fgf23* mRNA expression in *leptin*-deficient mice [240]. Further, it is shown that treatment with leptin potentiates $1,25(OH)_2D_3$-dependent upregulation of *Fgf23* expression in UMR106 cells [391]. Interestingly, it is shown that fenofibrate treatment decreases serum leptin levels in type 2 diabetic patients with hypertriglyceridemia [392] as well as *Leptin* mRNA expression in visceral adipose tissue of obese mice on HFD [393]. In this context, it is reasonable to speculate that treatment with fenofibrate might also influence FGF23 synthesis via a reduction in both leptin levels and expression.

PPARα and its agonists not only improve lipid profiles in dyslipidemia, but also increase matrix mineralization and osteoblast gene expression in pre-osteoblastic MC3T3-E1 cells [334, 394], osteoblast differentiation [328, 334], and bone mineral density [395]. In detail, treatment with PPARα agonist WY-14643 increases matrix calcification and induces gene expression of alkaline phosphatase (ALP), osteocalcin (OC) and bone sialoprotein (BSP) in MC3T3-E1 cells [394]. Consistent with this, fenofibrate also upregulates *Alp* and *Oc* gene expression and induces osteoblast differentiation by increasing PPARα-mediated bone morphogenic protein

(BMP) 2 expression in MC3T3-E1 cells [334]. Furthermore, BMP4 enhances osteoblast differentiation by modulating PPARα activity and PPARα agonists blunt the TNF-α-mediated suppression of osteoblast differentiation induced by BMP4 in C2C12 cells [328]. In addition, treatment with PPARα agonists in ovariectomized rats maintains bone mass and bone mineral density [395]. In contrast, FGF23 is shown to inhibit osteoblast differentiation and matrix mineralization [103–105]. Thus, treatment with FGF23 and α-Klotho decreases Ca^{2+} accumulation and *Alp*, *Oc*, and *Bsp* expression in MC3T3-E1 cells [105]. Thus, according to the results of **Paper 2**, the suppression of *Fgf23* expression by PPARα and its agonists fits well with the concept that PPARα and its agonists directly increase osteoblast differentiation and matrix mineralization and possibly indirectly through reduction of FGF23 and its mediated effects.

An increase in serum FGF23 with decreasing GFR is observed in patients with CKD [156, 157]. Of note, fenofibrate treatment is associated with a slower decline in estimated GFR compared with placebo in participants of the Action to Control Cardiovascular Risk in Diabetes trial [396] and the Fenofibrate Intervention and Event Lowering in Diabetes sub-study [397]. Moreover, treatment with fenofibrate reduces total cardiovascular events in patients with type 2 diabetes and renal impairment [398]. Interestingly, higher serum FGF23 are both associated with a greater risk for cardiovascular events in patients with CKD [97] and left ventricular mass index, which is associated with a greater risk of death in patients on hemodialysis [399]. In this context, it would be interesting to determine the extent to which a possible reduction in FGF23 levels in humans and a fenofibrate-induced slowing of GFR reduction in patients with declining renal function would affect concomitant comorbidities.

Taken together, **Paper 2** identified PPARα as a novel regulator of *Fgf23* gene expression in UMR106 cells. The PPARα agonists fenofibrate and WY-14643 showed dose-dependent suppressive effects on *Fgf23* gene expression. In addition, PPARα was shown to act on *Fgf23* expression, at least in part, via AMPK-dependent regulation of SOCE. As described, the use of PPARα agonists is established or shown to be beneficial in several pathologies, including those which are characterized by increased levels of circulating FGF23. Their use in these conditions could be beneficial both for direct treatment and indirectly via concomitant regulation of FGF23. Therefore, future studies should be conducted to investigate the *in-vivo* relevance of PPARα and its agonists.

4.3 Myostatin regulates the production of FGF23

Myostatin is a member of the TGF-β family and is also known as growth differentiation factor 8 [400]. Myostatin initiates its intracellular signaling by binding to ACVR2B (and to a lesser extent ACVR2A) and acts in complex with TGF-βRI as a negative regulator of muscle development [267, 400]. Since the identification of myostatin in 1997, a cytokine secreted by muscles (also called myokine), it is shown to have various paracrine and endocrine effects [401]. In addition, myostatin, among others, is a signaling factor in muscle-bone-crosstalk involved in communicating and mediating molecular interactions between these organs [401]. The data published in **Paper 3** of this thesis fit well into this concept, adding molecular insights and a novel function of myostatin as regulator of the Fgf23 synthesis in UMR106 cells.

Data published in **Paper 3** showed that *Acvr2b*, the main membrane receptor of myostatin [267], was more highly expressed in UMR106 osteoblast-like cells used, than the receptor variant *Acvr2a*. In contrast, IDG-SW3 osteocytes and MC3T3-E1 pre-osteoblastic cells showed high expression of *Acvr2a*, whereas *Acvr2b* expression was low. Interestingly, treatment of IDG-SW3 and MC3T3-E1 cells with recombinant myostatin did not increase *Fgf23* mRNA expression, whereas *Fgf23* expression was increased in a dose-dependent manner in UMR106 cells, as shown in **Paper 3**. These results are consistent with the observed higher binding affinity of myostatin to ACVR2B [267, 268]. In addition, myostatin is shown to complex with TGF-βRI after binding to ACVR2B, activating a TGF-β-like signaling pathway, whereas the binding of myostatin to ACVR2A is weak and only detectable when ACVR2A expression is much higher than that of ACVR2B [267, 268]. In line with this, the data published in **Paper 3** showed that myostatin failed to increase *Fgf23* expression in UMR106 cells in the presence of TGF-βRI inhibitor SB431542. Thus, it could be speculated that myostatin mediates its effects mainly via binding to ACVR2B and signals in a TGF-β-like pathway in UMR106 cells. This speculation is supported by the fact that *Fgf23* expression in IDG-SW3 and MC3T3-E1 cells was not affected by myostatin in **Paper 3**, possibly as a consequence of relatively low *Acvr2b* expression compared with UMR106 cells. As published in **Paper 3**, treatment with myostatin also increased the secretion of C-terminal and intact Fgf23 into the cell culture supernatant, suggesting that myostatin regulates biologically active FGF23.

Next, the underlying signal transduction was investigated in subsequent experiments in **Paper 3**. Given that p38MAPK was identified as a novel regulator of *Fgf23* expression in **Paper 1** but is also a known downstream effector of myostatin [269, 402, 403], we tested whether p38MAPK is involved in myostatin-dependent regulation of FGF23. The experiments in **Paper**

3 revealed that the induction of *Fgf23* transcription in UMR106 cells is abolished in the presence of p38MAPK inhibitor SB202190. This is in line with other studies, which show an abrogation of myostatin-mediated effects in the presence of p38MAPK inhibitors [269, 402, 404]. Thus, the data showed in **Paper 3** suggests that myostatin-dependent activation of p38MAPK in UMR106 is involved in the regulation of *Fgf23* transcription. Interestingly, *Kido et al*. show an abrogation of cadmium-induced *Galnt3* expression in UMR106 cells in the presence of p38MAPK inhibitor SB203580 [247]. Given that GALNT3-dependent O-glycosylation protects FGF23 from cleavage [40, 44], it could be speculated that myostatin possibly also regulates *Galnt3* expression in UMR106 cells via p38MAPK activity. This speculation is supported by the increased secretion of intact Fgf23 into the UMR106 cell culture supernatant observed in **Paper 3** in the presence of myostatin.

However, current data showed that myostatin was capable of increasing *Fgf23* expression even in the presence of a p38MAPK inhibitor compared with treatment with p38MAPK inhibitor alone, suggesting the involvement of additional effectors. In **Paper 1**, it was shown that p38MAPK regulates *Fgf23* transcription, at least in part, via NFκB activity. Moreover, NFκB activity itself is important for FGF23 formation [215, 230] and myostatin also acts via NFκB [405, 406]. Therefore, in subsequent experiments, we investigated the additional involvement of NFκB in the myostatin-mediated induction of *Fgf23* transcription. In this experiment, we observed an abrogation of the myostatin-induced *Fgf23* transcription in the UMR106 cells in the presence of the NFκB inhibitor withaferin A. Consistent with this, withaferin A also abolished p38MAPK activator anisomycin-dependent upregulation of *Fgf23* transcription as shown in **Paper 1**. Taken together, these results indicate that myostatin in UMR106 cells may signal directly via p38MAPK and NFκB, but also indirectly via the mutual regulation and activation of p38MAPK and NFκB. This assumption is supported by a recent study, which shows that myostatin, through its association with ACVR2B, enhances NFκB and p38MAPK activation, thereby promoting osteoclastogenesis [406].

In addition, NFκB increases *Fgf23* expression in UMR106 cells via upregulation of *Orai-1* and subsequent enhancement of SOCE [215]. Of note, TGF-β, whose family includes myostatin [264], mediates its effects in UMR106 cells via TGF-βRI and also induces *Fgf23* transcription via upregulation of SOCE [218]. In this context, we performed further experiments to investigate whether myostatin also affects *Fgf23* expression by regulating SOCE. Interestingly, in these experiments published in **Paper 3**, an attenuation of the myostatin-mediated increase of *Fgf23* expression was observed in UMR106 cells in the presence of SOCE inhibitor 2-APB. Thus, it could be speculated that myostatin influences *Fgf23* transcription in

UMR106 cells, at least in part, via p38MAPK and NFκB-mediated SOCE, as summarized in Fig. 5 in **Paper 3**. This speculation on a myostatin-mediated regulation of SOCE is supported by recent *in-vivo* findings, which show reduced STIM1 and Orai-1 protein levels, reduced Ca^{2+}-store content, and subsequently suppressed SOCE in muscles from myostatin deficient mice [407, 408].

NFκB is activated downstream of myostatin [409], mediating the expression of *Il-1β* and *Il-6* [410]. Moreover, treatment of C2C12 myotubes with myostatin increases mRNA expression and protein secretion of Il-6 via involvement of p38MAPK activity [411]. In addition, decreased Tnf-α protein levels in $Mstn^{Ln/Ln}$ mice and decreased adipose *Tnf-α*, *Il-6*, and *Il-1β* mRNA expression in $Mstn^{Ln/Ln}$ mice on HFD are found [412]. Further, treatment of human RA synovial fibroblasts with myostatin directly increases mRNA and protein production of TNF-α [413]. These data suggest a possible involvement of myostatin in the production of pro-inflammatory cytokines. Given that TNF-α and IL-1β, both dependent on NFκB activity [230], and IL-6 [235] are potent inducers of FGF23 formation, it could be speculated that myostatin also mediates the increase in Fgf23 synthesis in UMR106 cells via NFκB-related or direct expression of pro-inflammatory cytokines. Thus, it could be hypothesized that myostatin and FGF23 are also directly or indirectly related via mediation of pro-inflammatory stimuli. This assumption is supported by the fact that myostatin is both positively correlated with CRP and associated with the disease activity in patients with RA [414]. In addition, myostatin is positively associated with TNF-α, a prototypical pro-inflammatory cytokine in RA, in human RA synovial fibroblast tissues [413]. Moreover, both myostatin and IL-1β levels are higher and positively correlated in RA synovial fluid compared with osteoarthritis synovial fluid [415]. Interestingly, myostatin directly induces IL-1β production in RA synovial fibroblasts [415]. Further, serum myostatin concentrations are higher in patients with knee osteoarthritis than in control patients [416]. Interestingly, patients with osteoarthritis show higher FGF23 levels than controls [377] and FGF23 is correlated with inflammation in subjects with RA [378]. It is therefore tempting to speculate, that the higher FGF23 levels observed in patients suffering from RA result from a direct induction by myostatin or indirectly via myostatin-mediated production of pro-inflammatory cytokines such as TNF-α or IL-1β. This speculation is supported by the detected myostatin levels in RA patients, which range from 1.2-140 ng/mL [414], concentrations that are much higher than those used in **Paper 3** that resulted in a significant increase in Fgf23 mRNA and protein expression in UMR106 cells. In addition, in a recently published study, an increase in both myostatin and FGF23 is observed in patients with dermatomyositis, an idiopathic inflammatory myopathy [417]. Therefore, inhibition of

myostatin could be a therapeutic approach in such conditions to potentially lower FGF23 levels directly or indirectly by reducing myostatin-mediated expression of pro-inflammatory cytokines. In this context, it is noteworthy that myostatin deficiency or antibody-mediated inhibition leads to amelioration of arthritis severity in a mouse model of human RA [418]. Interestingly, fenofibrate, a PPARα agonist identified in **Paper 2** as a suppressor of *Fgf23* expression, reduces *Tnf-α* mRNA in liver and myostatin mRNA and protein expression in muscles of rats with arthritis [380].

Myostatin regulates myotube differentiation, protein degradation, muscle atrophy, and cachexia by reducing the phosphorylation of AKT and its downstream signaling [419–421]. This leads to activation of the transcription factor forkhead box protein O (FOXO) and expression of atrophy-related genes and subsequent activation of the ubiquitin-proteasome and autophagy-lysosome systems [419–423]. Remarkably, activation of the PI3K/AKT pathway and subsequent phosphorylation and inactivation of FoxO1 by insulin treatment in UMR106 cells is identified as an potent regulatory mechanism of FGF23 production [239]. Thus, the insulin-dependent reduction of *Fgf23* expression in UMR106 cells is attenuated in the presence of both PI3K and AKT inhibitors. In addition, insulin treatment increases phosphorylation of FoxO1 in UMR106 cells [239]. Consistent with these findings, pharmacological inhibition and siRNA-mediated knockdown of FoxO1 decrease *Fgf23* expression, whereas overexpression of *FoxO1* increases *Fgf23* expression in UMR106 cells [239]. Given that myostatin is able to induce *FoxO1* mRNA and protein expression and reduce FoxO1 phosphorylation in C2C12 cells [418], it is tempting to speculate that positive regulation of FoxO1 by myostatin is involved in the observed upregulation of *Fgf23* expression in the presence of myostatin (**Paper 3)**.

HFD fails to increase leptin levels in $Mstn^{Ln/Ln}$ mice, indicating a positive role of myostatin in leptin production [412]. Consistent with this, treatment with myostatin induces leptin mRNA and protein secretion in fully differentiated 3T3-L1 adipocytes [424]. Remarkably, leptin treatment is shown to increase both serum Fgf23 levels and bone-derived *Fgf23* mRNA expression in *leptin*-deficient mice [240] and also potentiate $1,25(OH)_2D_3$-dependent upregulation of *Fgf23* expression in UMR106 cells [391]. Therefore, it can be assumed that myostatin may influence FGF23 via regulation of leptin production *in-vivo*.

Treatment with myostatin induces the expression of *Sost* mRNA and its gene product in osteocytic Ocy454 cells [425]. Interestingly, a recent study finds that sclerostin directly stimulates Fgf23 mRNA expression and protein secretion [324], providing evidence for the speculation that myostatin may also influence *Fgf23* expression in bone cells via upregulation of sclerostin.

Myostatin not only induces loss of skeletal muscle, but also of bone mass [426]. Thus, myostatin is shown to inhibit osteoblast differentiation [425]. In contrast, inhibition of myostatin by follistatin increases osteogenic differentiation and calcification *in-vitro* [403]. In line with this, systemic inhibition of myostatin/activin A signaling results in protection from bone loss during spaceflight and enhances recovery of bone mass following spaceflight in mice [426]. Considering that FGF23 inhibits osteoblast differentiation and matrix mineralization [103–105], the observed myostatin-mediated increase in Fgf23 production in **Paper 3** fits well with the concept of suppressive effects of myostatin on bone mass via directly controlled mechanisms or possibly indirectly through regulation of FGF23 synthesis.

Interestingly, in a recent published study, it is observed that myostatin protein expression in rat myoblast cells is approximately 20-fold higher under high phosphate treatment, accompanied by lower protein synthesis and higher degradation, than in control [427]. CKD is accompanied by hyperphosphataemia [428] and high FGF23 levels [156], but also by muscle wasting [429]. Of note, a recent study shows suppression of myogenic differentiation *in-vitro* by high P_i treatment, while skeletal muscle atrophy is enhanced *in-vivo*, especially under conditions of muscle wasting such as in CKD [430]. In this publication, CKD mice on high phosphate diet show decreased gastrocnemius muscle weights accompanied by the highest Fgf23 levels compared to all other groups [430]. Interestingly, an inhibition of skeletal muscle differentiation by treatment with FGF23 and α-Klotho is reported [431]. It is highly remarkable that the mRNA and protein expression of myostatin is increased in the muscles of humans, mice or rats with CKD [411, 422, 432]. In addition, similar to FGF23 [156], myostatin levels are shown to increase with decreasing GFR in patients with CKD [433]. This is in line with the observation, that myostatin is higher in both patients with diabetic CKD [434] and chronic hemodialysis [435], than in control subjects. However, another study observed no significant difference in serum myostatin between hemodialysis and control patients [436]. Nevertheless, it is tempting to speculate that the increases in myostatin concentrations found and the associated muscle atrophy under conditions of CKD contribute directly or indirectly to the high FGF23 concentrations observed. This speculation is supported by the fact that patients receiving hemodialysis have plasma myostatin concentrations equal to (40.1 ± 8.3 ng/mL [435]) or exceeding (25.7 ± 12.8 µg/mL; [437]) those used in **Paper 3**, which significantly increased *Fgf23* expression in UMR106 cells. However, other measured plasma myostatin concentrations in CKD and dialysis patients [438] are lower than those used in **Paper 3**, which significantly induce *Fgf23* expression. Furthermore, activin A, another member of the TGF-β family that signals through ACVR2A receptors, is positively correlated with plasma Fgf23 concentration

in rats with CKD [439]. In addition, CKD is characterized by an increase of circulating pro-inflammatory cytokines activating the expression of both myostatin [411, 422, 438] and FGF23 [234, 237]. Considering that myostatin regulates FGF23 directly, as shown in **Paper 3**, or possibly indirectly by affecting the expression of pro-inflammatory cytokines under conditions of CKD, it might be beneficial to inhibit myostatin. This notion is supported by the observations of Zhang *et al*., who show higher levels of Il-6 and Tnf-α in mice with CKD, which are decreased after inhibition of myostatin, accompanied by decreased mRNA expression of *Il-6* and *Tnf-α* in the muscles of these mice [411]. In addition, the study of inhibition of myostatin in conditions of CKD and the potential benefits of this is the focus of several clinical trials [440]. The findings from these trials could also be beneficial in the treatment of possible myostatin-mediated regulation of FGF23.

In addition, physical exercise could be another therapeutic approach to lower myostatin, FGF23, and inflammation in conditions of CKD. Thus, myostatin mRNA is reduced after exercise training in patients with CKD [441] and exercise intervention decreases IL-6 levels in patients with moderate to severe CKD [442]. In addition, both dynamic and isometric resistance training results in decreased FGF23 levels in patients receiving hemodialysis [443]. These data provide evidence for potential benefits of exercise intervention that attenuates the levels of inflammatory factors, myostatin, and FGF23 and possibly attenuates their downstream reciprocal regulations.

In summary, studies published in **Paper 3** found a direct stimulatory effect of myostatin on Fgf23 mRNA and protein expression, identifying this myokine as a novel regulator of Fgf23 production in UMR106 cells. The investigations showed that p38MAPK and NFκB are involved, at least in part, in myostatin-mediated signaling. Future studies are needed to examine the *in-vivo* relevance of our findings and elucidate the extent to which myostatin also affects FGF23 and downstream phosphate metabolism here. However, as described, high levels of myostatin are observed in various pathologies, including the above, which are characterized by elevated FGF23 levels. Pharmacological inhibition of myostatin in such pathological conditions and the potential benefit thereof are the subject of current research. The use of myostatin inhibitors in these conditions could be beneficial both for direct treatment and indirectly via concomitant regulation of FGF23.

4.4 Conclusion

The aim of the present work was to contribute to a better understanding of the local and systemic regulation of FGF23 by identifying novel regulators in bone cells, the main synthesis site of this hormone. Furthermore, involved signaling pathways should be identified. The new regulators identified in this work, fit into existing gaps of the local signaling described so far and complement the previous knowledge about the complex regulation of FGF23 in bone cells. The newly identified regulators of FGF23 in this work and their interaction with already known signaling pathways that modulate FGF23 are shown in **Fig. 5**.

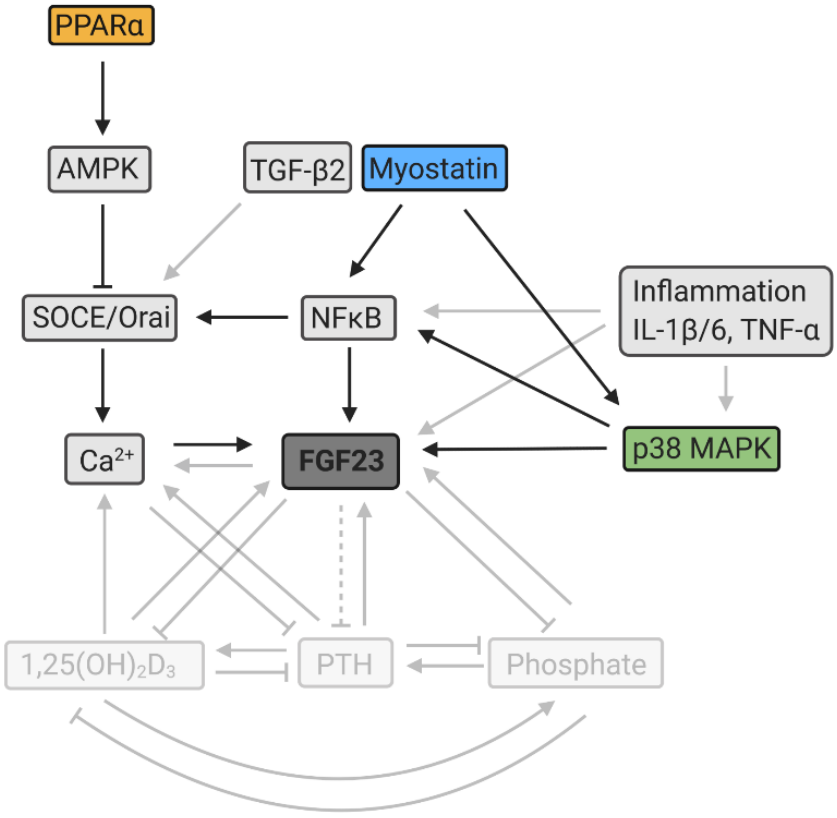

Figure 5: Newly identified regulators of FGF23 in bone cells and their interaction with known FGF23-modulating signaling pathways.
Figure according to [213]. p38MAPK: Paper 1; PPARα: Paper 2; Myostatin: Paper 3. For more details see text. Illustration was created with biorender.com.
Peroxisome proliferator-activated receptor alpha (PPARα); AMP-activated protein kinase (AMPK); Transforming growth factor-β2 (TGF-β2); Interleukin (IL); Tumor necrosis factor alpha (TNF-α); Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NFκB); Calcium (Ca^{2+}); Store-operated Ca^{2+} entry (SOCE); p38-mitogen-activated protein kinase (p38MAPK); Fibroblast growth factor 23 (FGF23); Parathyroid hormone (PTH); $1,25(OH)_2D_3$ (active vitamin D)

These molecular insights into the local regulatory mechanisms of FGF23 in bone cells can contribute to the better understanding and development of novel therapeutic approaches that could be used in FGF23-related diseases. The three studies conducted and published were able to comprehensively answer the key questions of this thesis:

I) What is the role of p38MAPK in the regulation of FGF23 synthesis and is the transcription factor NFκB involved?

Paper 1 identified p38MAPK as a potent regulator of *Fgf23* expression in UMR106 osteoblast-like cells and osteocytic IDG-SW3 cells. Thus, activation of p38MAPK increased, whereas inhibition of p38MAPK decreased *Fgf23* expression. Furthermore, p38MAPK-mediated regulation of *Fgf23* expression was shown to be dependent on NFκB activity, as inhibition of this transcription factor abrogated p38MAPK activator-mediated stimulation of *Fgf23* transcription. These data, published in **Paper 1**, fit well with the concept of p38MAPK activity localizing its action both upstream of the established regulator of FGF23 formation NFκB, but also potentially in known distinct cellular signaling cascades that regulate FGF23. Therefore, it is tempting to address in future studies the *in-vivo* relevance of p38MAPK activity in the regulation of FGF23 synthesis.

II) What is the impact of PPARα on FGF23 formation and is AMPK involved in the underlying signaling?

In **Paper 2**, PPARα was identified as a negative regulator of *Fgf23* expression. Thus, PPARα agonists were observed to suppress *Fgf23* expression in a dose-dependent manner, whereas pharmacological inhibition or siRNA-mediated knockdown of PPARα induced *Fgf23* expression. In addition, AMPK activity was found to be involved in PPARα-dependent regulation of *Fgf23* transcription. Consistent with a previous publication that identified AMPK as a regulator of FGF23 production via inhibition of Orai-1-mediated SOCE, **Paper 2** showed that AMPK-mediated control of SOCE is also involved in PPARα-dependent regulation of FGF23. Considering that the use of PPARα agonists is established in inflammatory conditions or dyslipidemia, which are also associated with dysregulated FGF23 levels, the data from **Paper 2** provide hints for future studies that should investigate the *in-vivo* relevance of PPARα and its agonists to FGF23 metabolism.

III) What is the relevance of myostatin in the regulation of FGF23?

Myostatin was identified in **Paper 3** as a potent stimulator of Fgf23 mRNA expression and protein production in UMR106 cells. Furthermore, UMR106 cells were observed to express ACVR2B, the major receptor for myostatin, more strongly than ACVR2A. Interestingly, in the additional pre-osteoblastic MC3T3-E1 cells and osteocytic IDG-SW3 cells examined, no stimulation of *Fgf23* expression was found in the presence of myostatin, which may be explained by the observation of contrasting expression of ACVR2B and ACVR2A in these cell lines compared with UMR106 cells. Investigation of the underlying signaling pathway revealed that myostatin-dependent regulation of *Fgf23* expression in UMR106 cells depends on the activity of p38MAPK, the novel regulator of *Fgf23* transcription identified in **Paper 1**. In addition, myostatin-mediated regulation of *Fgf23* expression in UMR106 cells was also found to depend on NFκB activity and possibly downstream SOCE. Considering that various pathologies are accompanied by higher levels of FGF23 and myostatin, future studies are needed to investigate myostatin and its inhibition regarding its role in the regulation of FGF23 *in-vivo*.

5 Summary

Fibroblast growth factor 23 (FGF23), a hormone secreted by bone, is an important regulator of the maintenance of phosphate homeostasis and vitamin D metabolism. Thus, FGF23 acts with its co-receptor α-Klotho mainly in the kidney and suppresses phosphate reabsorption and enzymatic formation of $1,25(OH)_2D_3$ (active vitamin D). In addition, FGF23 also acts on extrarenal tissues such as the liver, heart, parathyroid glands, and immune cells. FGF23 gained great clinical relevance by its identification as a causative factor in rare inherited diseases, characterized by phosphate wasting and hypophosphataemic rickets, but also by its involvement in the pathogenesis of chronic kidney disease and concomitant cardiovascular comorbidities. FGF23 is therefore discussed both as a potential biomarker for altered mineral metabolism, renal and related cardiovascular disease and mortality, but also as a clinical target.

Elucidation of the systemic and local regulation of FGF23 is the subject of research in recent years. Thus, several systemic modulators of FGF23 such as parathyroid hormone, $1,25(OH)_2D_3$, or alimentary phosphate and their reciprocal feedback loops were identified. Furthermore, inflammatory stimuli and cellular signaling cascades such as nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NFκB)- or AMP-activated protein kinase (AMPK)-regulated store-operated Ca^{2+} entry (SOCE) are identified to influence both transcriptional and post-translational regulation of FGF23 in bone cells. Nevertheless, the complex regulatory network of *FGF23* transcription and protein production is still incompletely understood.

Therefore, the studies published in this thesis will contribute to a better understanding of the local and systemic regulation of FGF23 by identifying novel regulators and their signaling in osteoblasts and osteocytes, the main site of synthesis of this hormone.

The studies performed focused on the following factors: p38 mitogen-activated protein kinase (MAPK), a serine/threonine kinase activated by stress-stimuli upstream of NFκB, a well-established stimulator of FGF23 formation; peroxisome proliferator-activated receptor (PPAR)-α, a transcription factor with anti-inflammatory properties that controls target genes for β-oxidation and triglyceride catabolism and activates the newly identified regulator of FGF23 AMPK; myostatin, a suppressor of muscle growth with signaling similar to transforming growth factor-β (TGF-β), which is also identified as regulator of FGF23.

It was shown that p38MAPK is a potent regulator of *Fgf23* transcription in UMR106 osteoblast-like cells and osteocytic IDG-SW3 cells. Inhibition of p38MAPK significantly decreased Fgf23 mRNA and protein expression in the cells examined. The stimulatory effect

of p38MAPK activation on *Fgf23* transcription in UMR106 cells was abrogated in the presence of NFκB inhibitors. This suggests that p38MAPK influences *Fgf23* expression in UMR106 cells, at least in part, via NFκB activity.

Moreover, it was shown that PPARα is a negative regulator of *Fgf23* transcription in UMR106 cells, as treatment with PPARα agonists, such as fenofibrate and WY-14643, decreased *Fgf23* in a dose-dependent manner, whereas inhibition of PPARα or siRNA-mediated knockdown increased *Fgf23* expression. Furthermore, treatment with PPARα agonist fenofibrate was shown to induce AMPK activity in UMR106 cells and PPARα agonist-induced suppression of *Fgf23* expression was abolished in the presence of AMPK inhibition. Furthermore, treatment with fenofibrate reduced SOCE in UMR106 cells, and it was observed that PPARα did not affect *Fgf23* expression in the presence of siRNA-mediated knockdown of *Orai-1*. Thus, PPARα agonists decrease *Fgf23* transcription in UMR106, at least in part, through AMPK-mediated suppression of SOCE. The use of fenofibrate in inflammatory conditions and dyslipidemia is widely established. More importantly, it appears to have the potential to reduce the higher FGF23 levels that are also observed in such conditions.

Furthermore, myostatin was shown to be a potent stimulator of Fgf23 mRNA and protein production in UMR106 cells. In addition, these cells were found to predominantly express the major myostatin receptor activin receptor type 2B (ACVR2B). Moreover, TGF-β type I receptor was found to be involved in myostatin-induced *Fgf23* expression. Myostatin increased the *Fgf23* transcription involving the newly identified regulator of *Fgf23* expression p38MAPK. Moreover, myostatin-mediated induction of *Fgf23* expression was also dependent, at least in part, on NFκB activity and possibly downstream SOCE. These findings provide a molecular mechanistic complement to the equally elevated levels of FGF23 and myostatin observed in various diseases and may be relevant to future studies investigating the utility of pharmacological inhibition of myostatin in such diseases.

In conclusion, p38MAPK, PPARα, and myostatin were identified as novel and potent regulators of FGF23 mRNA and protein synthesis in bone cells. The data published in this work provide new molecular insights into the underlying complex network of FGF23 regulation and reveal interactions between novel and known cellular and systemic modulators of FGF23.

6 Zusammenfassung

Der Fibroblasten-Wachstumsfaktor 23 (*fibroblast growth factor 23*, FGF23) ist ein vom Knochen gebildetes und sezerniertes Hormon, welches die Aufrechterhaltung der Phosphat-Homöostase und den Vitamin D-Metabolismus reguliert. So wirkt FGF23 mit seinem Co-Rezeptor α-Klotho überwiegend in der Niere und reduziert die Reabsorption von Phosphat, sowie die enzymatische Bildung von $1,25(OH)_2D_3$ (aktives Vitamin D). Darüber hinaus wirkt FGF23 aber auch auf extra-renale Gewebe, wie Leber, Herz, Nebenschilddrüse und Immunzellen, ein. FGF23 erlangte große klinische Relevanz durch seine Identifizierung als kausaler Faktor in seltenen Erbkrankheiten, welche durch extreme Phosphatverluste und hypophosphatämischer Rachitis charakterisiert sind, aber auch durch seine Beteiligung an der Pathogenese der chronischen Nierenerkrankung und begleitender kardiovaskulärer Komorbiditäten. FGF23 wird daher als potentieller Bio- und Risikomarker zum Nachweis von Mineralstoffwechselstörungen und für renale und damit verbundene kardiovaskuläre Krankheiten und Mortalität diskutiert. Im klinischen Kontext wird darüber hinaus auch die direkte Beeinflussung von FGF23 für therapeutische Anwendungen untersucht.

Die Aufklärung der systemischen und lokalen Regulation von FGF23 ist Gegenstand der Forschung der letzten Jahre. So wurden mehrere systemische Modulatoren von FGF23 wie Parathormon, $1,25(OH)_2D_3$ oder alimentäres Phosphat und deren gegenseitige Rückkopplungsschleifen identifiziert. Darüber hinaus wurden Entzündungsreize und zelluläre Signalkaskaden wie der *nuclear factor 'kappa-light-chain-enhancer' of activated B-cells* (NFκB)- oder AMP-aktivierte Proteinkinase (AMPK)-regulierte speichergesteuerte Ca^{2+}-Einstrom (*store-operated Ca^{2+} entry*, SOCE) identifiziert, die sowohl die transkriptionelle als auch die posttranslationale Regulation von FGF23 in Knochenzellen beeinflussen. Dennoch ist das komplexe regulatorische Netzwerk der *FGF23*-Gentranskription und Proteinproduktion noch immer unvollständig verstanden.

Daher sollen die in dieser Arbeit veröffentlichten Studien zu einem besseren Verständnis der lokalen und systemischen Regulation von FGF23 beitragen, indem neue Regulatoren und deren Signalwege in Osteoblasten und Osteozyten, dem Hauptort der Synthese dieses Hormones, identifiziert werden.

Die durchgeführten Studien untersuchten die regulatorischen Funktionen der p38-mitogenaktivierte Proteinkinase (p38MAPK), einer Serin/Threonin-Kinase die durch Stress-Stimuli aktiviert wird und NFκB moduliert, welcher ein bekannter Stimulator von FGF23 ist und des Peroxisom-Proliferator-aktivierte Rezeptors α (PPARα), ein Transkriptionsfaktors, der

Zielgene für die β-Oxidation und den Abbau von Triglyceriden kontrolliert und AMPK als den neu-identifizierten Regulator von FGF23 aktiviert. Zuletzt wurde Myostatin, ein Suppressor des Muskelwachstums mit einem dem Transformierenden Wachstumsfaktor-β (TGF-β) - ähnlichen Signalweg, welcher als Regulator von FGF23 identifiziert ist, untersucht.

Es konnte gezeigt werden, dass p38MAPK ein potenter Regulator der *Fgf23*-Transkription in UMR106 osteoblasten-ähnlichen Zellen und in IDG-SW3-Osteozyten ist. In den untersuchten Zellen wurde die Fgf23-mRNA- und Proteinexpression durch die Inhibition von p38MAPK signifikant gesenkt. Der stimulierende Effekt der Aktivierung von p38MAPK auf die Gentranskription von *Fgf23* in UMR106 Zellen wurde in Gegenwart von NFκB-Inhibitoren aufgehoben. Dies deutet darauf hin, dass p38MAPK die *Fgf23*-Expression in UMR106 Zellen, zumindest teilweise, über die NFκB-Aktivität beeinflusst.

Darüber hinaus konnte gezeigt werden, dass PPARα ein negativer Regulator der *Fgf23*-Transkription in UMR106 Zellen ist. So senkte die Behandlung mit PPARα-Agonisten die *Fgf23*-Genexpression dosisabhängig, wohingegen die Inhibition oder die siRNA-vermittelte Herunterregulation von *PPARα* die *Fgf23*-Expression erhöhten. Außerdem wurde gezeigt, dass die Behandlung mit dem PPARα-Agonisten Fenofibrat die AMPK-Aktivität in UMR106 Zellen induziert und die durch PPARα-Agonisten induzierte Reduktion der *Fgf23*-Expression unter AMPK-Inhibition aufgehoben war. Darüber hinaus senkte die Behandlung mit Fenofibrat SOCE in UMR106 Zellen und es wurde beobachtet, dass PPARα die *Fgf23*-Expression in Gegenwart der siRNA-vermittelten Herunterregulation von *Orai-1* nicht beeinflusste. Somit senken Agonisten von PPARα die *Fgf23*-Transkription in UMR106 Zellen, zumindest teilweise, über die AMPK-vermittelte Unterdrückung von SOCE. Fenofibrat wird klinisch bei pro-inflammatorischen Zuständen und Dyslipidämie angewendet. Interessanterweise scheint es auch das Potenzial zu haben, höhere FGF23-Werte, welche ebenfalls unter solchen Bedingungen beobachtet werden, zu reduzieren.

Außerdem wurde gezeigt, dass Myostatin ein potenter Stimulator der mRNA- und Proteinproduktion von Fgf23 in UMR106 Zellen ist. Darüber hinaus wurde festgestellt, dass UMR106 Zellen den wichtigsten Rezeptor von Myostatin, Activin Rezeptor Typ 2B (ACVR2B), exprimieren. Außerdem wurde festgestellt, dass der TGF-β-Typ I-Rezeptor an der Myostatin-induzierten *Fgf23*-Expression beteiligt ist. Myostatin wirkte auf die *Fgf23*-Gentranskription über die Beteiligung des neu-identifizierten Regulators der *Fgf23*-Expression, p38MAPK. Außerdem war die Myostatin-vermittelte Induktion der *Fgf23*-Expression, zumindest teilweise, auch von der NFκB-Aktivität und möglicherweise vom nachgeschalteten SOCE abhängig. Diese Befunde liefern Ansätze zu molekularen Mechanismen zu den bei

verschiedenen Krankheiten beobachteten gleichermaßen erhöhten Werten von FGF23 und Myostatin und könnten für zukünftige Studien relevant sein, die den Nutzen einer pharmakologischen Hemmung von Myostatin bei solchen Krankheiten untersuchen.

Zusammenfassend wurden p38MAPK, PPARα und Myostatin als neuartige und potente Regulatoren der mRNA- und Proteinsynthese von FGF23 in Knochenzellen identifiziert. Die in dieser Arbeit publizierten Daten liefern neue molekulare Einblicke in das zugrundeliegende komplexe Netzwerk der Regulation von FGF23 und zeigen Interaktionen zwischen den neuartigen und bekannten zellulären und systemischen Modulatoren von FGF23 auf.

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