Cellular stress regulates fibroblast growth factor 23 (FGF23) and αklotho

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Abbreviations

$1,25(OH)_2D_3$	calcitriol, active vitamine D_3
7-DHC	7-dehydrocholesterol
aa	amino acid
ADAM	A disintegrin and metalloproteinase domain-containing protein
ADHR	autosomal dominant hypophosphatemic rickets
AKI	acute kidney injury
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
APAF-1	apoptotic protease activating factor 1
ATP	adenosine triphosphate
BAD	BCL2-associated death promoter
BAK	BCL2 homologous antagonist/killer
BAX	BCL2-associated X
BCL-2	B cell lymphoma 2
BCL-XL	B cell lymphoma-extra large
CKD	chronic kidney disease
CTR	copper transporter
CVD	cardiovascular disease
CYP24A1	cytochrome P450 24A1
CYP27B1	cytochrome P450 27B1
CYP2R1	cytochrome P450 2R1
DBP	vitamin D binding protein
ELISA	enzyme-linked immunosorbent assay
ERK1/2	extracellular-signal regulated kinase 1 and 2
FAM20C	family with sequence similarity 20, member C
FGF	fibroblast growth factor
FGFR1	fibroblast growth factor receptor
FOXO	forkhead-box-protein O
Galnt3	polypeptide N-acetylgalactosaminyltransferase 3
GSH	glutathione
H_2O_2	hydrogen peroxide
HIF1α	hypoxia inducible factor

HK-2	human kidney cell line
HO•	hydroxyl radical
HPV	human papillomavirus
IFN-γ	interferon gamma
IGF-1	insulin-like growth factor
IGF-1R	insulin-like growth factor 1 receptor
IgG	immuneglobuline G
IL	interleukin
JNK	c-Jun N-terminal kinase
KL	αklotho
LLC-PK1	porcine kidney epithelial cell line
MAPK	mitogen activated protein kinase
MDCK	madin Darby canine kidney cell line
mTOR	mammalian target of rapamycin
NaP_iII	sodium-phosphate co-transporter 2
NCC	sodium chloride co-transporter
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHERF1	sodium-hydrogen exchanger regulatory factor 1
NRF2	nuclear factor erythroid 2-related factor 2
NRK-52E	normal rat kidney cell line
$O_2^{\bullet-}$	superoxide anion radical
OAT	organic anion transporter
OATP	organic anion transporting polypeptide
OCT2	organic cation transporter
PAC-1	procaspase activating compound 1
P_i	phosphate
PI3K	phosphatidylinositol-3-phosphate
PiT	phosphate transporter
PPARγ	peroxisome proliferator-activated receptor gamma
PTH	parathyroidhormone
R	amino acid arginine
RANK	receptor activator of nuclear factor κB
RANKL	receptor activator of nuclear factor κB ligand
RIPK1	receptor-interacting serine/threonine protein kinase 1

ROS	reactive oxygen species
SGK1	serum/glucocorticoid regulated kinase 1
sklotho	soluble klotho
SOCE	store-operated calcium entry
TGF-β	transforming growth factor beta
Thr	threonine
TIO	tumor-induced osteomalacia
TNFα	tumor necrosis factor alpha
TRPV	transient receptor potential cation channel subfamily V
TWEAK	TNF-like weak inducer of apoptosis
UMR106	rat osteoblast-like osteosarcoma cell line
VDR	vitamin D receptor
VDRE	vitamin D responsive element
X	any amino acid

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1 Introduction

Few decades ago, fibroblast growth factor 23 (*FGF23*) and αklotho knockout mice revealed a shared role in the metabolism of inorganic phosphate[1]. FGF23 or αklotho deficiency results in hyperphosphatemia with massive ectopic calcification[2,3]. These deposits cause a syndrome resembling human aging[2]. Thus, αklotho was named after the Greek goddess who spun the thread of life[2]. Beside its major role in tumor-induced osteomalacia (TIO)[4] or autosomal dominant hypophosphatemic rickets (ADHR)[5], aberrant regulation of FGF23 has been associated with various diseases without a clear relation to phosphate or bone metabolism[6-8] and also αklotho is involved in various disorders[9–11]. Cellular stress or subsequent senescence is a frequent event in severe tissue injury and diseases[12]. The aim of the present thesis was the elucidation of a regulatory mechanism of cellular stress on FGF23 and αklotho.

1.1 Features of FGF23

Fibroblast growth factors are a family of versatile signaling proteins with a broad spectrum of functions. Based on structural and evolutionary data, FGFs are divided into several subfamilies comprising 22 proteins in humans[13]. FGF23 is assigned to endocrine FGF family including FGF19/FGF15, FGF21, and FGF23, termed hormone-like FGFs[14]. Their mutual structure and the absence of a C-terminal heparin-binding domain found in paracrine FGFs enables secretion, circulation, and signal transduction of endocrine FGFs to distant target organs[15].

Produced primarily by osteoblasts and osteocytes in bone[16,17], FGF23 is an approximately 30 kDa glycoprotein with 251 amino acids (aa) and the gene sequence is located on human chromosome 12p13 comprising 3 exons[18,19]. Secreted FGF23 contains 227 aa, lacking a 24 aa hydrophobic signal peptide[18]. The N-terminal receptor binding site with a β-trefoil structure comprises 154 aa sharing homologies with other FGFs[20], whereas the 72 aa C-terminal sequence of FGF23 enables interaction with co-receptor αklotho[21].

In human blood, circulating FGF23 can be detected in two major forms: intact full-length FGF23 (aa 25-251) and a C-terminal fragment resulting from proteolytic cleavage (aa 180-251)[4,22]. Half-life of human intact FGF23 is approximately one hour[23]. Proteolytic cleavage is catalyzed by subtilisin-like pro-protein convertase furin between arginine$_{179}$ and phosphorylated serine$_{180}$ at consensus sequence $R_{176}XXR_{179}$[24,25]. This produces N- and C-terminal fragments, separating FGFR and klotho binding domains[26]. The exact role of these fragments is currently unclear but C-terminal fragments are suggested to antagonize FGF23 binding to FGFR1, thereby inhibiting its function on phosphate homeostasis[21]. Complex regulation of FGF23 cleavage points to a specified role of the fragments rather than inactive degradation products. Thus, cleavage of FGF23 may serve as a regulator of FGF23 signal transduction[21].

Posttranslational modification of FGF23 includes glycosylation and phosphorylation[25]. O-glycosylation at Thr_{178} by polypeptide N-acetylgalactosaminyltransferase 3 (GALNT3) prevents furin-mediated cleavage at $R_{176}XXR_{179}$ site, protecting FGF23 from proteolysis[27]. The family with sequence similarity 20, member C (FAM20C) is a protein kinase that phosphorylates Ser_{180} of the FGF23 sequence, thereby preventing GALNT3-mediated O-glycosylation and driving proteolysis[25]. Consequently, loss-of-function mutation in *GALNT3* decreases intact FGF23 levels promoting hyperphosphatemia[28], whereas loss-of-function mutation in *FAM20C* is accompanied by excess levels of intact FGF23 and hypophosphatemia[25].

FGF receptors (FGFR) are a family of receptor tyrosine kinases that contain an extracellular ligand-binding domain composed of three immunoglobulin-like loops, a single transmembrane domain, and the intracellular tyrosine kinase domain[29]. Endocrine FGFs require the co-receptors αklotho and βklotho for receptor binding and signaling[30–32]. FGF23 uses co-factor αklotho to form FGF23-FGFR-αklotho receptor complexes[26]. FGF23 shows the highest binding affinity for FGFR1 subtype c (FGFR1c), which appears to be the major physiologically relevant receptor in the kidney[32,33]. Upon FGF23-binding, FGFR-αklotho-FGF23 complex activates intracellular kinase activity, activating various signaling pathways e.g. mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinases 1/2 (ERK1/2) signaling[34].

1.2 αKlotho

αKlotho was originally discovered as an anti-aging factor because mice with a mutation in the αklotho (*KL*) gene show typical aging-related disorders including organ atrophy, tissue and vascular calcifications, arteriosclerosis, infertility, hyperphosphatemia, osteoporosis, and a short lifespan[2]. The αklotho gene and protein are highly homologous (>80 %) in mice, rat and human[35]. αKlotho is a single transmembrane protein predominantly expressed within the kidney[2], the parathyroid glands[36], and the brain[37]. It consists of one short intracellular, a transmembrane and two repeated extracellular sequences termed KL1 and KL2 domains[38]. Cleavage of the extracellular domain by α-secretases A desintegrin and metalloproteinase (ADAM)-10 and ADAM-17 releases soluble klotho (sklotho) into the circulation[39]. Soluble αklotho can be detected in serum, cerebrospinal fluid, and urine[9,40]. sKlotho levels decrease with increasing age[41,42] and low αklotho levels or decreased production are associated with severe diseases like CKD[43–45], cancer[46,47], or cardiovascular disorders[10,48].

The phenotype of homozygous αklotho knockout mice strongly resembles that of FGF23-deficient mice, suggesting a shared role in phosphate metabolism[1,2,49,50]. As mentioned above, αklotho functions as a co-factor for FGFR1 in renal tubule cells mediating stable interaction between FGF23 and FGFR1c[32]. Beside its action as a co-receptor of FGF23, αklotho has many beneficial effects e.g. anti-inflammatory[51,52], antioxidant[53], or anti-apoptotic functions[54]. Furthermore, high αklotho levels correlate with the relief of symptoms of numerous diseases including acute kidney injury (AKI)[55], chronic kidney disease (CKD)[56,57],

cardiovascular disease (CVD)[48,58], Alzheimer's disease[59], or sepsis[60]. The inhibition of wnt/β-catenin as well as insulin-like growth factor (IGF-1) signaling pathway strongly participates in the health-promoting effects of αklotho[61,62]. Activation of wnt signaling results in the accumulation of β-catenin, followed by its translocation into the nucleus where it activates target genes including cyclin D1 and myc-c, promoting cell proliferation[63]. IGF-1 is involved in postnatal growth and stimulates anabolic processes via IGF-1R[64], including cell proliferation and differentiation[65], and inhibits apoptosis by activating the phosphatidylinositol-3-phosphate (PI3K)/Akt and MAPK ERK1/2 pathways[66]. Up-regulation of the wnt/β-catenin or the insulin/IGF-1 pathway play important roles in malignancy[67,68] and their suppression is associated with anti-carcinogenic effects of αklotho[62,69,70].

1.3 Physiological effects of FGF23 and αklotho

Phosphate (P_i) is one of the most abundant minerals in the human body and more than 80 % is stored in the form of hydroxyapatite in bones or teeth[71]. In addition, phosphate is a component of nucleic acids and biological membranes, contributes to energy supply and storage in the form of adenosine triphosphate (ATP), or intracellular signaling by phosphorylation via kinase[71,72]. For most of these functions, constant intra- and extracellular phosphate concentrations are necessary[71]. Serum phosphate levels are regulated mainly by three specific hormones: parathyroid hormone (PTH), $1,25(OH)_2D_3$, and FGF23[73]. Intestinal absorption within the small intestine[74], renal excretion[75], and release from bone[76] are the most important regulatory mechanisms of serum phosphate concentration[73]. In the intestine, most phosphate is absorbed by sodium-dependent transporters of IIb type (NaP_iIIb)[77]. About eighty percent of the filtered phosphate is reabsorbed from the urine in the proximal and marginally in the distal tubule via sodium-dependent transporters NaP_iIIa, NaP_iIIc, and phosphate transporter PiT2[76,78–80].

FGF23 exerts its phosphaturic actions predominantly in renal proximal tubules, supported by FGFR and αklotho[32,81]. As shown in figure 1, ligand binding activates FGFR and induces phosphorylation of ERK1/2, and SGK1[32,81]. Downstream phosphorylation of sodium-hydrogen exchange regulatory factor 1 (NHERF1), which anchors NaP_iIIa in the tubular brush border membrane, leads to internalization and degradation of the phosphate transporter molecules[81–83]. The phosphorylation of NHERF1 and subsequent downregulation of NaP_iIIa was originally reported as an effect of PTH, pointing to a similar and possibly synergistic role of PTH and FGF23 in phosphate regulation[82,83]. Simultaneously, FGF23 down-regulates the production of NaP_iIIa and NaP_iIIc thereby decreasing renal phosphate reabsorption and reducing serum phosphate levels[33,81].

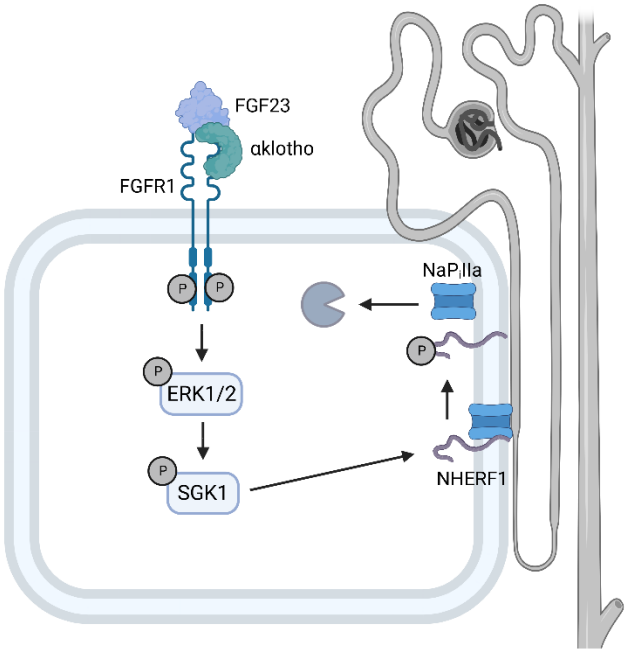

Figure 1: Effect of FGF23 on phosphate reabsorption in renal tubule cells[81–83]
Further details are provided in the text. FGF23 fibroblast growth factor 23; FGFR1 fibroblast growth factor receptor 1; ERK extracellular signal-related kinase; SGK serum/glucocorticoid regulated kinase; NHERF sodium-hydrogen exchanger regulatory factor; NaP_iIIa sodium-phosphate co-transporter 2a.

Conversely, high dietary phosphate intake stimulates FGF23 production[22,84]. Beside its effect on phosphate reabsorption, FGF23 decreases renal $1,25(OH)_2D_3$ production, thereby diminishing intestinal phosphate absorption[49,85,86]. The consequences, especially of chronic hyperphosphatemia are deranged bone growth, vascular as well as soft tissue calcification e.g. in heart and kidney, organ atrophy and an early death[3,49]. Chronic hypophosphatemia leads to rickets or osteomalacia, respectively[87].

In addition to phosphate, FGF23 regulates calcium reabsorption within the distal tubule in a αklotho-dependent way[88]. By activating ERK1/2 and SGK1, the abundance of TRPV5 (transient receptor potential cation channel subfamily V) calcium channels is increased, resulting in decreased urinary calcium excretion[88,89]. In line with this, FGF23/αklotho signaling increases expression of sodium-chloride co-transporter (NCC) and renal sodium reabsorption with subsequent plasma expansion, enhanced blood pressure, and cardiac hypertrophy[90]. This indicates a regulatory effect of FGF23 and αklotho not only on phosphate but also on calcium and sodium.

Vitamin D, a precursor of the steroid hormone $1,25(OH)_2D_3$ (calcitriol), is primarily synthesized in humans by UVB radiation-mediated conversion of 7-dehydrocholesterol in the skin[91,92]. Bound to circulating vitamin D binding protein (DBP)[93] vitamin D is transported to the liver and converted to 25-hydroxyvitamin D_3 (calcidiol) by the 25-hydroxylase CYP2R1[94]. $25(OH)D_3$ is then hydroxylated to

active $1,25(OH)_2D_3$ (calcitriol) by 1α-hydroxylase (CYP27B1) in renal tubule[95,96]. The major target of hormonally active $1,25(OH)_2D_3$ is the gastrointestinal tract, where it stimulates calcium and phosphate absorption[86,97]. $1,25(OH)_2D_3$ is inactivated by 24-hydroxylase (CYP24A1)[98]. 24-hydroxylase is stimulated[99] and 1α-hydroxylase is inhibited by $1,25(OH)_2D_3$ in a negative feedback loop[100] to prevent vitamin D toxicity associated with life-threatening hyperphosphatemia and hypercalcemia[101,102].

FGF23 decreases $1,25(OH)_2D_3$ on one hand by upregulating catabolic 24-hydroxylase and on the other hand by reducing 1α-hydroxylase[85]. The underlying intracellular signaling pathway is not completely known but is suggested to be mediated through FGFR3 and FGFR4 via ERK1/2 pathway[103,104]. Reduction of $1,25(OH)_2D_3$ consequently lowers intestinal phosphate absorption via NaP_iIIb[86], and intestinal absorption as well as renal reabsorption of calcium[97,105]. $1,25(OH)_2D_3$ stimulates FGF23 production to prevent hyperphosphatemia[106,107].

PTH maintains serum calcium and phosphate by osteoclast-mediated release from bone and similar to FGF23, by decreasing renal phosphate reabsorption[108,109]. PTH stimulates bone resorption by binding to PTH receptor on osteoblasts and osteocytes up-regulating the expression of receptor activator of nuclear factor κB ligand (RANKL)[110]. RANKL interacts with RANK receptor on osteoclast surface, triggering osteoclastogenesis and the release of bone resorption[111]. Additionally, PTH stimulates $1,25(OH)_2D_3$ synthesis[112], thereby increasing intestinal calcium and phosphate absorption[74]. PTH stimulates FGF23 production in osteoblasts preventing calcium and phosphate excess[113]. In turn, FGF23 inhibits PTH secretion in interaction with αklotho, which is also expressed and secreted in the parathyroid glands[114]. Similar to FGF23, PTH reduces the abundance of NaP_iIIa and NaP_iIIc in renal brush border membrane thereby reducing serum phosphate levels[82,108].

In summary, FGF23 is part of a feedback loop between bone and kidney including $1,25(OH)_2D_3$ and PTH for balancing phosphate levels (see Figure 2)[115]: $1,25(OH)_2D_3$ and PTH increase serum phosphate levels and stimulate FGF23 production in bone[107] whereas FGF23 suppresses $1,25(OH)_2D_3$ and PTH reducing phosphate levels[85,114], and $1,25(OH)_2D_3$ decreases PTH expression[116].

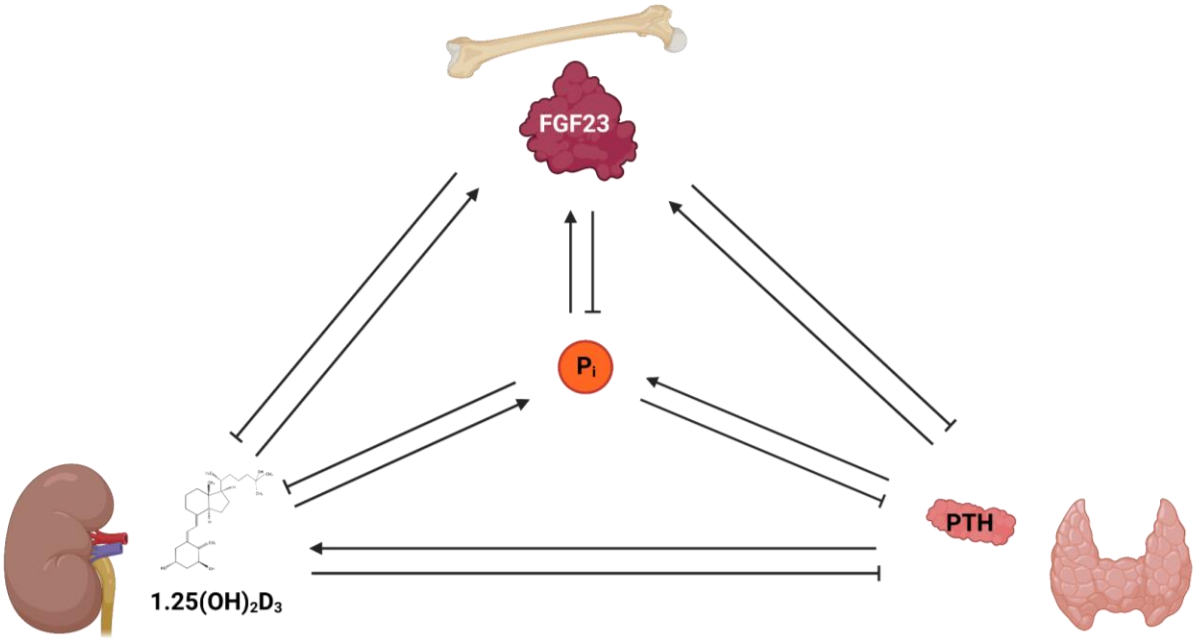

Figure 2: Phosphate regulation by FGF23, 1,25(OH)$_2$D$_3$, and PTH[115].
Further information is provided in the text. $1,25(OH)_2D_3$ calcitriol or active vitamin D_3; FGF23 fibroblast growth factor 23; P_i phosphate; PTH parathyroid hormone.

In other organs, FGF23 acts predominantly independently of αklotho and partially on a pathophysiologic basis. For instance, FGF23 induces left ventricular hypertrophy via FGFR4[117] and is associated with atrophy in the skeleton muscle[118]. Furthermore, FGF23 suppresses neutrophil activation and recruitment thereby impairing immune defense in CKD[119] and on the other hand, it stimulates the secretion of pro-inflammatory cytokine IL-6 in inflammatory airway diseases[120]. Additionally, there is a certain association of FGF23 and cancer[121]. FGF23 overexpression by tumor cells is reported from TIO[4] or oncogenic hypophosphatemic osteomalacia[122] but has also been observed in other malignancies including lung[123], breast[124], and colon cancer[125].

1.4 Regulation of FGF23 and αklotho

FGF23 synthesis in osteoblasts and osteocytes is transcriptionally and post-transcriptionally regulated by many different factors. $1,25(OH)_2D_3$ and PTH have already been described as regulators of FGF23 synthesis[107,126]. Beside these, also dietary calcium and phosphate intake increase FGF23 levels[22,84] possibly due to *Galnt3* up-regulation, preventing proteolytic cleavage of intact FGF23[127]. Furthermore, FGF23 production is stimulated by store-operated calcium ion entry (SOCE) into the cell via the calcium selective ion channel ORAI1 in the plasma membrane[128]. ORAI1 is activated during calcium deficiency in the endoplasmic reticulum (ER)[129]. Furthermore, FGF23 is negatively regulated by energy restriction via activation of adenosine monophosphate (AMP)-activated protein kinase (AMPK) pathway[130], whereas increased glucose or caloric intake stimulates FGF23 via mammalian target of rapamycin (mTOR)[131]. AMPK is a cytosolic protein, protecting cells from energy deficiency by inhibiting anabolic functions and mTOR[132,133]. In this process, AMP, a degradation product of ATP, functions as an energy sensor[133]. AMPK has been shown to suppress FGF23 production by decreasing the abundance of ORAI1 in the cell membrane and causes inhibition of SOCE[130]. On the other side, mTOR is a protein kinase involved in the PI3K pathway signaling serving as a biomarker for energy availability promoting anabolic processes[134]. FGF23 is positively regulated by a high glucose intake whereas the simultaneous inhibition of mTOR decreases FGF23[131].

Compared to FGF23, fewer regulating factors are known for αklotho. In general, αklotho expression and soluble klotho levels decrease under disease conditions including systemic inflammation[52], renal[9,45], and cardiovascular diseases[48,135]. Excess phosphate and wnt/β-catenin signaling are suggested to play a key role in disease-associated decline of αklotho[136]. For example, angiotensin II, a regulator of blood pressure via wnt/β-catenin signaling, is associated with cardiomyopathy[137] and suppresses renal αklotho synthesis[138]. Furthermore, αklotho is decreased by transforming growth factor β1 (TGF-β1), an inducer of renal fibrosis[57,139].

1.5 Current knowledge of the regulation of FGF23 and αklotho by cellular stress

There are numerous external stimuli like infections, toxins, extreme environmental conditions, or mechanical damage, as well as internal factors like inflammation or oxidative stress that challenge intracellular stress balance (see figure 3)[140,141]. The form, severity, and exposition time of stress stimuli, as well as the cell's adaptive capacity determines cell survival or death[142]. Subsequently, the mechanism of cell death e.g. apoptosis or necrosis, which is not always distinguishable, may influence the environment of the moribund cell[143–145].

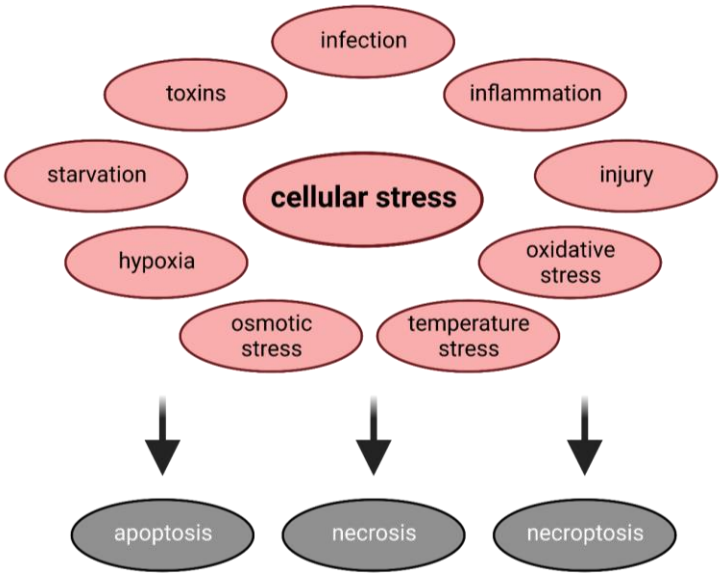

Figure 3: Internal and external inducers of cellular stress[140,141].
Further information is provided in the text.

Apoptosis degrades damaged or unwanted cells[146]. The so-called "programmed cell death", is a highly regulated process induced by oxidative stress[147], cytotoxic compounds[148], or radiation[149], and strongly depends on caspases mediating subsequent degradation of cellular components[150]. Apoptosis is characterized by the absence of inflammation[151] and the activity of caspase proteins[150]. Caspase-3 inactivates transcriptional activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB)[152], which is strongly involved in the regulation of cytokine production[153]. Additionally, recruited phagocytic cells suppress the secretion of inflammatory cytokines[154].

One important factor of apoptosis induction is tumor suppressor protein p53, which occurs at low levels in the cytosol and is rapidly degraded by the proteasome under unstimulated conditions[155,156]. In response to stress stimuli, p53 is strongly increased and phosphorylated[157] and regulates the transcription of various genes e.g. of pro-apoptotic protein B cell lymphoma 2 (BCL2)-associated X (BAX)[158]. The induction of apoptosis strongly depends on the interaction of BCL-2 protein family members, including pro-apoptotic

proteins BCL2-associated death promoter (BAD), BCL2 homologous antagonist/killer (BAK), and BAX, and anti-apoptotic BCL-2 and B cell lymphoma-extra large (BCL-XL) proteins[159,160]. Thereby, the ratio of pro- to anti-apoptotic proteins determines the outcome of an apoptotic stimulus[161]. Upon activation, BAK and BAX form homo-oligomers accumulating in outer mitochondrial membrane to form pores[162,163]. This results in a loss of mitochondrial membrane potential and cytochrome c release[162,164,165]. Cytochrome c binds apoptotic protease-activating factor 1 (APAF-1) and caspase-9 subsequently activating caspase-3[166]. The activation of caspases triggers typical signs of apoptosis such as DNA fragmentation[167], cell shrinkage[168], and the formation of membrane vesicles[169]. Pro-apoptotic proteins BAX and BAK are inhibited when complexed with anti-apoptotic proteins BCL-2 or BCL-XL[170]. Phosphorylation of BCL-2 prevents binding of BAX and triggers apoptosis[171]. BAD dimerizes with anti-apoptotic proteins BCL-XL or BCL-2, thereby preventing BAX inhibition and promoting apoptosis[170,172]. The apoptotic pathway includes but is not limited to the pro- and anti-apoptotic proteins described here and depicted in figure 4.

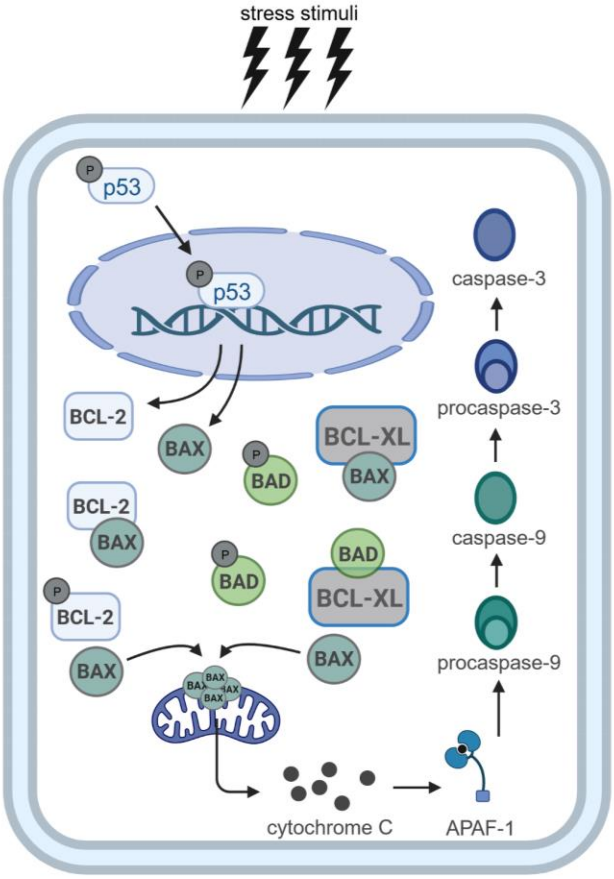

Figure 4: Components of apoptotic pathway following cellular stress[162–166,170–172].
Further details are described in the text. APAF-1 apoptotic protease-activating factor 1; BAD BCL2-associated death promoter; BAX BCL2-associated X; BCL-2 B cell lymphoma; P phosphorylation.

In contrast to apoptosis, necrotic cell death is associated with an inflammatory stress response[173]. Formally, necrosis is defined as a form of cell death with no signs of apoptosis or autophagy[174], proceeding

independently of caspases[144]. Morphologically, necrosis is characterized by a swelling of the cell[175] and membrane permeabilization followed by the bursting of the plasma membrane[176]. Major inducers of necrotic cell death are ROS[177], TNFα[178], or mechanical damage[179]. A special form of necrosis is necroptosis, referred to as programmed necrosis[180]. Like apoptosis, this is a highly regulated cell death mechanism associated with inflammatory processes[180]. The activation of caspases requires ATP whereas ATP-depleted cells undergo necrosis[166,181]. Thus, progressing cell damage results in necrotic cell death[182]. As one particular stimulus can induce both apoptosis or necrosis in the same cell but under different conditions, the differentiation between various forms of cell death is very challenging[182,183].

Inflammation is characterized by the recruitment of immune cells to the injured tissue and the release of large amounts of pro-inflammatory cytokines, e.g. tumor necrosis factor α (TNFα), interleukin 6 (IL-6), or IL-8 from immune and inflamed cells[184–186]. Many inflammatory cytokines are transcriptionally regulated by transcription factor NFκB[187,188] contributing to the regulation of inflammation and immunity in virtually all cell types[189]. Inflammation affects mineral and bone metabolism and especially chronic inflammation results in bone resorption and osteoporosis[190–192]. Acute inflammation, initiated by bacterial infection or cytokine stimulation strongly induces C-terminal but not intact FGF23 production via NFκB[193]. This may be due to increased hypoxia inducible factor 1α (HIF1α) expression in acute inflammation[194,195] which increases furin production[196] reinforcing FGF23 cleavage[25]. Furthermore, acute inflammation promotes iron deficiency and hypoxic conditions, which are both reported to increase FGF23 levels, in case of hypoxia via HIF1α[195]. In chronic inflammation, both intact and C-terminal FGF23 levels are increased[195] decreasing bone density[192]. In addition to inflammatory cytokines, NFκB also stimulates FGF23 secretion by up-regulating Orai1 and SOCE[128,197]. NFκB is activated by stress stimuli via p38 MAPK[198,199], and cytokines including TGF-β[200], TNFα[201], or IL-1β[202] and have been shown to positively regulate p38, which stimulates FGF23 production in bone cells[203].

Renal diseases such as AKI and CKD are strongly linked to inflammation induced e.g. by nephrotoxic drugs or cellular injury, and patients show excess FGF23 levels[187,204,205]. Progression of renal injury results in chronic inflammation, fibrosis, and goes along with a loss of renal function[206,207]. AKI and CKD are associated with a decrease in αklotho production[44,208], which contributes to an unfavorable outcome[209,210]. αKlotho is decreased by inflammatory cytokines e.g. TNFα[211] and its serum concentration is low in patients suffering from inflammatory diseases such as chronic obstructive pulmonary disease[212] or CVD[213] and may serve as a biomarker for systemic inflammation[214].

Cellular stress is often promoted by reactive oxygen species (ROS), generating oxidative stress[215]. ROS, $O_2^{\bullet-}$, HO•, and H_2O_2, are generated by the reduction of oxygen in the organism[215]. A tight balance between ROS formation and antioxidant scavenger molecules such as glutathione and vitamin C, or antioxidant enzymes determines the oxidative stress response[216]. Oxidative stress stimulates FGF23 synthesis by

activating MAPK ERK1/2 and NFκB signaling[217]. Comparable to inflammation, αklotho decreases under the influence of oxidative stress[218].

2 Objective of the present work

Cellular stress in the form of inflammation, oxidative stress and eventually apoptosis or necrosis play important roles in various diseases, e.g. in inflammatory bowel disease, diabetes, or Alzheimer's disease[219–221], or as a consequence of cancer therapy[222,223]. FGF23 and αklotho levels have been reported to determine the outcome of severe diseases[224-226]. Thus, it is of particular significance to investigate the correlation between FGF23, αklotho, and severe disorders and elucidate the therapeutic or diagnostic relevance of FGF23/αklotho signaling.

To investigate the regulation of FGF23 and αklotho by cellular stress, we chose different stress stimuli and compound classes that exert different cytotoxic mechanisms. Cisplatin is a cytotoxic drug used to treat many solid tumors e.g. in reproductive organs, breast, lung, and esophagus[227–229]. Its full spectrum of action has not been resolved yet, but it is known that cisplatin forms DNA inter- and intrastrand crosslinks as well as DNA-protein adducts[230,231]. By contrast, doxorubicin is an anthracycline drug used in a wide range of solid and hematological cancer types with antineoplastic actions and especially cardiotoxic side effects[232,233]. It intercalates into genomic and mitochondrial DNA, thereby disturbing topoisomerase II-mediated DNA-processing resulting in double-strand breaks and apoptosis[233–235]. Paclitaxel is a natural compound occurring in the bark of yew trees[236] and is used for the treatment of several malignancies including breast[237], ovarian[238], or lung cancer[239]. Paclitaxel affects the dissociation of microtubules during mitosis, resulting in mitotic arrest of cancer cells[240,241]. Furthermore, we applied direct apoptotic inducers procaspase-activating compound 1 (PAC-1) and serum starvation to investigate the effect on FGF23 or αklotho. PAC-1 is a caspase-3 activator that complexes zinc ions inhibiting the enzymatic activity of procaspase-3 and active caspase-3, to induce apoptosis[242]. As procaspase-3 is up-regulated in many types of cancer[243–246], PAC-1 alone or in combination with chemotherapeutic drugs is a promising approach to induce apoptosis in cancer cells[247–249]. Serum starvation is known to induce apoptosis in a wide range of cells probably by the withdrawal of growth factors[250–253].

In paper 1, we determined the transcriptional regulation of *FGF23* in UMR106 osteoblast-like osteosarcoma cells after 24- and 48-h-incubation with cisplatin, doxorubicin, PAC-1, or serum-depleted media. Simultaneously, we measured the impact of the aforementioned stress stimuli on cell number and viability. To investigate whether inflammatory stress is induced by chemotherapeutic agents, we assessed the expression level of *IL6* and its contribution to the regulation of FGF23 by using IL-6 signaling inhibitor SC144. As explained in chapter 1.5, cellular stress and inflammation are frequently associated with the

activation of NFκB, a known regulator of FGF23. Thus, we determined if NFκB is activated by cellular stress and whether it is involved in the regulation of FGF23 by using NFκB inhibitors wogonin and withaferin A. In conclusion, this paper investigated the regulation of FGF23 by cellular stress and the involvement of inflammatory signaling.

In paper 2, we assessed the transcriptional regulation of αklotho after incubation of canine distal tubular cell line MDCK and rat proximal tubular cell line NRK-52E with cisplatin, paclitaxel, doxorubicin, PAC-1, or serum deprivation. In parallel, we assessed cell number and viability, as well as the induction of apoptosis or necrosis using a combined apoptosis/necrosis assay. Apoptosis was additionally assayed by investigating transcriptional regulation of apoptotic proteins BAD, BAX, and BCL-2. With regard to the intracellular signaling involved in αklotho regulation, we considered peroxisome proliferator γ (PPARγ), a known regulator of αklotho, to be involved in the αklotho regulation in MDCK cells. Furthermore, FGFR1 mRNA and protein levels were investigated to see whether αklotho regulation is accompanied by FGFR1 stimulation. For the ELISA detection of αklotho protein we used human proximal tubular cell line HK-2. At last, we compared αklotho levels in human serum of cancer patients before and after chemotherapy administration. In conclusion, paper 2 assessed the influence of cellular stress on renal αklotho expression particularly with regard to apoptosis.

In summary, the aims of the present thesis are (**i**) elucidating a regulatory mechanism of cellular stress on FGF23 or αklotho, (**ii**) investigating, whether FGF23 or αklotho are influenced by certain forms of cellular stress or by particular signaling components of the cellular stress response, and (**iii**) investigating the regulation of FGF23 and αklotho as a consequence of a certain cell death mechanisms.

3 Experimental

3.1 Paper 1: Up-Regulation of Fibroblast Growth Factor 23 Gene Expression in UMR106 Osteoblast-like Cells with Reduced Viability

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Article

Up-Regulation of Fibroblast Growth Factor 23 Gene Expression in UMR106 Osteoblast-like Cells with Reduced Viability

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Abstract: Fibroblast growth factor 23 (FGF23) controls vitamin D and phosphate homeostasis in the kidney and has additional paracrine effects elsewhere. As a biomarker, its plasma concentration is associated with progression of inflammatory, renal, and cardiovascular diseases. Major stimuli of FGF23 synthesis include active vitamin D and inflammation. Antineoplastic chemotherapy treats cancer by inducing cellular damage ultimately favoring cell death (apoptosis and necrosis) and causing inflammation. Our study explored whether chemotherapeutics and other apoptosis inducers impact on *Fgf23* expression. Experiments were performed in osteoblast-like UMR106 cells, *Fgf23* gene expression and protein synthesis were determined by qRT-PCR and ELISA, respectively. Viability was assessed by MTT assay and NFκB activity by Western Blotting. Antineoplastic drugs cisplatin and doxorubicin as well as apoptosis inducers procaspase-activating compound 1 (PAC-1), a caspase 3 activator, and serum depletion up-regulated *Fgf23* transcripts while reducing cell proliferation and viability. The effect of cisplatin on *Fgf23* transcription was paralleled by *Il-6* up-regulation and NFκB activation and attenuated by Il-6 and NFκB signaling inhibitors. To conclude, cell viability-decreasing chemotherapeutics as well as apoptosis stimulants PAC-1 and serum depletion up-regulate *Fgf23* gene expression. At least in part, Il-6 and NFκB may contribute to this effect.

Keywords: cisplatin; apoptosis; $1,25(OH)_2D_3$; klotho; inflammation

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1. Introduction

Cells that make up bone, osteoblasts, and osteocytes produce fibroblast growth factor 23 (FGF23), a protein with classical endocrine, but also paracrine effects [1,2]. As a hormone, it targets renal sodium phosphate co-transporter NaP_iIIa, the main phosphate transporter of the proximal tubule, thereby enhancing urinary elimination of phosphate [3]. Moreover, FGF23 down-regulates *CYP27B1*, the renal key enzyme for the activation of vitamin D [4]. Therefore, FGF23 lowers the plasma concentration of active vitamin D ($1,25(OH)_2D_3$), which itself is a major regulator of phosphate metabolism [5]. Further endocrine effects of FGF23 are effective in the parathyroid gland, where FGF23 reduces parathyroid hormone (PTH) expression and secretion [6]. These classical endocrine effects require a complex of a FGF receptor (FGFR) and co-receptor αKlotho, a transmembrane protein with high expression in the kidney and parathyroid gland [7–9]. A certain motif with FGF23-independent endocrine and paracrine effects can be released from αKlotho upon cleavage, called soluble Klotho (sKl) [7,10]. The correct interplay of FGF23 and αKlotho in the regulation of phosphate and vitamin D metabolism is critical: mice deficient for FGF23 or αKlotho age rapidly and exhibit premature aging-associated diseases with death at young age, whereas overexpression of αKlotho extends life span by about 30% [11–13].

Elevations of the plasma FGF23 concentration were found in many clinical conditions including renal [14,15], cardiovascular [16–19], and inflammatory diseases [20]. Particularly in chronic kidney disease (CKD), changes in FGF23 level can be detected very early and correlate with outcome [21].

For this reason, regulation of FGF23 production and secretion is of high interest. Regulators of FGF23 thus far disclosed include dietary phosphate [22], PTH [23], 1,25$(OH)_2D_3$ [24], insulin [25], erythropoietin [26], or inflammation [27]. Pro-inflammatory cytokines such as interleukin-6 (Il-6) [28], tumor necrosis factor alpha (TNFα) [29] or transcription factor complex NFκB (nuclear factor kappa-light-chain-enhancer of activated B-cells) are major drivers of *FGF23* expression [30].

For most malignancies, chemotherapy is part of therapy either at certain stages, or along with other approaches (e.g., surgery, radiation) [31]. Common chemotherapeutics are cytotoxic drugs damaging cells and inducing apoptosis [32]. Among them are anthracyclines (e.g., doxorubicin) that intercalate with DNA [33] or platinum derivatives (e.g., cisplatin) inhibiting DNA replication by DNA cross-linking [34]. Initiation of apoptosis ultimately results in the activation of executioner caspase 3, which can directly be activated by procaspase-activating compound 1 (PAC-1) [35]. Lack of growth factors also induces apoptosis, which can be accomplished by serum depletion in cell culture [36].

Chemotherapeutics induce strong inflammation [37]. Moreover, chemotherapy with platinum derivatives is nephrotoxic [38] whereas anthracyclines are cardiotoxic [39]. In view of the strong *FGF23* expression-inducing properties of pro-inflammatory pathways [27] and kidney or cardiovascular damage elevating FGF23 plasma levels, we hypothesized that chemotherapeutic drugs may up-regulate *FGF23* expression. This may result in higher FGF23 plasma levels in patients undergoing chemotherapy and may have clinical relevance. Therefore, this study aimed to explore the impact of antineoplastic drugs and apoptosis stimulants on FGF23 in vitro. Moreover, we aimed to elucidate underlying mechanisms.

2. Materials and Methods

2.1. Cell Culture

Rat osteoblast-like UMR106 cells (CRL-1661; ATCC, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 25 mM glucose and 1 mM pyruvate (Gibco, Life Technologies, Thermo Scientific, Darmstadt, Germany), supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, Life Technologies) at 5% CO_2 and 37 °C. Serum depletion was accomplished for 24 h or 48 h by incubating the cells in culture medium with 1% or 0% FBS and additional 10 nM 1,25$(OH)_2D_3$ (Tocris, Bioscience, Bristol, UK) to enhance *Fgf23* expression [40]. Cells were seeded into 6-well plates (Greiner Bio-One, Frickenhausen, Germany) for 24 h. Subsequently, cisplatin, PAC-1 or doxorubicin (all from Tocris Bioscience) were added at the indicated concentrations for 24 or 48 h or the FBS concentration was reduced as described above. Il-6 signaling was blocked through gp130 inhibitor SC144 (1 µM, Tocris Bioscience). NFκB inhibitors withaferin A (Tocris Bioscience) and wogonin (Merck, Darmstadt, Germany) were used at concentration of 500 nM and 100 µM, respectively, where indicated.

To study cell proliferation, cells were trypsinized after 24 h or 48 h, respectively, and counted on a Neubauer hemocytometer.

2.2. Quantitative Real Time PCR

Total RNA was isolated from UMR106 cells using RNA-Solv reagent (Omega Bio-Tek, Norcross, GA, USA), and 1.2 µg thereof was used for cDNA synthesis with the GoScript Reverse Transcription System and random primers (Promega, Mannheim, Germany) on a Biometra TAdvanced thermal cycler (Analytik Jena, Jena, Germany).

Two µL cDNA was subjected to quantitative real-time PCR (qRT-PCR) with the CFX Connect Real-Time PCR Detection System (Bio-Rad, Feldkirchen, Germany). The reaction mix contained 0.25 µM (*Fgf23*) or 0.5 µM (*TATA-binding protein (Tbp), Il6, Rela*) of each primer, 10 µL GoTaq qPCR Master Mix (Promega), and sterile water to 18 µL per sample.

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

Fgf23: TAGAGCCTATTCAGACACTTC and CATCAGGGCACTGTAGATAG;

Tbp: ACTCCTGCCACACCAGCC and GGTCAAGTTTACAGCCAAGATTCA;

Il6: CAGAGTCATTCAGAGCAATAC and CTTTCAAGATGAGTTGGATGG;
Rela: GCACCCCACCATCAAGATCAA and CTTGCTCCAGGTCTCGCTTC.

Fgf23, *Il6* and *Rela* transcript levels were normalized to transcript levels of housekeeping gene *Tbp* [41–43] and evaluated with the $2^{-\Delta Ct}$ method.

2.3. Viability Assay (MTT Assay)

Cells were seeded into 96-well plates and treated for 24 or 48 h with cytostatic agents cisplatin or doxorubicin or apoptosis inducers PAC-1 or serum deprivation. Subsequently, cells were incubated with 0.5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, Schnelldorf, Germany) for 1 h. Next, MTT solution was removed, cells lyzed in dimethyl sulfoxide (DMSO; AppliChem, Darmstadt, Germany), and absorption measured at 550 nm and 690 nm (reference) on a FluoStar Omega plate reader (BMG Labtech, Ortenberg, Germany). Results are given as percentage of viable cells compared to control cells.

2.4. Enzyme Linked Immunosorbent Assay (ELISA)

Cell culture supernatants were collected and concentrated using Vivaspin® 2 ultrafiltration columns (Sartorius, Göttingen, Germany). C-terminal FGF23 protein concentration was then determined by ELISA according to the manufacturer's protocol (Immutopics, San Clemente, CA, USA).

2.5. Western Blot

UMR106 cells were seeded into T25 cell culture flasks (Greiner Bio-One) and cultured for 24 h under standard conditions, then treated with 10 µM cisplatin or vehicle for another 24 h. Next, cells were lyzed using RIPA buffer (Cell Signaling Technology, Frankfurt, Germany) supplemented with protease and phosphatase inhibitor cocktail and EDTA (Halt, Thermo Scientific), total protein concentration measured by Bradford assay (Bio-Rad), and 30 µg of total protein subjected to 10% SDS-PAGE and standard Western Blotting. The following antibodies were used: anti-phospho-p65-NFκB (Ser536; 93H1), anti-GAPDH (D16H11), and anti-rabbit IgG HRP-linked antibody (all from Cell Signaling Technology). For visualization, membranes were incubated for 2 min with Westar Nova 2.0 (GAPDH) or Westar Supernova (phospho-p65-NFκB) ECL substrate (both from Cyanagen, Bologna, Italy). The densitometrical analysis was performed on a C-Digit® Blot scanner (Li-Cor, Lincoln, NE, USA) and phospho-p65-NFκB bands were normalized to GAPDH bands using the Image Studio™ software (Li-Cor).

2.6. Statistics

Data are shown as arithmetic means ± standard error of the mean (SEM) with n representing the number of independent experiments. Normal distribution was tested using Shapiro–Wilk normality test. Effects on cell number and viability and western blots were analyzed with one-sample t-test or one-sample Wilcoxon signed rank test, respectively. Two groups were analyzed with student's t-test, Welch's test, or Mann–Whitney U test. More than two groups were analyzed with one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, Dunnett T3 test, or with non-parametric Kruskal–Wallis test followed by Dunn–Bonferroni post hoc test. Differences were considered significant if $p < 0.05$. Statistics were made using IBM SPSS Statistics (Version 27.0; Armonk, NY, USA).

3. Results

To investigate whether chemotherapeutics impact on *Fgf23* expression, we performed experiments in UMR106 osteoblast-like cells. In a first series of experiments, the cells were treated with platinum derivative cisplatin, an antineoplastic drug used in the treatment of a variety of malignancies, and *Fgf23* transcript levels were determined by qRT-PCR. As demonstrated in Figure 1A, cisplatin enhanced *Fgf23* gene expression in UMR106 cells in

a dose-dependent manner within 24 h. By the same token, exposure to cisplatin reduced number (Figure 1B) and viability (Figure 1C) of UMR106 cells following a 24-h exposure.

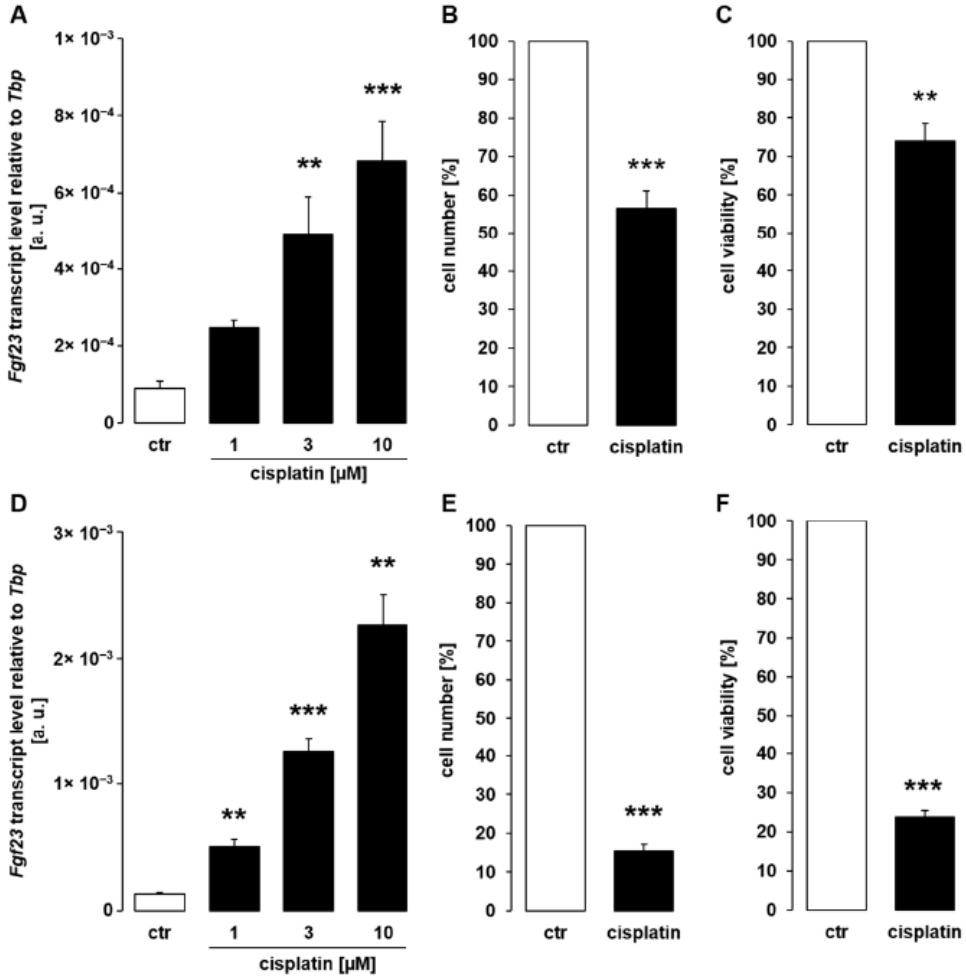

Figure 1. Cisplatin induced fibroblast growth factor 23 (*Fgf23*) expression in UMR106 cells. (**A**,**D**): Arithmetic means ± SEM of *Fgf23* transcript abundance relative to *TATA-binding protein* (*Tbp*) in UMR106 cells treated with vehicle control (ctr) or cisplatin at the indicated concentrations for 24 h ((**A**), $n = 6$; ANOVA followed by Dunnett's multiple comparison test) or 48 h ((**D**), $n = 6$; one-way ANOVA followed by Dunnett T3 multiple comparison test). (**B**–**F**): Arithmetic means ± SEM of the number ((**B**); $n = 7$; one-sample *t*-test; (**E**), $n = 6$; one-sample *t*-test) or viability ((**C**); $n = 6$; one-sample *t*-test; (**F**); $n = 5$; one-sample *t*-test) of UMR106 cells treated without or with 10 µM cisplatin for 24 h (**B**,**C**) or 48 h (**E**,**F**). All values are relative to the respective values of vehicle-treated cells. ** $p < 0.01$, *** $p < 0.001$ indicate significant difference from control cells. a. u., arbitrary units; ctr, control.

To check whether upregulation of *Fgf23* gene expression is a stress reaction only observable at 24 h, we extended exposure time in a further series of experiments. According to Figure 1D, also a 48-h exposure of UMR106 cells resulted in dose-dependent upregulation of *Fgf23* gene expression. Cell number (Figure 1E) and viability (Figure 1F), however, were more strongly reduced upon a 48-h exposure to cisplatin compared to a 24-h incubation (Figure 1B,C).

The next series of experiments was carried out to investigate whether anthracyclines, chemotherapeutic drugs that inhibit topoisomerase and intercalate with DNA [33], are similarly capable of inducing *Fgf23* gene expression. UMR106 cells exposed to doxorubicin (0.03–0.3 µM) for 24 h exhibited enhanced *Fgf23* gene expression in a dose-dependent manner (Figure 2A). Similar to cisplatin, doxorubicin also compromised cell proliferation (Figure 2B) and viability (Figure 2C). Again, we tested whether a longer exposure similarly up-regulated *Fgf23*. As a result, incubation of UMR106 cells with doxorubicin for 48 h killed virtually all cells (Figure 2D). Hence, *Fgf23* transcripts were not detectable after 48 h.

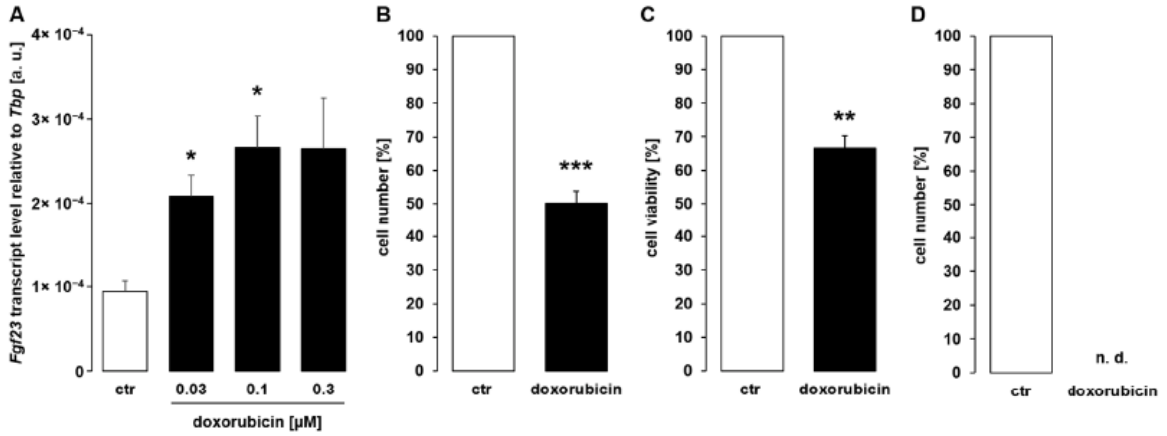

Figure 2. Doxorubicin enhanced *Fgf23* expression in UMR106 cells. (**A**): Arithmetic means ± SEM of *Fgf23* transcript abundance relative to *Tbp* in UMR106 cells treated for 24 h with vehicle control (ctr) or doxorubicin at the indicated concentrations ($n = 6$; one-way ANOVA followed by Dunnett T3 multiple comparison test). (**B–D**): Arithmetic means ± SEM of the number ((**B**); $n = 5$; one-sample *t*-test; (**D**); $n = 4$) or viability ((**C**); $n = 5$; one-sample *t*-test) of UMR106 cells treated without or with 0.1 µM doxorubicin for 24 h (**B**,**C**) or 48 h (**D**). All values are relative to the respective values of vehicle-treated cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate significant difference from vehicle-treated cells. a.u., arbitrary units; ctr, control; n.d., not detectable.

Our results indicate that cytotoxic reagents up-regulate *Fgf23* gene expression in UMR106 cells. In order to test whether this effect is mimicked by direct stimulation of apoptosis, PAC-1, an activator of apoptosis-initiating executioner caspase 3, was applied. As demonstrated in Figure 3A, similar to chemotherapeutics, PAC-1 dose-dependently up-regulated *Fgf23* gene expression in UMR106 cells within 24 h. This effect was paralleled by compromised cell proliferation (Figure 3B) and viability (Figure 3C), as well. A 48-h exposure to PAC-1 did not significantly modify *Fgf23* transcripts in UMR106 cells (Figure 3D) while suppressing cell proliferation (Figure 3E) and viability (Figure 3F).

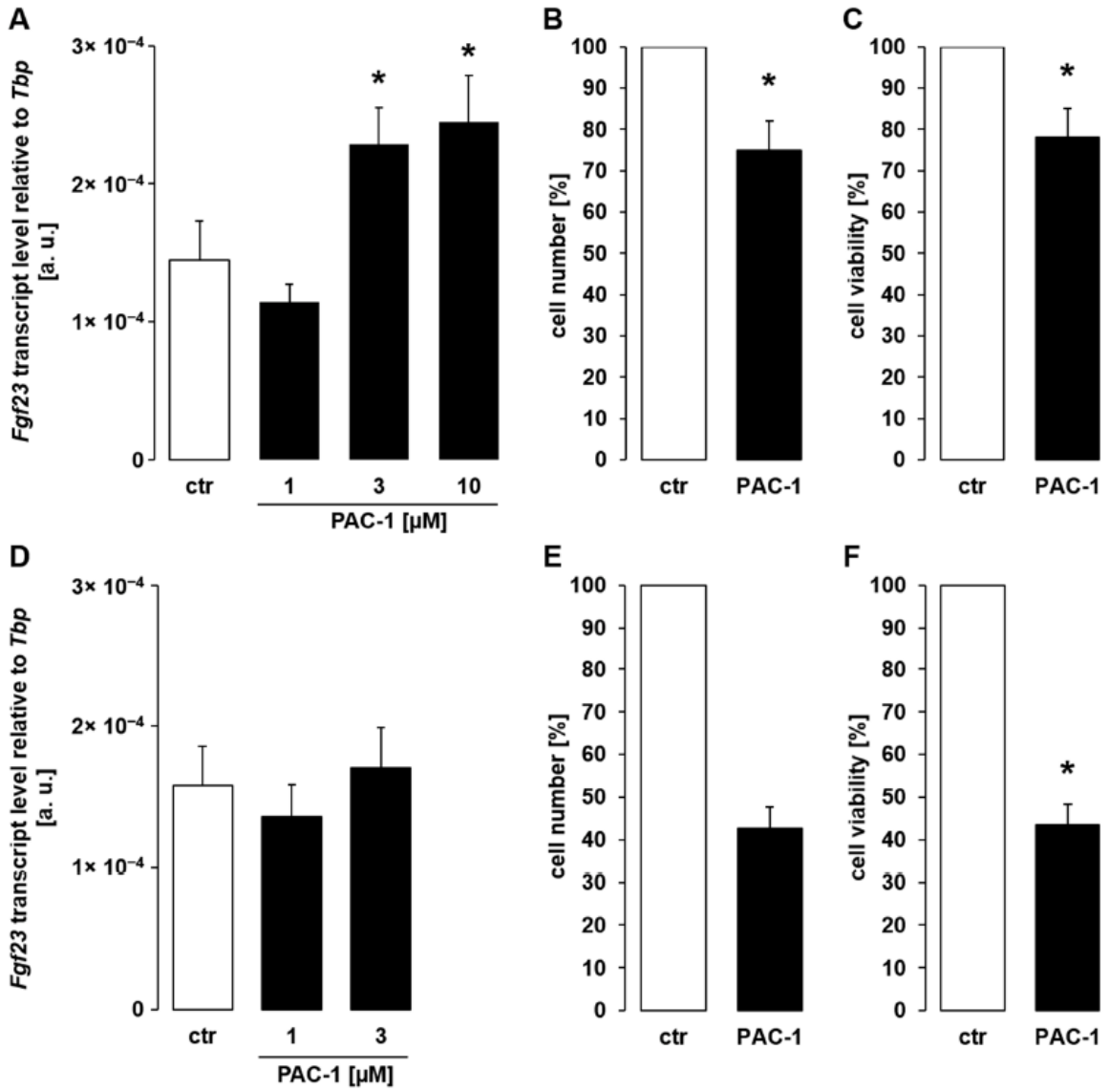

Figure 3. Procaspase-activating compound 1 (PAC-1) stimulated *Fgf23* expression in UMR106 cells. (**A**,**D**): Arithmetic means ± SEM of *Fgf23* transcript abundance relative to *Tbp* in UMR106 cells treated for 24 h ((**A**); $n = 10$; Kruskal–Wallis test followed by Dunn–Bonferroni test) or 48 h ((**D**); $n = 10$; one-way ANOVA) with vehicle control (ctr) or PAC-1 at the indicated concentrations. (**B**–**F**): Arithmetic means ± SEM of the number ((**B**); $n = 5$; one-sample *t*-test; (**E**); $n = 4$; one-sample Wilcoxon signed rank test) or viability ((**C**); $n = 5$; one-sample Wilcoxon signed rank test; (**F**); $n = 5$; one-sample Wilcoxon signed rank test) of UMR106 cells treated with vehicle control (ctr) or 3 µM PAC-1 for 24 h (**B**,**C**) or 48 h (**E**,**F**). All values are relative to the respective values of control-treated cells. * $p < 0.05$ indicates significant difference from vehicle-treated cells. a. u., arbitrary units; ctr, control.

Since direct apoptosis inducer PAC-1 enhanced *Fgf23* gene expression in UMR106 cells, we performed a further series of experiments to study whether another stimulant of apoptosis, depletion of cell growth factors, also affects *Fgf23* transcription. To this end, we incubated UMR106 cells for 24 h under normal conditions (10% FBS), under conditions of

reduced FBS (1%), and without FBS in the presence of 10 nM $1,25(OH)_2D_3$. Serum depletion resulted in a strong up-regulation of *Fgf23* gene expression (Figure 4A). Again, the effect was paralleled by decreased proliferation (Figure 4B) and viability (Figure 4C) of UMR106 cells. The stimulatory effect of serum depletion on *Fgf23* transcripts was followed by enhanced secretion of C-terminal FGF23 protein into the cell culture supernatant (Figure 4D). Also, 48 h serum depletion up-regulated *Fgf23* gene expression (Figure 4E), an effect again paralleled by reduced proliferation (Figure 4F) and viability (Figure 4G).

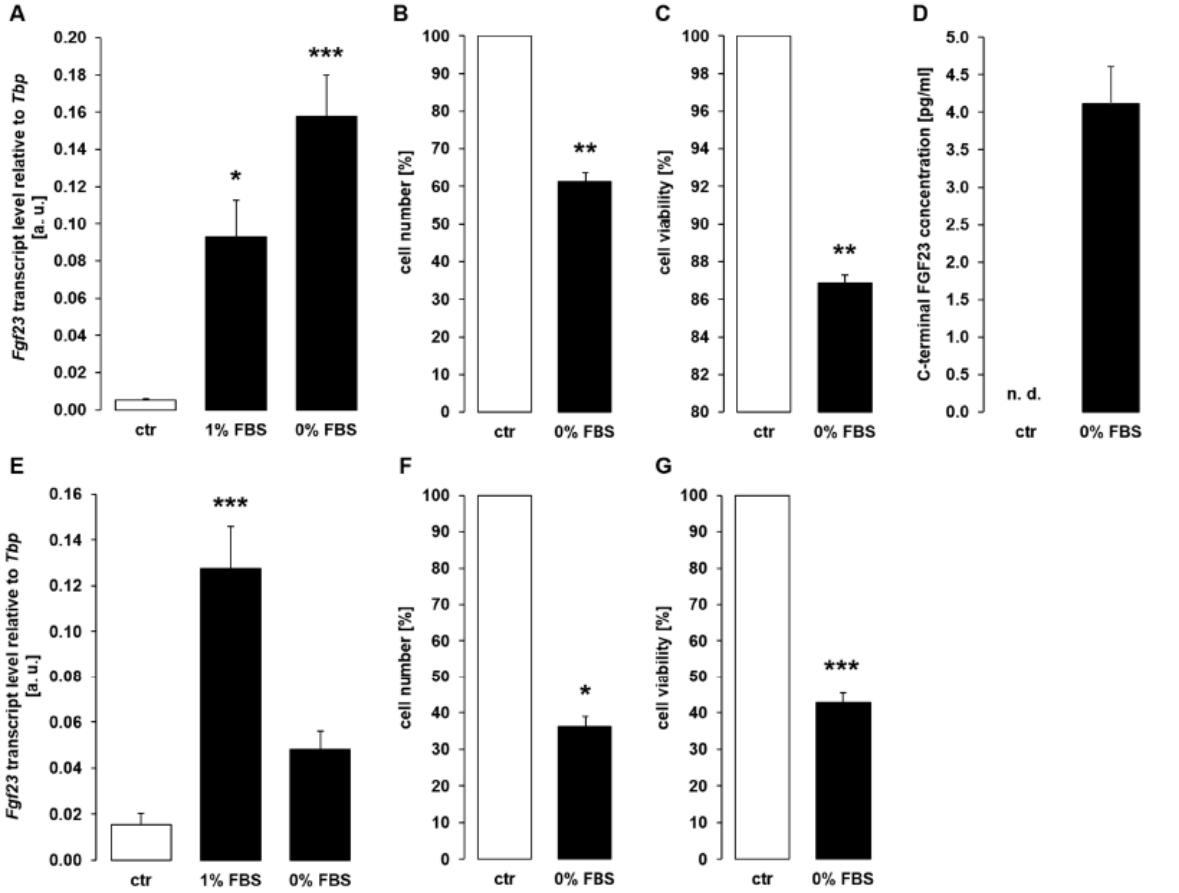

Figure 4. Serum depletion induced *Fgf23* expression and secretion in osteoblast-like UMR106 cells. (**A**): Arithmetic means ± SEM of *Fgf23* transcript level relative to *Tbp* in UMR106 cells incubated for 24 h in medium containing 10% (ctr), 1%, or 0% fetal bovine serum (FBS) ($n = 6$; Kruskal–Wallis test followed by Dunn–Bonferroni post hoc test). (**B**,**C**): Arithmetic means ± SEM of the number ((**B**); $n = 4$; one-sample *t*-test) or viability ((**C**); $n = 4$; one-sample *t*-test) of UMR106 cells incubated for 24 h without FBS relative to the respective value of cells incubated in 10% FBS. (**D**): Arithmetic means ± SEM of C-terminal FGF23 protein concentration in the supernatant of UMR106 cells incubated with 10% FBS (ctr) or without FBS for 24 h ($n = 7$). (**E**): Arithmetic means ± SEM of *Fgf23* mRNA levels relative to *Tbp* levels of UMR106 cells treated for 48 h with medium containing 10% (ctr), 1%, or 0% FBS ($n = 7$; Kruskal–Wallis followed by Dunn–Bonferroni test). (**F**,**G**): Arithmetic means ± SEM of cell number ((**F**), $n = 6$; one-sample Wilcoxon signed rank test) or cell viability ((**G**), $n = 5$; one-sample *t*-test) of UMR106 cells incubated in culture medium with 10% FBS (ctr) or without FBS for 48 h. In all experiments, cell culture medium contained 10 nM $1,25(OH)_2D_3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate significant difference from control cells. a. u., arbitrary units; ctr, control; n. d., not detectable.

Pro-inflammatory cytokines including Il-6 are major stimuli of *Fgf23* expression, and chemotherapy has been shown to enhance inflammation [44]. A further series of experiments, therefore, aimed to explore the role of Il-6 for antineoplastic drug-dependent up-regulation of *Fgf23*. As illustrated in Figure 5, a 24-h exposure of UMR106 cells to 10 µM cisplatin (Figure 5A) or 0.3 µM doxorubicin (Figure 5B) readily stimulated *Il6* gene expression. Importantly, SC144, an Il-6 signaling inhibitor blocking gp130, significantly attenuated cisplatin-induced *Fgf23* transcription (Figure 5C)

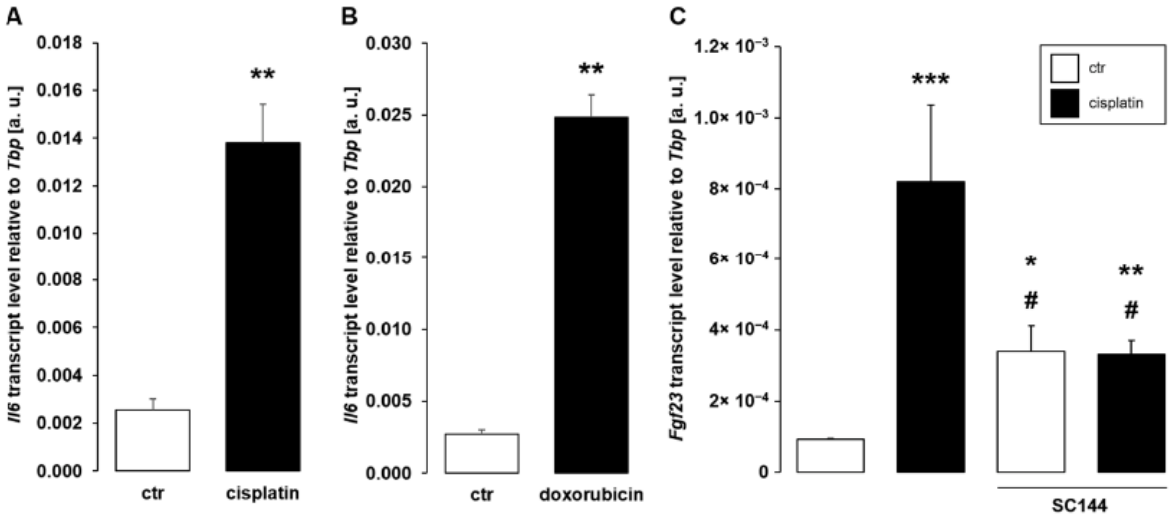

Figure 5. Interleukin-6 (IL-6) signaling inhibitor SC144 attenuated cisplatin-induced *Fgf23* gene expression in UMR106 cells. (**A**,**B**): Arithmetic means ± SEM of interleukin-6 (*Il6*) mRNA levels relative to *Tbp* in UMR106 cells treated without (ctr) or with 10 µM cisplatin ((**A**), $n = 6$; Welch's test) or 0.3 µM doxorubicin ((**B**), $n = 6$; Mann–Whitney U test) for 24 h. (**C**): Arithmetic means ± SEM of *Fgf23* transcript levels relative to *Tbp* in UMR106 cells treated without (ctr) or with 10 µM cisplatin in the presence or absence of 1 µM Il-6 signaling inhibitor SC144 ($n = 9$; Kruskal–Wallis followed by Dunn–Bonferroni test) for 24 h. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate significant differences from vehicle-treated cells (1st bar); # $p < 0.05$ indicates significant difference from absence of SC144 (2nd bar vs. 4th bar). a. u., arbitrary units; ctr, control.

Downstream signaling of pro-inflammatory stimuli may eventually result in the activation of transcription factor complex NFκB, an important driver of FGF23 production [30]. Further experiments, therefore, focused on the involvement of NFκB in the stimulation of *Fgf23* by cisplatin. Within 24 h, treatment of UMR106 cells with 10 µM cisplatin resulted in enhanced *Rela* expression, the gene encoding p65 subunit of NFκB (Figure 6A). As detected by Western Blotting, cisplatin (10 µM, 24 h) significantly stimulated phosphorylation of p65 (Figure 6B). Moreover, treatment with doxorubicin (0.3 µM, 24 h) enhanced *Rela* expression (Figure 6C). Hence, cisplatin and doxorubicin induced NFκB activity in UMR106 cells. A last series of experiments explored whether NFκB activity is required for the effect of cisplatin on *Fgf23*. To this end, UMR106 cells were treated with and without cisplatin and NFκB inhibitors wogonin or withaferin A for 24 h. As depicted in Figure 6D, wogonin significantly attenuated the cisplatin-induced effect on *Fgf23* gene expression. Similarly, withaferin A blunted cisplatin-induced up-regulation of *Fgf23* (Figure 6E).

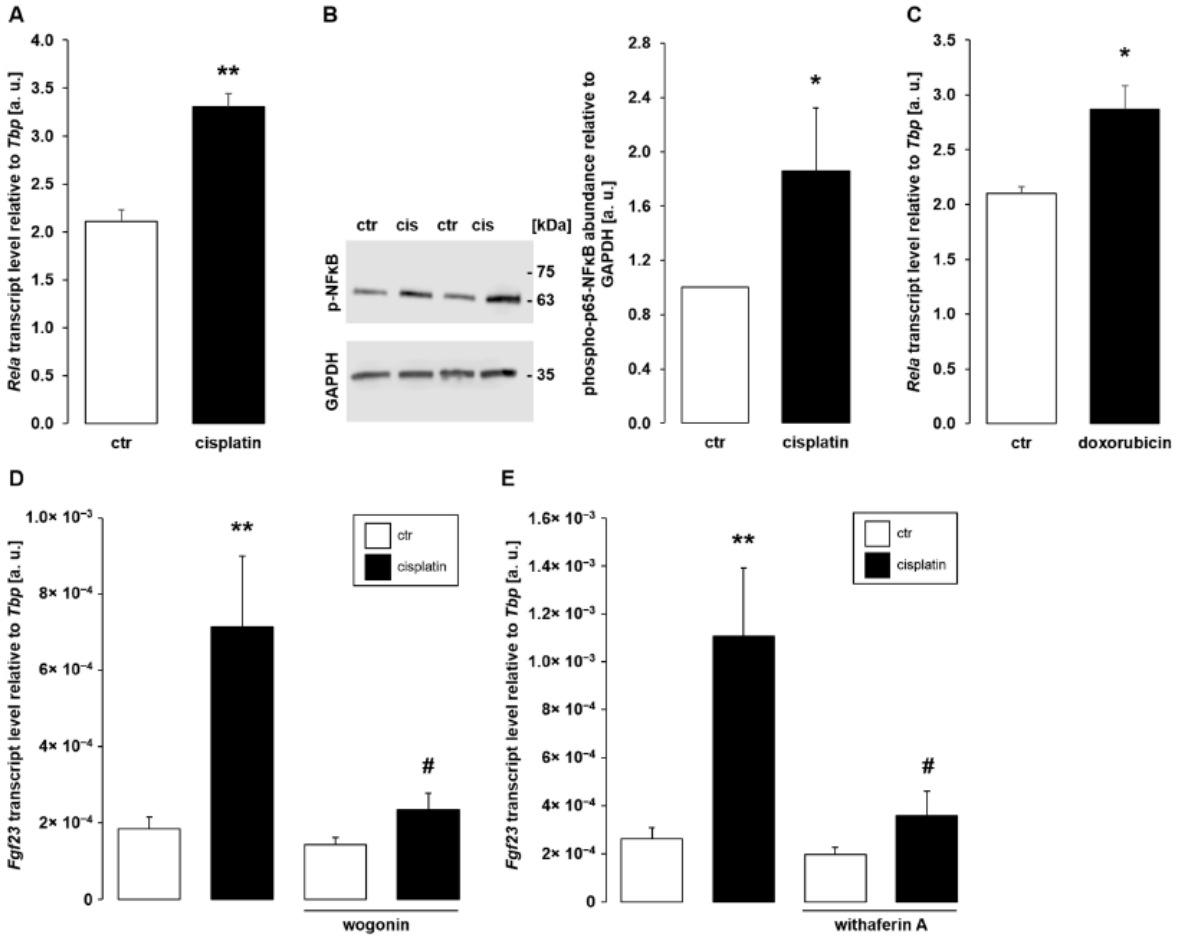

Figure 6. The contribution of NFκB to the *Fgf23* effect of cisplatin. (**A**): Arithmetic means ± SEM of NFκB subunit p65 (*Rela*) gene expression relative to *Tbp* in UMR106 cells incubated without (ctr) or with 10 µM cisplatin for 24 h ($n = 4$; student's *t*-test). (**B**): Left panel: Original Western Blot demonstrating the abundance of phospho-p65-NFκB and GAPDH in UMR106 cells treated with (cis) or without (ctr) 10 µM cisplatin for 24 h. Right panel: Arithmetic means ± SEM of phospho-p65-NFκB relative to GAPDH abundance ($n = 8$; one-sample Wilcoxon signed rank test). (**C**): Arithmetic means ± SEM of *Rela* expression relative to *Tbp* in UMR106 cells incubated for 24 h without (ctr) or with 0.3 µM doxorubicin ($n = 4$; student's *t*-test). (**D**,**E**): Arithmetic means ± SEM of *Fgf23* transcript abundance relative to *Tbp* in UMR106 cells treated for 24 h with vehicle control (ctr, white bars) or 3 µM cisplatin (black bars) in the presence or absence of 100 µM wogonin ((**D**); $n = 9$; Kruskal–Wallis test followed by Dunn–Bonferroni test) or 500 nM withaferin A ((**E**); $n = 9$; Kruskal–Wallis test followed by Dunn–Bonferroni test). * $p < 0.05$, ** $p < 0.01$ indicate significant difference from vehicle-treated cells (1st bar). # $p < 0.05$ indicates significant difference from the absence of NFκB inhibitors wogonin and withaferin A, respectively (2nd bar vs. 4th bar). a. u., arbitrary units; ctr, control.

4. Discussion

According to our study, two cytotoxic drugs with different cellular targets used in the treatment of several malignancies as well as apoptosis inducers PAC-1 and serum depletion stimulated *Fgf23* gene expression in UMR106 osteoblast-like cells within 24 h. The effect was paralleled by a reduction in cell viability and proliferation as deduced from cell number.

UMR106 osteoblast-like cells were chosen for our study because under physiological conditions, bone is the major site of FGF23 production [45] and these cells are a versatile tool employed in many studies to unravel the regulation of FGF23 [25,46–49].

Incubation of UMR106 cells with cisplatin or in serum-depleted medium for 48 h also resulted in enhanced *Fgf23* expression. Prolonged incubation with doxorubicin, however, killed all cells. In contrast to 24 h, 48-h exposure of the cells to PAC-1 did not significantly modify *Fgf23* expression, possibly because PAC-1-dependent apoptosis induction occurs much earlier and late apoptotic cells cannot up-regulate *Fgf23* gene expression any longer.

Cisplatin, doxorubicin, PAC-1 as well as serum depletion have in common that they cause cellular damage reducing cell number and viability, which may ultimately result in cell death. Cisplatin is effective by interfering with DNA replication [50], doxorubicin inhibits topoisomerase and intercalates with DNA [51], PAC-1 directly stimulates apoptotic cell death through executioner caspase 3 [35], whereas serum depletion favors apoptotic cell death due to lack of essential growth factors [36]. Although the mechanism of cell damage is different, the up-regulation of *Fgf23* gene expression is consistent for all four inducers of cellular injury. This important finding may point to a role of FGF23 in cellular stress, cell death, and survival. Indeed, FGF23-Klotho signaling favors cell proliferation and inhibits apoptosis, elicited by vitamin D, through phosphoinositide-3 kinase (PI3K) signaling [52]. Moreover, FGF23 exerts many effects through serum and glucocorticoid-dependent kinase 1 (SGK1) [53]. SGK1 is an important mediator of pro-survival signaling inhibiting apoptosis [54]. Moreover, in acute kidney injury (AKI), FGF23 has turned out to stimulate cell proliferation promoting regeneration of injured tubules through influencing SDF-1/CXCR4 signaling [55]. In tumor cells, namely prostate cancer, FGF23 similarly stimulates cell proliferation [56]. According to these studies, FGF23 has pro-survival/anti-apoptotic properties. Hence, up-regulation of FGF23 in cell stress as demonstrated in our study may help the cell activate pro-survival signaling. Alternatively, FGF23 may not only be a disease biomarker, but *Fgf23* gene expression may also indicate injury on cellular level or even serve as a marker for moribund cells. Definitely, further research is required to elucidate this.

In UMR106 cells, basal *Fgf23* expression is low unless the cells are pretreated with $1,25(OH)_2D_3$ which strongly up-regulates *Fgf23* expression [24]. Therefore, it must be kept in mind that although *Fgf23* transcripts significantly increased upon treatment with cisplatin, doxorubicin, or PAC-1, yet the cellular FGF23 protein concentration remained below the detection limit of ELISA. Serum depletion experiments were accomplished in the presence of 10 nM $1,25(OH)_2D_3$, hence, C-terminal FGF23 protein in the cell culture supernatant could be detected by ELISA and was significantly up-regulated in serum-depleted cells compared to control cells.

Chemotherapy is known to induce inflammation [37]. We demonstrated that both cisplatin and doxorubicin induce pro-inflammatory cytokine Il-6 within 24 h. Importantly, Il-6 is a stimulator of FGF23 [28]. In line with this, Il-6 signaling inhibitor SC144 significantly blunted cisplatin-induced *Fgf23* gene expression. Moreover, expression and phosphorylation of NFκB subunit p65 were up-regulated by cisplatin. Accordingly, wogonin and withaferin A, inhibitors of NFκB, significantly blunted cisplatin-induced up-regulation of *Fgf23* expression. This is in line with the pivotal role of NFκB and generally inflammation for the stimulation of FGF23 production. Importantly, cisplatin is a powerful inducer of NFκB activity [57], which may also contribute to treatment resistance [58] or nephrotoxicity [59]. Doxorubicin also induces inflammation by activating NFκB [60,61]. Hence, it appears likely that chemotherapy-induced inflammation involving Il-6 and NFκB is a major contributor to the up-regulation of *Fgf23* expression. In our experiments, wogonin and withaferin A tended to decrease *Fgf23* transcript levels in untreated cells, a difference, however, not reaching statistical significance. Presumably, the effect of NFκB inhibition on Fgf23 is smaller in cells with low basal *Fgf23* expression in the absence of $1,25(OH)_2D_3$ stimulation than in cells pre-treated with $1,25(OH)_2D_3$ to up-regulate *Fgf23* expression [30].

Direct executioner caspase-3-activator PAC-1 also up-regulated *Fgf23* gene expression. The same holds true for serum depletion, which favors apoptosis through growth factor deficiency [62]. However, caspase 3 activation and subsequent apoptosis are rather associated with decreased NFκB activity and not with a pro-inflammatory response [63]. Hence, additional mechanisms elucidated by future studies can clearly be expected to be also involved in the up-regulation of *Fgf23* expression of injured cells.

Taken together, the induction of cellular injury through cytotoxic drugs, serum depletion, or caspase 3 activation resulting in decreased proliferation and viability leads to the up-regulation of *Fgf23* gene expression. This effect can in part, but not fully, be explained by IL-6 up-regulation and NFκB activation.

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3.2 Paper 2: Impact of cytotoxic agents or apoptosis stimulants on αklotho in MDCK, NRK-52E and HK2 kidney cells

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Research Paper

Impact of cytotoxic agents or apoptosis stimulants on αklotho in MDCK, NRK-52E and HK2 kidney cells

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ABSTRACT

αKlotho is a transmembrane protein acting as a co-receptor for FGF23, a bone hormone regulating renal phosphate and vitamin D metabolism. αKlotho expression is controlled by PPARγ. Soluble αklotho (sKL) regulates cellular signaling impacting stress resistance and death. αKlotho deficiency causes early onset of aging-associated diseases while its overexpression markedly increases lifespan. Cellular stress due to cytotoxic therapeutics or apoptosis induction through caspase activation or serum deficiency may result in cell death. Owing to αklotho's role in cellular stress and aging, this study explored the effect of cytotoxic agents or apoptosis stimulants on cellular αklotho expression. Experiments were performed in renal MDCK, NRK-52E and HK-2 cells. Gene expression was determined by qRT-PCR, sKL by ELISA, apoptosis and necrosis by annexin V binding and a fluorescent DNA dye, and cell viability by MTT assay. Cytostatic drugs cisplatin, paclitaxel, and doxorubicin as well as apoptosis induction with caspase 3 activator PAC-1 and serum deprivation induced αklotho and *PPARG* gene expression while decreasing viability and proliferation and inducing apoptosis of MDCK and NRK-52E cells to a variable extent. PPARγ antagonism attenuated up-regulation of αklotho in MDCK cells. In HK-2 cells, αklotho gene expression and sKL protein were down-regulated by chemotherapeutics. SKL serum levels in patients following chemotherapy were not significantly changed. In summary, potentially fatal stress results in up-regulation of αKlotho gene expression in MDCK and NRK-52E cells and down-regulation in HK-2 cells. These results indicate that different renal cell lines may exhibit completely different regulation of αklotho.

INTRODUCTION

The αklotho gene product was discovered in mice in 1997 as a protein with strong anti-aging properties [1, 2]. Mice almost completely lacking αklotho exhibit a dramatically shortened life span of a few weeks only whilst suffering from a broad range of diseases and symptoms mimicking human aging [1]. Observed abnormalities affect nearly every organ and tissue [1] and include frequent aging-associated diseases including fibrosis [3, 4], lung emphysema [5], multiple organ atrophy [1], or hearing loss [6, 7]. The accelerated aging of αklotho-deficient mice is paralleled by massive calcification in most tissues [1, 8]. Importantly, the reduction of dietary phosphate or vitamin D intake of the animals almost completely normalizes their phenotype pointing to a dominant role of phosphate and vitamin D excess in their rapid aging [9, 10]. Indeed, αklotho protein has important functions in the homeostasis of these nutrients [11]: It is a trans-membrane protein predominantly expressed in the kidney that enhances the binding affinity of fibroblast

growth factor 23 (FGF23) for its membrane receptor [12, 13]. FGF23 is a proteohormone released by bone cells that inhibits phosphate reabsorption and $1,25(OH)_2D_3$ (biologically active vitamin D) synthesis in the kidney [14, 15] and has gained attention as a marker indicating disease [16, 17]. Hence, the lack of αklotho or FGF23 results in abnormally high serum phosphate and $1,25(OH)_2D_3$ levels that account for enhanced calcification and contribute to rapid aging and early death to a large extent [18].

In addition to its significance as a co-receptor for FGF23, FGF23-independent endocrine and paracrine effects of αklotho have been revealed [19–21]. These are mainly due to soluble klotho (sKL) that is produced through the cleavage of transmembrane αklotho [22]. SKL can be detected in body fluids including serum, urine, or cerebrospinal fluid [23, 24]. Endocrine or paracrine actions of sKL include the direct regulation of ion channels [25] or important signaling pathways (e.g., IGF, Wnt, or TGF-β1 signaling) [2, 26, 27]. αKlotho exerts anti-neoplastic [28], anti-inflammatory [29, 30], anti-fibrotic [3], and anti-oxidant effects [31, 32] and has been proven organoprotective, e.g., in the kidney [33, 34]. In several tumor cell lines and cancer mouse models, higher expression of αklotho is associated with beneficial, potentially lifespan-expanding effects [35, 36]. And indeed, overexpression of αklotho results in a 30% longer lifespan of mice uncovering αklotho as a very powerful anti-aging factor [2]. Also in human centenarians, single nucleotide polymorphisms (SNPs) of the αklotho gene may be effective [37]. Moreover, lower αklotho levels are associated with poorer outcome in kidney or cardiovascular disease in men [33, 38–40].

Chemotherapy with platinum derivative cisplatin, anthracycline doxorubicin, or paclitaxel is standard of care in many forms of cancer. Although the three compounds differ in their cellular targets, they have in common that they exert cytotoxic effects which compromise proliferation and may ultimately result in apoptotic cell death [41–43]. Apoptosis of cultured cells without prior cell damage may be induced by activation of executioner caspase 3 with PAC-1 or by growth factor deprivation through serum depletion [44, 45].

In view of the versatile effects of αklotho on cell survival and death [46, 47], this study aimed to investigate whether cytotoxic drugs or initiation of apoptosis affect αklotho gene expression in three different renal cell lines and in patients receiving chemotherapy.

RESULTS AND DISCUSSION

As a first step, MDCK and NRK-52E cells were used to study αklotho gene expression. MDCK cells were treated with antineoplastic platinum derivative cisplatin for 24 h, and αklotho mRNA levels were analyzed by qRT-PCR. As illustrated in Figure 1A, cisplatin up-regulated αklotho gene expression in MDCK cells, an effect reaching significance at 3 μM cisplatin. The effect was not paralleled by decreased viability of MDCK cells even at 10 μM cisplatin (Figure 1B), but by reduced cell proliferation (Figure 1C). We determined the rate of apoptosis and necrosis by means of an assay analyzing annexin V binding and a DNA-binding dye which is impermeable to the membrane of intact cells. As illustrated in Figure 1D, cisplatin induced apoptosis without significantly influencing necrosis of MDCK cells. In another series of experiments, NRK-52E cells were treated without or with cisplatin for 24 h, and αklotho gene expression, viability, proliferation, and apoptosis/necrosis were assessed. Again, cisplatin (10 μM) significantly enhanced αklotho expression (Figure 1E), an effect paralleled by decreased cell viability (Figure 1F) and proliferation (Figure 1G). Again, cisplatin induced apoptosis without significantly stimulating necrosis of NRK-52E cells (Figure 1H).

Further experiments were performed to elucidate whether cytostatic compound paclitaxel also affects αklotho. To this end, MDCK cells were incubated with different concentrations of paclitaxel for 24 h or with vehicle control, respectively. It is shown in Figure 2A that 120 nM paclitaxel significantly stimulated the abundance of αklotho mRNA. By the same token, 120 nM paclitaxel significantly lowered the viability (Figure 2B) and proliferation (Figure 2C) of MDCK cells. These effects were paralleled by enhanced apoptosis and necrosis (Figure 2D). We also studied the effect of 120 nM paclitaxel in NRK-52E cells. This concentration of the antimitotic agent significantly up-regulated αklotho gene expression within 24 h (Figure 2E), too, whilst down-regulating viability (Figure 2F) and proliferation (Figure 2G) of NRK-52E cells. Similar to MDCK cells, paclitaxel induced apoptosis and necrosis in NRK-52E cells (Figure 2H).

As a third common antineoplastic drug, we tested anthracycline doxorubicin. A 24 h-exposure to 100 nM or 300 nM doxorubicin led to a significant increase in the abundance of αklotho transcripts in MDCK cells (Figure 3A). Doxorubicin treatment (300 nM) did not significantly affect viability (Figure 3B) but reduced proliferation (Figure 3C) of MDCK cells. Doxorubicin induced apoptosis while slightly reducing the number of necrotic cells (Figure 3D). In NRK-52E cells, 300 nM doxorubicin readily stimulated αklotho expression within 24 h (Figure 3E) and compromised cell viability (Figure 3F) as well as proliferation (Figure 3G).

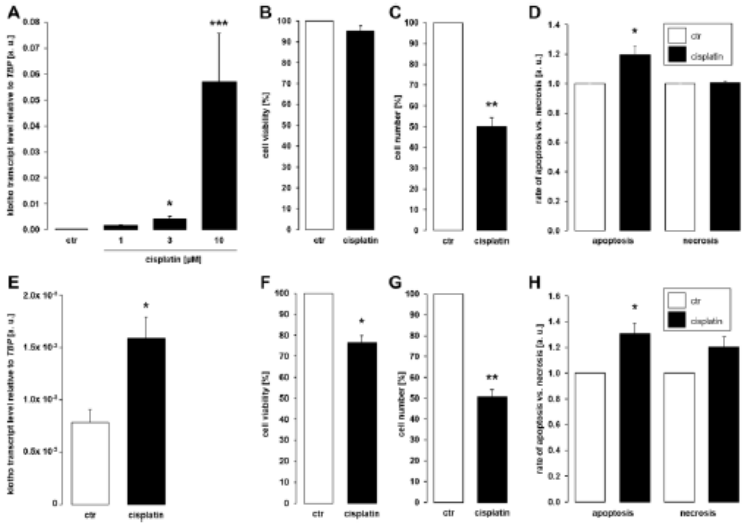

Figure 1. Cisplatin upregulates αklotho expression in MDCK and NRK-52E cells. (**A**) Arithmetic mean ± SEM of αklotho transcript levels normalized to *TBP* in MDCK cells treated with cisplatin at the indicated concentration for 24 h ($n = 5$; *Friedman ANOVA* followed by *Dunn-Bonferroni* post-hoc test). (**B, C**) Arithmetic mean ± SEM of MDCK cell viability (**B**) or number (**C**) upon treatment without or with 10 µM cisplatin for 24 h (**B**: $n = 5$, *one-sample t*-test; **C**: $n = 4$, *one-sample t*-test). (**D**) Rate of apoptosis and necrosis of MDCK cells treated with or without 10 µM cisplatin for 24 h ($n = 6$, *one-sample t* test) (**E**) Arithmetic mean ± SEM of αklotho transcript levels relative to *TBP* in NRK-52E cells incubated without or with 10 µM cisplatin for 24 h ($n = 5$, *paired t*-test). (**F, G**) Arithmetic mean ± SEM of NRK-52E cell viability (**F**) or number (**G**) upon treatment without or with 10 µM cisplatin for 24 h (**F**: $n = 5$, *one-sample t*-test; **G**: $n = 4$, *one-sample t*-test). (**H**) Rate of apoptosis and necrosis of NRK-52E cells treated with or without 10 µM cisplatin for 24 h ($n = 5$, *one-sample t* test) $^{*}p < 0{,}05$, $^{**}p < 0.01$, $^{***}p < 0.001$ indicate significant difference from vehicle control; Abbreviations: a. u.: arbitrary units; ctr: control.

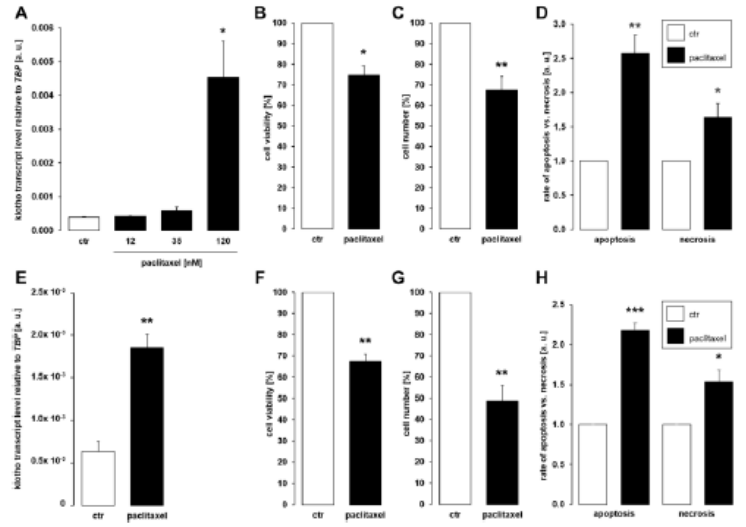

Figure 2. Paclitaxel induces αklotho expression in MDCK and NRK-52E cells. (**A**) Arithmetic mean ± SEM of αklotho transcript levels normalized to *TBP* in MDCK cells treated with paclitaxel at the indicated concentration for 24 h ($n = 5$; *Friedman ANOVA* and *Dunn-Bonferroni* post-hoc test). (**B, C**) Arithmetic mean ± SEM of MDCK cell viability (**B**) or number (**C**) upon treatment without or with 120 nM paclitaxel for 24 h (**B**: $n = 4$, *one-sample t*-test; **C**: $n = 5$, *one-sample t*-test). (**D**) Rate of apoptosis and necrosis of MDCK cells treated with 120 nM paclitaxel or vehicle control for 24 h ($n = 6$, *one-sample t* test). (**E**) Arithmetic mean ± SEM of αklotho transcript levels relative to *TBP* in NRK-52E cells incubated without or with 120 nM paclitaxel for 24 h ($n = 5$, *paired t*-test). (**F, G**) Arithmetic mean ± SEM of NRK-52E cell viability (**F**) or number (**G**) upon treatment without or with 120 nM paclitaxel for 24 h (**F**: $n = 5$, *one-sample t*-test; **G**: $n = 5$, *one-sample t*-test). (**H**) Rate of apoptosis and necrosis of NRK-52E cells treated with or without 120 µM paclitaxel for 24 h ($n = 5$, *one-sample t* test). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ indicate significant difference from vehicle control; Abbreviations: a. u.: arbitrary units; ctr: control.

Apoptosis and necrosis were enhanced by doxorubicin in NRK52-E cells (Figure 3H).

Since different classes of cytostatic drugs with pro-apoptotic properties similarly enhanced αklotho expression in MDCK and NRK-52E cells within 24 h, we sought to explore whether direct apoptosis induction also affects αklotho. To this end, we treated the cells with and without caspase 3 activator PAC-1 for 24 h. As demonstrated in Figure 4A, 10 µM PAC-1 induced αklotho expression in MDCK cells, an effect paralleled by decreased cell viability (Figure 4B) and proliferation (Figure 4C). PAC-1 enhanced apoptosis without significantly modifying necrosis (Figure 4D). Also in NRK-52E cells, PAC-1 treatment (10 µM) resulted in a significant surge in αklotho transcripts within 24 h (Figure 4E) and decreased their viability (Figure 4F) and proliferation (Figure 4G). The rates of apoptosis and necrosis were significantly higher in NRK-52E cells upon exposure to PAC-1 (Figure 4H).

Depriving cells of growth factors through serum depletion similarly favors apoptosis [45]. We therefore aimed to test whether αklotho expression is affected by serum depletion. As depicted in Figure 5A, a 24 h-incubation of MDCK cells in the absence of serum significantly up-regulated αklotho gene expression without significantly impacting on cell viability (Figure 5B) and proliferation (Figure 5C). Serum depletion up-regulated apoptosis whereas necrosis-dependent fluorescence was reduced in serum-starved cells (Figure 5D). In NRK-52E cells, serum depletion did not significantly affect αklotho mRNA levels within 24 h (Figure 5E). However, viability and proliferation were moderately but significantly lower in NRK-52E cells incubated in the absence of serum compared to control cells (Figure 5F, 5G). Serum depletion induced apoptosis and did not significantly affect necrosis in NRK-52E cells (Figure 5H).

Next, we analyzed gene expression of pro-apoptotic molecules BAD, BAX, and the ratio of BAX/BCL-2 expression in MDCK cells. As illustrated in Figure 6, treatment with cisplatin (Figure 6A, 6E, 6I) or doxorubicin (Figure 6C, 6G, 6K) up-regulated BAD, BAX and BAX/BCL-2 expression. Paclitaxel induced up-regulation of BAX, but did not significantly modify BAD and BAX/BCL-2 (Figure 6B, 6F, 6J) whilst PAC-1 significantly enhanced expression of BAX and BAX/BCL-2, but did not significantly change BAD expression (Figure 6D, 6H, 6L).

We performed further experiments to identify the mechanism underlying enhanced αklotho expression in

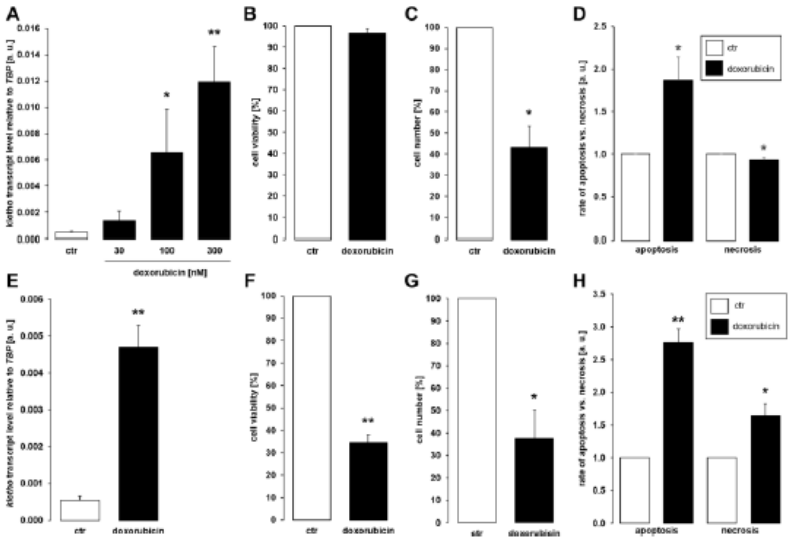

Figure 3. Doxorubicin enhances αklotho expression in MDCK and NRK-52E cells. (A) Arithmetic mean ± SEM of αklotho transcript levels normalized to *TBP* in MDCK cells treated with doxorubicin at the indicated concentration for 24 h ($n = 5$; *Friedman ANOVA* followed by *Dunn-Bonferroni post-hoc* test). (B, C) Arithmetic mean ± SEM of MDCK cell viability (B) or number (C) upon treatment without or with 300 nM doxorubicin for 24 h (B: $n = 5$; *one-sample t*-test; C: $n = 4$; *one-sample t*-test). (D) Rate of apoptosis and necrosis of MDCK cells treated with or without 300 nM doxorubicin for 24 h ($n = 6$, *one-sample t* test). (E) Arithmetic mean ± SEM of αklotho transcript levels relative to *TBP* in NRK-52E cells incubated without or with 300 nM doxorubicin for 24 h ($n = 5$, *paired t*-test). (F, G) Arithmetic mean ± SEM of NRK-52E cell viability (F) or number (G) upon treatment without or with 300 nM doxorubicin for 24 h (F: $n = 4$, *one-sample t*-test; G: $n = 4$, *one-sample t*-test). (H) Rate of apoptosis and necrosis of NRK-52E cells treated with or without 300 nM doxorubicin for 24 h ($n = 5$, *one-sample t* test) $^{*}p < 0.05$, $^{**}p < 0.01$ indicate significant difference from vehicle control; Abbreviations: a. u.: arbitrary units; ctr: control.

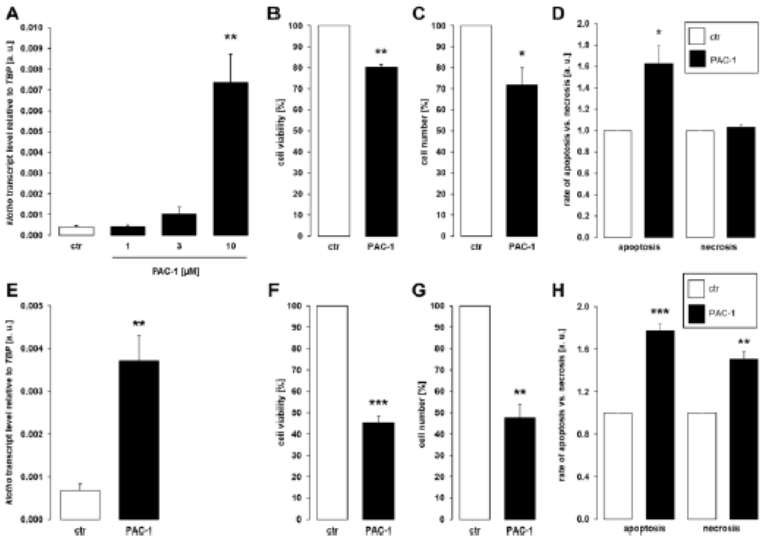

Figure 4. αKlotho gene expression is stimulated by procaspase activating compound 1 (PAC-1) in MDCK and NRK-52E cells. (A) Arithmetic mean ± SEM of αklotho transcript levels normalized to *TBP* in MDCK cells treated with PAC-1 at the indicated concentration for 24 h ($n = 6$; *Friedman ANOVA* followed by *Dunn-Bonferroni* post hoc test). (B, C) Arithmetic mean ± SEM of MDCK cell viability (B) or number (C) upon treatment without or with 10 µM PAC-1 for 24 h (B: $n = 4$, *one-sample t*-test; C: $n = 6$, *one-sample t*-test). (D) Rate of apoptosis and necrosis of MDCK cells treated with or without 10 µM PAC-1 for 24 h ($n = 6$, *one-sample t* test). (E) Arithmetic mean ± SEM of αklotho transcript levels relative to *TBP* in NRK-52E cells incubated without or with 10 µM PAC-1 for 24 h ($n = 6$, *paired t*-test). (F, G) Arithmetic mean ± SEM of NRK-52E cell viability (F) or number (G) upon treatment without or with 10 µM PAC-1 for 24 h (F: $n = 5$, *one-sample t*-test; G: $n = 4$, *one-sample t*-test). (H) Rate of apoptosis and necrosis of NRK-52E cells treated with or without 10 µM PAC-1 for 24 h ($n = 5$, *one-sample t* test). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ indicate significant difference from vehicle control; Abbreviations: a. u.: arbitrary units; ctr: control.

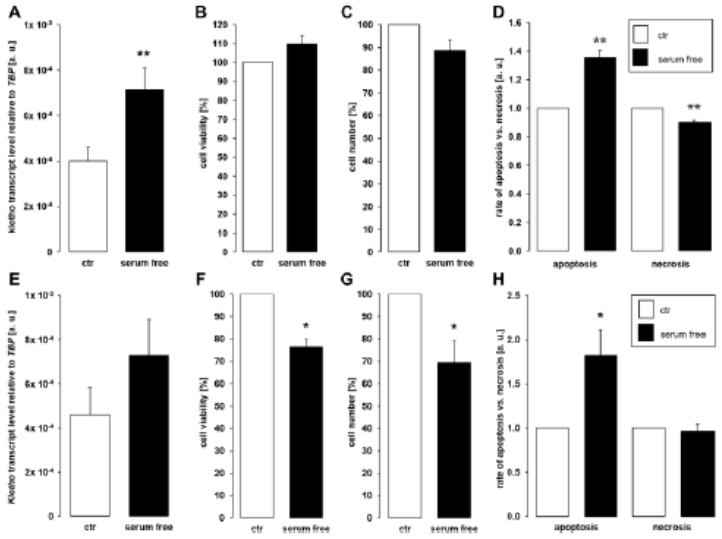

Figure 5. Serum deprivation up-regulates αklotho expression in MDCK cells. (A) Arithmetic mean ± SEM of αklotho transcript levels relative to *TBP* in MDCK cells incubated for 24 h with or without 5% fetal bovine serum (FBS; $n = 5$; *paired t*-test). (B, C) Arithmetic mean ± SEM of MDCK cell viability (B) or number (C) upon incubation with or without 5% FBS for 24 h (B: $n = 4$, *one-sample t*-test; C: $n = 6$, *one-sample t*-test). (D) Rate of apoptosis and necrosis of MDCK cells cultured with or without 5% FBS for 24 h ($n = 6$, *one-sample t* test). (E) Arithmetic mean ± SEM of αklotho transcript levels relative to *TBP* in NRK-52E cells incubated for 24 h with or without 5% newborn calf serum (NBCS) ($n = 8$, *paired t*-test). (F, G) Arithmetic mean ± SEM of NRK-52E cell viability (F) or number (G) upon incubation with or without 5% NBCS for 24 h (F: $n = 5$, *one-sample t*-test; G: $n = 4$, *one-sample t*-test). (H) Rate of apoptotis and necrosis of NRK-52E cells cultured with or without 5% NBCS for 24 h ($n = 5$, *one-sample Wilcoxon* test). $^{*}p < 0.05$, $^{**}p < 0.01$ indicates significant difference from control cells; Abbreviations: a. u.: arbitrary units; ctr: control.

MDCK and NRK-52E cells exposed to chemotherapeutics or apoptosis stimulants. Since transcription factor PPARγ is pivotal for klotho expression [48] and has been demonstrated to be up-regulated by cisplatin [49], we analyzed *PPARG* expression. As a result, treatment with cisplatin (Figure 7A, 7F), paclitaxel (Figure 7B, 7G), doxorubicin (Figure 7C, 7H), and PAC-1 (Figure 7D, 7I) enhanced *PPARG* expression in both, MDCK and NRK52-E cells. Moreover, serum starvation enhanced *PPARG* in NRK-52E (Figure 7J), but not in MDCK cells (Figure 7E).

In order to confirm that PPARγ is indeed required for cisplatin to up-regulate αklotho expression, we exposed MDCK cells to cisplatin in the presence and absence of PPARγ antagonist SR202. As illustrated in Figure 8, SR-202 significantly blunted cisplatin-dependent up-regulation of αklotho. Hence, PPARγ contributes to enhancement of αklotho expression, but may not fully explain it.

Transmembrane αklotho forms a complex with FGFR1 to serve as a receptor for FGF23. A further series of experiments sought to clarify whether the effect of

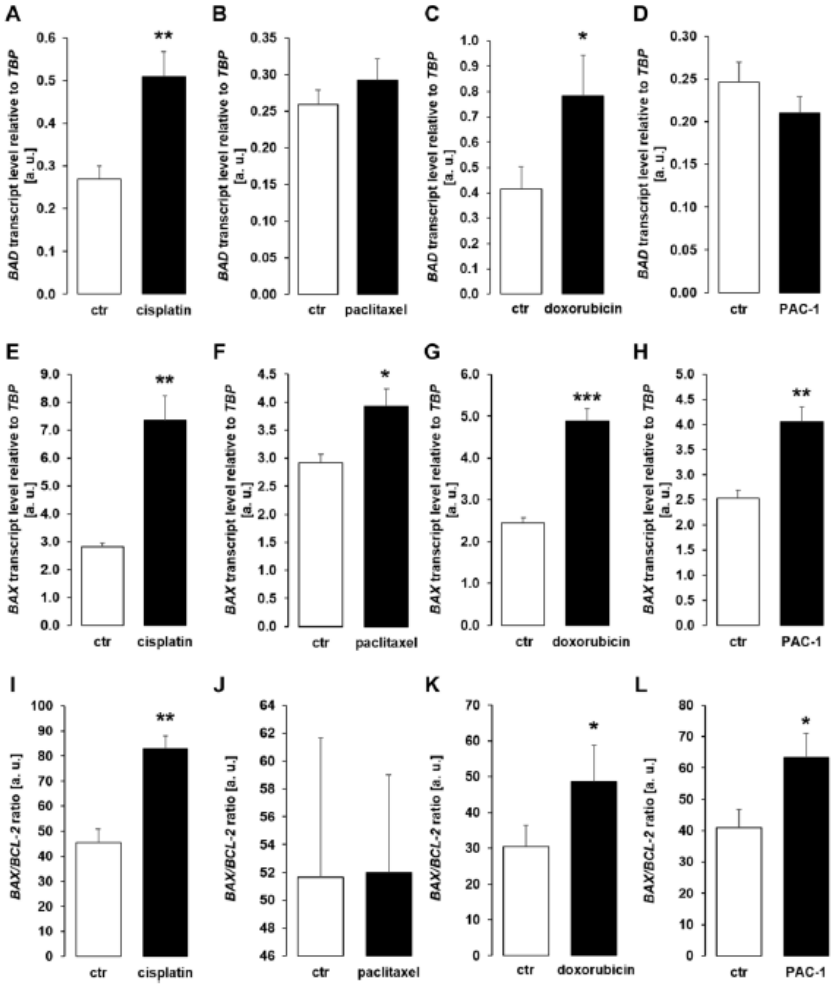

Figure 6. Cytotoxic agents and PAC-1 up-regulate apoptotic proteins BAD and BAX in MCDK cells. (A–D) Arithmetic mean ± SEM of *BAD* transcript levels relative to *TBP* in MDCK cells incubated for 24 h without or with 10 μM cisplatin (A; *n* = 5; *paired t*-test), 120 nM paclitaxel (B; *n* = 5, *paired t*-test), 300 nM doxorubicin (C; *n* = 5, *paired t*-test), or 10 μM PAC-1 (D; *n* = 6, *paired t*-test). (E–H) Arithmetic mean ± SEM of BAX transcripts relative to TBP in MDCK cells treated without or with 10 μM cisplatin (E; *n* = 5, *Wilcoxon signed-rank* test), 120 nM paclitaxel (F; *n* = 5, *paired t*-test), 300 nM doxorubicin (G; *n* = 5, *Wilcoxon signed-rank* test), or 10 μM PAC-1 (H; *n* = 6, *paired t*-test). (I–L) Arithmetic mean ± SEM of BAX to BCL-2 mRNA ratio in MDCK cells incubated for 24 h without or with 10 μM cisplatin (I; *n* = 5, *paired t*-test), 120 nM paclitaxel (J; *n* = 5, *paired t*-test), 300 nM doxorubicin (K; *n* = 5, *paired* t-test), or 10 μM PAC-1 (L; *n* = 6, *paired t*-test). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ indicate significant difference from vehicle control; Abbreviations: a. u.: arbitrary units; ctr: control.

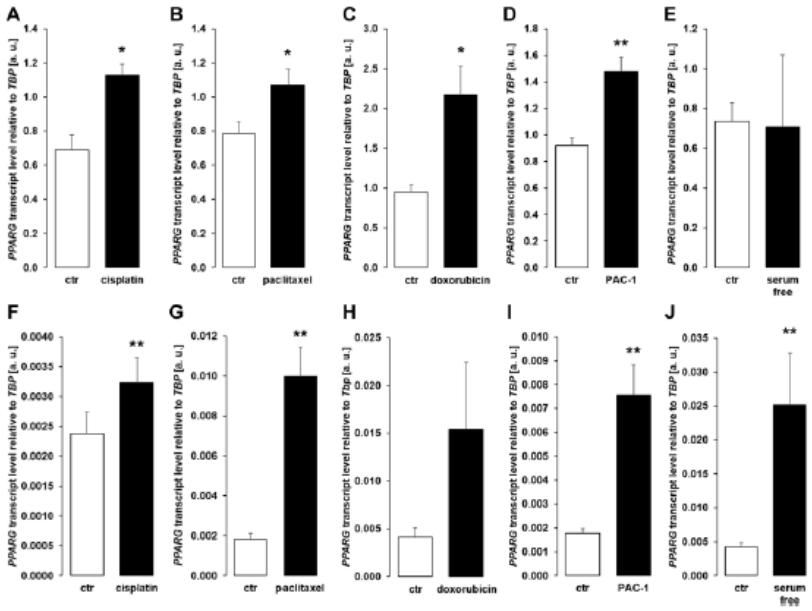

Figure 7. Cytotoxic agents and apoptosis inducers up-regulate *PPARG* in MDCK and NRK-52E cells. (A–E) Arithmetic mean ± SEM of *PPARG* transcript levels normalized to *TBP* in MDCK cells treated with or without 10 µM cisplatin (A; *n* = 5; *paired t*-test), 120 nM paclitaxel (B; *n* = 5; *paired t*-test), 300 nM doxorubicin (C; *n* = 5, *paired t*-test), 10 µM PAC-1 (D; *n* = 6, *paired t*-test), or with and without 5% FBS in the culture medium (E; *n* = 5, *Wilcoxon signed-rank* test) for 24 h. (F–J) Arithmetic mean ± SEM of *PPARG* mRNA levels relative to *TBP* in NRK-52E cells treated for 24 h with or without 10 µM cisplatin (F; *n* = 8; *paired t*-test), 120 nM paclitaxel (G; *n* = 5; *paired t*-test), 300 nM doxorubicin (H; *n* = 7, *paired t*-test), 10 µM PAC-1 (I; *n* = 6, *paired t*-test), or incubated with or without 5% NBCS in the culture medium (J; *n* = 6, *Wilcoxon signed-rank* test). $^{*}p < 0.05$, $^{**}p < 0.01$ indicate significant difference from vehicle control; Abbreviations: a. u.: arbitrary units; ctr: control.

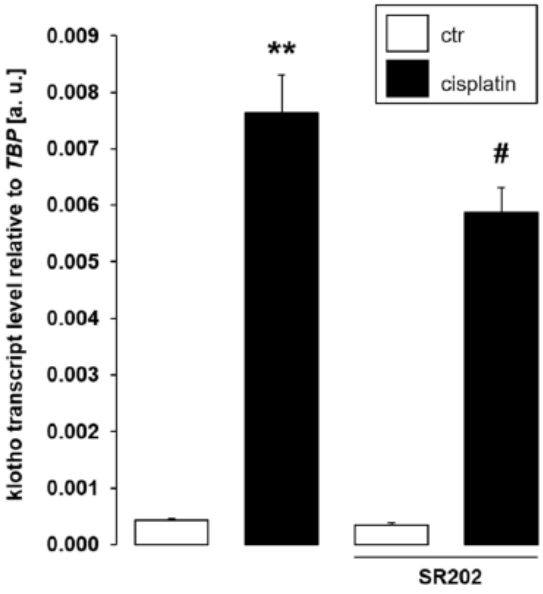

Figure 8. Selective PPARγ antagonist SR-202 blunts cisplatin-dependent αklotho gene expression in MDCK cells. Arithmetic mean ± SEM of αklotho transcripts relative to TBP in MDCK cells treated with 3 µM cisplatin or vehicle control in the absence (left bars) or presence (right bars) of 200 µM PPARγ antagonist SR-202 for 24 h (*n* = 8, *repeated measures ANOVA* followed by *Dunnett* post hoc test). $^{**}p < 0.01$ indicates significant difference from vehicle control (1st bar vs. 2nd bar), #indicates significant difference from the absence of PPARγ inhibitor SR-202 (2nd bar vs. 4th bar); Abbreviations: a. u.: arbitrary units; ctr: control.

chemotherapeutics and apoptosis stimulants also affect FGFR1 and/or FGF23 expression in MDCK cells. As demonstrated in Figure 9A, 9B, cisplatin up-regulated FGFR1 expression and protein. Similar effects on FGFR1 expression were observed following incubation with doxorubicin (Figure 9C), PAC-1 (Figure 9D), and upon incubation in serum-free medium (Figure 9E). The expression of FGF23, which is mainly expressed in bone, could not be detected in unstimulated (Ct value: > 40, n = 5) MDCK. Cisplatin-treated MDCK cells exhibited lower Ct values for FGF23, however expression was still very low (Ct value: 37.1 ± 1.85, n = 5).

ELISA-based quantification of αklotho protein is particularly feasible in human cells. Therefore, we performed further experiments in human proximal tubular cell line HK-2. We treated these cells with the cytotoxic agents and apoptosis stimulants in a way similar to MDCK and NRK-52E cells and measured αklotho transcripts as well as sKL protein by ELISA.

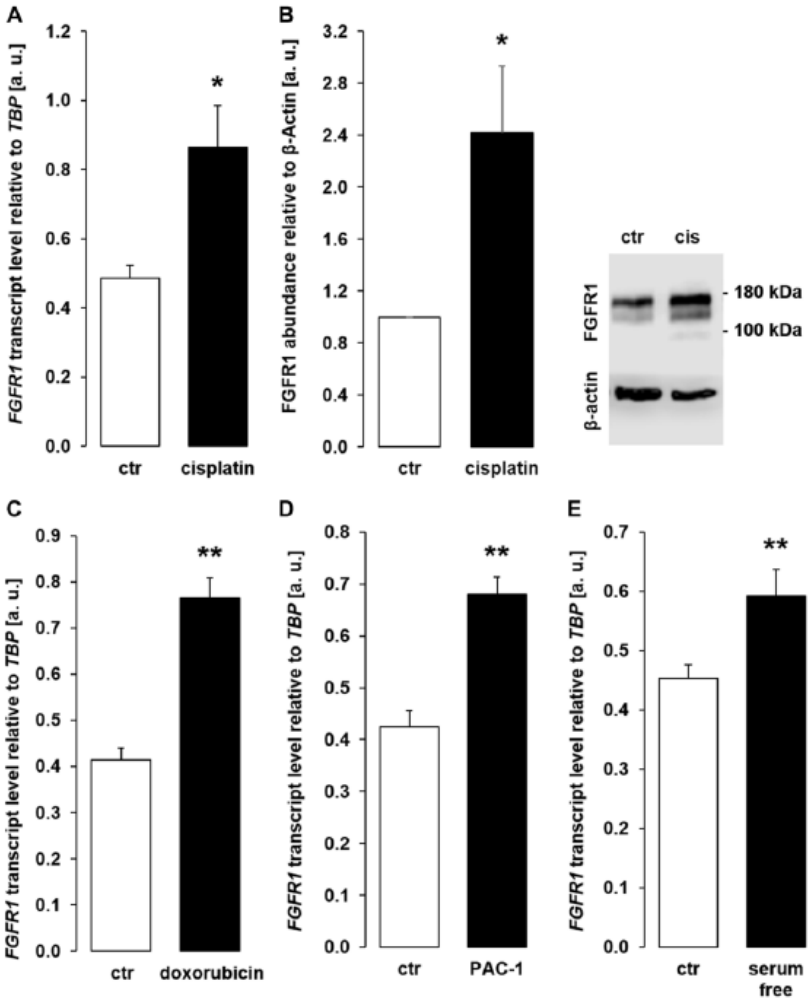

Figure 9. Cisplatin, doxorubicin, PAC-1, and serum depletion up-regulate FGFR1 in MDCK cells. (A) Arithmetic mean ± SEM of *FGFR1* mRNA levels relative to *TBP* in MDCK cells treated with or without 10 μM cisplatin for 24 h (n = 5, *paired* t-test). (B) Left panel: Arithmetic mean ± SEM of FGFR1 protein abundance normalized to the abundance of β-actin in MDCK cells following treatment with or without 10 μM cisplatin for 24 h (n = 7, *one-sample* t-test). Right panel: Original Western Blot demonstrating the abundance of FGFR1 in MDCK cells treated with (cis) or without (ctr) 10 μM cisplatin for 24 h. (C) Arithmetic mean ± SEM of *FGFR1* transcript levels relative to *TBP* in MDCK cells treated with or without 300 nM doxorubicin for 24 h (n = 4, *paired* t-test). (D) Arithmetic mean ± SEM of *FGFR1* transcript level relative to *TBP* in MDCK cells treated with or without 10 μM PAC-1 for 24 h (n = 5, *paired* t-test). (E) Arithmetic mean ± SEM of *FGFR1* transcripts relative to *TBP* in MDCK cells incubated without or with 5 % FBS in culture medium for 24 h (n = 5, *paired* t-test). $^{*}p < 0.05$, $^{**}p < 0.01$ indicate significant difference from vehicle control; Abbreviations: a. u.: arbitrary units; cis cisplatin; ctr: control.

Surprisingly, cisplatin (Figure 10A), paclitaxel (Figure 10C), doxorubicin (Figure 10E), and serum-free incubation (Figure 10G) significantly down-regulated αklotho gene expression. In line with this, sKL protein concentration was lower in the cell culture supernatant of HK-2 cells upon incubation with cisplatin (Figure 10B), doxorubicin (Figure 10F), and in the absence of serum (Figure 10H) and virtually unchanged upon exposure to paclitaxel (Figure 10D).

As a last step, we analyzed sKL in serum samples from patients before and after chemotherapy (Table 1) and found that the serum sKL concentration was not significantly different after chemotherapy compared to samples obtained before therapy (Figure 11).

According to our study, αklotho expression was up-regulated by antineoplastic cytostatic agents cisplatin, paclitaxel, and doxorubicin in MDCK and NRK-52E cells within 24 h. Moreover, caspase 3 activator PAC-1 enhanced αklotho expression in both cell lines, whereas serum depletion was only effective in MDCK cells. Caspase 3 activation and serum depletion can be expected to induce apoptosis [44, 45]. In sharp contrast, the same treatment resulted in down-regulation of both, αklotho transcripts and sKL protein, in HK-2 cells. The serum concentration of sKL was not significantly affected by chemotherapy.

Treatment with antineoplastic agents induces cellular stress through different mechanisms: Cisplatin impairs DNA replication by enabling inter- and intrastrand crosslink adducts [41], anthracycline derivative doxorubicin is a topoisomerase II inhibitor and DNA intercalator [42], and paclitaxel is an antimitotic agent that prevents spindle assembly by interacting with

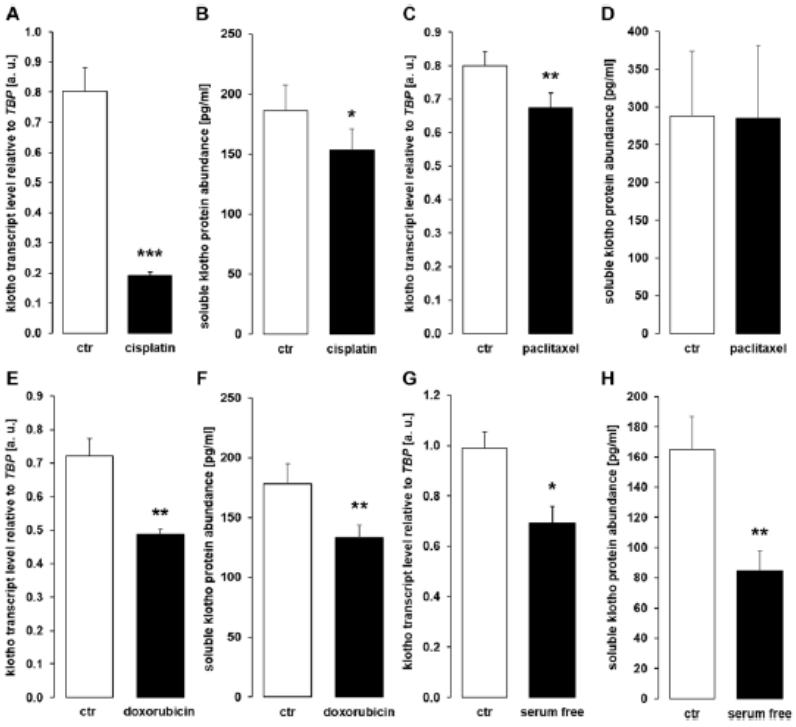

Figure 10. Cytostatic drugs and serum deprivation reduce αklotho gene expression and soluble klotho (sKL) protein secretion in HK-2 cells. (**A**) Arithmetic mean ± SEM of αklotho mRNA levels relative to *TBP* in HK-2 cells treated with or without 10 μM cisplatin for 24 h (n = 8, *paired* t-test). (**B**) Arithmetic mean ± SEM of sKL concentration in the supernatant of HK-2 cells treated with 10 μM cisplatin or vehicle control for 24 h (n = 6, *paired* t-test). (**C**) Arithmetic mean ± SEM of αklotho transcript levels relative to *TBP* in HK-2 cells treated with or without 120 nM paclitaxel (n = 6, *paired* t-test) for 24 h. (**D**) Arithmetic mean ± SEM of sKL concentration in the cell culture supernatant of HK-2 cells treated with or without 120 nM paclitaxel for 24 h (n = 6, *paired* t-test). (**E**) Arithmetic mean ± SEM of αklotho transcript levels relative to *TBP* in HK-2 cells treated with or without 300 nM doxorubicin for 24 h (n = 5, *paired* t-test). (**F**) Arithmetic mean ± SEM of sKL concentration in the cell culture supernatant of HK-2 cells treated with or without 300 nM doxorubicin for 24 h (n = 4, *paired* t-test). (**G**) Arithmetic mean ± SEM of αklotho mRNA levels relative to *TBP* in HK-2 cells incubated with (ctr) or without 10 % FBS in the culture medium for 24 h (n =5, *paired* t-test). (**H**) Arithmetic mean ± SEM of sKL concentration in the HK-2 cell culture supernatant after incubation with or without 10% FBS for 24 h (n = 5, *paired* t-test). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ indicate significant difference from vehicle control; Abbreviations: a. u.: arbitrary units; ctr: control.

Table 1. Patients' characteristics.

Patient no.	Age	Sex	Diagnosis	Chemotherapy	Cycle of chemotherapy
1	59	m	colon adenocarcinoma	folinic acid, fluorouracil, oxaliplatin, bevacizumab	18
2	62	m	colon carcinoma	folinic acid, fluorouracil, oxaliplatin	2
3	74	m	esophageal carcinoma	fluorouracil, folinic acid, oxaliplatin, docetaxel	4
4	70	m	pancreatic carcinoma	folinic acid, fluorouracil, irinotecan, oxaliplatin	6
5	79	m	esophageal carcinoma	fluorouracil, folinic acid, oxaliplatin, docetaxel	4
6	78	m	esophageal carcinoma	folinic acid, fluorouracil, oxaliplatin	6
7	73	f	pancreatic adenocarcinoma	folinic acid, fluorouracil, irinotecan, oxaliplatin	6
8	61	f	lung carcinoma	nivolumab, ipilimumab, carboplatin, pemetrexed	1
9	80	m	esophageal carcinoma	fluorouracil, folinic acid, oxaliplatin, docetaxel	1

tubulin [50]. Ultimately, the cellular impairments induced by these drugs may result in apoptotic cell death, a consequence intended in therapeutic use of these agents in the treatment of different types of cancer [51]. In line with this, cisplatin, doxorubicin, and paclitaxel reduced viability and proliferation of MDCK and NRK-52E cells, albeit to a variable extent. Moreover, the treatment was followed by induction of apoptosis and partially by secondary necrosis. In these two cell lines, apoptosis was paralleled by a marked upregulation of αklotho gene expression. In addition, expression of pro-apoptotic genes BAD, BAX, and BAX/BCL-2 ratio was induced by the chemotherapeutic agents, albeit to a variable extent. Also, direct induction of apoptotic cell death in the absence of cytotoxic drugs up-regulated αklotho mRNA levels in MDCK and

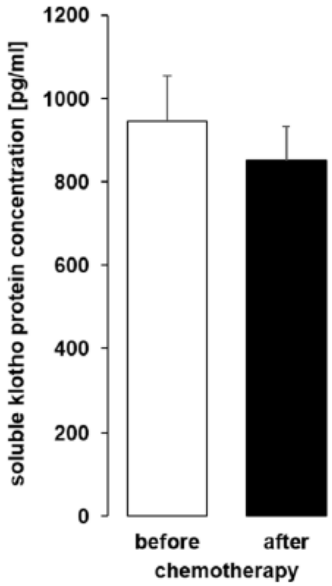

Figure 11. The serum concentration of soluble klotho (sKL) in patients with cancer before and after administration of a cycle of chemotherapy. Arithmetic mean ± SEM of sKL serum concentration (n = 9; *paired* t-test) in patients 24 ± 4 h before and after administration of a cycle of chemotherapy.

NRK-52E cells. According to these results, αklotho expression was upregulated in injured and potentially moribund MDCK and NRK-52E cells prior to their putative death.

Several of the effects of αklotho on major intracellular signaling pathways can be expected to be pro-apoptotic: Inhibition of IGF-1 and insulin signaling [52] as well as Wnt signaling [53]. Also αklotho's role as a tumor suppressor fits to the concept of αklotho being pro-apoptotic [52]. Accordingly, our findings, i.e., up-regulation of αklotho in MDCK and NRK-52E cells prone to death, may be a novel aspect of the cellular machinery which is part of the initiation and/or execution of apoptosis. Other effects of αklotho including increased anti-oxidant resistance [54], further anti-apoptotic properties [55], or reduced inflammation [29] may rather be associated with being pro-survival. In view of the latter aspect of αklotho signaling, up-regulation of αklotho in damaged and/or dying cells as revealed by our study could therefore be interpreted as an attempt to enhance cellular stress resistance and possibly overcome the injury. In line with this, αklotho has been shown to counteract another form of cell death, necroptosis [46]. Definitely, further studies are necessary to decipher the precise role of increased αklotho expression in cells exposed to potentially deadly noxae.

In an attempt to identify the mechanism underlying αklotho up-regulation in MDCK and NRK-52E following exposure to cytotoxic agents or other apoptosis stimulants, we uncovered a role for transcription factor PPARγ. PPARγ has been demonstrated to be relevant for αklotho expression [48] and is upregulated itself by cisplatin [49]. In line with this, we could confirm that the chemotherapeutics up-regulate *PPARG* in both, MDCK and NRK-52E cells. Moreover, using PPARγ antagonist SR-202 we demonstrated that the cisplatin effect on αklotho in MDCK cells is indeed dependent on PPARγ albeit other factors are likely to be involved, too.

In the kidney, transmembrane αklotho forms a complex with FGFR1, yielding the receptor for bone-derived hormone FGF23 [12]. In line with stimulation of αklotho expression, the cytotoxic agents and apoptosis inducers also up-regulated FGFR1 in MDCK cells.

While our experiments clearly demonstrated up-regulation of αklotho in apoptotic MDCK and NRK-52E cells and uncovered PPARγ as a factor explaining, at least in part, this effect, a completely different response was found in HK-2 cells: The same treatment down-regulated both, αklotho gene expression and sKL concentration in the cell culture supernatant. Several factors may contribute to this discrepancy: Firstly, HK-2 is a human proximal tubule cell line from normal kidney that has been immortalized with human papilloma virus (HPV 16) E6/E7 genes, and these two genes are part of its genome [56]. In contrast, MDCK and also NRK-52E cells are spontaneously immortalized cells [57]. As a matter of fact, E6 and E7 genes used to immortalize HK-2 cells render them more resistant to apoptotic stimuli [58], an effect that may help explain the different response of HK-2 cells observed in our study. Secondly, it also appears possible that the origin of the cells (MDCK cells: dog, NRK-52E: rat, HK-2: human) also contributes to the different response [59]. Thirdly, the renal localization of αklotho may play a role: It is expressed in proximal and, at a higher level, in distal tubule. Renal phosphate handling mainly occurs in the proximal tubule, but its regulation is more dependent on αklotho in the distal tubule [60, 61]. MDCK cells are from distal tubule [62], whereas NRK-52E cells are from proximal tubule [63] as are HK-2 cells [64]. Therefore, the different origin of the cell lines may also contribute to the contrasting results. Moreover, it has to be kept in mind that renal cell lines are only models that do not reflect all aspects of kidney physiology [65]. Therefore, our diverging results using the three different kidney cell lines also underscores that care must be taken when studying αklotho in cell culture.

In a pilot human study, we studied the impact of one cycle of chemotherapy on serum sKL in patients suffering from different types of cancer. We did not observe a significant change of sKL after chemotherapy. It is a major limitation of this small pilot study that patients with different forms of cancer, different chemotherapeutic regimens and different treatment cycles were included. Hence, several aspects may be relevant for our finding: Different forms of cancer themselves impact on αklotho [66]. Moreover, the disease stage and also the number of chemotherapy cycles may influence the effect on αklotho. Although distal tubule is thought to be the main source of sKL [61], also proximal tubule may produce sKL. Given the different response of distal tubular MDCK and proximal tubular HK-2 cells to chemotherapeutics, it appears to be possible that divergent effects also play a role in the human kidney. Definitely, further human studies are warranted to define possible effects of cytotoxic agents on sKL.

Since αklotho plays a particular role in patients with severe disease (e.g., CKD patients [67]), it would of course be of high clinical interest to know whether different responses of αklotho to chemotherapeutics are of clinical relevance and may reflect a different

Table 2. Primers.

Gene	Species	Primer sequence (5′ → 3′)
klotho	dog	AAATGAAGCTCTGAAAGCC and AATGATAGAGGCCAAACTTC
TBP	dog	CCTATTACCCCTGCCACACC and GCTCCCGTACACACCATCTT
klotho	rat	CAACTACATTCAAGTGGACC and CAGTAAGGTTTTCTCTTCTTGG
TBP	rat	ACTCCTGCCACACCAGCC and GGTCAAGTTTACAGCCAAGATTCA
klotho	human	TGGAAACCTTAAAAGCCATCAAGC and CCACGCCTGATGCTGTAACC
TBP	human	TGCACAGGAGCCAAGAGTGAA and CACATCACAGCTCCCCACCA
PPARG	dog	CCTCACGAAGAGCCTTCCAA and CCGGAAGAAGCCCTTGCAT
PPARG	rat	GAAGCTGTGAACCACTAATATCCA and GCTCTTGTGAACGGGATGTCT
FGFR1	dog	AGACAGGTAACAGTGTCGGC and ACGGTTGGGTTTGTCCTTGT
BAD	dog	CCAGTGAGCAGGAAGACTCC and TTCCTTCATCCTCGTCGGTC
BAX	dog	GATGGCAACTTCAACTGGGG and AAGCACTCCAGCCACAAAGA
BCL-2	dog	GGTGAACTGGGGGAGGATTG and TCAAACAGAGGCTGCATGGT

response to the treatment. This should be addressed in further studies.

In conclusion, our study shows that the expression of αklotho gene is stimulated in MDCK or NRK-52E cells exposed to cytotoxic chemotherapeutics cisplatin, doxorubicin or paclitaxel or treated with apoptosis inducers PAC-1 or serum depletion. The effect is, at least in part, dependent on PPARγ. In contrast, the same treatment down-regulates αklotho gene expression and sKL protein in HK-2 cells.

MATERIALS AND METHODS

Cell culture

Madin-Darby Canine Kidney cells (MDCK; CCL-34, ATCC, Manassas, VA, USA) were cultured at 37°C and 5% CO_2 in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12; (Gibco, Life Technologies, Darmstadt, Germany) plus 5% fetal bovine serum (FBS; Gibco), 1% glutamine, and 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco). NRK-52E (CRL-1571, ATCC) cells were cultured in DMEM (Gibco) with 5% newborn calf serum (NBCS; Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco) at 37°C and 5% CO_2. Human HK-2 cells (CRL-2190, ATCC) were cultured in DMEM with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C and 5% CO_2. For the experiments, cells were first seeded into 6-well plates (Greiner Bio-One, Frickenhausen, Germany) for 24 h. Subsequently, cisplatin, PAC-1, doxorubicin (all from Tocris Bioscience, Bristol, UK), or paclitaxel (MP Biomedicals, Eschwege, Germany) were added for 24 h consent indicated. For serum starvation, culture medium was replaced by serum free medium. After 24 h, cells were either trypsinated and counted with a Neubauer hemocytometer or analyzed for RNA isolation. Selective PPARγ inhibitor SR-202 (Biomol, Hamburg, Germany) was added to the culture medium along with cisplatin at 200 µM. Cell culture supernatants were collected and frozen for further use.

Quantitative real time PCR

RNA isolation was accomplished by means of RNA-Solv reagent (Omega Bio-Tek, Norcross, GA, USA). For cDNA synthesis 1.2 µg of total RNA was transcribed with the GoScript Reverse Transcription System and random primers (Promega, Mannheim, Germany). Quantitative real time PCR (qRT-PCR) using 2 µl of total cDNA was performed in reaction mixes containing 0.25 µM (αklotho) and 0.5 µM (TATA-binding protein, TBP) of each primer, 10 µl GoTaq qPCR Master Mix (Promega), and sterile water.

The primers used in qPCR analysis are provided in Table 2. αklotho, *PPARG, FGFR1, BAD, BAX, and BCL-2* mRNA levels were normalized to *TBP* mRNA.

Viability assay (MTT assay)

Cells were seeded into 96-well plates and treated as described for 24 h and for another hour with 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT; Sigma-Aldrich, Schnelldorf, Germany). Thereafter, the MTT solution was replaced by dimethyl sulfoxide (DMSO; AppliChem, Darmstadt, Germany), and absorption was measured at 550 nm and 690 nm (reference) on a FluoStar Omega plate reader (BMG Labtech, Ortenberg, Germany). Results were

normalized to vehicle-treated cells and are given as percentage of viable cells.

ELISA

HK-2 supernatants and patients' serum samples were subjected to ELISA for measurement of soluble αklotho protein according to the manufacturer's protocol (IBL, Hamburg, Germany).

Apoptosis and necrosis assay

The rate of apoptosis and necrosis was measured using the RealTime-Glo Annexin V Apoptosis and Necrosis Assay (Promega) according to the manufacturer's protocol.

Western blotting

MDCK cells were cultured in T25 cell culture flasks (Greiner Bio-One) for 24 h under standard conditions, then incubated with or without 10 µM cisplatin for another 24 h. After cell lysis using RIPA buffer (Cell Signaling Technology, Frankfurt, Germany) supplemented with protease and phosphatase inhibitor cocktail and EDTA (Halt, Thermo Scientific), total protein concentration was measured by Bradford assay (Bio-Rad). Thirty µg of total protein were subjected to standard 10% SDS-PAGE and Western Blotting. The following antibodies were used: anti-FGF receptor 1 (D8E4), anti-β-actin (8H10D10), anti-rabbit IgG HRP-linked (all from Cell Signaling Technology), and anti-mouse IgG HRP-linked antibody (Abcam, Cambridge, UK). For visualization, membranes were incubated for 2 min with Westar Nova 2.0 (β-actin) or Westar Supernova (FGFR1) ECL substrate (both from Cyanagen, Bologna, Italy). Densitometrical analysis was performed on a C-Digit® Blot scanner (Li-Cor, Lincoln, NE, USA) and FGFR1 bands were normalized to β-actin bands using the Image Studio™ software (Li-Cor).

Patients

Serum samples were collected from cancer patients of the Department of Oncology, University Hospital of Martin-Luther-University Halle-Wittenberg, Halle (Saale), Germany. The study was approved by the ethics committee of Martin-Luther-University (approval no. 2014–75). Blood samples were collected 20 ± 4 h before and after chemotherapy, centrifuged and frozen at −70°C until analysis. Patient characteristics are depicted in Table 1.

Statistics

Data represent arithmetic mean ± standard error of the mean (SEM) with n denoting the number of independent experiments. Groups were tested for normal distribution using Shapiro-Wilk test. The cell number and viability experiments were analyzed with one-sample *t*-test or alternatively with one-sample Wilcoxon signed rank test, as appropriate. Data with more than two groups were analyzed with repeated measures analysis of variance (ANOVA) followed by Dunnett's multiple comparison test or with non-parametric Friedman ANOVA and Dunn-Bonferroni post-hoc test. If $p < 0.05$, differences were considered significant. SPSS software was used for statistical data evaluation (IBM Version 27.0; Armonk, NY, USA).

AUTHOR CONTRIBUTIONS

S. M., M. F., and L. H. conceived and designed research; S. M., and L. W., conducted experiments; B.E. and D.C. provided material; S.M. analyzed and visualized data; S. M., and M. F. wrote the manuscript; all authors read and approved the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest related to this study.

ETHICAL STATEMENT AND CONSENT

The study was approved by the ethics committee of Martin-Luther-University (approval no. 2014–75). All patients gave informed consent prior to inclusion into the study.

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4 Discussion

Chemotherapy is the most common method to treat malignant cancer diseases[256]. To compare the action of different cytostatic substances on FGF23/αklotho signaling, we selected three different classes of compounds: DNA-intercalating platinum derivative cisplatin, doxorubicin, an inhibitor of topoisomerase II, and paclitaxel as an inducer of cell cycle arrest[230,233,240]. The common mode of action of these cytostatic drugs is the induction of apoptosis in cancer cells[235,240,257]. To investigate the direct impact of apoptosis on FGF23 or αklotho, we additionally selected caspase 3-activator PAC-1[249] and removed serum from the cell culture media for a defined period of time to induce apoptosis[251]. In paper 1, we investigated the influence on *FGF23* expression in UMR106 osteoblast-like cells whereas Paper 2 describes the regulation of αklotho by the aforementioned apoptotic stimulators. The experimental design and results are reported within the papers.

4.1 Paper 1: Cytostatic drugs and apoptosis inducers as regulators of FGF23

In paper 1, we first investigated the transcriptional regulation of *FGF23* following incubation with cisplatin, doxorubicin, PAC-1, and serum depletion in UMR106 osteoblast-like osteosarcoma cell line, which is well established for studying FGF23[203,258,259]. Cisplatin induced a dose-dependent up-regulation of *FGF23* mRNA within 24 and 48 h. Simultaneously, cell proliferation and viability decreased. Similarly, doxorubicin increased *FGF23* gene expression in a dose-dependent manner, while cell number and viability significantly decreased after 24 h. After 48 h, all cells were dead which was probably due to the strong cytotoxic effect of doxorubicin as confirmed by others[260,261]. The induction of apoptosis confirmed by diminished cell viability of osteosarcoma cell lines has already been reported as a frequent effect of cisplatin and doxorubicin[262–265]. Specifically, cisplatin has been reported to initiate apoptosis through ERK1/2 activation, followed by half-life extension and phosphorylation of p53[266–268]. Doxorubicin has similarly been reported to activate ERK1/2 signaling[261]. *FGF23* is a target gene of ERK1/2 signaling[258], which might explain, at least in part, its up-regulation by cisplatin and doxorubicin. Conversely, FGF23/αklotho signaling activates PI3K and SGK1 signaling, stimulating cell proliferation and preventing cell apoptosis[81,269,270]. In mice suffering from AKI, FGF23 ameliorates renal function and prevented cell senescence in an αklotho-independent manner[271]. Thus, the up-regulation of *FGF23* may be a measure to protect cells from apoptosis.

Next, we used apoptotic compound PAC-1 to mimic direct apoptosis induction and study the consequence on *FGF23* expression. In summary, PAC-1 stimulated *FGF23* gene expression by simultaneously decreasing number and viability of UMR106 cells after 24 h. *FGF23* up-regulation could not be observed after 48 h, but viability further declined. Reduced viability caused by PAC-1 treatment has been observed in other, predominantly cancer cell lines[272], with caspase-3-dependent apoptosis being the predominant form of cell death[242,249]. Peterson et al. reported, that PAC-1 activates caspase-3 within less than one hour[242], and

the finding that PAC-1 did not affect *FGF23* expression after 48 h implies that FGF23 is up-regulated during initial apoptosis but not in late-phase apoptosis or secondary necrosis. Likewise, serum reduction (1 % FBS) and complete withdrawal markedly increased FGF23 mRNA and protein levels after 24 h while reducing cell viability and proliferation. After 48 h, cell viability and proliferation were strongly diminished. 1 % FBS still increased *FGF23* expression, whereas complete withdrawal had no effect. This might again be explained by FGF23 up-regulation in initial but not in end-stage apoptosis, as serum depletion causes caspase-3 activation after 4-8 h[250]. Furthermore, increasing concentrations or incubation times of a stress stimulus accelerate the transition from apoptotic to necrotic cell death[182]. Thus, 1 % FBS may not yet lead to necrotic cell death after 48 h. The experiments with reduced FBS or under serum-free conditions were carried out in the presence of 10 nM $1,25(OH)_2D_3$ in the respective culture media to stimulate FGF23 expression and secretion[107,273], otherwise FGF23 protein concentration is not detectable in ELISA. However, $1,25(OH)_2D_3$ does not prevent apoptosis induction by serum depletion in UMR106 cells[274]. Domazetovic et al. confirmed our assumption, that 24 h serum starvation induces apoptosis in bone cells through the activation of caspase-3[275]. The stimulation of *FGF23* expression upon serum starvation has also been observed by others, partially mediated via MAPK c-Jun N-terminal kinase (JNK) and ERK1/2 signaling, as well as NFκB[217]. Some tumor cells develop chemotherapy and stress resistance upon serum deprivation[276-278]. In line with this, increased FGF23 and FGFR1 amounts are associated with cancer progression and therapy resistance[279–281], leading to the assumption, that the *FGF23* up-regulation in UMR106 cells protects cells from cellular stress by cytostatic drugs or growth factor withdrawal.

Increased FGF23 synthesis under inflammatory conditions such as CKD[119], pediatric inflammatory bowel disease[192], or systemic inflammation[195] is a frequent observation. IL-6 plays an important role in acute inflammation by recruiting and stimulating lymphocytes[282]. Consequently, we investigated *IL6* mRNA levels following cisplatin and doxorubicin treatment in UMR106 cells. Following a 24 h-incubation, *IL6* gene expression significantly increased and co-treatment of cisplatin with IL-6 signaling inhibitor SC144 attenuated the cisplatin-induced stimulation of *FGF23*. This indicates the presence of inflammatory processes in cisplatin and doxorubicin-induced cell death. The induction of IL-6 synthesis by cisplatin or doxorubicin has already been observed in other studies[261,283,284] and refers to necrotic cell death[173]. This may be due to secondary necrosis, which is a common issue in cell culture where phagocytic cells are absent[173,285], or necroptosis, referred to as programmed necrosis which has recently been observed as a consequence of cisplatin and doxorubicin treatment[286,287]. Pro-inflammatory cytokine IL6 has been reported to stimulate FGF23 in UMR106 cells and *in vivo*[288]. In conclusion, the up-regulation of *FGF23* after cisplatin or doxorubicin incubation is partially dependent on IL-6 signaling underlining the presence of necrotic cell death.

By targeting mitochondria, cisplatin has been reported to impair glycolysis resulting in intracellular ATP restriction[289,290]. Apoptosis is an ATP-dependent mode of cell death whereas necrosis occurs at low intracellular ATP content[181]. Consequently, intracellular ATP content correlates directly with the rate of apoptosis and inversely with necrosis[181,182] and this may explain the presence of necrotic cell death upon cisplatin incubation. Since doxorubicin has been observed to activate AMPK, a sensor of energy shortage and increased intracellular AMP levels[133,291], it may similarly induce necrotic senescence through ATP restriction[181].

As both, *IL6* and *FGF23* are target genes of NFκB[128,292], we investigated whether cisplatin and doxorubicin stimulate *FGF23* mRNA via NFκB. As shown in paper 1, cisplatin and doxorubicin incubation increased mRNA levels of NFκB subunit *RELA* and NFκB phosphorylation in UMR106 cells. NFκB inhibitors wogonin[293] and withaferin A[294] markedly reduced cisplatin-mediated *FGF23* induction. Although apoptotic cell death is usually not associated with NFκB activity and inflammation[151,152,173], cisplatin and doxorubicin have already been reported to activate NFκB in malignant and normal cells[295–298]. This further underlines the influence of inflammation on the increase in *FGF23* expression. In UMR106 cells, NFκB stimulates FGF23 synthesis[128,197]. Additionally, excess NFκB activity is involved in AKI and CKD[299,300] which are both characterized by excess FGF23 levels[225,301]. Furthermore, due to its nephrotoxic impact, therapeutic cisplatin administration causes AKI[302]. This shows a clear association between NFκB activity, renal diseases, and enhanced *FGF23* expression. In conclusion of our experiments, cisplatin and doxorubicin increase *FGF23*, at least in part, via NFκB.

Other inflammatory cytokines which are involved in cisplatin or doxorubicin-induced inflammation include TNFα, IL-1β, or TGF-β[184,303–307]. Consequently, inflammatory cytokine production may be induced in UMR106 cells exposed to cisplatin or doxorubicin as described in paper 1. All of these cytokines have been reported to increase FGF23 production[308–310] and thus, might participate in the up-regulation of FGF23 in our experiments. Cisplatin or doxorubicin-mediated TGF-β signaling is involved in the activation of p53 and apoptosis[263,311]. TGF-β stimulates FGF23 secretion in osteoblast-like cells via SOCE[308]. TGF-β is strongly involved in renal fibrosis[312,313] and FGF23 excess has equally been linked to the development of fibrosis[314,315].

Another target gene of NFκB, TNFα, is an important pro-inflammatory cytokine secreted by activated macrophages[199,316] and normal tissue cells under inflammatory conditions[317,318]. The cytotoxic effects of cisplatin and doxorubicin have been reported to partially depend on TNFα[184,261,305]. In line with NFκB and IL-6, TNFα increases FGF23 production[309] and is strongly involved in AKI[319] and CKD[320], which are both characterized by excess FGF23 levels[225,321]. In conclusion, FGF23 might be generally increased via inflammatory cytokines following chemotherapeutic drug administration. However, this does not explain

its up-regulation by PAC-1 and serum depletion. Thus, additional mechanisms must be involved in cellular stress response.

Apoptosis is frequently associated with oxidative stress[322]. Cisplatin not only causes nuclear DNA damage, but also accumulates in mitochondria inducing the generation of ROS[290,323], even in osteosarcoma cells[324]. The binding of ROS scavenger glutathione (GSH) protects cells from cisplatin-induced cytotoxicity[325]. Thus, oxidative stress is probably induced in UMR106 cells treated with cisplatin, and this may account for the up-regulation of *FGF23* mRNA. This may be supported by the inhibitory effect of wogonin on cisplatin-induced stimulation of *FGF23*, as wogonin also inhibits nuclear factor erythroid 2-related factor 2 (NRF2), a regulator of antioxidant proteins[293,326]. The cytotoxic effect of doxorubicin similarly depends on the stimulation of oxidative stress in cancer and healthy cardiac and kidney cells[235,327,328]. PAC-1-induced apoptosis correlates with an increase in mitochondrial oxidative stress in cancer cells[329,330]. Furthermore, ROS production can be stimulated via inflammatory signaling, e.g. by TNFα[331] or TGF-β[332]. Domazetovic et al. conducted experiments similar to those described in paper 1 but linked the up-regulation of FGF23 by serum depletion to the influence of oxidative stress[217]. Likewise, excessive phosphate concentration in UMR106 culture media has been reported to stimulate *FGF23* expression by increasing intracellular amount of ROS[258]. Therefore, generation of ROS might be responsible for the increase in *FGF23* by all cytotoxic stimuli. In turn, FGF23 increases NRF2 in osteoblasts in a αklotho-independent way, thereby stimulating the production of antioxidant scavengers or enzymes and reducing oxidative stress[333]. In conclusion, the up-regulation of *FGF23* as a consequence of oxidative stress may promote cell protection by increasing antioxidant proteins.

Inflammation and oxidative stress are frequently associated with HIF1α stabilization[334]. This transcription factor usually responds to hypoxic conditions to regulate target genes involved in angiogenesis[335], erythropoiesis[336] as well as tissue regeneration after injury[337,338], cellular stress resistance and survival[339,340]. HIF1α stabilization is a frequent event following cisplatin or doxorubicin administration and correlates with chemotherapy resistance[339,341]. Beside the activation of caspase-3, stabilization and accumulation of active HIF1α is an observed effect of PAC-1 in cancer cells[342]. In line with this, also serum deprivation has been shown to induce HIF1α production in cancer cells promoting resistance against starvation stress[343]. Wogonin, used against NFκB activation in UMR106 cells, has additionally been reported to down-regulate HIF1α[344]. This implies, that HIF1α may be stabilized in UMR106 cells exposed to cytotoxic stimuli used in paper 1. Subsequently, HIF1α is a strong activator of FGF23 production and co-overexpressed in tumors resected from TIO patients[345]. HIF1α protects cells from oxidative stress and apoptosis[334,346] possibly via up-regulation of FGF23 promoting cell survival[333]. Thus, all cytotoxic treatments may regulate *FGF23* via HIF1α to increase cell resistance. Interestingly, FGF23-cleaving protease furin is up-regulated under hypoxic conditions via HIF1α activity[196] suggesting that only C-terminal FGF23 increases after HIF1α

stabilization[25]. In line with this, osteocytes exposed to pro-inflammatory cytokines TNFα, TNF-like weak inducer of apoptosis (TWEAK), IL-1β, or bacterial lipopolysaccharides produce excess amounts of C-terminal, but not intact FGF23[193]. Acute renal inflammation in mice is associated with decreased serum iron as well as greatly increased C-terminal but weakly increased intact FGF23 production[195]. In this context, C-terminal FGF23 has been reported to alleviate iron shortage and acute inflammation[347] and inhibit FGF23-FGFR1 receptor interaction to block phosphaturic actions of FGF23[21]. This may be supported by the observation, that phosphate levels are increased in AKI and correlate with increased C-terminal FGF23 and mortality hazard[225,348]. These reports indicate an important bidirectional influence of FGF23 in acute inflammation, independent of its phosphate-regulating function.

In acute bone injury, TNFα activates p38 MAPK and NFκB in bone cells[199] which subsequently stimulates FGF23 synthesis[201,203]. Excessive FGF23 levels are associated with suppressed osteoblast differentiation and bone synthesis[349]. Especially chronic inflammation goes along with bone loss[191,350]. Cisplatin has been reported to inhibit bone formation[351] and also doxorubicin inhibits osteoblasts while promoting osteoclastogenesis[352] causing bone loss in mice[353]. In line with this, serum deprivation stimulates osteoclastogenesis resulting in increased bone resorption, an effect which is reinforced by FGF23 excess[217,354,355]. Thus, the observed up-regulation of *FGF23* by cisplatin or doxorubicin treatment possibly explains, at least in part, bone loss during chemotherapy[353,356,357]. In summary, cellular stress through cytotoxic substances or apoptosis induction stimulates inflammation, oxidative stress, and HIF1α stabilization, and all these stress responses may increase FGF23 to promote cell protection.

4.2 Paper 2: Chemotherapeutic drugs and apoptosis stimulants regulate αklotho

Beside their cytostatic effects on cancer cells, chemotherapeutic drugs often exert organotoxic, and especially nephrotoxic properties which is one of the main factors responsible for dose limitations[233,358,359]. The first step in renal toxicity is the absorption and accumulation of cytotoxic compounds in tubular epithelial cells via different transport mechanisms[360]. Cisplatin is predominantly excreted in the urine[361], entering the renal tubular epithelium basolateral via organic transporter 2 (OCT2)[362], copper transporter 1(CTR1)[363], or organic anion transporters OAT1 and OAT3[364]. Doxorubicin is absorbed by renal tubular cells via organic anion transporter polypeptide 1 (OATP1)[365]. In the cellular uptake of paclitaxel OAT2[366] and OATP1[367,368] are involved. Nephrotoxicity is characterized by tubular inflammation, necrosis, and apoptosis, accompanied by declined glomerular filtration resulting in the accumulation of waste products e.g. urea and creatinine and the loss of electrolytes[222,369]. The impairment of kidney cells may subsequently affect αklotho, which is predominantly produced in proximal and distal tubule cells[370]. Thus, paper 2 investigates the regulatory impact of cytotoxic and apoptotic compounds on αklotho in three different renal cell lines.

In the first set of experiments, αklotho regulation was investigated in canine distal tubule cell line MDCK and rat proximal tubule cell line NRK-52E following a 24 h-incubation with cisplatin, paclitaxel, doxorubicin, PAC-1, or under serum depletion. Both cell lines are well established for *in vitro* studies concerning αklotho[208,371–373]. Cisplatin up-regulated αklotho transcripts in MDCK and NRK-52E cells. In both cell lines, cisplatin decreased the cell number, whereas cell viability was only attenuated in NRK-52E cells. A combined apoptosis and necrosis assay revealed that cisplatin predominantly induced apoptosis, which was confirmed by up-regulation of pro-apoptotic *BAD*, *BAX*, and *BAX*/*BCL-2* ratio. In line with this, also paclitaxel up-regulated αklotho gene expression in MDCK and NRK-52E cells, simultaneously diminishing cell viability and proliferation. In contrast to cisplatin, paclitaxel induced apoptosis and to a lesser extent, necrosis in both cell lines, simultaneously increasing expression of pro-apoptotic *BAX* gene. As a third cytotoxic drug, anthracycline doxorubicin up-regulated αklotho mRNA in MDCK and NRK-52E cells while reducing cell viability and proliferation to varying degrees. In MDCK cell line, the dominating mode of cell death after doxorubicin application was apoptosis, however in NRK-52E cells, necrosis occurred too. Doxorubicin markedly increased *BAD*, *BAX*, and *BAX*/*BCL-2* ratio, indicating apoptosis induction[374]. Beside their action on cancer cells[257,375,376], cisplatin, paclitaxel, and doxorubicin induce apoptosis and decrease cell proliferation and viability in different non-tumorigenic cells[261,359,377]. The induction of apoptotic cell death is frequently confirmed by detecting the up-regulation of pro-apoptotic *BAD* or *BAX* as well as down-regulation of anti-apoptotic *BCL-2*[375,378,379].

Caspase-3 activator PAC-1 up-regulated αklotho mRNA levels while decreasing cell proliferation and viability in MDCK and NRK-52E cells. In MDCK cells, the mode of cell death was predominantly

apoptosis, while in NRK-52E cells also necrosis was detectable. Along with αklotho, PAC-1 increased *BAX* and *BAX*/*BCL-2* ratio in MDCK cells, confirming apoptotic cell death[378]. PAC-1 directly activates caspase-3[242] which is associated with decreased cell viability due to apoptotic cell death[249,272]. The presence of necrotic cell death implies secondary necrosis due to the lack of phagocytic cells in the cell culture[285]. Although BAX activation precedes and triggers caspase-3 activation in apoptosis[380], we observed that PAC-1 increased *BAX* and *BAX*/*BCL-2* mRNA in our experiments. This might be due to a PAC-1-mediated, caspase-3-independent activation of p53, BAK, and BAX observed by others, which has not been completely elucidated yet[329,381]. In summary, cisplatin, paclitaxel, doxorubicin, and PAC-1 may all induce apoptosis via p53, which is a regulator of *BAX* and *BCL-2* genes[158]. Serum depletion increased gene expression of αklotho along with apoptotic cell death in MDCK cells. Cell viability and proliferation markedly decreased after 24 h. The induction of apoptosis in different cells exposed to serum depletion has also been reported by other researchers[250,275] confirming our results. In NRK-52E cells, the rise in αklotho expression after serum depletion was not significantly changed, although cell proliferation and viability decreased due to exclusively apoptotic cell death. The reason may be a slower intracellular drug accumulation caused by a slower metabolism of NRK-52E compared to MDCK cells. This is confirmed by the approximate doubling times of 45 h in NRK-52E[382] and 18 h in MDCK cells[383]. Thus, NRK-52E cells possibly need longer starvation periods to up-regulate αklotho. Apoptotic proteins of the BCL-2 family are partially regulated on the basis of transcription but in case of BAD and BCL-2 also through phosphorylation[158,384,385]. As we did not observe transcriptional regulation of *BAX*, *BAD*, or *BCL-2* after serum deprivation, apoptosis may be induced via phosphorylation of BCL-2 protein, but this remains to be determined.

Cisplatin, paclitaxel, doxorubicin, PAC-1, or serum deprivation all reduced cell proliferation and viability and induced apoptosis in MDCK and NRK-52E cells. Due to its nephrotoxic properties, cisplatin has already been linked to AKI[187,222,386]. Doxorubicin has equally been reported to induce AKI by increasing wnt signaling, and increasing TGF-β as well as angiotensin II abundance in kidney cells[387]. In case of paclitaxel, nephrotoxic effects have been reported[359] but little is known about the molecular mechanisms. Renal tubular cell apoptosis is a characteristic feature in AKI[388]. Consequently, it is suggested, that cisplatin, paclitaxel, and doxorubicin but also PAC-1 and serum deprivation induced AKI-like conditions in MDCK and NRK-52E cells. Furthermore, AKI is tightly associated with inflammatory processes[389]. In line with this, cisplatin, paclitaxel, or doxorubicin stimulate the secretion of pro-inflammatory cytokines e.g. TNFα, IL-6, or IL-1β[261,305,390]. As mentioned before, this may be due to necroptotic cell death[286,287,391]. Cytokines including interferon γ, TNFα, or TWEAK have been reported to down-regulate αklotho[211,392]. In line with this, low αklotho levels have been observed in inflammatory disorders like AKI[393], CVD[213], systemic

inflammation[394], and colitis[392]. Therefore, the up-regulation of αklotho in MDCK and NRK-52E cells exposed to cytotoxic noxae is in contrast to many other investigations.

However, increased αklotho production has also been described in a mouse auditory cell line exposed to cisplatin[395]. Ototoxicity is a common side effect of cisplatin[396,397]. The authors suggested a protective role of the αklotho up-regulation and underlined its significance as a biomarker predicting cellular injury[395]. Likewise, skeletal muscle injury strongly increased the amount of αklotho in tissue and serum of young mice (4-6 months)[398]. Simultaneously, αklotho levels of old mice (22-24 months) remained unchanged after injury[398]. This indicates an age-dependent effect of αklotho regulation but although αklotho declines with progressing age, differential regulation has barely been adressed[41]. Furthermore, one group reported higher αklotho levels in individuals who had already suffered from a myocardial infarction compared to individuals without former infarction history[399]. This may point to an important role of αklotho as a biomarker of myocardial damage but also to its therapeutic effect, since αklotho shows cardioprotective effects[58,400,401]. All these reports have in common, that αklotho is up-regulated following local injury probably as a novel aspect to protect or restore normal cell function. The fact that αklotho is almost undetectable in normal muscle cells but strongly enhanced upon injury[398], supports the thesis that it has no regular function in most healthy tissues but participates in pathophysiology.

Beneficial effects of αklotho have not only been observed in the heart but also in AKI[387] as well as fibrosis, where it inhibits TGF-β signaling by blocking TGF-β receptor and thereby ameliorates renal function[402]. αKlotho suppresses TNFα-mediated activation of renal NFκB and subsequent production of pro-inflammatory cytokines[51]. Consequently, up-regulation of αklotho expression may serve to protect the cells from injury progression and to restore physiological function.

Transcription factor PPARγ regulates insulin sensitivity[403] and adipogenesis[404] and is a positive regulator of αklotho[371]. Thus, we investigated whether cytotoxic compounds affect PPARγ expression in MDCK and NRK-52E cells. And in fact, PPARγ mRNA was stimulated upon cisplatin, paclitaxel, or PAC-1 treatment in both cell lines, whereas doxorubicin stimulated PPARγ only in MDCK and serum depletion increased PPARγ expression only in NRK-52E cells. SR202, a selective PPARγ antagonist, reduced cisplatin-mediated αklotho stimulation in MDCK cells indicating that αklotho up-regulation by cisplatin is partially due to PPARγ stimulation. Cisplatin, paclitaxel, or serum deprivation treatment has already been reported to up-regulate PPARγ expression[405]. PPARγ signaling is transduced via PPAR-responsive element within the αklotho gene[371]. In contrast to our results, doxorubicin has been reported to decrease PPARγ in adipocytes[406], which may indicate a differential regulation of PPARγ in different tissues. This could also be the reason for differential PPARγ regulation in MDCK and NRK-52 E cells exposed to doxorubicin or serum depletion: MDCK originates from the distal tubule[407] whereas NRK-52E cells are isolated from the proximal tubule[382]. PPARγ activation promotes insulin sensitivity[403] whereas αklotho suppresses

insulin/IGF-1 signaling promoting insulin resistance of adipocytes[408]. This may indicate a highly regulated feedback mechanism in glucose metabolism, which needs to be further investigated.

Due to the fact, that αklotho serves as a co-receptor of FGFR1[32], we further investigated whether up-regulation of αklotho is associated with an increase in *FGFR1* expression. As depicted in Paper 2, cisplatin, doxorubicin, PAC-1, or serum depletion significantly increased *FGFR1* mRNA in MDCK cells. The same applied for FGFR1 protein in cell lysates after cisplatin-incubation. The overexpression of FGFR1 in several tumor cells correlates with resistance to chemotherapy[281,409,410]. Thus, FGFR1 up-regulation in MDCK cells may indicate resistance against cytotoxic treatments. Furthermore, we observed detectable levels of *FGF23* mRNA in MDCK cells after cisplatin treatment whereas FGF23 was undetectable in vehicle treated cells. Amplification of FGF/FGFR signaling stimulates PI3K/Akt pathway and subsequently promotes cell proliferation and suppresses apoptosis[411,412]. In line with this, FGF23/αklotho signaling prevents $1,25(OH)_2D_3$-mediated apoptosis in the kidney via PI3K/Akt pathway[269]. Thus, simultaneous up-regulation of αklotho and FGF23 in MDCK cells presumably protects the cell against cytotoxicity.

In order to assess αklotho protein levels, we used human proximal tubular cell line HK-2, which is another well-established model for investigating αklotho[413,414]. In contrast to MDCK and NRK-52E cells, αklotho mRNA and soluble klotho protein in the cell culture supernatant decreased after 24 h-incubation with cisplatin and doxorubicin. Paclitaxel-treatment only diminished mRNA, but not protein levels. PAC-1 did not affect αklotho expression and protein secretion in HK-2 cells, whereas serum depletion significantly decreased αklotho mRNA and protein amount. Similar to MDCK and NRK-52E cells, cisplatin has been reported to induce apoptosis in HK-2 cells, confirmed by caspase and p53 activation, and *BAX* and *BAD* up-regulation[415]. Likewise, doxorubicin induces apoptosis in HK-2 cells via p53 activation[261]. It is therefore likely to assume that apoptotic cell death was induced in HK-2 cells.

To evaluate the differential regulation of αklotho in MDCK, NRK-52E, and HK-2 cells, it is necessary to consider the different characteristics of these cells concerning immortalization, species, and sensitivity. Immortalization of HK-2 cells has been achieved by transfection with human papillomavirus 16 (HPV16) E6/E7 genes which were discovered to immortalize epithelial cells without significantly changing their phenotype or specific cell functions[416,417]. E6 gene product binds p53 and promote its proteasomal degradation, resulting in unlimited cell proliferation[418]. In contrast to this, MDCK and NRK-52E are spontaneously immortalized[382,407,419]. Garcia-Perez et al. observed higher sensitivity of HK-2 cells against oxidative stress compared to LLC-PK1 cells, which is a spontaneously immortalized porcine kidney cell line[420,421]. Specifically, ROS production in HK-2 cells far exceeded ROS levels in LLC-PK1 cells while antioxidant glutathione levels in HK-2 cells were depleted and antioxidant enzymes were up-regulated after ochratoxin A-treatment, which is a strong indication for oxidative stress[421]. Ochratoxin A is a mycotoxin with nephrotoxic properties that induces apoptosis in kidney cells[422,423]. Another study observed decreased

sensitivity of immortalized renal cell lines including NRK-52E compared to primary kidney cells exposed to ochratoxin A[424]. In a direct comparison of HK-2 with renal cancer cell lines, cisplatin induced apoptosis in HK-2 cells to a larger extent than in renal cancer cells[425], hinting at increased resistance of cancer cell lines to cisplatin. However, since immortalized cell lines share aspects of normal and cancer cells[426], particularly with regard to initiation and execution of apoptosis, results cannot always be transferred to native cells. In conclusion, these reports hint at increased sensitivity of HK-2 cells against nephrotoxicity compared to MDCK and NRK-52E cells. Therefore, HK-2 cells presumably better reflect the conditions in an organism. However, cell culture is only a model and does not completely reflect the physiology within the kidney of a living organism[427].

Furthermore, the species of origin of the cells e.g. rat, dog, and human might differ with regard to drug intake and efflux transporters as well as susceptibility, influencing the execution and outcome of apoptotic signals. For instance, HK-2 cells do not express transporters involved in drug intake including OCT2, OAT1, OAT2, OAT3 but express OATP and CTR1 transporter[428,429], NRK-52E express at least OCT2 and CTR1[427,429], whereas for MDCK cells, no data could be found. In mice, an age-dependent regulation of αklotho during muscle injury has been reported[398]. Likewise, Handl et al. observed passage-dependent susceptibility of HK-2 cells towards cisplatin[430]. Furthermore, also the sex of the donor organism might impact αklotho regulation, although αklotho levels in primates and mice showed contradictory sex-dependent correlation[431,432]. HK-2 cells are derived from a male subject[433], MDCK cells originate from a female dog[407] whereas the sex of the NRK-52E donor animal is not known[382]. Therefore, female organisms may tend to a positive regulation of αklotho whereas males rather down-regulate αklotho. This hints to an association between αklotho and sex hormones *in vivo*. And in fact, female αklotho knockout mice have significantly decreased estrogen levels and hyperphosphatemia, whereas estradiol supplementation decreased renal abundance of NaP_iIIa and NaP_iIIc as well as serum phosphate[434]. Furthermore, estradiol reduced oxidative stress induced by αklotho deficiency[434]. In a cell culture, estrogen is supplemented via serum component[435]. However, estrogen does not completely explain the regulation of αklotho as it is equally affected in serum free culture media.

In our experiments, αklotho mRNA and protein levels did not change upon PAC-1 treatment. Procaspase-3 is frequently up-regulated in cancer cells[244,246] but might be at normal level in HK-2 cells and therefore, caspase-3 activation and apoptosis induction occurred only to a small degree. Likewise, PAC-1-induced apoptosis has been reported to be much stronger in cancer, than in normal blood cells[436]. This implies, that αklotho is only regulated by apoptosis induction and PAC-1 induces apoptosis in HK-2 cells only to a minor degree. In conclusion, the αklotho up-regulation in MDCK and NRK-52E cells might provide cellular stress protection to restore normal function in cancer-like cells. In turn, since renal αklotho levels are decreased

by cisplatin or doxorubicin *in vivo*, this is suggested as the physiologic response of renal tubular cells to cytotoxic noxae[211,437]. However, this topic requires further intensive investigation.

To additionally assess the impact of cytotoxic drugs on soluble klotho levels in the human organism, we examined αklotho serum concentration of patients receiving chemotherapy 24 h before and after drug administration. We observed no significant change in αklotho concentration before and after drug administration. This might be due to heterogeneous drug combinations, varying treatment cycles, and heterogeneous patient and cancer characteristics influencing systemic αklotho amounts. Three independent studies observed that mice or rats injected with a single dose of cisplatin or doxorubicin had significantly decreased αklotho expression and lower protein abundance compared to controls[211,437,438]. αKlotho overexpression during cisplatin-based chemotherapy reduces cisplatin uptake via OCT2 resulting in lower caspase-3 activation and smaller BAX/BCL-2 ratio, indicating reduced apoptosis[438]. Likewise, αklotho gene transfer has been confirmed as a promising tool to improve renal function in AKI in mice[439]. Consequently, the impact of chemotherapy on αklotho needs to be further investigated, as a decrease in αklotho may affect cancer progression outcome and mortality[224,440].

αKlotho exerts numerous protective and anti-apoptotic functions on non-cancerous cells[54,441–443]. However, in cancer cells it suppresses excessive proliferation and promotes apoptosis[224,444–446]. Growth factor signaling is frequently overexpressed in cancer e.g. IGF-1 or FGFR/FGF[68,412,447] induce ERK1/2 phosphorylation and subsequent PI3K/Akt activation[411,448,449]. Active Akt phosphorylates BAD, thereby preventing it to bind to BCL-XL and this consistently suppresses apoptosis[450]. Additionally, IGF-1 up-regulates anti-apoptotic BCL-2[451] and BCL-XL[452]. αKlotho suppresses IGF-1 signaling in cancer cells[62,70,449], thereby promoting apoptosis[444]. Overexpression of wnt/β-catenin pathway is another mechanism of cancer cells to promote excessive cell proliferation[67,446]. By suppressing wnt/β-catenin signaling, αklotho inhibits tumor growth and promotes apoptosis e.g. in liver cancer[69,453]. Thus, another important mechanism of cancer cells to prevent cell death is the downregulation of αklotho synthesis[11,453,454]. In summary, the overexpression of growth factor signaling, down-regulation of αklotho, or loss of function of pro-apoptotic factors such as p53 strongly promotes chemotherapeutic drug resistance[11,409,455]. On the other hand, αklotho supplementation or overexpression has been shown to sensitize cancer cells to chemotherapy by overcoming drug resistance[456,457].

Resistance to cisplatin, paclitaxel, or doxorubicin has also been linked to HIF1α activation in cancer cells[339,344,458,459]. αKlotho overexpression has been reported to decrease HIF1α levels in colon cancer which is associated with a decrease in cisplatin-resistance[11,460]. *In vitro*, Cisplatin[461], paclitaxel[462,463], doxorubicin[339], PAC-1[342], and serum deprivation[343] have been reported to activate HIF1α signaling. In general, HIF1α stabilization plays an important role in the adaptation of cells to stress to prevent further damage[464]. Cisplatin-induced AKI decreases renal vascular perfusion and renal blood pressure causing

hypoxia and HIF1α activation[465,466]. Likewise, with progression of doxorubicin-induced kidney injury in mice the abundance of HIF1α increases[467]. AKI and CKD are associated with reduced αklotho levels[44,208] and therefore αklotho may correlate negatively with HIF1α stabilization, as has been observed in colorectal cancer[460]. Hypoxia has been shown to down-regulate αklotho expression via HIF1α signaling in retinal cells[468] but did not change αklotho production in the kidney[469]. HIF1α may contribute to the decrease in αklotho synthesis observed in HK-2 cells. During hypoxia, this may be a measure to increase cell proliferation by decreasing αklotho-mediated suppression of wnt/β-catenin or IGFR signaling[449,453]. In conclusion, αklotho may be of therapeutic value to overcome HIF1α-mediated chemotherapy resistance[339] and in the treatment of chemotherapy-induced AKI[470].

Cytotoxic properties of cisplatin, paclitaxel, or doxorubicin on cancer cells are frequently linked to oxidative stress[147,235,323]. However, excessive ROS production has also been observed in kidney tissue exposed to chemotherapeutic drugs[303,328,376,471,472]. Likewise, serum deprivation increases ROS and reduces GSH production in HK-2 cells[322]. PAC-1 has been shown to induce the production of ROS in cancer cell lines[329] but its effect in normal cells is not clear. αklotho production is negatively affected by hydrogen peroxide or inducers of ROS *in vitro* and *in vivo*[218,473]. In turn, αklotho suppresses ROS formation[53] and stimulates production of radical scavenger GSH and antioxidant enzymes via NRF2 and forkhead-box-protein O3 (FOXO3)[474,475]. In conclusion, oxidative stress may be another reason for the decrease in αklotho in HK-2 cells. Conversely, in MDCK and NRK cells αklotho up-regulation might be a protective mechanism to diminish oxidative stress and promote cell survival.

Oxidative stress[476,477], Hypoxia[476], and starvation stress[478] activate intracellular energy sensor AMPK. By targeting mitochondria, cisplatin impairs glycolysis resulting in intracellular ATP restriction[289,290]. High levels of ATP degradation-product AMP are responsible for the activation of energy sensor AMPK[133]. Like cisplatin, doxorubicin and serum depletion have been reported to activate AMPK in different non-tumorigenic cells[291,478–480]. In lung cells, AMPK has been shown to reduce inflammation and positively regulate αklotho[481] and in neuronal cells, αklotho increases due to energy restriction[482,483]. In line with this, αklotho deficiency is associated with AMPK downregulation in smooth muscle cells[484]. This indicates that AMPK activation via oxidative stress or HIF1α may positively regulate αklotho production. Additionally, PPARγ is a positive regulator of AMPK as well as αklotho[371,485] and we confirmed PPARγ signaling to be partially responsible for the increase in αklotho in MDCK cells.

The AMPK increase in renal tubular cells exposed to cisplatin correlates with down-regulation of mTOR[486,487]. Comparably, doxorubicin inhibits mTOR signaling in cardiomyocytes and ventricular tissue[488]. As mTOR is a negative regulator of αklotho[489], chemotherapy-induced mTOR inhibition might up-regulate αklotho in MDCK and NRK-52E cells. However, there are also reports of decreased AMPK activity after cisplatin treatment e.g. in HK-2 cells[490] or mice[491]. By reviewing the influence of doxorubicin

on AMPK, Timm et al. noted a similar discrepancy between several studies[492]. This may be due to a hypoxia-induced, AMP-independent activation of AMPK as observed by others[476]. In the reported mechanism, hypoxia-induced ROS formation and subsequent SOCE results in an AMPK activation[476]. As mentioned before, the intracellular ATP content correlates directly with the number of apoptotic cells in a culture[182]. The ATP content decreases with increasing concentration or incubation time of a cytotoxic substance and subsequently, apoptotic cell death changes into necrotic cell death[182]. Therefore, cytotoxic stimuli possibly knockdown AMPK early or at low concentrations of cytotoxic stimuli and activate AMPK with increasing concentration and incubation time. This subsequently affects αklotho in a negative way during the apoptotic phase of cellular injury and stimulates αklotho at the necrotic phase. Transferred to the cell culture model used in paper 2, HK-2 cells were in the apoptotic phase whereas NRK-52E and MDCK are already in the necrotic phase of cell death. This may be supported by the mean doubling times of the cell lines: MDCK 18 h[383], NRK-52E 45 h[382] and HK-2 about 54 h[430] with metabolic rates of MDCK > NRK-52E > HK-2. However, the negative regulation of αklotho by inflammatory cytokines[211] or during inflammatory disorders[52,212] partially contradicts this assumption. In summary, this points to a very sensitive role of energy metabolism on the induction of apoptotic or necrotic cell death and subsequently on the regulation of αklotho.

5 Conclusion

The initial aims of the present thesis were (**i**) the elucidation of a regulatory mechanism of cellular stress on FGF23 or αklotho, (**ii**) whether FGF23 or αklotho are influenced by certain forms of cellular stress or by particular signaling components of the cellular stress response, and (**iii**) if FGF23 and αklotho regulation may also be a consequence of apoptotic or necrotic senescence.

The present work was the first to investigate a direct regulation of FGF23 and αklotho by chemotherapeutic drugs or apoptosis induction *in vitro* which probably involves cellular stress mechanisms. There are several forms of cellular stress upon treatment with cisplatin, doxorubicin, paclitaxel, PAC-1, or serum depletion reported in the literature. Especially inflammatory, injury, oxidative, hypoxic, and starvation stress have been extensively discussed. Since the different conditions are closely interrelated, it is difficult to evaluate the impact of only one stress response on FGF23 or αklotho. On the one hand, UMR106 osteoblast-like cells exposed to cytotoxic stimuli reacted with an up-regulation of FGF23, which is usually associated with cancer progression[279] and cell protection[269]. In renal cells, stress stimulants cisplatin, paclitaxel, doxorubicin, PAC-1, or serum depletion increased αklotho expression in MDCK and NRK-52E cells whereas αklotho expression decreased in HK-2 cells exposed to chemotherapeutics or serum depletion. The cause for the differential regulation in these cell lines can only be discussed. It is possible that excessive αklotho

production plays a protective role e.g. anti-apoptotic[439] or anti-inflammatory[493]. However, the αklotho decrease in HK-2 cells confirms many other observations, in which αklotho was reduced under conditions of injury[393], disease[9,48] or cellular stress[218,394]. The different forms of cellular stress may induce various signaling pathways associated with increased stress resistance, e.g. HIF1α[334], TGF-β[341], or AMPK[494], but also FGF23 and αklotho exert protective functions[269,333,438]. Especially the chemotherapeutic drugs cisplatin, paclitaxel, and doxorubicin induce inflammatory stress related to necrotic cell death[153,173,359,495]. All stimuli used drive oxidative stress[147,235,471] and HIF1α signaling[461,463,467], which are known positive regulators of FGF23[258,345] and may thus account for the FGF23 stimulation. However, oxidative stress is reported to decrease αklotho[218] whereas αklotho reduces ROS and increases the antioxidant state[474,475]. αKlotho up-regulation might thus be a mechanism to protect the cell from inflammation[493], hypoxia[469], and ROS[474]. Conversely, down-regulation is frequently reported under disease conditions[213] and might prevent hypophosphatemia[496] or promote cell proliferation[497]. All stress stimulants used in this study are able to induce apoptotic cell death[235,240,253,257,272], but necrosis or necroptosis cannot be excluded, especially by chemotherapeutic drugs[286,287]. Due to the activation of a variety of different signaling pathways following cisplatin and doxorubicin incubation, it is difficult to conclude that FGF23 is up-regulated solely by one factor but rather by the combination of pro-inflammatory cytokine induction, oxidative stress, and HIF1α activation. On the one hand, the up-regulation of FGF23 protects the cell itself[333], but in the bone environment FGF23 inhibits bone formation and promotes bone resorption[349]. Increased systemic FGF23 levels result in decreased renal phosphate reabsorption[33], decreased $1,25(OH)_2D_3$ production with decreased intestinal phosphate absorption and overall in hypophosphatemia and the risk of osteomalacia or osteoporosis[85,86,355]. The simultaneous kidney injury, caused by chemotherapy or adjuvant apoptosis inducers may further derange mineral homeostasis[356]. However, αklotho up-regulation promotes cell resistance and restores function of damaged renal tissue[393]. αKlotho attenuates many functions associated with excessive FGF23 production such as AKI[55], fibrosis[402], and CVD[400] and the overall morbidity and mortality risk declines with higher αklotho levels[226,440]. Conversely, decreased αklotho production attenuates renal function and aggravates disease outcome[209,224].

Taken together, the negative effects of excess FGF23 production upon cellular stress may be compensated by increased αklotho synthesis. However, excess FGF23 and αklotho may cause hypophosphatemia with the risk of bone loss[498]. Thus, down-regulation of αklotho may also be plausible to prevent phosphaturia. In conclusion, the interaction of FGF23 and αklotho under stress conditions need to be further investigated.

The present thesis provided insight in a very sensitive context between chemotherapy-based cellular stress and the regulation of phosphate metabolism. However, beneficial effects of FGF23 and αklotho may exceed their significance in phosphate metabolism. The studies indicated a diagnostic potential to measure FGF23

during chemotherapy with regard to secondary bone loss. Furthermore, αklotho supplementation may be a promising approach to attenuate nephrotoxicity and sensitize cancer cells to cytostatic drugs.

6 Outlook

In future research, the discussed intracellular mechanisms need to be addressed with regard to FGF23 and αklotho regulation by cellular stress. In detail, inflammatory cytokines, oxidative stress, HIF1α, or AMPK and their impact on phosphate levels need to be enlightened. The association between FGF23 or αklotho and inflammation has been extensively investigated but whether FGF23 actively drives inflammation or the role of decreased αklotho during inflammation is still unclear and requires clarification. In this context, the time course of apoptotic and necrotic cell death should be carefully studied with regard to NFκB, AMPK, PPARγ, or HIF1α, as well as furin regulation. Furthermore, research is necessary to thoroughly investigate the differential regulation of αklotho in MDCK, NRK-52E, and HK-2 cells with regard to concentration and incubation times of cytotoxic substances. At last, diagnostic significance of FGF23 as well as therapeutic value of αklotho supplementation are promising fields of research.

7 Summary

Cellular stress is defined as the impairment of regular cell function by internal or external stimuli including critical temperatures, energy deficiency, infections, mechanic injury, or chemical noxae. The present thesis aims to investigate the influence of cellular stress on the expression of *FGF23* and αklotho. FGF23 is predominantly produced in bone and regulates the phosphate excretion in the kidney. Thereby, αklotho functions as a co-receptor for FGF23. By binding to the FGF receptor-αklotho complex, FGF23 reduces the reabsorption of phosphate from the tubular lumen by decreasing the abundance of sodium-phosphate co-transporters. Furthermore, FGF23 decreases the synthesis of $1,25(OH)_2D_3$, active vitamin D, and increases its degradation. $1,25(OH)_2D_3$ is a regulator of intestinal phosphate absorption and therefore, FGF23 additionally reduces dietary phosphate uptake. Chronically elevated FGF23 is associated with numerous disorders such as kidney disease or CVD. Beside its function as a co-receptor of FGFR, αklotho has many beneficial FGF23-independent functions. It has originally been identified as an anti-aging hormone, as a loss-of-function mutation in the αklotho gene causes numerous aging-like symptoms such as vascular and tissue calcification, osteoporosis, sterility, and an early death. The present papers investigated the influence of cytostatic drugs cisplatin, paclitaxel, and doxorubicin as well as apoptosis inducers PAC-1 and serum depletion on the regulation of FGF23 and αklotho. In UMR106 rat osteoblast-like osteosarcoma cells, a 24 or 48 h-treatment with cisplatin, doxorubicin, PAC-1, or serum reduction and depletion significantly up-regulated *Fgf23* expression. Under serum depletion, also FGF23 protein secretion was increased. In addition to FGF23, cisplatin and doxorubicin also increased gene expression of pro-inflammatory cytokine *Il6* hinting at the presence of necrotic cell death. By inhibiting Il-6 membrane receptor gp130 it has been shown, that FGF23 stimulation partially depended on IL-6 signaling. The stimulation of FGF23 by inflammatory mediators including IL-6, TNFα, TGF-β, or IL-1β has already been reported by others. Furthermore, inflammatory diseases such as rheumatoid arthritis, CKD, or inflammatory bowel disease are associated with excess FGF23 serum concentrations. In this regard, we investigated gene expression and activation of the transcription factor NFκB, which regulates numerous inflammatory functions. Cisplatin and doxorubicin increased the expression of NFκB subunit *Rela* and cisplatin also stimulated the phosphorylation of NFκB. Independently, NFκB inhibitors wogonin and withaferin A attenuated cisplatin-mediated stimulation of FGF23 indicating, that FGF23 excess was in part promoted by NFκB signaling. These investigations confirmed a strong impact of cisplatin or doxorubicin-induced inflammation on FGF23 synthesis, whereas PAC-1 and serum depletion have reported to directly induce apoptosis, which is commonly not associated with inflammation. Known factors, induced by all cytotoxic substances used here, are the formation of ROS and activation of HIF1α. Both are positive regulators of FGF23, leading to the conclusion, that cellular stress might regulate FGF23 via HIF1α or oxidative stress. FGF23 excess results in increased bone resorption and suppressed bone formation. Likewise, also chemotherapeutic drugs and serum deficiency reduce bone

density. Therefore, the stimulation of FGF23 may cause or further stimulate bone resorption. In paper 2, the influence of the cytostatic drugs cisplatin, paclitaxel, and doxorubicin as well as apoptosis inductors PAC-1 or serum depletion on αklotho expression in renal MDCK, NRK-52E, and HK-2 cells has been investigated. In fact, all cytotoxic compounds stimulated gene expression of αklotho while decreasing cell proliferation and viability. By using a combined apoptosis and necrosis assay, we confirmed the induction of apoptosis but also necrosis to a variable extent. Additionally, the transcriptional regulation of apoptotic proteins of the BCL-2 family was assessed and confirmed apoptosis stimulation. Transcription factor PPARγ is a known positive regulator of αklotho. In MDCK cells, we detected a significant influence of cisplatin-mediated stimulation of *PPARγ* mRNA on the αklotho increase. Furthermore, cisplatin, doxorubicin, PAC-1, and serum deprivation also up-regulated FGFR production in MDCK cells. In cancer cells, overexpression of FGFR is associated with enhanced resistance against chemotherapeutic drugs. Consequently, αklotho and FGFR1 stimulation may be a protective mechanism to prevent hyperphosphatemia during diseases. However, human HK-2 cells treated with cisplatin, paclitaxel, doxorubicin, or serum depletion significantly down-regulated αklotho expression and protein secretion. PAC-1 did not change the expression or production of αklotho in HK-2 cells, which might be explained by the minor effect of PAC-1 on non-carcinogenic cells lacking an overexpression of procaspase-3. The differential regulation of αklotho in MDCK and NRK-52E versus HK-2 cells by cytotoxic stress might have numerous causes. For instance, there is evidence of an increased sensitivity of HK-2 cells to stress stimuli but a better comparability to the animal model. However, immortalized cell lines can not completely reflect the conditions of native tissue especially with regard to cell death. Furthermore, the species, sex or age of the donor organism as well as passage number of the cells and drug transporter expression might impact αklotho regulation. Additionally, the mode of cell death determined by intracellular ATP homeostasis and its regulation of AMPK might play an important role in αklotho regulation. However, all these theories need to be further addressed. In summary, inflammation, ROS formation, or the activation of HIF1α are all reported to correlate in a negative manner with αklotho production or serum levels. αklotho down-regulation may be a tool to increase cell proliferation or prevent hypophosphatemia. In contrast, AMPK activation by intracellular ATP restriction may positively regulate αklotho to promote cell protection and avoid hyperphosphatemia.

8 Zusammenfassung

Zellulärer Stress ist definiert als eine Beeinflussung der regulären Zellfunktion durch innere oder äußere Einflüsse wie kritische Temperaturen, Energiedefizite, Infektionen, mechanische Verletzungen oder chemische Noxen. Die vorliegende Arbeit dient dem Ziel, den Einfluss von zellulärem Stress auf die Expression von FGF23 und αKlotho zu untersuchen. FGF23 ist ein vorwiegend im Knochen produziertes Hormon, dass in der Niere die Phosphatausscheidung reguliert. Durch Bindung an den Komplex aus FGF Rezeptor und Ko-Rezeptor αKlotho wird die Rückresorption von Phosphat aus dem Tubuluslumen reduziert. Außerdem senkt FGF23 die Produktion von $1,25(OH)_2D_3$, dem aktiven Vitamin D, und erhöht gleichzeitig dessen Abbau. $1,25(OH)_2D_3$ reguliert im Dünndarm die Resorption von Phosphat. Durch die Wirkung von FGF23 wird also zusätzlich weniger Phosphat aus der Nahrung aufgenommen. Chronisch erhöhte FGF23-Serumkonzentrationen sind mit Erkrankungen wie renalen oder kardiovaskulären Erkrankungen assoziiert. αKlotho hat neben der Funktion als Ko-Rezeptor für FGF23 noch weitere, FGF23-unabhängige Wirkungen. Es wurde ursprünglich als anti-Alterungshormon entdeckt, da eine *loss-of-function* Mutation im αKlotho-Gen zahlreiche altersassoziierte Probleme wie massive Kalziumablagerungen in Geweben und Blutgefäßen, Osteoporose, Sterilität und eine frühe Sterblichkeit hervorruft. Im Rahmen der beiden Veröffentlichungen wurde untersucht, inwiefern die Zytostatika Cisplatin, Doxorubicin und Paclitaxel, sowie die Apoptoseinduktion durch PAC-1 oder Serumentzug die Regulation von FGF23 und αKlotho beeinflussen. In UMR106, einer osteoblastenähnlichen Osteosarkom-Zelllinie wurde durch eine 24- oder teilweise 48-stündige Behandlung mit Cisplatin, Doxorubicin, PAC-1 und durch Serumreduktion oder –Entzug die *FGF23*-Expression signifikant stimuliert. Gleichzeitig sanken die Viabilität und Proliferation der Zellen. Mittels Serumentzug konnte zusätzlich die Erhöhung der FGF23-Proteinkonzentration im Überstand gezeigt werden. Parallel zu FGF23 wurde die Expression des pro-inflammatorischen Zytokins *IL6* durch Cisplatin und Doxorubicin erhöht und die Hemmung des IL-6 Membranrezeptors gp130 zeigte, dass die Stimulation von FGF23 zumindest zu einem Teil durch IL-6 vermittelt wurde. Andere Arbeitsgruppen konnten bereits vorher zeigen, dass IL-6 und andere pro-inflammatorische Zytokine und Entzündungsmediatoren wie TNFα, TGF-β oder IL-1β die Genexpression und Synthese von FGF23 stimulieren. Außerdem werden entzündliche Erkrankungen wie rheumatoide Arthritis, CKD oder chronisch-entzündliche Darmerkrankungen mit erhöhten FGF23 Serumkonzentrationen assoziiert. In diesem Zusammenhang wurde zusätzlich die Expression und Aktivierung des Transkriptionsfaktors NFκB untersucht, der zahlreiche Entzündungsfaktoren reguliert. Cisplatin und Doxorubicin steigerten die Genexpression der NFκB Untereinheit *Rela* und Cisplatin wurde erhöhte zusätzlich die Phosphorylierung von NFκB in UMR106 Zellen. Die NFκB-Inhibitoren Wogonin und Witheraferin A konnten separat voneinander die Stimulation von FGF23 durch Cisplatin unterbinden, was zeigte, dass die FGF23-Stimulation teilweise auf einer Aktivierung von NFκB beruhte. Die Versuche

zeigten einen starken Einfluss von Entzündungsprozessen auf die FGF23-Stimulation durch Cisplatin und Doxorubicin. PAC-1 und Serumentzug induzieren direkt eine Apoptose, die üblicherweise nicht mit Entzündungsprozessen einhergeht. Mögliche Faktoren, die im Zuge der Apoptose durch alle verwendeten Substanzen beeinflusst werden, sind die Bildung von ROS und die Aktivierung von HIF1α. Beides sind bekannte Regulatoren von FGF23. Insofern könnten apoptotische Zellen über HIF1α oder oxidativen Stress die Bildung von FGF23 anregen. Als Konsequenz der FGF23-Steigerung wird die Knochenbildung unterdrückt und vermehrt Knochenmasse abgebaut. Dementsprechend sind auch Chemotherapeutika und Serumentzug Faktoren, die für eine Reduktion der Knochenmasse bekannt sind. Wie in Veröffentlichung 1 gezeigt, könnte dies durch die Stimulation von FGF23 mitverursacht oder verstärkt werden. In der zweiten Veröffentlichung wurde der Einfluss der Chemotherapeutika Cisplatin, Paclitaxel und Doxorubicin sowie der Apoptoseinduktoren PAC-1 und Serumentzug auf αKlotho in renalen MDCK, NRK-52E und HK-2 Zellen untersucht. In MDCK- und NRK-Zellen stimulierten alle zytotoxischen Substanzen die Genexpression von αKlotho und beeinträchtigten gleichzeitig die Zellproliferation und –viabilität. Mittels kombiniertem Apoptose-Nekrose-Test wurde die Induktion von Apoptose aber teilweise auch Nekrose nachgewiesen. Zusätzlich bestätigte die transkriptionelle Stimulation von apoptotischen Proteinen der BCL-2 Familie die Induktion der Apoptose. Ein bekannter Positivregulator von αKlotho ist der Transkriptionsfaktor PPARγ. Diesem konnte in MDCK-Zellen ein signifikanter Einfluss auf die Genexpression von αKlotho durch Cisplatin nachgewiesen werden. Parallel zu αKlotho wurde auch die Produktion von FGFR1 durch Cisplatin, Doxorubicin, PAC-1 und Serumentzug in MDCK-Zellen stimuliert. Die Hochregulierung von FGFR1 ist in Krebszellen mit einer verstärkten Resistenz gegenüber Chemotherapeutika assoziiert und deutet folglich auf einen protektiven Mechanismus hin. In menschlichen HK-2 Zellen wurde die Genexpression und Proteinsekretion von αKlotho durch die Behandlung mit Cisplatin, Paclitaxel, Doxorubicin und Serumenzug überraschenderweise verringert. PAC-1 zeigte keinen Effekt auf die HK-2-Zellen, was vermutlich durch eine geringe Wirkung von PAC-1 auf nicht-kanzerogene Zellen durch die fehlende Überregulation von Procaspase-3 herrührt. Die unterschiedliche Regulation von αKlotho in MDCK-, NRK- und HK-2-Zellen durch zytotoxischen Stress kann zahlreiche Ursachen haben. Zum Einen gibt es Hinweise auf eine erhöhte Sensibilität der HK-2-Zellen und eine bessere Vergleichbarkeit zum Tiermodell. Allerdings können immortalisierte Zellen die Bedingungen in nativen Geweben nur teilweise reflektieren, besonders in Hinblick auf den Zelltod. Desweiteren kann auch die Spezies, das Geschlecht oder Alter des Spenderorganismus, beziehungsweise die Anzahl der Passagen der Zellkultur eine Rolle bei der Expression der Medikamententransporter spielen und somit Einfluss auf αKlotho nehmen. Außerdem könnte die Form des Zelltods, die durch den ATP-Haushalt der Zelle und die Regulation von AMPK bestimmt wird, αKlotho beeinflussen. Entzündung, die Bildung von ROS sowie die Aktivierung von HIF1α korrelieren alle in negativer Weise mit der renalen αKlotho Produktion bzw. Serumkonzentrationen. Die Herunterregulierung von αKlotho könnte zur Förderung der Zellproliferation beitragen oder eine

Hypophosphatämie verhindern. Eine Hochregulierung von αKlotho könnte dem Schutz der Zellfunktion bzw. der Vermeidung einer Hyperphosphatämie dienen.

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