GSK3-controlled sympathetic activity in FGF23 production and FGF23 gene regulation by actin cytoskeleton reorganization

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Abul Fajol

Zusammenfassung

Der Fibroblast Growth Factor (FGF23) ist ein Hormon, das im Knochen gebildet wird und die Phosphatausscheidung steigert. Es ist ein wichtiger Regulator des Vitamin D-Stoffwechsels und des Phosphathaushalts. Es wird im Knochen gebildet und wirkt hauptsächlich in der Niere. FGF23 hemmt das Enzym 25-Dihdroxyvitamin D 1α-hydroxylase und reduziert somit die blidung des biologisch aktiven Vitamin d-Hormones und stimuliert dessen Abbau durch die 25-Dihydroxyvitamin D 24-hydroxylase. Es blockiert die renale Phosphatreabsorption im proximalen Nierentubulus und erniedrigt damit die Serumphosphatkonzentration sowie die Konzentration von aktivem Vitamin D. Die Glykogensynthasekinase (GSK3) ist eine ubiquitär exprimierte Serin-/Threoninkinase. Sie ist in eine Vielzahl von zellulären Prozessen eingebunden, z.B. in den Glykogenstoffwechsel, die Transkription und Translation, Proliferation und Überleben der Zellen, die Regulation des Zell-Zyklus, die reorganisation des Zytoskeletts und die Apoptose. Die GSK3 ist ein Signalmolekül des PI3Kinase-Stoffwechsels. Sie wird durch den Insulin-abhängigen PKB/Akt-Signalweg phosphoryliert und dadurch blockiert. Transgene Mäuse, welche PKB-insensitives GSK3α/β *(gsk-3^{kI})* exprimieren, zeigen erhöhte Aktivität des sympathischen Nervensystems und Phosphaturie sowie erniedrigte Knochendichte. Es ist bereits bekannt, dass der Sympathikus die FGF23-Sekretion fördert. Im ersten Teil der Studie wurde die Rolle der GSK-kontrollierten Sympathikusaktivität für die Bildung von FGF23 und die Regulation des Phosphatstoffwechsels untersucht. Serum FGF23, Adrenalinausscheidung im urin, Vanillinmandelsäure, Phosphat und Kalziumausscheidung waren signifikant höher in *gsk-3^{kI}* Mäusen im Vergleich zu *gsk-3^{WT}* Mäusen. Die Serum-FGF23 und 1,25$(OH)_2D_3$-Konzentrationen waren niedriger in *gsk-3^{KI}* Mäusen als in *gsk-3^{WT}* Mäusen. Die Mäuse wurden eine Woche mit einem β-Blocker (propranolol) im Trinkwasser behandelt. Die Propranololbehandlung erniedrigte den Serum-FGF23, den renalen Phosphat-und Kalziumverlust und erhöhte die Serum Phosphatkonzentration in *gsk-3^{KI}* Mäusen. Damit wurde gezeigt, dass die PI3Kinase-insensitive GSK3 an der Regulation der FGF23-Bildung, des Vitamin D Metabolismus und somit auch des Mineralstoffwechsels durch das sympathische Nervensystem mitwirkt. Im zweiten Teil der Studie Rolle der Reorganisation des Aktin-Zytoskeletts für

die Kontrolle der FGF23 Produktion untersucht. Es ist bekannt, dass $1,25(OH)_2D_3$ und NF-κB die FGF23-Bildung steigern. Um die Rolle der $1,25(OH)_2D_3$-induzierten Aktinpolymerisation für die Fgf23 Transkription in UMR 106 Osteoblasten ähnlichen Zellen zu untersuchen, wurde die Dynamik der Aktinpolymerisation durch Western Blot und konfokale Mikroskopie verfolgt und die Fgf23-Transkription mit quantitativer RT-PCR gemessen. Es konnte gezeigt werden, dass die Induktion der FGF23-Produktion durch $1,25(OH)_2D_3$ in Aktinpolymerisation resultiert. Dieser Effekt wurde durch die pharmakologische Hemmung des NF-kB durch Wogonin blockiet. Cytochalasin B, eine Substanz, welche Aktinfilamente depolymerisiert, hat die $1,25(OH)_2D_3$ induzierte Fgf23 Transkription behindert. Dieses Resultat deuten auf eine entscheidende Rolle des Aktinzytoskeletts für die Fgf23-Transkription hin. Zur Identifikation der zugrundeliegenden Signaltransduktion wurden die Zellen mit dem Rac1-inhibitor NSC 23766 und den PAK-inhibitor IPA3 behandelt. Beide Inhibitoren blockierten die $1,25(OH)_2D_3$ induzierte FGF23 Expression, so dass der Rac1/PAK-Signalweg an dem Effekt beteiligt ist. Die vorliegenden Ergebnisse liefern einen starken Beweis dafür, dass die Rac1 regulierte Aktinreorganisation einen wichtigen Beitrag für die 1,25(OH)2D3-induzierte FGF23-Bildung liefert.

Summary

Fibroblast growth factor (FGF) 23 is a bone derived phosphaturic hormone. It is a potent regulator of vitamin D metabolism and phosphate homeostasis. FGF23 is secreted from the bone and exerts its function on the kidney. Further, it inhibits 25-dihydroxyvitamin D 1α-hydroxylase and reduces formation of active vitamin D and stimulates 25-dihydroxyvitamin D 24-hydroxylase, which in turn favors degradation of vitamin D. FGF23 inhibits renal phosphate reabsorption in the proximal tubules, thereby lowering serum phosphate and active vitamin D. To exert its renal function, FGF23 requires klotho as a co-receptor. Both FGF23 and klotho deficiency, lead to vascular calcification, hyperphosphatemia, hypercalcemia, muscular atrophy and profound aging like phenotypes. Glycogen synthase kinase (GSK) 3 is a serine-threonine kinase, which is ubiquitously expressed and involved in a variety of cellular process including; glycogen metabolism, transcription, translation, proliferation, survival, cell cycle regulation, cytoskeleton reorganization and apoptosis. GSK3 is a downstream signaling molecule of phosphoinositide-3 kinase (PI3 kinase)/PKB/Akt pathway. Mice expressing PKB insensitive GSK3α/β (*gsk-3*^{ki}) show enhanced sympathetic nervous activity and phosphaturia with low bone density. The sympathetic nervous system was shown to stimulate FGF23 release. In this thesis, I investigated the role of GSK3-controlled sympathetic activity in the production of FGF23 and phosphate metabolism. Serum FGF23, urinary epinephrine, Vanillylmandelic acid (VMA), phosphate and calcium excretion were significantly higher in *gsk-3*^{ki} mice compared to *gsk-3*^{WT} mice. Serum FGF23 and $1,25(OH)_2D_3$ concentrations were lower in *gsk3*^{KI} mice than in *gsk-3*^{WT} mice. Mice were treated with β-blocker (propranolol) for one week resulted in decreased serum FGF23 and renal phosphate and calcium loss and elevated serum phosphate concentration in *gsk-3*^{KI} mice. Thus, these data suggest that PI3K insensitive GSK3 participates in the regulation of FGF23 formation, vitamin D metabolism and, thereby mineral metabolism by sympathetic nervous system.

Next, I explored the role of actin cytoskeleton reorganization in controlling FGF23 production. Previous findings have suggested that $1,25(OH)_2D_3$ and NF-κB stimulates FGF23 production. Therefore, the role of $1,25(OH)_2D_3$-induced actin polymerization on Fgf23 expression in UMR 106 osteoblast-like cells was explored. Actin polymerization

dynamics was determined by Western blotting and confocal imaging and Fgf23 transcript levels was measured by qRT-PCR. Western blotting and confocal imaging data showed 1,25$(OH)_2D_3$ induces actin polymerization in UMR 106 osteoblast-like cells. Thus induction of FGF23 production by 1,25$(OH)_2D_3$ resulted in actin polymerization, an effect blocked by the pharmacological inhibitor of NF-κB wogonin (100 μM). Cytochalasin B (100 nM) is a actin microfilament disrupting agent which abolished 1,25$(OH)_2D_3$-induced Fgf23 gene transcription, pointing a role of actin cytoskeleton in Fgf23 expression. Both Rac1 inhibitor NSC23766 (50 μM) and IPA3 (10 μM) blocked 1,25$(OH)_2D_3$-induced Fgf23 expression, suggesting the mechanism involved in actin cytoskeleton- controlled Fgf23 expression in UMR 106 cells involves Rac1 small GTPase signaling. These results provide strong evidence that actin reorganization regulated by Rac1 signaling contributes to 1,25$(OH)_2D_3$-induced Fgf23 gene transcription.

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Abbreviations

ABP	Actin binding protein
ADHR	Autosomal dominant hypophosphatemic rickets
AOPP	Advanced oxidation protein products
BMD	Bone mineral density
CKD	Chronic kidney disease
CVD	Cardiovascular disease
Cyp24a1	1,25-dihydroxyvitamin D3 24-hydroxylase
Cyp27b1	25-hydroxyvitamin D 1- α hydroxylase
DT	Distal tubules
Egr-1	Early growth response gene-1
ERK	Extracellular signal-regulated kinase
Es RAGE	Endogenous soluble receptor of advance glycation end product
ESRD	End stage renal disease
FGF23	Fibroblast growth factor 23
FGFR	Fibroblast growth factor receptor
GSK3	Glycogen synthase kinase 3
hsCRP	High-sensitivity C reactive protein
IL	Interleukin
IRS1	Insulin receptor substrate 1
IRS2	Insulin receptor 2
LPS	Lipopolysaccharide
LVH	Left ventricular hypertrophy
MAPK	Mitogen-activated protein kinase
NADPH	Nicotinamide adenine dinucleotide phosphate
NaPi2a	Sodium-dependent phosphate cotransporter 2a
NaPi2c	Sodium-dependent phosphate cotransporter 2c
NF-κB	Nuclear factor kappa B
PCT	Proximal convoluted tubule
PDK	Phosphoinositide-dependent kinase

PH	Pleckstrin homology
PHEX	Phosphate regulating endopeptidase homolog, X-linked
PI3K	Phosphatidylinositol 3 kinase
PKB	Protein kinase B
PTG	Parathyroid gland
PTH	Parathyroid hormone
RAS	Renin-angiotensin system
Rac1	Ras-related C3 botulinum toxin substrate 1
ROS	Reactive oxygen species
SH2	Src-homology domain
SGK	Serum and glucocorticoid inducible kinase
SPC	Subtilisin-like proprotein convertase
TGF-β	Transforming growth factor beta
TIO	Tumor-induced osteomalacia
TNF	Tumor necrosis factor
VDR	Vitamin D receptor

1. Introduction

Fibroblast growth factors (FGF) are a large family of secreted proteins. There are 22 members of this family which have been found in both human and mice (1). FGFs function by binding to FGF receptors (FGFR) and transduce signals by different pathways which are involved in the regulation of many cellular processes including differentiation, cell proliferation or migration (2).

1.1 FGF23

Fibroblast growth factor (FGF) 23, a member of the FGF19 subfamily of the fibroblast growth factor, has been proposed as a potent regulator of mineral ion homeostasis (3-6). FGF23 is a ~ 32 kDa secreted protein of 251 amino acids, which has a hydrophobic signal sequence (~24 amino acids), a N-terminal FGF core homology domain (155 amino acids), and a C-terminal domain (72 amino acids). Human cDNA encoding FGF23 (251 amino acids) highly resembles (~72% amino acid identity) to mouse FGF23. Human *Fgf23* gene is localized on the chromosome 12p13 and is mainly expressed in osteocytes (7-10). The C-terminal domain is necessary for the interaction with the FGFR. The proteolytic cleavage site (176RXXR179) is found between N- and C-terminal domain (11) (Fig.1). The FGF23 protein is proteolytically cleaved and this processing occurs intracellularly, either before or during the secretion of FGF23 (12). Abnormalities of bone and mineral metabolism are associated with increased risk of mortality in chronic kidney diseases (CKD) including; hyperphosphatemia (13, 14), hypo- and hypercalcemia (15), hypo and hyperparathyroidism (16) and hyperphosphatasemia (17). Inorganic phosphate is an important factor in intracellular signaling, DNA synthesis and metabolism. In spite of its biological significance, regulation of phosphate homeostasis is not clear. A mutation in phosphate regulating endopeptidase homolog, X-linked (PHEX) causes X-linked hypophosphatemia (XLH) (18) and autosomal dominant hypophosphatemic rickets (ADHR) is caused by mutation

in FGF23 (19, 20), have been considered important regulators of phosphate homeostasis.

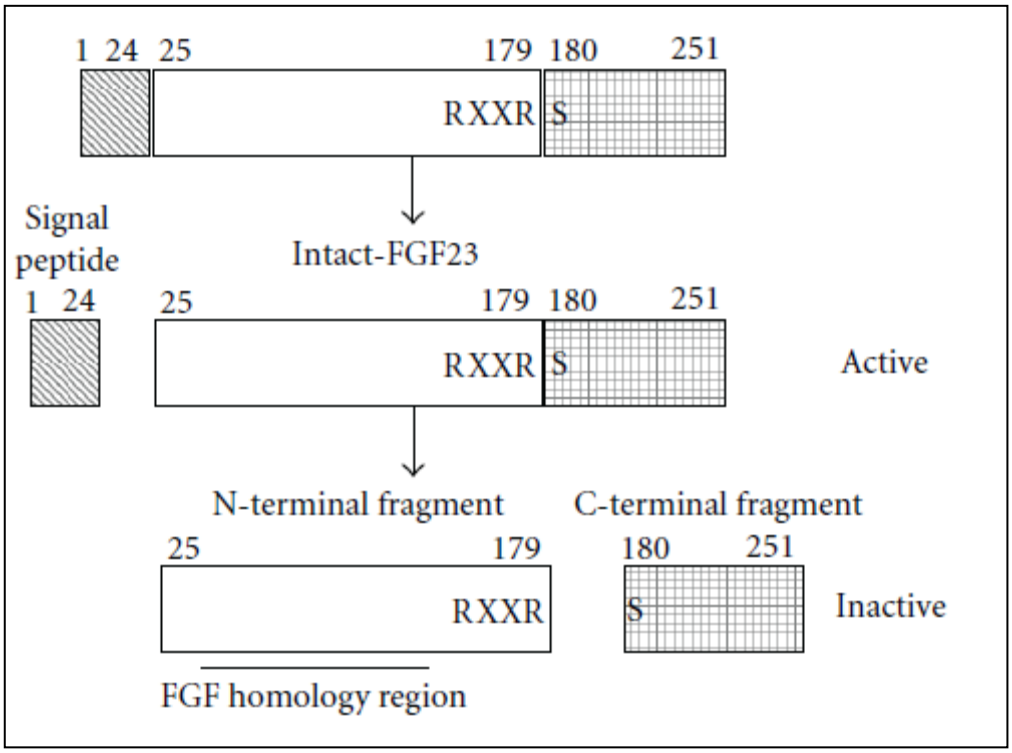

Fig. 1: Structure of FGF23. FGF23 is a secreted protein consists of 251 amino acids. C-terminal FGF23 is the inactive form which is proteolytically cleaved between Arg^{179} and Ser^{180} from intact FGF23 which is the biologically active form, adapted from Saito T *et al, Int J Pediatr Endocrinol.* 2009 (21).

1.2 Discovery of FGF23

Hypophosphatemic diseases in the many parts of the world have been caused by malnutrition and vitamin D deficiency. The most common cause of vitamin D resistant rickets is X-linked hypophosphatemic rickets (XLH) and other diseases, include autosomal dominant hypophosphatemic rickets (ADHR) and tumor-induced osteomalacia (TIO) with similar phenotypes (22). The murine analogue of human (FGF23) was cloned for the first time in 2000 in mice (7). It has been shown that ADHR

is caused by mutation on chromosome 12p13.3 and the mutated gene encodes the 251 amino acid human FGF23 protein (6, 19, 20). Analysis of the structure revealed that mutations occurred in ADHR were situated within a subtilisin-like proprotein convertase (SPC) cleavage site in FGF23 that renders the protein resistant to degradation, resulting in increased serum levels of active FGF23 and hypophosphatemia due to increasing urinary Pi loss in ADHR patients (6, 12, 23). Tumor-induced osteomalacia (TIO) was thought as a vitamin D resistant osteomalacia causing hypophosphatemia which was cured by the removal of tumor (22, 24). Transplantation of these tumors into wild type mice caused hypophosphatemia with a production of a phosphaturic factor that can suppress $1,25(OH)_2D_3$ and decrease renal Pi reabsorption (25, 26). Further, a mutation in FGF23 is responsible for ADHR, at the same time the FGF23 protein was shown to be produced in TIO (20, 27, 28).

1.3 Source of FGF23

FGF23 is expressed primarily in bone, thymus, brain, lymph nodes and to a lesser extent in heart, skeletal muscle, spleen, thyroid/parathyroid gland, lung and testis (4, 7, 8, 10, 28). In bone, FGF23 is mainly localized to osteocytes and osteoblasts (8, 10). In the brain, expression of FGF23 was found in the ventrolateral thalamic nuclei (7).

1.4 Phenotypes of Fgf23 mice

1.4.1 Fgf23 deficient mice

To interpret the physiological roles of FGF23 in mice, *Fgf23* knockout mice were generated by Shimada et al., in 2004 (3). FGF23 is produced from bone (29, 30) and its biological function depends on the interaction with a cofactor protein called Klotho (31-33). *Fgf23* knockout mice suffer from severe hyperphosphatemia and elevated levels of

$1,25(OH)_2D_3$ due to enhanced renal 1α-hydroxylase expression and abnormalities in skeletal muscle (4). Genetic deletion of Fgf23 results in aging like phenotypes similar to human premature aging, including growth retardation, infertility, atherosclerosis, massive soft tissue calcifications, atrophy of multiple organ systems together with hypoglycemia and increased peripheral insulin sensitivity, disorder of phosphate and vitamin D metabolism, pulmonary emphysema, osteoporosis, and a severely shortened lifespan (3, 4, 34).

1.4.2 Fgf23 transgenic mice

A previous study suggested that recombinant FGF23 was able to enhance urinary phosphate excretion and lower serum $1,25(OH)_2D_3$ when given *in vivo* (3). Overexpressing human wild-type FGF23 in transgenic mice exhibited hypophosphatemia and enhanced renal phosphate excretion which was accompanied by reduced expression of the sodium-dependent phosphate cotransporter types IIa (NaPi-2a) and IIc (NaPi-2c) in the kidney (35). Renal phosphate loss resulted in skeletal abnormalities and reduced bone mineral density (BMD) in these transgenic mice (35, 36). Similarly, transgenic mice over expressing mutant form of FGF23 (human (R176Q)), which is resistant to degradation by furin-like proteases, displayed more pronounced hypophosphatemia and rickets/osteomalacia compared to animals expressing the wild-type FGF23 (37-39).

1.5 Function of FGF23

1.5.1 FGF23 and Klotho

FGF23 is produced by the bone and acts on kidney and transduces signals through FGF receptors (FGFR) (4, 10, 40, 41). There are four FGFRs and several subtypes formed as a result of alternative splicing. Although FGFRs differ in tissue expression, activity and ligand binding, all members of the receptor belong to tyrosine kinase family (42). However, binding affinity of FGF23 to its receptors (FGFRs) is poor (32). Following administration of recombinant FGF23 in mice, it was found that FGF23 binds to klotho, an aging counteracter, membrane-bound protein and induces the early growth response gene -1 (Egr-1) expression and ERK phosphorylation in the kidney, the parathyroid and pituitary gland (32). Klotho deficient mice and Fgf23 deficient mice both display high $1,25(OH)_2D_3$ levels, hyperphosphatemia, a shortened life span and severe aging phenotypes (3, 4, 43). Kurosu et al. have shown that Klotho complexed with FGFR1c, 3c and 4, FGF23 bound tightly and transduce signal to the target organ (31). An *in vivo* study demonstrated that Klotho converted the FGFR1c to a functional FGF23 receptor and Klotho acts as a co-receptor for FGF23 signaling (32). The importance of the FGFR1 in FGF23 signaling has further been supported by *in vivo* studies of FGFR3/4 null mice and conditional knock-out of FGFR1 mice (44). FGF23 signaling, requires FGFR1c and Klotho form a complex with FGF23 (Fig. 2), which is trimerized and stabilized by heparin sulphates as seen in other FGFR signaling complexes (45)

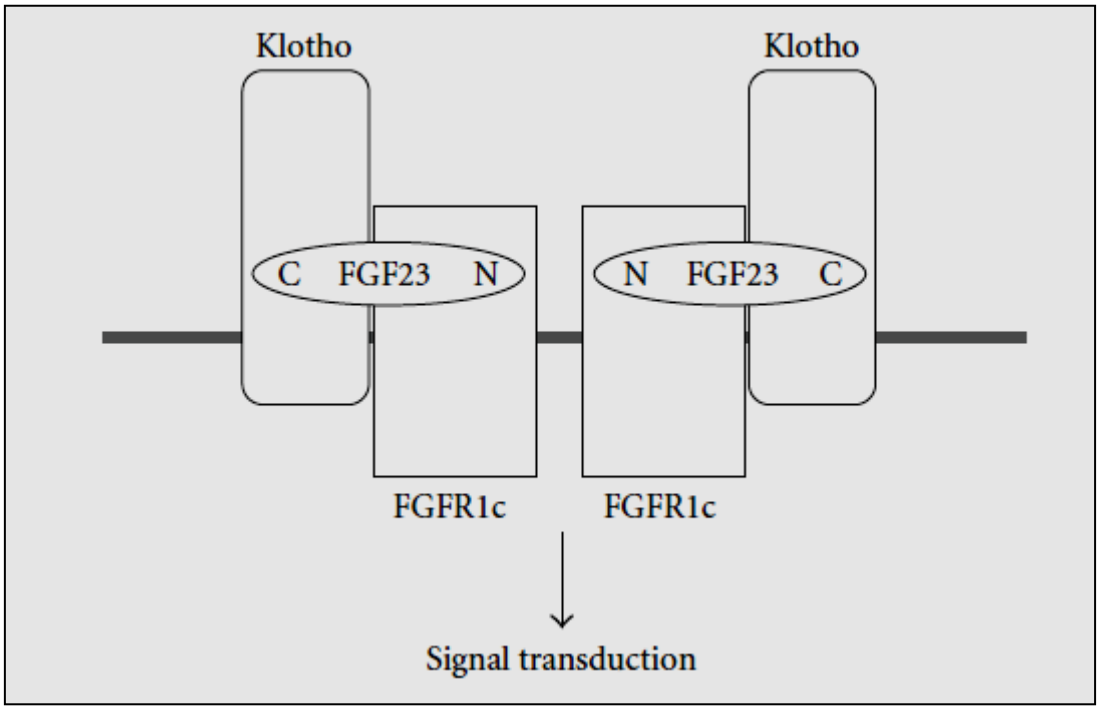

Fig. 2: Formation of heterotrimer complex of FGF23 with FGFR and klotho to transmit signal. To mediate renal effects, FGF23 bind to its receptor which requires Klotho as a co-receptor and form a complex, adapted from Saito T *et al, Int J Pediatr Endocrinol.* 2009 (21).

1.5.2 Renal functions of FGF23

Excessive FGF23 leads to hypophosphatemia, abnormal vitamin D metabolism, impaired growth and rickets/osteomalacia (35, 38, 46). Fgf23 deficient mice results in hyperphosphatemia, excess $1,25(OH)_2D_3$ and calcification of soft tissues (3, 8). The kidney is the major target organ of FGF23 actions, deregulation of FGF23 can lead to various disorders. Renal functions of FGF23 are given below:

1.5.3 Vitamin D metabolism

FGF23 functions as counter regulatory hormone of vitamin D (47). Cyp27b1 and Cyp24a1 are key renal enzymes which are responsible for the synthesis of the bioactive form of vitamin D and degradation of the bioactive form of vitamin D respectively (21, 40). FGF23 can influence circulating vitamin D levels through suppression of 25-

hydroxyvitamin D 1-α-hydroxylase (*Cyp27b1*) and stimulation of 1,25-dihydroxyvitamin D 24-hydroxylase (*Cyp24a1*) in the proximal tubules which results in decreased serum concentration of $1,25(OH)_2D_3$, the biologically-active form of vitamin D (28, 35, 38, 48-50). FGF23 stimulates 1,25-dihydroxyvitamin D 24-hydroxylase Cyp24a1 expression (50) that leads to the reduction of serum $1,25(OH)_2D_3$ levels are vitamin D receptor (VDR) dependent (51). Recombinant FGF23 injected into wild type mice reduces renal expression of Cyp27b1 dose-dependently and its effect on Cyp27b1 gene expression is ERK1/2 dependent (52). On the other hand, up regulation of Cyp27b1 mRNA expression is associated with higher FGF23 levels (53, 54). These studies suggest that the effect of FGF23 on the enzymes that are responsible for synthesis and degradation of $1,25(OH)_2D_3$ depends on the circulatory FGF23 concentrations (3, 38, 50, 55).

1.5.4 Phosphate reabsorption

Cross-organ communication between kidneys, intestine and bones play a central role in phosphate homeostasis (56). It adjusts intestinal absorption of phosphate from the diet and renal reabsorption / excretion of phosphate that typically regulate phosphate balance. Phosphate reabsorption is taken place in the renal proximal tubule (Fig. 3) (40). The intestinal absorption and renal reabsorption of phosphate is mediated by sodium-dependent phosphate transporter system that includes NaPi-2a (kidney), NaPi-2b (intestine) and NaPi-2c (kidney) (57-61). It has been reported that FGF23 inhibits the expression of NaPi-2a and NaPi-2c which induces renal phosphate excretion (44, 50). Transgenic mice over expressing Fgf23 have severe hypophosphatemia due to the reduction of renal NaPi co transporters expression (36, 38). On the other hand, Fgf23 knockout mice suffer from hyperphosphatemia and soft tissue calcification with enhanced renal NaPi-2a abundance (4, 62). Reduced renal NaPi-2a expression restores the biological effects of FGF23 in Fgf23 null mice significantly reversed hyperphosphatemia to hypophosphatemia and prevent calcification (63)

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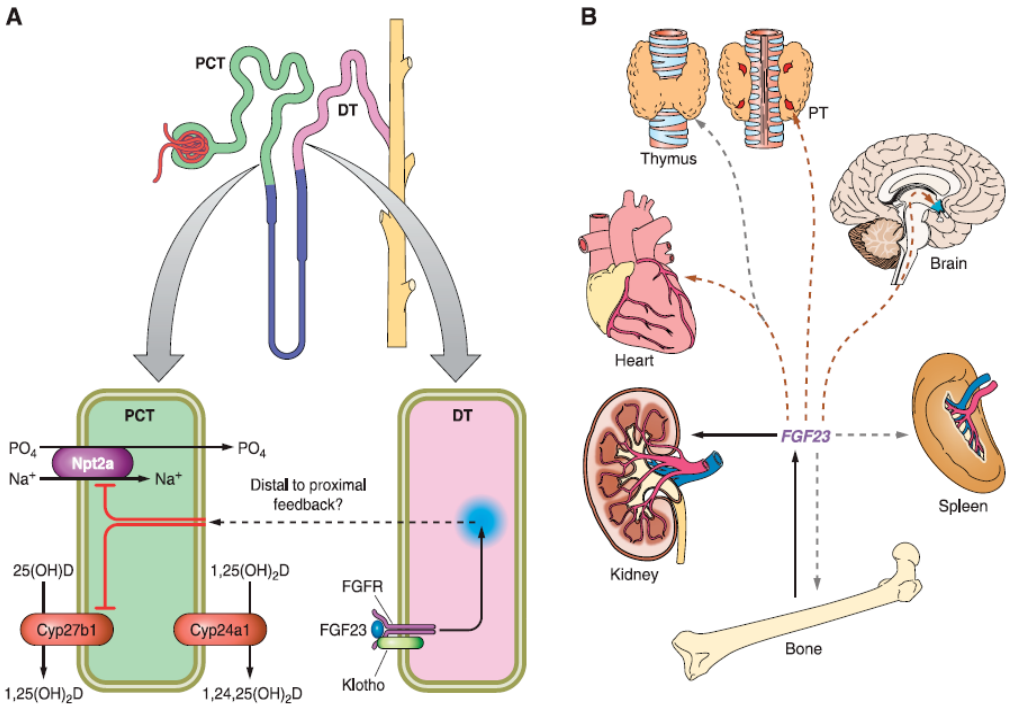

Fig. 3: Schematic illustration for the functions of FGF23 in kidney and extra renal organs. (A) FGF23 activates FGFR/Klotho complex which lead to the inhibition of tubular phosphate reabsorption by inhibiting Npt2a. FGF23 inhibits Cyp27b1 in the proximal convoluted tubule (PCT), a key enzyme responsible for $1,25(OH)_2D_3$ synthesis and stimulate Cyp24a1 which favor catabolism of $1,25(OH)_2D_3$. Therefore, both phosphate and $1,25(OH)_2D_3$ levels are decreased. (B) Extra renal targets of FGF23 are those tissue or organ which express FGFR and klotho i.e Bone, heart, brain, thymus, spleen and parathyroid gland (PT) which mediate possible effects of FGF23 **,** adapted from Martin A *et al*, *Physiol Rev. 2012* (40).

1.6 FGF23 and diseases

1.6.1 Possible role of FGF23 in the premature aging

Pathophysiology of aging is a complex biological process that involves multi-organ and multi-system pathologies (64-66). *Klotho* mutant mice display multiple aging-like features including shortened lifespan, infertility, skin atrophy, soft tissue calcification, atherosclerosis, muscles wasting, disorder of mineral ion metabolism and aberrant vitamin D metabolism (67, 68). A recent study suggests that aging like phenotypes in

klotho knockout mice are the consequence of abnormal mineral metabolism due to inability of *Fgf23* gene function in these mice (69). Ablation of *Fgf23* shares same phenotypes as found in the *klotho* null mice (70). Thus, common phenotypes of these mice are due to either absence (*Fgf23* null mice) or lack of FGF23 activity (*klotho* mutant mice) (71).

1.6.2 Hypervitaminosis-D and premature aging in *Fgf23* mutant mice

Both *klotho* and *Fgf23* deficient mice show up-regulation of 25-hydroxyvitamin D 1-α-hydroxylase (*Cyp27b1*) in the kidney and thereby increased serum levels of $1,25(OH)_2D_3$ (3, 4, 72), which are associated with aging like-features. The premature aging like-phenotypes in these mutants were rescued by feeding a vitamin-D deficient diet (73, 74) or genetically disrupting vitamin-D activities from *Fgf23* mice (70, 73). By deleting 1-α hydroxylase gene from *Fgf23* null mice, which is responsible for calcitriol synthesis, most of the phenotypes were ameliorated in *Fgf23/1-α hydroxylase* double knockout mice (70, 75). Thus, most of the aging like-features in *Fgf23* and *klotho* null mice are due to abnormal mineral metabolism caused at least in part by hypervitaminosis-D (70, 76, 77).

1.6.3 Hyperphosphatemia and aging

Renal NaPi2a regulates plasma and urinary phosphate balance (60, 62). Renal abundance of NaPi2a is up-regulated by the stimulation of $1,25(OH)_2D_3$ and is decreased by FGF23 and PTH (60, 78). Similarly NaPi2b expression in the intestine is increased by $1,25(OH)_2D_3$ (79). It has already been shown that NaPi2a renal expression is increased in *Fgf23* mutant mice (72) and similar results were also found in *klotho* deficient mice (80). Accelerated aging directly or indirectly depend on the hyperphosphatemia in experimental animals, as feeding them a low-phosphate or low-vitamin D diet greatly expands their life span (81). The *klotho* deficient mice show excessive phosphate retention which cause hyperphosphatemia and thereby reduced survival (67, 68, 82-87). Age-related phenotypes of *klotho* deficient mice could be

suppressed and thereby extending lifespan by reducing phosphate burden *via* inhibiting renal NaPi2a activities by generating *NaPi2a/klotho* double knockout mice (86). When these mice were fed a with high phosphate diet these mice experienced accelerated aging like phenotypes, as seen in the klotho deficient mice (86, 88). The *klotho* deficient mice are infertile. Phosphate restriction of these mice regained fertility, as evidenced in *NaPi2a/klotho* double knockout mice (88). When these mice were given high phosphate diet, they became infertile, pointing that phosphate may affect reproductive abilities and thereby accelerate aging process (86).

1.6.4 FGF23 and chronic kidney disease (CKD)

Elevated levels of FGF23 is one of the earliest indications of abnormal bone-mineral metabolism in CKD (89). Serum FGF23 levels increase as a result of progressive loss of renal function and this level can be increased by 100-1000 fold times higher than in healthy controls by the time patients require dialysis (90). A recent study revealed that FGF23 levels increase as glomerular filtration rate (GFR) declines during the progression of CKD (91) and this report demonstrated that the elevation of FGF23 develops before that of parathyroid hormone (PTH) and serum phosphate (89). Even though with elevated levels of FGF23, CKD patients do suffer from hyperphosphatemia (92). Patients with CKD can develop secondary hyperthyroidism due to failure of FGF23 to maintain normal serum phosphate levels. However, the mechanism of increased FGF23 levels in patients with CKD is not fully known. Elevated FGF23 levels in patients with CKD could results from reduced renal clearance of FGF23 (93) and enhanced production of FGF23 that counteract hyperphosphatemia. This hypothesis is supported by phosphate loading which results in increased FGF23 levels (94). Calcitriol therapy in patients with CKD might also lead to increasing levels of FGF23 (95). It has been reported that both phosphorous and calcitriol could increase serum FGF23 levels (76). As elevated FGF23 levels are associated with increased excretion of phosphate and low vitamin D (47, 96), therefore, the elevated levels of FGF23 in patients with CKD can develop secondary hyperthyroidism by decreasing $1,25(OH)_2D_3$ (96, 97). The interaction of FGF23 and parathyroid hormone is a complex process that has not yet been clearly

understood. Several studies have demonstrated that PTH stimulates FGF23 production (98, 99), however FGF23 was also shown to reduce PTH synthesis (100, 101).

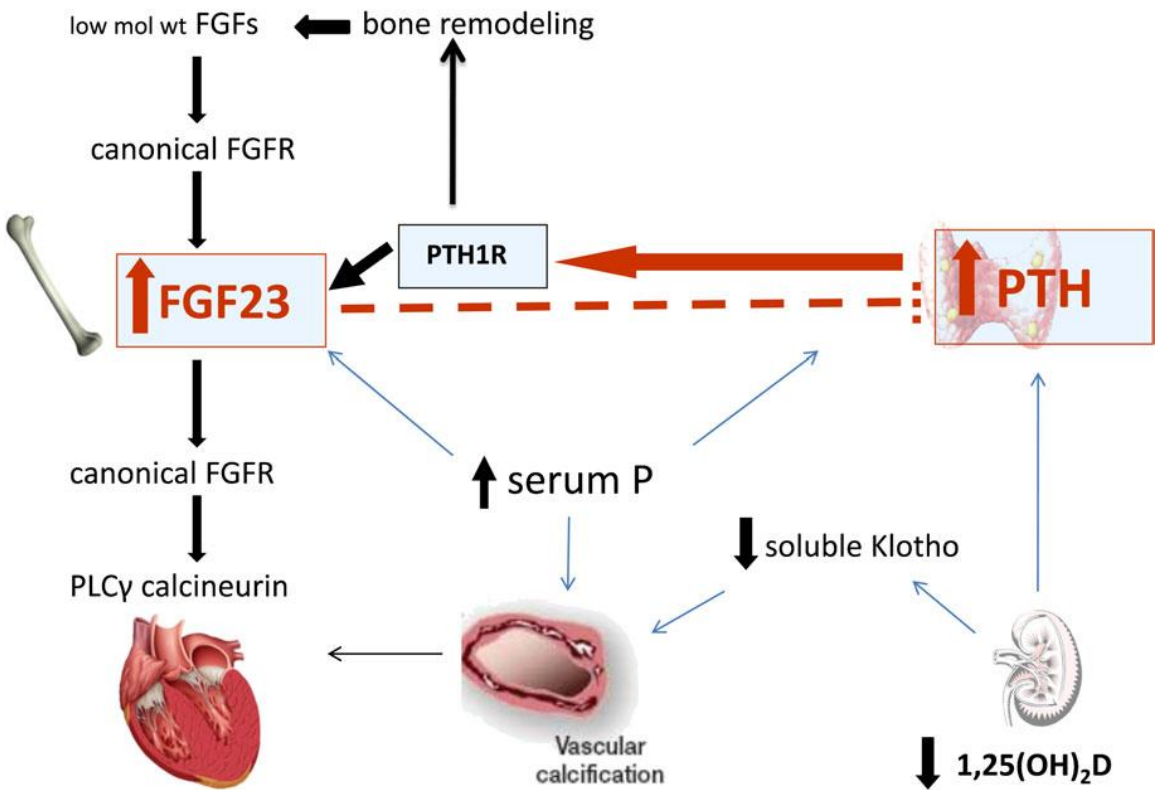

Fig. 4: Various factors involved in CKD-MBD. In advanced CKD, dietary phosphate and PTH stimulate FGF23 release and FGF23 decreases serum calcitriol that is which in turn secrete PTH. The higher level of PTH also releases low-molecular weight FGFs through parathyroid hormone receptor (PTHR) that act on canonical FGFRs to increase FGF23. The high FGF23 levels act on FGFRs in the heart activating PLCγ calcineurin contributing to the left ventricular hypertrophy (LVH) in these patients. Hyperphosphatemia and reduced soluble klotho are factors involved in vascular calcification of soft tissue of CKD patients, adapted from Silver *J et al, Nephrol Dial Transplant, 2012* (102).

1.6.5 FGF23 and cardiovascular diseases (CVD)

Elevated FGF23 levels are associated with CVD, including left ventricular hypertrophy (LVH), arterial stiffness, vascular calcifications, endothelial dysfunction and increased levels of inflammatory markers (103-107). LVH is a common cardiovascular disorder and the major risk factor for cardiovascular death in patients with end stage renal disease (ESRD) (108-110). FGF23 is associated with LVH (104). However, it is still ill-defined, whether the effect is direct or indirect on heart. An *in-vivo* study has shown that FGF23 directly induces LVH through the activation of FGF receptors (103) and the effect was klotho independent, as myocardial cells do not express klotho, a co-receptor of FGF23. However, Agarwal et al. have shown that klotho deficient mice did not have LVH in comparison to 1-α hydroxylase deficient mice (111). Several other studies have raised question if there is a direct effect of FGF23 on the myocardium (112-114). Further studies are required to answer this question.

1.6.6 Inflammation and oxidative stress

Inflammatory markers are known risk factors for cardiovascular diseases in CKD patients. *In vivo* experiments have reported that FGF23 increases the production of inflammatory markers i.e lipocalin-2, TGF-β and TNF (115). It has been shown that FGF23 is strongly correlated with interlukin-6, TNF-α, high-sensitivity C-reactive protein (hsCRP), endogenous soluble receptor of advanced glycation end products (esRAGE), advanced oxidation protein products (AOPP) in CKD patients (106, 116).

1.6.7 FGF23 and vascular calcification

Vascular calcification is the accumulation of phosphate and calcium in the blood vessels and soft tissue. The major factor of mortality in CKD is cardiovascular diseases and vascular calcification (117, 118). Accumulating evidence suggests that high serum phosphate levels associated with vascular calcification in CKD patients (119, 120). Transport of Pi by Na-dependent phosphate transporter (NaPiII) up-regulates

osteogenic genes and causes vascular calcification (121, 122). High serum FGF23 is associated with declining kidney function. Clinical observations in a several studies have found a positive association with serum FGF23 and vascular calcification in patients with CKD and ESRD (123-125)

1.7 Regulation of FGF23

In normal individuals, low levels of FGF23 are detected in the circulation, but levels are increased in response to phosphate loading, vitamin D administration, renal failure, and in several hereditary and acquired hypophosphatemic disorders (20, 76, 89, 126)

1.7.1 Regulation by $1,25(OH)_2D_3$

$1,25(OH)_2D_3$ is the most important regulator of FGF23. *In vivo* studies, have shown $1,25(OH)_2D_3$ increases circulating FGF23 levels. $1,25\ (OH)_2D_3$ increases the transcription of FGF23 in osteoblasts／osteocytes and this action is mediated by a vitamin D receptor (VDR), while disturbance of $1,25(OH)_2D_3$ pathway decreases circulating FGF23 in mice (47, 76, 95). It has been suggested that FGF23 acts as a counter regulatory hormone for $1,25(OH)_2D_3$, which maintains phosphate balance by vitamin D-mediated suppression of PTH and increased intestinal phosphate absorption (127). In the gastrointestinal tract $1,25(OH)_2D_3$ enhances calcium and phosphate absorption (Fig. 5). Increases in calcium, as well as $1,25(OH)_2D_3$ itself suppress PTH in the parathyroid gland, which in turn acts on the kidney and enhance renal calcium excretion to maintain neutral calcium balance (40, 47, 127). However, reduced PTH levels, limits kidney function to excrete increased phosphate absorbed from the gastrointestinal tract (47). FGF23 expression can be regulated by both VDR-dependent and VDR-independent signaling pathway. VDR null mice exhibited undetectable FGF23 levels, pointing the importance of VDR in the regulation of FGF23 (48, 128). On the other hand, dietary phosphate increased FGF23 levels in VDR null mice, suggesting

that regulation of FGF23 is also mediated by a VDR-independent pathway (35, 36, 50, 129).

1.7.2 Regulation by phosphate

The effect of dietary phosphorus on serum FGF23 levels and production by osteocytes /osteoblasts are conflicting.

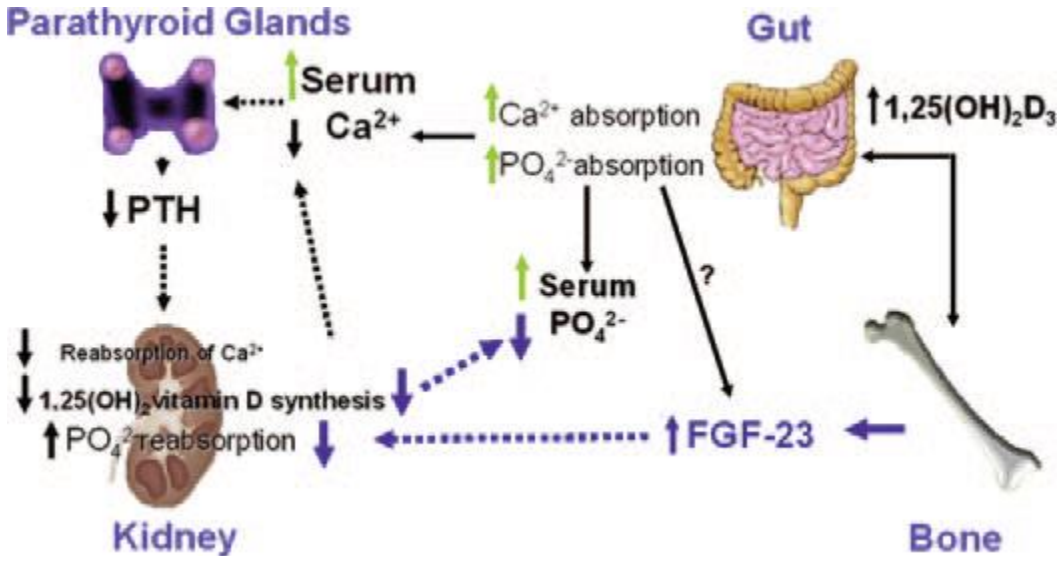

Fig. 5: Schematic diagram showing relationship between FGF23, vitamin D, PTH, calcium and phosphorus. $1,25(OH)_2D_3$ causes suppression of PTH and increases intestinal calcium absorption. $1,25(OH)_2D_3$ also stimulates phosphate absorption. Regulation of FGF23 production by dietary phosphate in bone likely occurs through unknown intermediate steps, because hyperphosphatemia *per se* does not directly stimulate FGF23 production by osteoblasts, adapted from Liu S *et al, J Am Soc Nephrol. 2006* (47).

Several observations have been shown that phosphate loading increases serum FGF23 levels in mice and in human subjects (76, 130, 131), whereas, two other studies have failed to demonstrate increased in serum FGF23 levels in response to phosphate loading (90, 132). Phosphate restriction has been shown to decrease circulating FGF23 levels (133). Based on current understanding it appears that the mechanism of FGF23 regulation by phosphate may be complex and indirect.

1.7.3 Regulation by PTH

In a mice model of primary hyperparathyroidism, FGF23 levels are increased (98), while decreased FGF23 has been shown after parathyroidectomy (134). PTH increased FGF23 mRNA levels directly in rat osteoblast-like UMR 106 cells (135). However, PTH failed to increase FGF23 production in ROS 17/2.8 osteoblast-like cells (47) or in calvarial culture (136). Patients with hypoparathyroidism (137) and PTH null mice (138) exhibit increased levels of FGF23. On the other hand vitamin D deficient mice display extremely low levels of FGF23, despite elevated levels of PTH (51, 139) and PTH null mice can restore FGF23 production after injection of vitamin D (47). Thus, regulation of FGF23 by PTH may be dependent on vitamin D.

1.7.4 Regulation by bone-derived factors

Bone is the main site of expression of FGF23 pointing the probability of important local regulators of FGF23. PHEX is expressed mainly by osteoblasts and osteocytes in bone (40). Increased production of FGF23 by osteocytes, has been found in a study due to inactivating mutations of PHEX (8), indicating that PHEX may somehow regulate the biosynthesis of FGF23. Initially, FGF23 was presumed to be a substrate for PHEX but recent findings suggest that FGF23 is not a direct substrate for PHEX, rather PHEX can regulate Fgf23 gene expression possibly through the action of unknown PHEX substrates or other downstream effectors (10). Like PHEX, dentin matrix protein (DMP) 1 is also predominantly expressed by osteoblast and osteocytes in bone. DMP1 regulates extracellular matrix mineralization (140). Inactivation mutation of DMP1 displays increased FGF23 production in bone (141).

1.7.5 Other regulators

Recently, iron has been considered a regulator of FGF23. But still it’s role is enigmatic (142-146). Sympathetic nervous system is also involved in the regulation of FGF23, since FGF23 expression is triggered by sympathetic activation (147).

1.8 GSK3 signaling

1.8.1 GSK3

Glycogen synthase kinase-3 (GSK3) is a downstream signaling molecule of PI3K and PKB/Akt and is a serine/threonine protein kinase that participates in the regulation of glycogen metabolism (148). GSK3 was first identified as a protein kinase that phosphorylates and thus inactivates glycogen synthase, the key enzyme in glycogen biosynthesis (149). GSK3 was isolated and purified from rabbit skeletal muscle (150). Woodgett JR et al. have found by cloning that GSK3 has two closely related isoforms, GSK3α and GSK3β, which are expressed ubiquitously in mammalian tissues (151, 152). Besides glycogen metabolism, GSK3 has important roles in the regulation of cellular processes including; cell proliferation, differentiation microtubule dynamics, cell cycle and apoptosis (153). Aberrant GSK3 signaling plays roles in the pathophysiology of diabetes, cancer, inflammation, neurological disorders and cancer (154, 155).

1.8.2 Regulation of GSK3 by insulin and growth factors

It was first thought that inhibition of GSK3 might be the cause of insulin-induced dephosphorylation of glycogen synthase (156) and later on it was confirmed that insulin stimulates the dephosphorylation of glycogen synthase at sites where phosphorylated by GSK3 (157) and as a result insulin inhibits GSK3 acutely (158, 159). Well known mechanism through which GSK3 is inhibited by insulin results from its phosphorylation at Ser^{9} in GSK3β and Ser^{21} in GSKα and that is catalyzed by PKB (160). Insulin stimulates PI3K which, in turn activate PKB/Akt and serum and glucocorticoid inducible kinase (SGK) isoforms (161, 162). In a cascade of signal transduction GSK3 is phosphorylated and thus inactivated by PKB (163) and SGK1 (164). By activating protein phosphatase insulin may stimulates dephosphorylation of glycogen synthase (165) (Fig. 6).

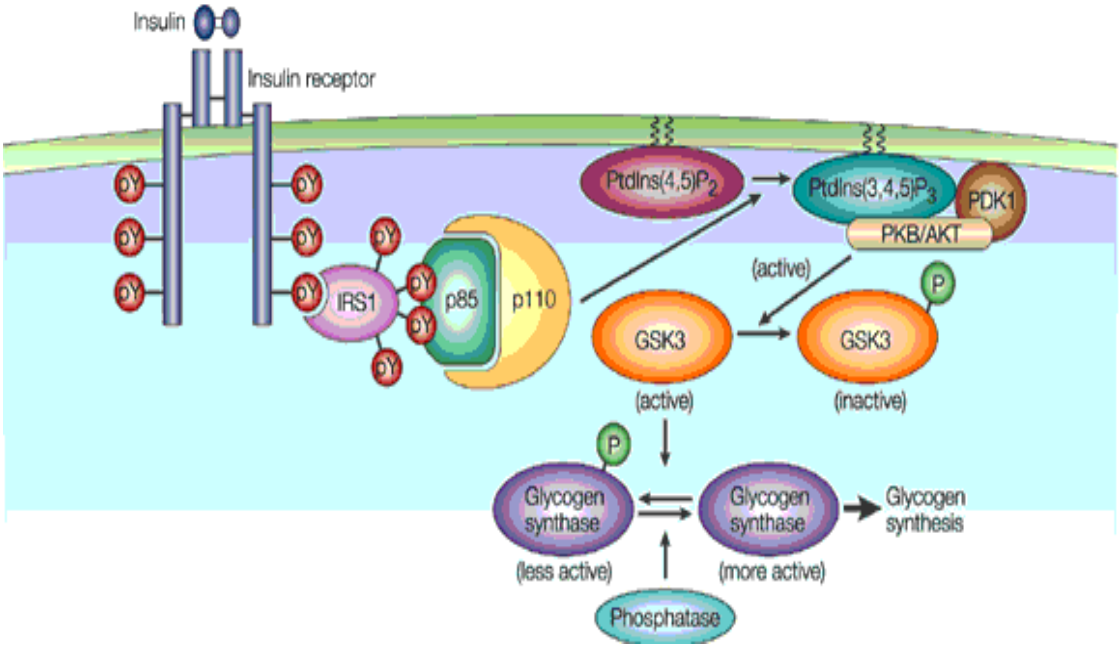

Fig. 6: Inhibition of GSK3 by insulin signaling pathway which regulate glycogen synthesis and protein synthesis. Insulin binds to its receptor and activates the intrinsic protein tyrosine kinase activity of the receptor. Phosphotyrosine (pY) residue interacts with insulin receptor substrate proteins (IRS1 and IRS2) and recruiting them to the plasma membrane. Then, insulin receptor substrate (IRS) interacts with p85 subunit of PI3K and recruits them to the plasma membrane. PI3K catalyses the formation of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3) from PtdIns(4,5)P_2. PtdIns(3,4,5)P_3 binds to PDK1 and PKB/Akt, engage them at the membrane and allowing PDK1 to activate PKB/Akt. Once PKB/Akt is activated, in turn, phosphorylates and inhibits GSK3, adapted from Cohen P et *al, Nat. Rev. Mol. Cell Biol. 2001* GSK3 (148).

1.8.3 GSK3 and sympathetic nervous activity

Insulin regulates glucose metabolism. It also plays a role in renal phosphate handling (166). Cellular insulin effects are mediated by PI3K dependent PKB/SGK isoform activation (161, 162). To reveal physiological importance of PKB/SGK dependent regulation of GSK3, gene-targeted mice were generated in which serine residue was replaced by alanine (GSK3α$^{21A/21A}$, GSK3ß$^{9A/9A}$) within the PKB/Akt phosphorylation

site. GSK3α/β mice carrying this mutation make the kinase insensitive to the inhibitory effect of PKB/Akt. Therefore, *gsk-3*^{KI} mice carrying mutations are protected from the inhibitory effect of insulin (167). In mice carrying these mutations (*gsk-3*^{KI}), plasma phosphate concentration was significantly lower and urinary phosphate excretion significantly higher than in the corresponding wild type mice (*gsk-3*^{WT}) (168). It has been reported that arterial blood pressure is enhanced in *gsk-3*^{KI} mice as compared to wild type mice (169, 170) and by the treatment with α-adrenergic antagonist prazosin enhanced blood pressure could be lowered which indicate to having sympathetic nerve activity (171). Epinephrine is a marker of sympathetic nervous activity. *Gsk-3*^{KI} mice show increased levels of plasma and urine epinephrine levels (172).

1.9 Actin cytoskeleton

Actin cytoskeleton is a cellular network within the cytoplasm which provides the cell shape, functional support and mechanical strength (173, 174), directs locomotion, regulates chromosome separation during mitosis and meiosis, intracellular transport of organelles in cells (175, 176). There are three main components of actin cytoskeleton in eukaryotic cells (actin microfilaments, microtubules and intermediate filaments). Actin microfilament is composed of monomeric (G-actin) and filamentous (F-actin) actin that constitute actin cytoskeleton along with ABP (actin-binding protein) (177). Actin microfilament consisting of highly polarized plus (barbed) end and minus (pointed) end. Actin exists within the cells in a dynamic transition of polymerization and deploymerization. At steady state F-actin grows fast at the barbed end by the adding of ATP-bound G-actin than does the pointed end where depolymerization occur by dissociation of ADP-bound G-actin. Actin can be polymerized or depolymerized by exchange of ATP or hydrolysis of ATP (178, 179).

1.9.1 Rho GTPase and actin cytoskeleton

Rho GTPase is a crucial regulator in the actin signaling pathway that links extracellular or intracellular signals to the assembly of actin cytoskeleton. Actin reorganization, following the modification of actin polymerization is a cellular response initiated by various signals including growth factors, cytokines and hormones (Fig. 7) which transmit signal through Rho-family GTPase in particular Rho, Rac and Cdc42 (180, 181). Actin cytoskeleton dependent cellular process including cytokinesis (182), cell migration (183) and endo/exocytosis (184) is severely impaired during dominant negative mutation in Rho-GTPase. Cofilin is phosphorylated by LIM kinase which reduces its actin-binding activity. LIM kinase is phosphorylated by ROCK that is regulated by Rho-GTPase (185, 186).

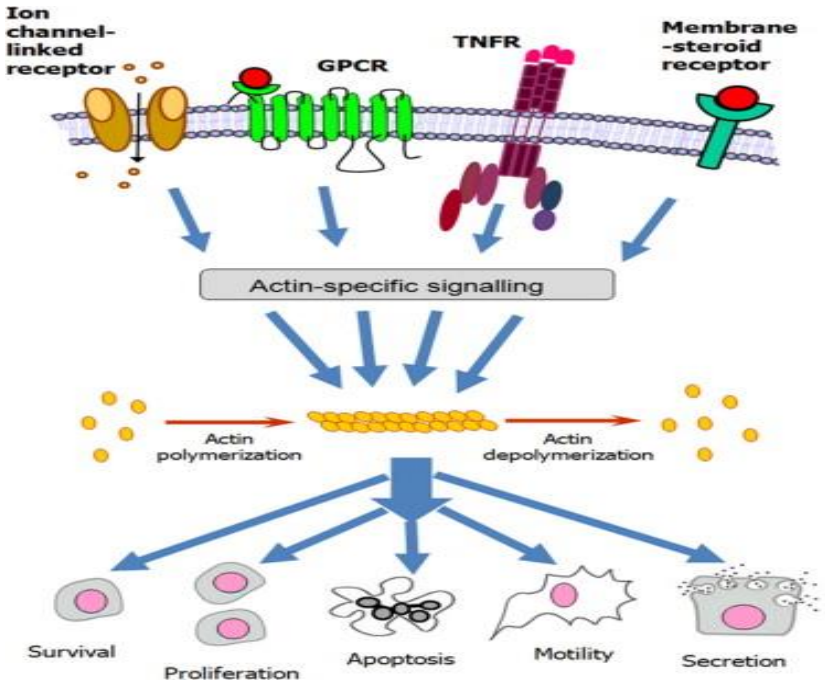

Fig. 7: Signal transduction to the actin cytoskeleton triggers a variety of cell response. Extracellular stimuli lead to the activation of membrane receptors which transmits signals to the actin cytoskeleton by specific actin signaling molecules. Alternation of actin cytoskeleton dynamics have been implicated in cellular outcomes including survival, secretion, proliferation and apoptosis and motility, Adapted from Papakonstanti EA and Stournaras C, *FEBS let, 2008*(187).

1.9.2 Rho GTPase and PI3K

Importance of PI3-kinase in cell survival and in actin restructuring has been described (188, 189). Rho GTPase associates with PI-kinase and control actin cytoskeleton reorganization. PI3K phosphorylates inisitol and forms the lipid products PIP2 and PIP3. These lipids then activate the signaling cascade of downstream effectors PKB/Akt or small GTPase Cdc42 and Rac1 (188). Rac1, in turn, promotes actin polymerization and changes cell morphology (190, 191)

1.9.3 Actin cytoskeleton and diseases

The dynamic reorganization of actin cytoskeleton is very crucial for cellular processes. During infection many pathogens hijack host cell actin cytoskeleton and disrupt cytoskeleton rapidly (192). Alternation in the actin cytoskeleton dynamics govern cellular outcomes which have been linked to human diseases, including muscular dystrophy (193, 194), liver diseases (195) and cancer metastasis and invasion (196, 197). Altered regulation of actin cytoskeleton is associated with neurological disorders and ischemic kidney disease. In Alzheimer's disease deregulation of ADF/cofilin was observed (198, 199).

2. Aim of the study

FGF23 is a phosphaturic hormone which regulates mineral homeostasis and vitamin D metabolism. Mice expressing PKB/Akt resistant *gsk-3*^{ki} show hypophosphatemia, renal phosphate loss and sympathetic nervous activation. It has been reported that insulin dependent PI3K signaling involved in the regulation of phosphate transport. Phosphaturia in these mice could be due to the inhibitory effects of GSK3 on the renal phosphate transporter NaPi2a, but in addition, FGF23 could be involved. Sympathetic activation of the nervous system and vitamin D stimulate FGF23 secretion. Vitamin D has been implicated in actin cytoskeleton reorganization. Based on this hypothesis, the present study was conducted to investigate

1. Whether GSK3-controlled sympathetic activity is involved in the regulation of FGF23 production.
2. Whether FGF23 gene transcription is regulated by actin cytoskeleton.

3. Materials and methods

3.1 Materials

3.1.1 Chemicals and reagents

Name	Company name and country of origin
$1,25(OH)_2D_3$	Enzo life science, New York, USA
30% Acrylamide	Carl Roth, Karlsruhe, Germany
Amersham hyperfilm	GE Healthcare, München, Germany
Ammonium persulfate	Sigma Aldrich, St. Louis, USA
Anti-Klotho (rat polyclonal IgG antibody)	Kyowa Hakko Kirin Co., Ltd., Japan
Anti-GAPDH	Cell Signaling Technology, USA
Anti-rabbit HRP- conjugate antibody	Cell Signaling Technology, USA
Anti-rat HRP conjugate linked antibody	Cell Signaling Technology, USA
Bradford protein assay	Bio-Rad, München, Germany
Bovine serum albumin (BSA)	Carl Roth, Karlsruhe, Germany
Chloroform	Carl Roth, Karlsruhe, Germany
Cytochalasin B	TOCRIS, Bristol, UK
Diethylether	Roth, Karlsruhe, Germany
DMEM	Invitrogen, Darmstadt, Germany
DTT	Invitrogen, Darmstadt, Germany
EDTA	Sigma Aldrich, St. Louis, USA
Ethanol	Roth, Karlsruhe, Germany
EGTA	Sigma Aldrich, St. Louis, USA
Enhanced chemiluminescence (ECL)	GE Healthcare, München, Germany
FBS	Gibco, Life Technologies, Darmstadt, Germany

Glucose, corn sugar	Sigma Aldrich, St. Louis, USA
Glycine	Carl Roth, Karlsruhe, Germany
Glycerol	Carl Roth, Karlsruhe, Germany
IPA3	Tocris, Bristol, UK
Isopropyl alcohol	Carl Roth, Karlsruhe, Germany
Methanol	Carl Roth, Karlsruhe, Germany
Sodium chloride (NaCl)	Carl Roth, Karlsruhe, Germany
Nitrocellulose membranes	Whatman, Maidstone, UK
Non-fat milk powder	Carl Roth, Karlsruhe, Germany
NP-40	Carl Roth, Karlsruhe, Germany
PBS	PAA Laboratories, Cölbe, Germany
Penicillin/Streptomycin	PAA Laboratories, Cölbe, Germany
PeqGold protein marker	PeqLab, Erlangen, Germany
peqGold TriFast	PeqLab, Erlangen, Germany
PMSF	Sigma Aldrich, St. Louis, USA
Propranolol	Sigma Aldrich, St. Louis, USA
Protease inhibitor cocktail	Roche Diagnostics, Mannheim, Germany
PVDF membrane	ThermoScientific,Waltham,Massachusetts,USA
Roti Loading Dye (4x)	Carl Roth, Karlsruhe, Germany
Rac1 inhibitor NSC 23766	Tocris, Bristol, UK
Phalloidin	Life Technologies, USA
RIPA lysis buffer	Cell Signaling Technology, USA
SDS	Carl Roth, Karlsruhe, Germany
Sodiumdeoxycholate	Sigma Aldrich, St. Louis, USA
Sodium fluride	Carl Roth, Karlsruhe, Germany
Sodium pyrophosphate	Sigma Aldrich, St. Louis, USA
Sodium ortho vanadate	Sigma Aldrich, St. Louis, USA
Stripping buffer	Carl Roth, Karlsruhe, Germany
TEMED	Carl Roth, Karlsruhe, Germany
Tris-base	Sigma Aldrich, St. Louis, USA

Trypsin-EDTA	PAA Laboratories, Cölbe, Germany
TritonX100	Carl Roth, Karlsruhe, Germany
Tween 20	Carl Roth, Karlsruhe, Germany
Wogonin	Enzo life science

3.1.2 Kits

1. 1,25$(OH)_2D_3$ EIA Kit (Immunodiagnostic Systems, Boldon, UK).
2. FGF23 (c-term) ELISA Kit (Immutopics International, California, USA).
3. Intact FGF23 ELISA kit (Kainos Laboratories, Tokyo, Japan).
4. Creatinin PAP,enzymatic (LT-SYS, Labor Technik, Germany).
5. Creatinine Jaffe, Kinetic (LT-SYS, Labor Technik, Germany).
6. Phos (inorganic phosphorus) (Roche Diagnostics, Mannheim, Germany).
7. Mouse PTH 1-84 ELISA Kit (Immunotpics International, California, USA).
8. Vanillylmandelic acid (VMA) ELISA Kit (IBL international, Hamburg, Germany).
9. Epinephrine ELISA kit (LDN, Germany).
10. SuperScript III First-Strand Synthesis kits (Invitrogen, Darmstadt, Germany).

3.1.3 Equipments

Name	**Company name and country of origin**
CFX96 real-time system	Bio-Rad Laboratories, München, Germany
BioPhotometer	Eppendorf, Hamburg, Germany
Biorad chemidoc XRS	Bio-Rad Laboratories, München, Germany
Electrophoresis cell	Bio-Rad Laboratories, München, Germany
Electrophoresis power supply	Bio-Rad Laboratories, München, Germany
Electrophoretic transfer cell	Bio-Rad Laboratories, München, Germany
Eppendorf centrifuge 5417c	Eppendorf, Hamburg, Germany
Eppendorf thermomixer 5436	Eppendorf, Hamburg, Germany
ELISA plate reader (Power Wave XS2)	BioTek, Winooski, VT, USA

Heraeus cell culture hood	Thermo Fisher Scientific, Waltham MA, USA
Heraeus cell culture incubator	Thermo Fisher Scientific, Waltham MA, USA
Homogenize rotor	Carl Roth, Karlsruhe, Germany
Non-Invasive blood pressure amplifier	IITC life sciences, Los Angeles, USA
pH meter	Sartorius, Göttingen, Germany
Power labs dual bio amps (ML135)	ADInstruments GmbH, Germany
Metabolic cage	Techniplast, Hohenpeissenberg, Germany
Flame photometry (ELEX 6361)	Eppendorf, Hamburg, Germany
Primus PCR instrument 25	Peqlab, Erlangen, Germany
Zeiss LSM 5 EXCITER confocal microscopy	Carl Zeiss, Germany

3.2 Methods

3.2.1 Cells

Rat osteosarcoma cells UMR 106 were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 4,5 g/l glucose, supplemented 10% fetal bovine serum FBS (Gibco, Life Technologies, Darmstadt, Germany), 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco, Life Technologies, Darmstadt, Germany) at 5% CO_2 and 37^0 C . UMR 106 cells were pretreated with 100 nM $1,25(OH)_2D_3$. After 42 h cells were treated with 100 nM cytochalasin B, 50 µM Rac1 inhibitor NSC 23766 and 10 µM IPA3 (TOCRIS, Bristol, UK), or with vehicle only for 6 h, and thereafter were harvested for RT-PCR. For some experiments, cells were treated with or without 150 µM propranolol (Sigma Aldrich, St. Louis, USA) over night (14-16 h) in the presence of 100 nM $1,25(OH)_2D_3$. For Western blotting cells were treated with 100 nM $1,25(OH)_2D_3$ for 15 min, 30 min and 24 h. For some experiments, cells were incubated with or without 100 µM NFκB inhibitor wogonin (Enzo Life Sciences, Germany) for 24 h and in addition treated with 100 nM $1,25(OH)_2D_3$ or with vehicle for another 15 min. Alternatively, cells were treated with 50

µM Rac1 inhibitor NSC 23766 and 10 µM PAK1 inhibitor IPA3 in presence or absence of 100 nM $1,25(OH)_2D_3$ for 15 min.

3.2.2 Quantification of mRNA expression

Total RNA was isolated using peqGOLD TriFast (PeqLab Biotechnologie GmbH, Erlangen, Germany) reagent, a method which is based on a chloroform extraction protocol. The mRNA was transcribed with SuperScriptIII Reverse Transcriptase (Invitrogen, Darmstadt, Germany) using random hexamers (Invitrogen, Darmstadt, Germany). Quantitative RT-PCR was performed on a BioRad iCycler iQTM Real-Time PCR Detection System (Bio-Rad Laboratories, München, Germany) using the following primers:

Tbp (TATA box-binding protein):

Forward primer (5'-3'): ACTCCTGCCACACCAGCC

Reverse primer (5'-3'): GGTCAAGTTTACAGCCAAGATTCA

Fgf23

Forward primer (5'-3'): TGGCCATGTAGACGGAACAC

Reverse primer (5'-3'): GGCCCCTATTATCACTACGGAG

The final volume of the PCR reaction mixture was 20 µl and contained: 2 µl cDNA, 1 µM of each primer, 10 µl GoTaq qPCR Master Mix (Promega, Mannheim, Germany) and sterile water up to 20 µl. qPCR conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 58°C for 30 s. Calculated mRNA expression levels were normalized to the expression levels of *Tbp* of the same cDNA sample as internal reference. All PCRs were performed in duplicate. Relative quantification of gene expression was performed using the $\Delta\Delta c_t$ method.

3.2.3 Confocal microscopy

For actin staining, UMR 106 cells were cultured on glass chamber slides (BD biosciences) for 24 h and treated with or without 1,25(OH)2D3 (100 nM) for 15 min, 30 min and 24 h. After washing twice with PBS, cells were fixed with 4% PFA for 15 min at room temperature and then permeabilized with 0.03% Triton-X100 for 10 min. The cells were blocked with 3% BSA in PBST and incubated with rhodamine-phalloidin (1:200, Life Technologies, USA) for F-actin staining and with DRAQ-5 dye (1:3000, Biostatus, Leicestershire, UK) for nuclei staining for 30 min in the dark. After three washing steps, slides and coverslips were mounted with ProLong Gold antifade reagent (Life Technologies, USA). Images were taken on a Zeiss LSM 5 EXCITER confocal laser scanning microscope (Carl Zeiss, Germany) with a water immersion Plan-Neofluar 40/1.3 NA DIC.

3.3 Mice

All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities (Baden-Württemberg). Experiments have been performed with gene-targeted mice carrying a mutant GSK3α,β, in which the codon encoding Ser9 of GSK3ß gene was changed to encode nonphosphorylable alanine (GSK3ß$^{9A/9A}$), and simultaneously the codon encoding Ser21 of GSK3α was changed to encode the nonphosphorylable GSK3α$^{21A/21A}$ thus yielding the GSK3α/β$^{21A/21A/9A/9A}$ double knock in mouse (*gsk-3*^{KI}) as described previously (167). The mice were compared to corresponding wild type mice (*gsk-3*^{WT}). In this study, I have used *gsk-3*^{KI} mice and *gsk-3*^{WT} mice. The animals were genotyped by PCR using standard method. If not stated otherwise, mice were fed a control diet (Altromin 1310) and adlib access to drinking water.

3.3.1 Propranolol treatment

GSK3 wild type (*gsk-3*^{WT}) and GSK3 knock in (*gsk-3*^{KI}) mice were treated with propranolol at dose of 500 mg/L for 7 days in drinking water. Blood was drawn before and after treatment with propranolol. Mice were fed a control diet (Altronin 1310). The mice were studied at the age of 2-5 months.

3.3.2 Blood chemistry

To obtain serum or plasma, animals were lightly anesthetized with diethyl ether (Roth, Karlsruhe, Germany) and about 50 - 200 µl of blood were drawn by puncturing the retro-orbital plexus with capillaries before and after treatment with β-blocker propranolol at the dose of 500mg/L in drinking water for one week. Serum or plasma was separated by centrifugation of blood at 7000 rpm for 10 min at 4^0C and kept at -80°c until measurement.

3.3.3 FGF23 measurement

Serum FGF23 was measured by ELISA (Immutopics International, San Clemente, USA) according to the manufacturer´s instructions. Briefly, for the measurement of C-term FGF23 25 µl standard, control or samples were loaded into the designated or mapped well coated with anti-FGF23. Biotinylated antibody and HRP-conjugated antibody were added to each well in 1:1 ratio and the plate was sealed and then covered with aluminium foil to avoid exposure to light. After incubation at room temperature for 3 h on a rotator at 180-220 rpm, wells were washed three times with washing buffer. ELISA HRP substrate was added to each well and incubated another half an hour. After incubation period, stop solution was added and reading took at 450 nm against a reagent blank.

3.3.4 Calcitriol measurement

An EIA kit was employed to determine plasma concentrations of $1,25(OH)_2D_3$ (IDS, Boldon, UK). All experiments have done according to the manufacturer's instructions.

3.3.5 Plasma PTH measurement

Plasma PTH concentration was measured by using mouse PTH 1-84 ELISA kit (Immutopics International, San Clemente, USA). Manufacturer's instructions were followed. Briefly, 20µl of plasma samples, standard and control were used into the designated antibody coated well. After that, mouse PTH 1-84 biotinylated antibody and mouse PTH 1-84 HRP conjugated antibody were added in 1:1 ratio into each well. The plate was covered with plate sealer and then wrapped with aluminium foil and incubated for 3 h at room temperature on a horizontal rotator at the speed of 180-220 rpm. Wells were washed five times with wash buffer and added 100 µl of ELISA HRP substrate into each well and incubated another half an hour. After adding ELISA stop solution into each of the wells, absorbance was measured by ELISA plate reader.

3.3.6 Serum calcium, phosphate and creatinine

Following serum parameters were measured. Serum Ca^{2+} was determined by flame photometry. Inorganic phosphate was measured by photometric method (Roche, Mannheim, Germany) and serum creatinine concentration by using an enzymatic colorimetric method (Labor & Technik, Berlin, Germany). All experiments have done according to the manufacturer's instruction.

3.3.7 Metabolic cage study

Both *gsk-3*^{KI} mice and *gsk-3*^{WT} mice were placed individually in metabolic cages (Techniplast, Hohenpeissenberg, Germany) for the collection of 24 h urine to evaluate renal excretion. Mice were allowed a 2-3 days habitation period and intake of food and drinking water, urinary flow rate and body weight were recorded regularly to make sure that the mice were adapted to the new environment. Subsequently, 24 h of urine was collected for 5 consecutive days to obtain the urinary parameters. Urine was collected under water-saturated oil and metabolic cages were siliconized to ensure smooth urine collection. After collecting base line urine mice were treated with propranolol in drinking water at the dose of 500 mg/L for 7 days and urine was collected. Mice were fed control diet. Urine aliquots were rapidly frozen and store at -80 until measurement.

3.3.8 Urinary phosphate, calcium and creatinine

The phosphate concentration was determined colorimetrically using commercial diagnostic kits (Roche Diagnostics, Mannheim, Germany). The urinary concentration of Ca^{2+} was measured by flame photometry (ELEX 6361, Eppendorf) or by a photometric method according to the manufacturer's instructions (Dri-chem clinical chemistry analyzer FUJI FDC 3500i, Sysmex, Norsted, Germany). The creatinine concentration in urine was determined using the Jaffe reaction (LT-SYS, Labor Technik, Germany).

3.3.9 Measurement of urinary vanillylmandelic acid (VMA) and epinephrine

For the measurement of renal excretion both *gsk-3*^{KI} mice and *gsk-3*^{WT} mice were placed individually in the metabolic cages to adapt new environment on the first two days for collecting 24 h urine. Mice were again housed from day 2-day 5 of propranolol treatment. Small amount of 6N HCL were added to the urine collection tube to acidify the urine and next 24 h acidic urine was collected. To ensure smooth urine collection,

metabolic cages were siliconized and urine was collected under water-saturated oil. Urinary epinephrine (Labor Diagnostika Nord, Nordhorn, Germany) and vanillylmandelic acid (IBL international, Hamburg, Germany) concentration were determined by using ELISA kits

3.4 Blood pressure measurement

The arterial blood pressure was determined by a non-invasive tail-cuff system (IITC life Science, Woodlands Hills, CA, USA). To get the systolic blood pressure of mice, I took average of several days readings for the respective mouse. To reduce the stress of mice some precautions were taken. Mice were placed in clean plastic animal holder. Before taking reading mice were given proper training. Tail of the mouse was positioned inside a tail cuff with a photoelectric sensor. We determined systolic blood pressure before and after treatment with β-blocker propranolol. All recordings and data analysis were obtained by using a computerized data acquisition system and software (PowerLab 4/26).

3.5 Measurement of F/G actin ratio by Triton X-100 fractionation

For measurements of the monomeric (Triton soluble) and polymerized (Triton insoluble) actin, UMR 106 cells were incubated for different time points with or without and/or with vitamin D (100 nM), wogonin (100 µM), Rac1 inhibitor NSC 23766 (50 µM) and PAK inhibitor IPA3 (10 µM) where indicated. Then, cells were harvested. Actin cytoskeleton determination by Triton X-100 was described previously (200). Briefly, cells were incubated with 130 µl of Triton extraction buffer containing (0.3% TritonX-100, 5 mM Tris, pH 7.4, 2 mM EGTA, 300 mM sucrose, 2 µM phalloidin, 1 mM PMSF, 10 µg/ml leupeptin, 20 µ/ml aprotinin, 1 mM sodium orthovanadate, and 50 mM NaF for 5-7 min on ice. The supernatant (G-actin) containing soluble protein was removed. The Triton insoluble fraction remaining on the culture plate was scraped and lysed with RIPA buffer (Cell Signaling, Frankfurt, Germany). An equal amount of protein of each fraction was subjected

to 10% SDS-PAGE. The proteins were transferred onto PVDF membranes which were then blocked with 5% nonfat dry milk powder in TBS-T for 1 h at room temperature. Next, the membrane was incubated overnight at 4°C with pan-actin primary antibody (1:1000 in 5% BSA with TBS-T, Cell Signaling) and washed 3-5 times with TBS-T. Then, incubation with secondary anti-rabbit horseradish peroxidase-conjugated antibody was carried out for 1 h at room temperature (1:2000; Cell Signaling). Blots were developed by commercial ECL (Thermo Scientific) or home made ECL reagent and band intensities were quantified by chemidoc Quantity one software.

3.6 Western blotting

To determine protein abundance, mice were anesthetized with diethyl ether. The kidneys were removed and immediately snap-frozen in liquid nitrogen. The kidneys of mice were homogenized with an electric homogenizer at 4°C on ice in lysis buffer (54.6 mM HEPES, 2.69 mM Na4P2O7, 360 mM NaCl, 10% [vol/vol] Glycerol, 1% [vol/vol] NP40) or RIPA (Cell signalling) containing phosphatase and protease inhibitors (Complete mini, Roche, Mannheim, Germany). Homogenates were kept on ice for 30 minutes and homogenates were centrifuged at 14000 rpm for 30 min at 4^0C. Protein concentration was quantified by Bradford protein assay (Bio-Rad, München, Germany). 40 µg tissue lysate was separated by SDS-PAGE (8% Tris-Glycine), transferred to PVDF membranes (Thermo scientific, Germany), blocked for 1 h in blocking buffer (5% fat-free milk in Tris-buffered saline (TBS) containing 0.1% Tween), and incubated overnight at 4^0c with rat anti-klotho (1:1000 in 5% fat free milk in TBST; kindly provided by Akiko Saito, Kyowa Hakko Kirin Co., Ltd., Japan) antibody. After incubation with a horseradish peroxidase-conjugated anti-rat or anti rabbit secondary antibody (1:2000 in 5% fat free milk in TBST; Cell Signaling, Freiburg, Germany), the bands were visualized with enhanced chemiluminescence according to the manufacturer's instructions or by using home-made ECL solution. For loading control, the membrane was probed with GAPDH antibody (1:2000 in 5% BSA in TBST; Cell Signaling, Frankfurt Germany). Densitometric analysis of klotho was performed using Quantity One software (Bio-Rad Laboratories).

3.6.1 Solutions

Table 1. **Running buffer (10X)**

Tris base	250 mM
Glycine	1.9 M
SDS	1%
dH_2O	Up to 1 litre

Table 2. **Transfer buffer (10X)**

Tris base	198 mM
Glycine	1.5 M
dH_2O	Up to 1 litre

Table 3. **TBS (10X)**

Tris base	200 mM
NaCl	1.3 M
dH_2O	Up to 1 litre, pH 7.6

Table 4. Solution A for ECL (200 ml)

Tris (0.1 M)	2.42 g
Luminol	50 mg
dH_2O	200 ml
Adjust pH	8.6

Table 5. Solution B for ECL (10 ml)

Para hydroxyl cumeric acid	11 mg
DMSO	10 ml

Table 6. Working solution for ECL

Solution A	1 ml
Solution B	100 µl
Hydrogen peroxide (H_2O_2)	0.3 µl

3.7 Statistics

Data are provided as means ± SEM, n represents the number of independent experiments. All data were tested for significance using unpaired Students t-test or ANOVA. Only results with $p < 0.05$ were considered statistically significant.

4. Results

Part 1 Results

4.1.1 Enhanced serum FGF23 in *gsk-3^{KI}* mice

It has been shown that *gsk-3^{KI}* mice suffer from hypophosphatemia and renal phosphate wasting as well as low serum levels of $1,25(OH)_2D_3$ (168). FGF23 is derived from bone and exerts its phosphate wasting effect in the kidney and thereby reduces serum phosphate. Thus, the serum C-terminal FGF23 and intact FGF23 concentration was measured. Both C-terminal FGF23 and intact FGF23 were significantly higher in *gsk-3^{KI}* mice compared to *gsk-3^{WT}* mice (Fig. 8).

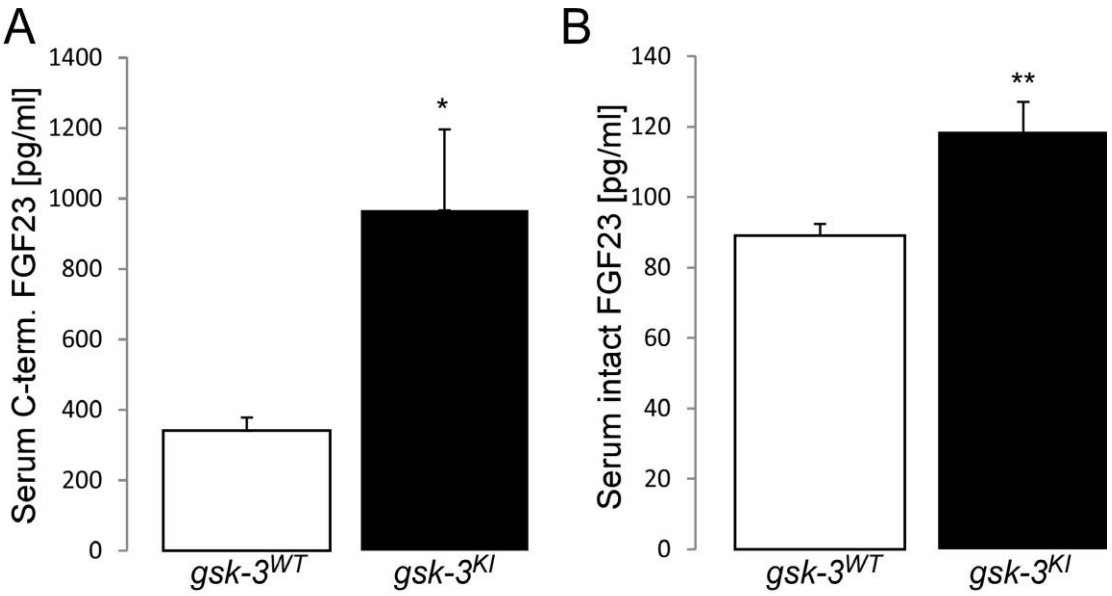

Fig. 8: The serum FGF23 level is elevated in *gsk-3^{KI}* mice compared to *gsk-3^{WT}* mice.
Arithmetic means ± SEM of the serum C-terminal FGF23 (A; n=5) and intact FGF23 (B; n=6) concentration determined in *gsk-3^{WT}* mice (white bars) and *gsk-3^{KI}* mice (black bars). ** p<0.05, **p<0.01.*

4.1.2 Renal klotho abundance in *gsk-3^{KI}* mice

FGF23 binds to its receptor to exert renal function. FGFR receptor requires α-klotho as a co-receptor. It has been reported that reduced expression of α-klotho is associated with high serum concentration of FGF23 (201) in klotho *knock out* mice. Therefore, renal abundance of klotho was determined by Western blotting. I did not find any difference in renal α-klotho expression between the genotypes (Fig. 9). Hence, α-klotho does not account for elevated serum FGF23 in *gsk-3^{KI}* mice.

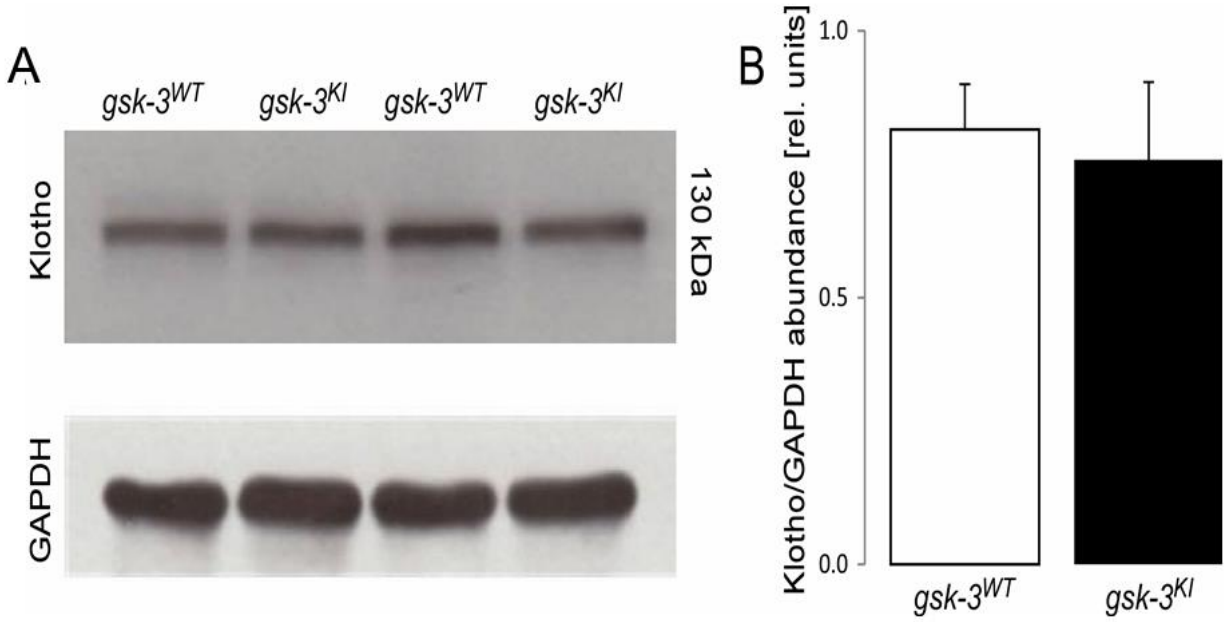

Fig. 9: Renal α-Klotho abundance in *gsk-3^{KI}* mice and *gsk-3^{WT}* mice.

A. Original Western blots demonstrating renal Klotho (upper panel) and GAPDH (lower panel) abundance.

B. Densitometric analysis of the Western Blot. Arithmetic means ± SEM (n = 6).

4.1.3 Sympathetic nervous activity in *gsk-3^{KI}* mice

Recently, it has been reported that sympathetic activation is a trigger of FGF23 release (147). Epinephrine and norepinephrine are the neurotransmitters of sympathetic nervous system (202). Vanillylmandelic acid (VMA) is an end-stage metabolite of the catecholamines, epinephrine and norepinephrine. Therefore, urinary VMA is a biomarker of sympathetic activation. To this end, I determined urinary epinephrine and VMA excretion by ELISA. In keeping with a previous report (172), I found significantly higher urinary epinephrine and VMA excretion in *gsk-3^{KI}* mice compared to *gsk-3^{WT}* mice (Fig. 10), which indicating increased activity of the sympathetic nervous system in *gsk-3^{KI}* mice.

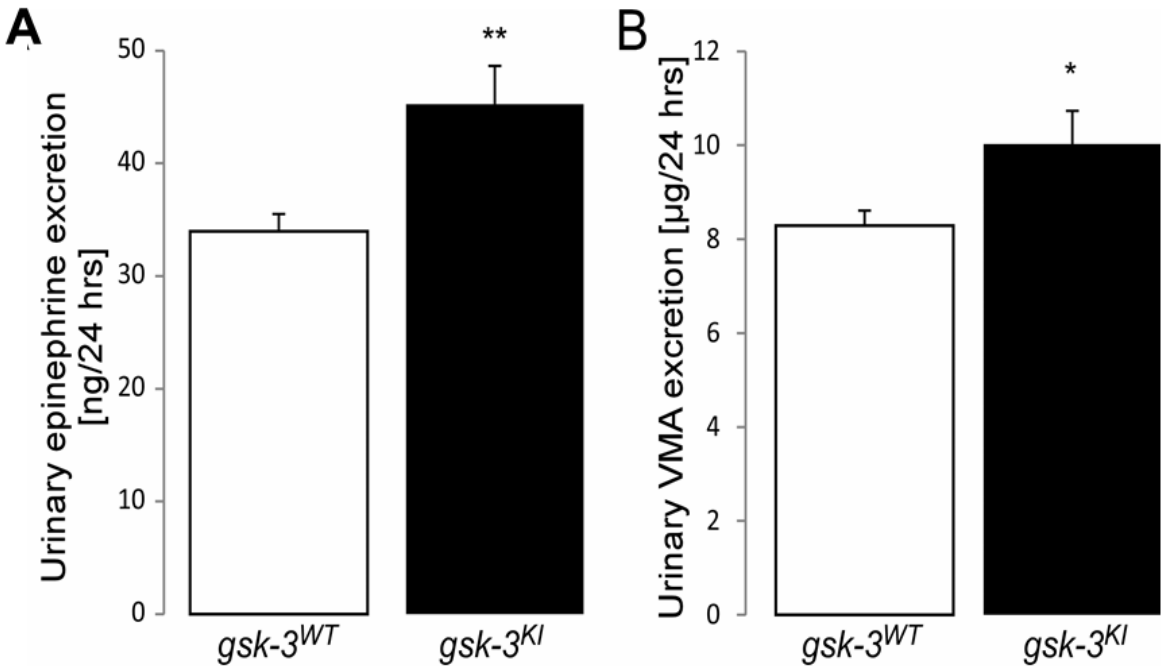

Fig. 10: Enhanced urinary excretion of epinephrine and vanillylmandelic acid (VMA) in *gsk-3^{KI}* mice compared to *gsk-3^{WT}* mice.

Arithmetic means ± SEM (n = 7) of the urinary 24 h-epinephrine (**A**) and 24 h-VMA (**B**) excretion of *gsk-3^{WT}* mice (white bar) and *gsk-3^{KI}* mice (black bar). ** p<0.05, ** p<0.01.*

4.1.4 High blood pressure in *gsk-3^{KI}* mice

In the next series of experiments, systolic blood pressure was measured in *gsk-3^{kI}* mice and *gsk-3^{WT}* mice by tail-cuff method. As a consequence of higher sympathetic nerve activation, blood pressure was significantly higher in *gsk-3^{kI}* mice compared to *gsk-3^{WT}* mice. β-blocker propranolol treatment significantly reduced blood pressure of *gsk-3^{KI}* mice to the level of *gsk-3^{WT}* mice (Fig. 11).

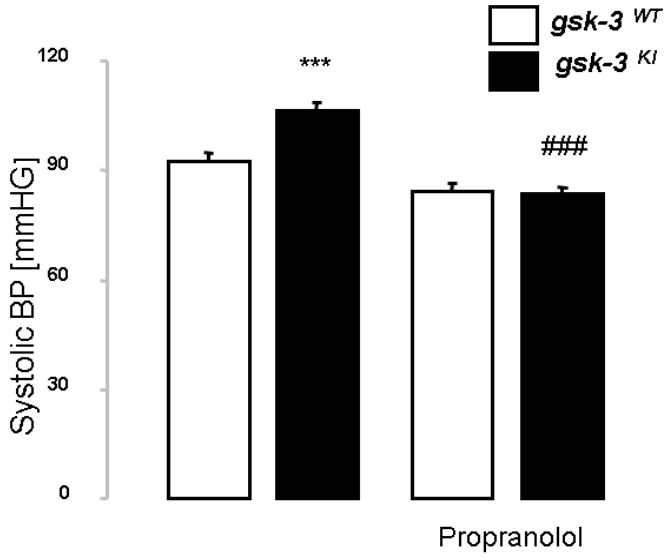

Fig. 11: β-blocker propranolol lowers systolic blood pressure in *gsk-3^{KI}* mice.
Arithmetic means ± SEM of systolic blood pressure (n=12) in *gsk-3^{WT}* mice (white bars) and *gsk-3^{KI}* mice (black bars) determined before (left bars) and after one week of treatment with β-blocker propranolol (500 mg/L) (right bars). *** *(p<0.001)* indicate significant difference between the genotypes, ### *(p<0.001*) indicates significant difference from the absence of propranolol treatment.

4.1.5 Glomerular filtration rate (GFR)

As blood pressure may influence glomerular filtration rate of kidney (GFR), I measured creatinine clearance to determine GFR. Before propranolol treatment GFR was significantly higher in *gsk-3*^{KI} mice than in *gsk-3*^{wt} mice. Propranolol reduced GFR between the genotypes, a difference not reaching statistical significance (Fig. 12).

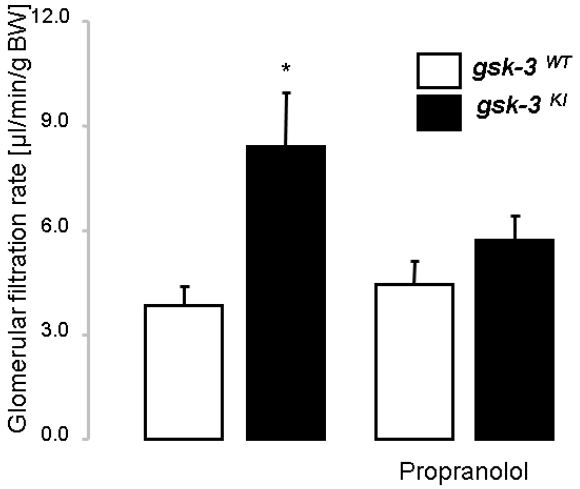

Fig. 12: Effects of β-blocker propranolol on GFR in *gsk-3*^{KI} mice.

Arithmetic means ± SEM of GFR (n=19) in *gsk-3*^{WT} mice (white bars) and *gsk-3*^{KI} mice (black bars) determined before (left bars) and after one week of treatment with β-blocker propranolol (500 mg/L) (right bars). * *(p<0.05)* indicates significant difference between the genotypes.

4.1.6 β-blocker propranolol normalized enhanced production of FGF23 in *gsk-3^{kI}* mice

Next, I investigated whether sympathetic activation is responsible for increased FGF23 production and leads to phosphaturia in *gsk-3^{KI}* mice. To verify this, *gsk-3^{KI}* mice and *gsk-3^{WT}* mice were treated with β-blocker propranolol for one week at dose of 500 mg/L in drinking water. The serum C-terminal FGF23 concentration was significantly higher in *gsk-3^{KI}* mice compared to *gsk-3^{WT}* mice before propranolol treatment (Fig. 13). Elevated serum C-terminal FGF23 in *gsk-3^{KI}* mice was reduced significantly by β-blocker propranolol treatment (500 mg/L) and abrogated the difference in serum FGF23 between the genotypes (Fig. 13). Serum intact FGF23 was also significantly higher in *gsk-3^{KI}* mice than in *gsk-3^{WT}* mice. Propranolol treatment also reduced intact FGF23 in lesser extent and mimics the difference between the genotypes (Fig. 14).

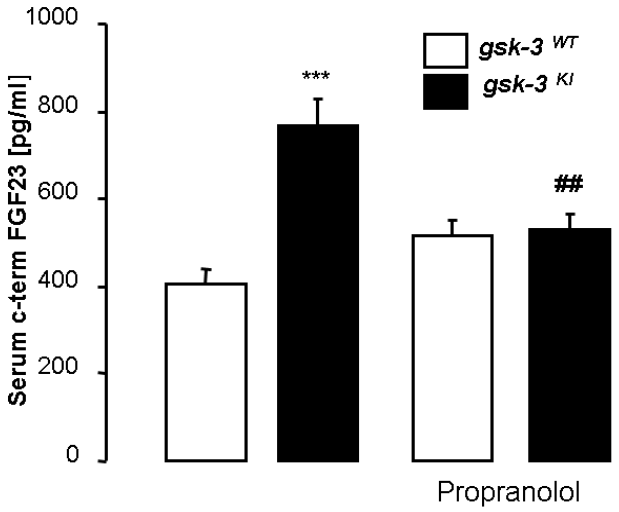

Fig. 13: β-blocker propranolol reduces the serum C-term FGF23 level in *gsk-3^{KI}* mice.

Arithmetic means ± SEM of the serum C-terminal FGF23 (n=20) concentration in *gsk-3^{WT}* mice (white bars) and *gsk-3^{KI}* mice (black bars) measured before (left bars) and after one week of treatment with β-blocker propranolol (500 mg/L) (right bars*).* *** *(p<0.001)* indicate significant difference between the genotypes, ## *(p<0.01)* indicates significant difference from the absence of propranolol treatment.

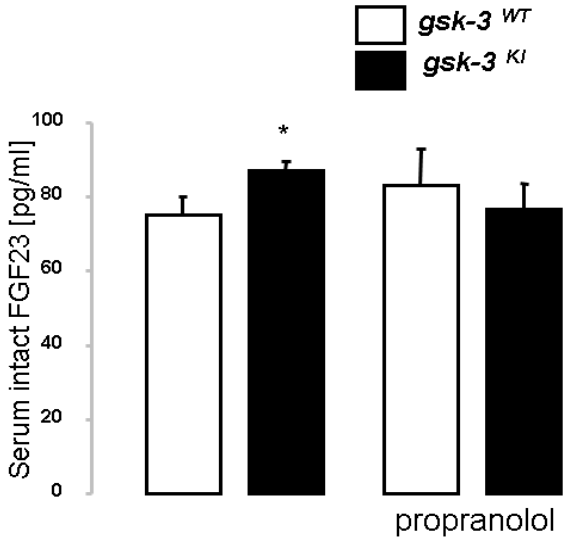

Fig. 14: Effects of β-blocker propranolol on the serum intact FGF23 level in *gsk-3^{KI}* mice

Arithmetic means ± SEM of the serum intact FGF23 (n=12) concentration in *gsk-3^{WT}* mice (white bars) and *gsk-3^{KI}* mice (black bars) measured before (left bars) and after one week of treatment with β-blocker propranolol (500 mg/L) (right bars). * *(p<0.05)* indicates significant difference between the genotypes.

4.1.7 The effect of propranolol on Fgf23 gene transcription in UMR 106 cells

These data suggests that sympathetic nervous activation is responsible for elevated FGF23 levels in *gsk-3^{KI}* mice. In a recent study, Kawai M *et al*, have shown that β-receptor agonist isoproterenol up-regulates Fgf23 gene transcription in UMR 106 cells (147). Thus, to define the molecular mechanism, UMR 106 osteoblast-like cells were treated with β-blocker propranolol overnight (14 h) to determine whether propranolol affects Fgf23 gene transcription by qRT-PCR. Overnight incubation with 150 µM propranolol significantly down regulated Fgf23 mRNA transcription compared to control cells (Fig. 15). Thus, propranolol inhibits FGF23 formation both *in vivo* and *in vitro.*

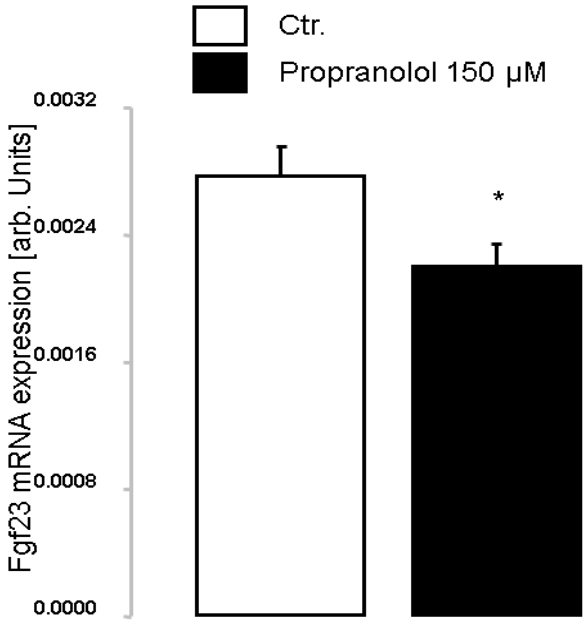

Fig. 15: β-blocker propranolol down-regulates Fgf23 transcription in UMR 106 cells.

Arithmetic means ± SEM of Fgf23 mRNA expression in UMR 106 cells treated with or without 150 µM propranolol for 16 h in the presence of $1,25(OH)_2D_3$ (n=15) Ctr. (white bars) and Propranolol (black bars). * *(p<0.05)* indicates significant difference from the absence of propranolol treatment.

4.1.8 Reduced $1,25(OH)_2D_3$ in *gsk-3^{KI}* mice

FGF23 down-regulates renal 1α hydroxylase (Cyp27b1), key enzyme responsible for active vitamin D ($1,25(OH)_2D_3$) synthesis and up-regulates 25-hydroxyvitamin D 24-hydroxylase (Cyp24a1) thus reducing the formation and enhancing the inactivation of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). Therefore, the serum $1,25(OH)_2D_3$ concentration was measured by ELISA. As expected, elevated serum levels of FGF23 was paralleled by low serum levels of $1,25(OH)_2D_3$ in *gsk-3^{KI}* mice before propranolol treatment. However, propranolol treatment did not change the serum $1,25(OH)_2D_3$ concentration (Fig. 16).

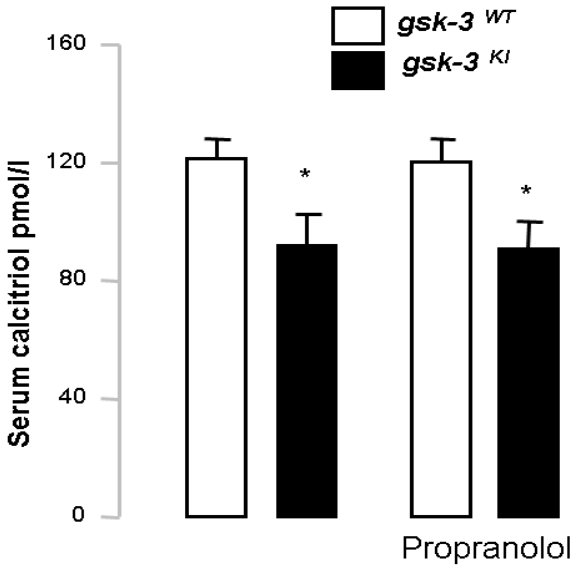

Fig. 16: Effects of β-blocker propranolol on the serum calcitriol level in *gsk-3^{KI}* mice.
Arithmetic means ± SEM of $1,25(OH)_2D_3$ (n=11-12) concentration in *gsk-3^{WT}* mice (white bars) and *gsk-3^{KI}* mice (black bars) measured before (left bars) and after one week of treatment with β-blocker propranolol (500 mg/L) (right bars). * *(p<0.05)* indicates significant difference between the genotypes.

4.1.9 Hypophosphatemia and phosphaturia in *gsk-3^{KI}* mice

High serum FGF23 inhibits tubular phosphate and increases renal phosphate wasting (phosphaturia). Therefore, I measured serum phosphate and renal phosphate excretion before and after treatment with propranolol. Consistent with previous reports (168), we found serum phosphate was significantly lower in *gsk-3^{KI}* mice compared to *gsk-3^{WT}* mice before treatment with propranolol (Fig. 17A). In spite of low serum phosphate (Hypophosphatemia), *gsk-3^{KI}* mice had significantly higher renal phosphate excretion than *gsk-3^{WT}* mice before propranolol treatment (Fig. 17B). I treated *gsk-3^{KI}* mice and *gsk-3^{WT}* mice with propranolol for one week in drinking water (500 mg/L). Propranolol treatment significantly alleviated hypophosphatemia of *gsk-3^{KI}* mice (Fig. 17A) and at the same time reduced renal phosphate excretion (Fig. 17B). Interestingly, treatment with β-blocker propranolol significantly increased the serum phosphate level and decreased renal phosphate excretion in *gsk-3^{WT}* mice (Fig 17).

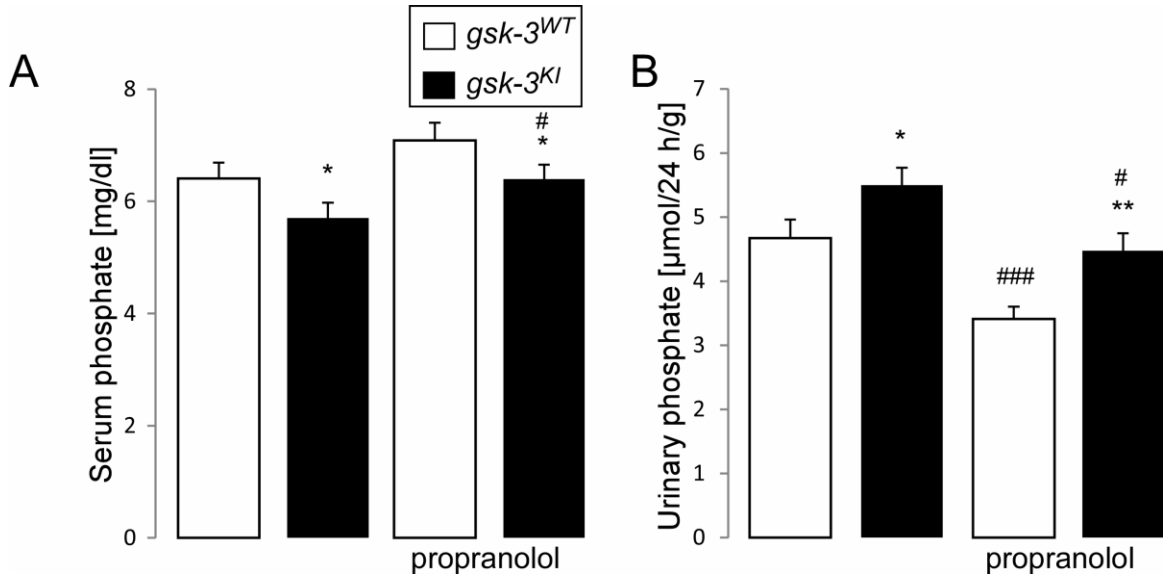

Fig. 17: β-blocker propranolol decreases the renal phosphate wasting of *gsk-3^{KI}* mice.

Arithmetic means ± SEM of the serum phosphate concentration (**A**; n=19) and renal phosphate excretion (**B**; n=19) in *gsk-3^{WT}* mice (white bars) and *gsk-3^{KI}* mice (black bars) measured before (left bars) and after one week of treatment with β-blocker propranolol (500 mg/L) (right bars). *,** *(p<0.05, p<0.01)* indicate significant difference between the genotypes, #,### *(p<0.05, p<0.001)* indicate significant difference from the absence of propranolol treatment.

4.2.1 Hypoparathyroidism in *gsk-3^{KI}* mice

PTH inhibits renal tubular phosphate reabsorption in the kidney. Therefore, phosphaturia could be caused by suppressing renal phosphate reabsorption by PTH. PTH also regulates calcium and vitamin D. Therefore, I measured plasma PTH levels in *gsk-3^{KI}* mice and *gsk-3^{WT}* mice by ELISA before and after treatment with propranolol (500 mg/L). In the line with a previous report (168), I found low plasma PTH levels in *gsk-3^{KI}* mice compared to *gsk-3^{WT}* mice. However, propranolol treatment did not affect plasma PTH concentration (Fig. 18).

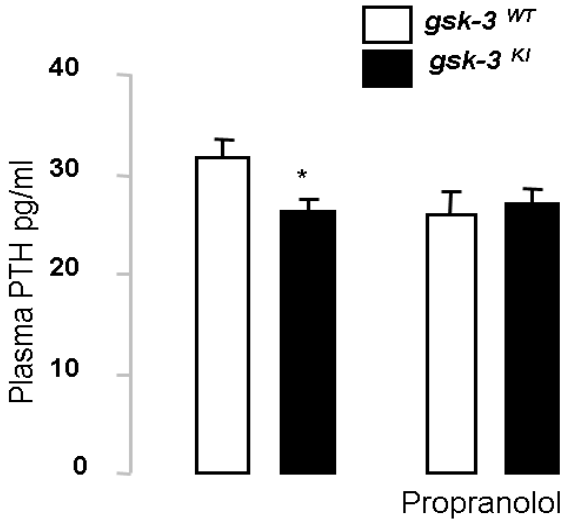

Fig. 18: Effects of β-blocker propranolol on the plasma PTH level in *gsk-3^{KI}* mice.
Arithmetic means ± SEM of PTH (n=11-12) concentration in *gsk-3^{WT}* mice (white bars) and *gsk-3^{KI}* mice (black bars) measured before (left bars) and after one week of treatment with β-blocker propranolol (500 mg/L) (right bars). * *(p<0.05)* indicates significant difference between the genotypes

4.2.2 Calciuria in *gsk-3^{KI}* mice

Gsk-3^{KI} mice have been reported to suffer from calciuria (168). In the next series of experiments, I explored whether propranolol influences serum calcium and renal calcium wasting in *gsk-3^{KI}* mice and *gsk-3^{WT}* mice. Prior to propranolol treatment I did not find any difference between *gsk-3^{KI}* mice and *gsk-3^{WT}* mice in serum calcium concentration (Fig. 19A). However, renal calcium excretion was higher in *gsk-3^{KI}* mice compared to *gsk-3^{WT}* mice (Fig. 19B). Propranolol treatment had no effects on serum calcium concentration in both genotypes. But propranolol significantly reduced renal calcium excretion (calciuria) in *gsk-3^{KI}* mice (Fig. 19B). At the same time, renal calcium excretion moderately enhanced in *gsk-3^{WT}* mice by the treatment with propranolol.

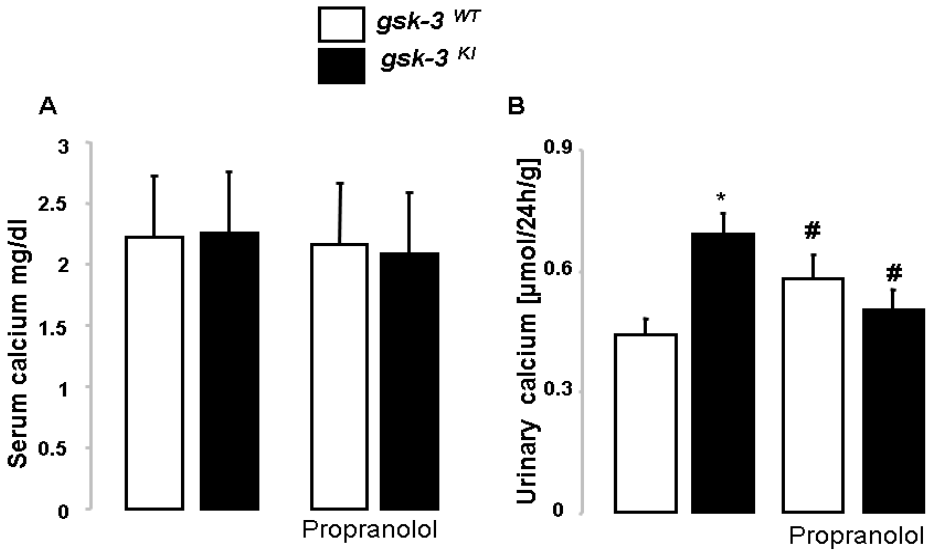

Fig. 19: β-blocker propranolol decreases the renal calcium excretion of *gsk-3^{KI}* mice.

Arithmetic means ± SEM of the serum calcium concentration (**A**; n=16-17) and renal calcium excretion (**B**; n=19) in *gsk-3^{WT}* mice (white bars) and *gsk-3^{KI}* mice (black bars) measured before (left bars) and after one week of treatment with β-blocker propranolol (500 mg/L) (right bars). **(p<0.05)* indicates significant difference between the genotypes, *# (p<0.05)* indicates significant difference from the absence of propranolol treatment.

Part 2 Results

4.3 The effect of 1,25(OH)$_2$D$_3$ on actin cytoskeleton reorganization in UMR 106 cells

This study addressed the role of the organization of the actin cytoskeleton in Fgf23 transcription in UMR 106 osteoblast-like cells. 1,25(OH)$_2$D$_3$ is a well known stimulator of FGF23 formation. In the first series of experiments, UMR 106 cells were incubated with 1,25(OH)$_2$D$_3$ (100 nM) and analyzed the changes in the actin cytoskeleton by Western blotting and confocal imaging. Fig. 20A and 20B illustrate that treatment with 1,25(OH)$_2$D$_3$ resulted in a polymerization of the actin cytoskeleton in UMR 106 cells as indicated by a decrease of the G-actin over F-actin ratio. This effect was evident upon 15 min treatment (Fig. 20A) and persisted for at least 24 h (Fig. 20B), indicating an early but persistent effect of 1,25(OH)$_2$D$_3$ on actin reorganization.

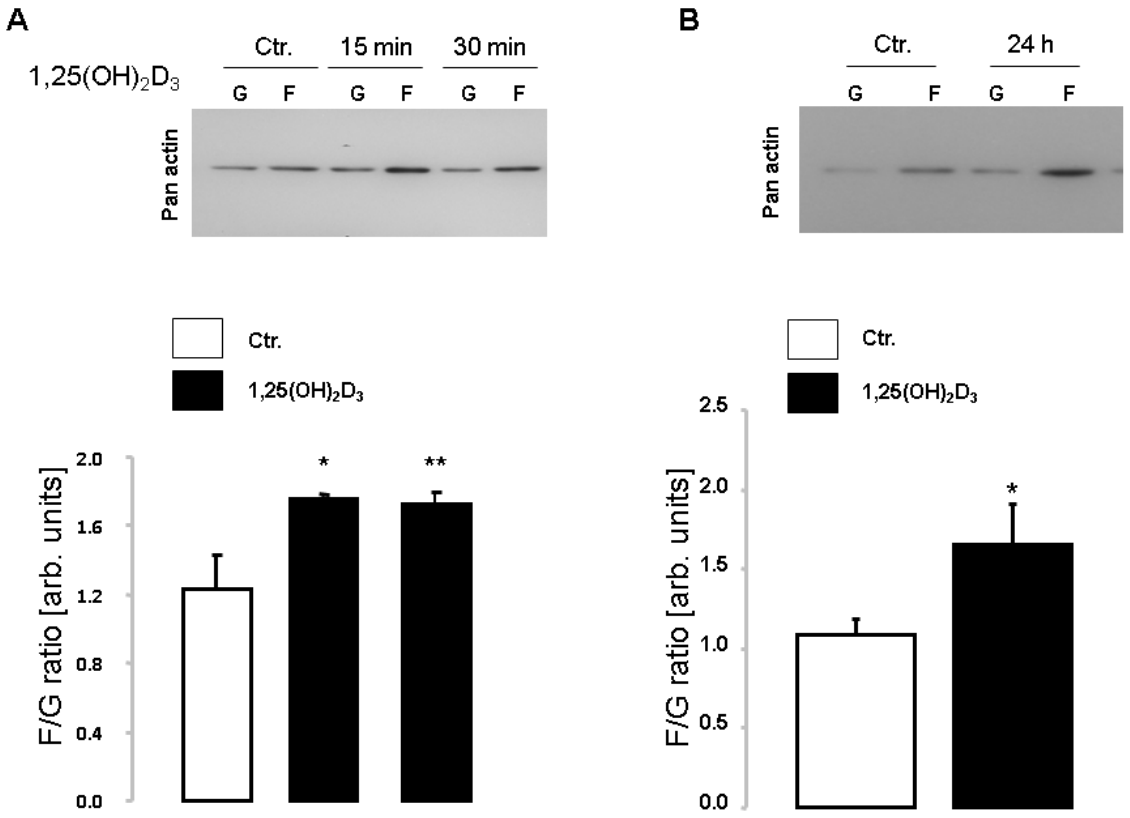

Fig. 20: 1,25(OH)$_2$D$_3$ induces polymerization of the actin cytoskeleton in UMR 106 cells.

A: Original Western blot demonstrating G-actin and F-actin abundance in UMR 106 cells left untreated (Ctr.) or treated for 15 or 30 min with 1,25(OH)$_2$D$_3$ (100 nM). Lower panel: Arithmetic means ± SEM (n=6) of the ratio of filamentous (F) over soluble (G) actin in UMR 106 cells.

B: Original Western blot demonstrating G-actin and F-actin abundance in UMR 106 cells left untreated (Ctr.) or treated for 24 h with 1,25(OH)$_2$D$_3$ (100 nM). Lower panel: Arithmetic means ± SEM (n=15) of the ratio of filamentous (F) over soluble (G) actin in UMR 106 cells. *$p < 0.05$.

Confocal microscopy imaging also fully supported these findings and demonstrated a profound effect of $1,25(OH)_2D_3$ on actin cytoskeleton reorganization with the formation of stress fibers (Fig. 21).

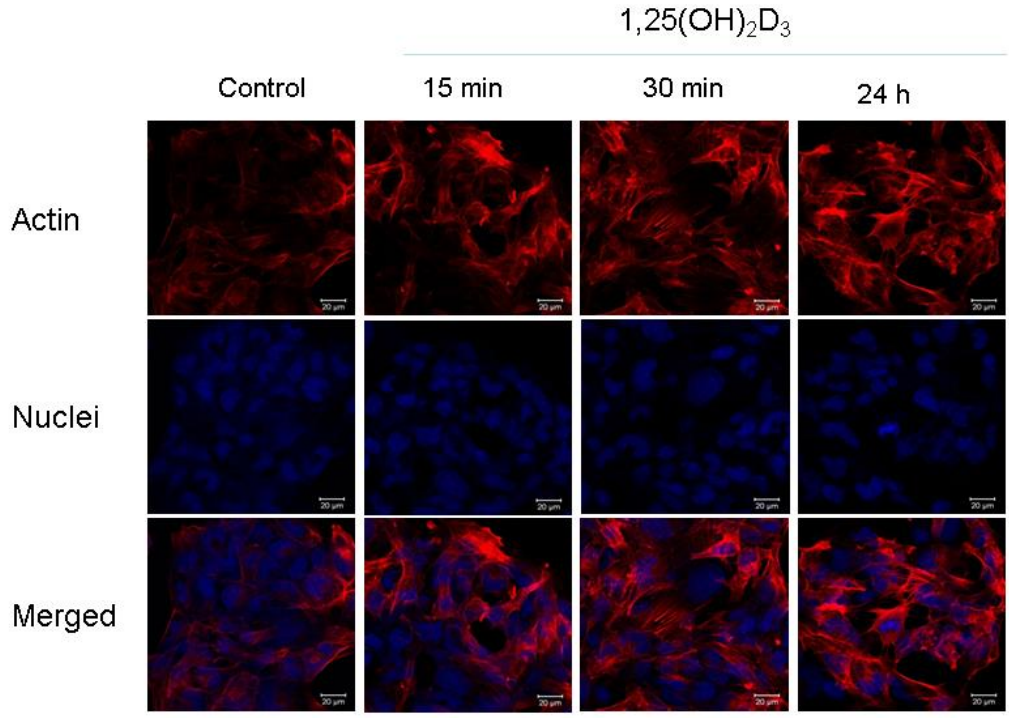

Fig. 21: Confocal microscopy image illustrating $1,25(OH)_2D_3$-induced actin stress fiber formation in UMR 106 cells.

Confocal microscopy images demonstrating actin staining (upper images), nucleus staining (middle images) or the merged staining (lower images) in UMR 106 cells left untreated or treated with $1,25(OH)_2D_3$ (100 nM) for 15 min, 30 min, or 24 h (n=3).

4.4 1,25(OH)$_2$D$_3$-induced actin polymerization is abolished by NF-κB inhibitor wogonin

Recently, it has been demontrated that proinflamatory cytokines are involved in the release and expression of Fgf23 which is transcription factor NF-κB dependent (203). On the other hand, NF-κB signaling has been shown to be involved in TNFα (204) and steroid hormones (205)-induced actin reorganization in various cell models. Therefore, in order to test whether inhibition of FGF23 expression by NF-κB inhibition also affects actin cytoskeleton I analyzed Western blot. As illustrated in (Fig. 22) NF-κB inhibitor wogonin (100 µM) totally blocked 1,25(OH)$_2$D$_3$-induced polymerization of actin cytoskeleton.

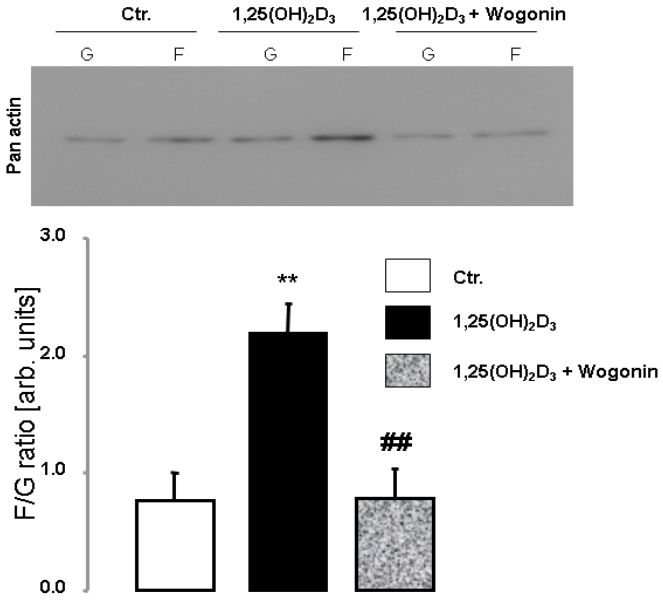

Fig. 22: 1 NF-κB inhibitor wogonin blocked 1,25(OH)$_2$D$_3$-induced actin polymerization in UMR 106 cells

Original Western blot showing G-actin and F-actin abundance in UMR 106 cells left untreated (Ctr.) or treated for 15 min with 1,25(OH)$_2$D$_3$ (100 nM) in the presence or absence of NF-κB inhibitor wogonin (100 µM). Arithmetic means ± SEM (n=6) of the ratio of filamentous (F) over soluble (G) actin in UMR 106 cells (lower panel). ** *p<0.01*, ## indicates significant difference from 1,25(OH)$_2$D$_3$ alone *(p<0.01).*

4.5 The effect of cytochalasin B on Fgf23 mRNA expression

Cytochalasin B is a mycotoxin which block actin polymerization. Therefore, the effect of cytochalasin B on the mRNA expression of Fgf23 was analyzed by qRT-PCR. As shown in (Fig. 23), $1,25(OH)_2D_3$-induced up-regulation of Fgf23 gene transcription largely inhibited when cells were incubated with cytochalasin B (100 nM) for 6 h that block actin reorganization.

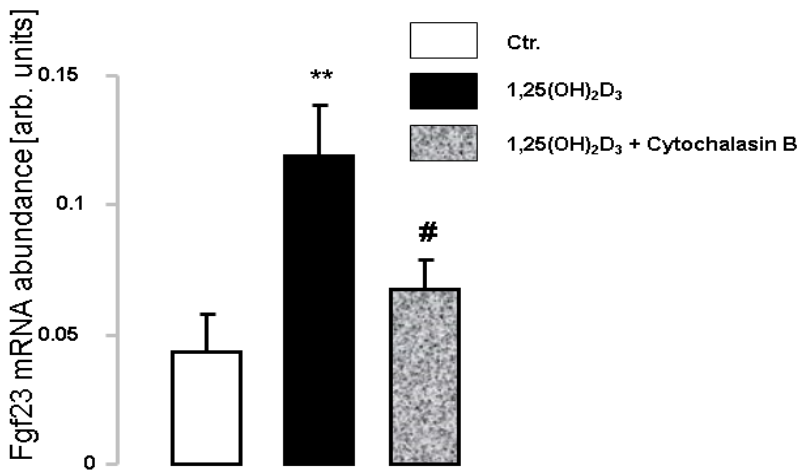

Fig. 23: $1,25(OH)_2D_3$-induced Fgf23 transcription is inhibited by actin-disrupting agent cytochalasin B.

Arithmetic means ± SEM (n = 6) of Fgf23 mRNA expression in UMR 106 cells following a 6 h treatment with vehicle alone or with $1,25(OH)_2D_3$ (100 nM) without or with cytochalasin B (100 nM). ** *p<0.01*, # indicates significant difference from $1,25(OH)_2D_3$ alone *(p<0.05).*

4.6 Rac1 and PAK1 inhibitor blocked 1,25(OH)$_2$D$_3$-induced Fgf23 mRNA expression

This result reveals the role of actin cytoskeleton in FGF23 transcription regulation. Rac1 regulates actin cytoskeleton restructuring (206). To elucidate underlying mechanism I treated UMR 106 cells with a Rac1 inhibitor NSC 23766 (50 µM) that blocks actin polymerization and Fgf23 transcript levels were determined. As illustrated in Fig. 24A, 1,25(OH)$_2$D$_3$ stimulated up-regulation of Fgf23 gene transcription significantly decreased by Rac1 inhibitor. Rac1 has been known to activate downstream PAK1, which in turn regulate actin cytoskeleton reorganization. As shown in Fig. 24B, PAK inhibitor IPA3 (10 µM) treatment was followed by a significant decrease of Fgf23 transcription to control levels, further implies that Rac1 controlled actin reorganization is directly involved in 1,25(OH)$_2$D$_3$-induced modulation of Fgf23 gene transcription.

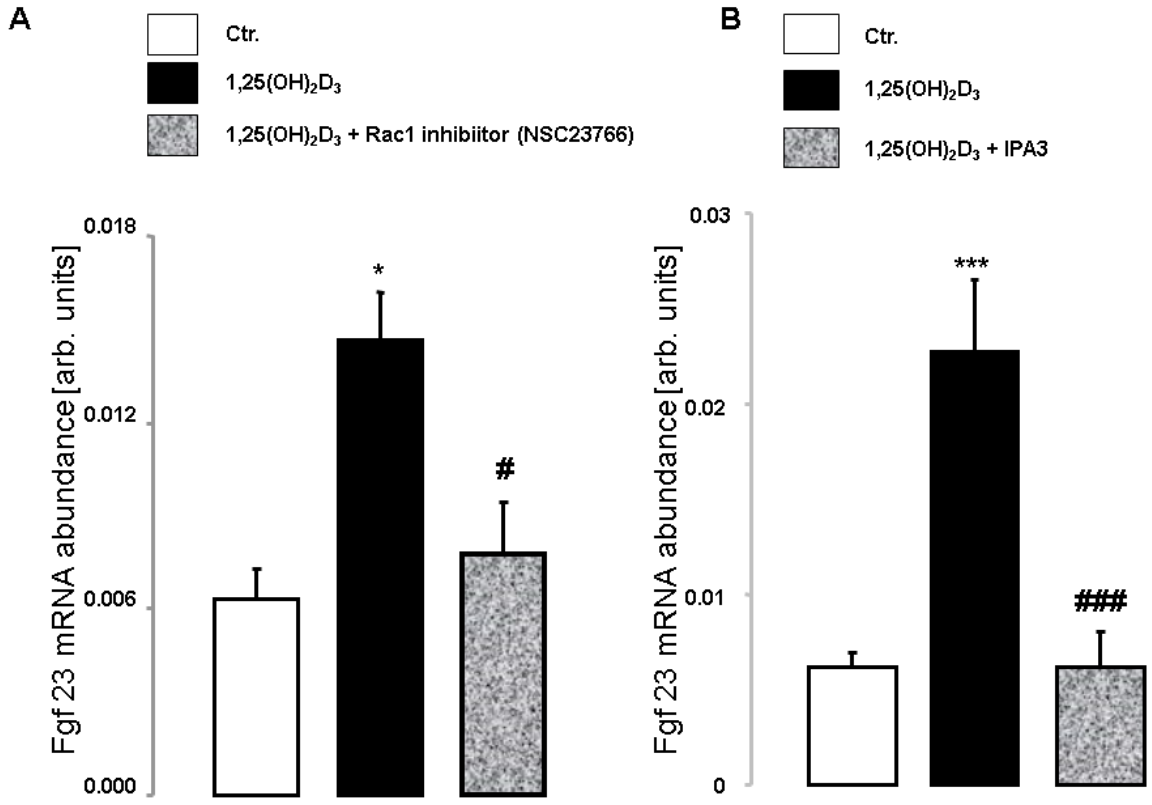

Fig. 24: $1,25(OH)_2D_3$-induced Fgf23 transcription is inhibited by Rac1 and PAK1 inhibitors.

A: Arithmetic means ± SEM (n=7) of Fgf23 mRNA abundance in UMR 106 cells following a 6 h treatment with vehicle alone or with $1,25(OH)_2D_3$ (100 nM) without or with Rac1 inhibitor NSC 23766 (50 µM).

B: Arithmetic means ± SEM (n=6) of Fgf23 mRNA abundance in UMR 106 cells following a 6 h treatment with vehicle alone or with $1,25(OH)_2D_3$ (100 nM) without or with PAK1 inhibitor IPA3 (10 µM).

**, *** p<0.05, p<0.001* #, ### indicate significant difference from $1,25(OH)_2D_3$ alone *(p<0.05, p<0.001).*

Rac1 inhibitor blocked 1,25(OH)$_2$D$_3$-induced actin polymerization (Fig. 25), suggesting that actin restructuring *via* polymerization upon 1,25(OH)$_2$D$_3$ treatment could control Fgf23 gene transcription in UMR 106 cells. On the other hand, the effect of IPA3 was moderate and did not reach to the statistical significance (Fig. 25).

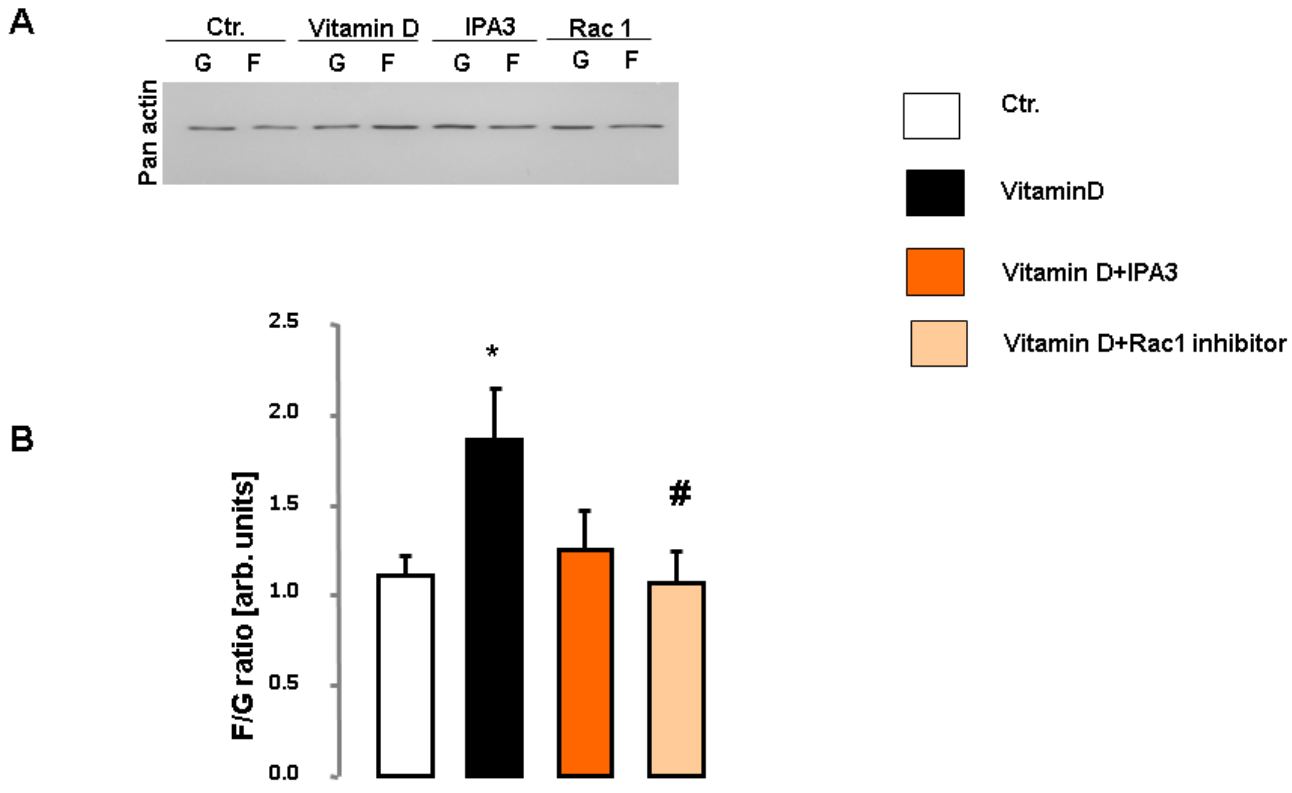

Fig. 25: 1,25(OH)$_2$D$_3$-induced actin polymerization is blocked by Rac1and PAK1 inhibitors

Original Western blot illustrating G-actin and F-actin abundance in UMR 106 cells left untreated (Ctr.) or treated for 15 min with 1,25(OH)$_2$D$_3$ (100 nM) without or with PAK1 inhibitor IPA3 (10 µM) or with Rac1 inhibitor NSC 23766 (50 µM). Lower panel: Arithmetic means ± SEM (n=5) of the ratio of filamentous (F) over soluble (G) actin in UMR 106 cells. * *p<0.05* # indicates significant difference from 1,25(OH)$_2$D$_3$ alone *(p<0.05).*

5. Discussion

The present observations reveal that protein kinase B (PKB/Akt)-sensitive GSK3 is critical for the formation of FGF23 by regulating the activity of the sympathetic nervous system and further demonstrated that the role of actin cytoskeleton on the formation of FGF23 in the UMR 106 osteoblastic cells.

In this study, I analyzed *gsk-3*^{KI} mice and compared with corresponding wild type mice. GSK3 is a downstream signaling molecule of PI3K signaling pathway. It is serine-threonine kinase which is phosphorylated and thus, inactivated by insulin through PKB/Akt/SGK signaling. *Gsk-3*^{KI} mice carrying a GSK3α/β mutation that makes the kinase insensitive to the inhibitory effect of PKB/Akt (*gsk-3*^{KI} mice), as a result, it is not phosphorylated by either PKB/Akt or SGK1. Though inactivation of GSK3 is mediated by the action of insulin through PI3K/PKB/Akt-dependent mechanism, *gsk-3*^{KI} mice are viable and do not suffer from insulin resistance and insulin action on muscle glycogen synthase is abolished in *gsk-3*^{KI} mice (167). However, *gsk-3*^{KI} mice have previously been reported to suffer from phosphaturia, calciuria and lower bone density (168). These effects were in part due to a direct inhibitory effect of GSK3 on the main phosphate transporter of the kidney, NaPiIIa and reduced activity of phosphate transporter in *gsk-3*^{KI} mice (168). These results further provide evidence that the renal phosphate wasting of *gsk-3*^{KI} mice may be in addition because of the elevated serum FGF23 level in these mice, as FGF23 is a phosphatonin and it inhibits renal phosphate reabsorption by reducing phosphate transporter (NaPi2a) and causes phosphaturia.

FGF23 ELISA was employed to measure either C-terminal FGF23 or only intact FGF23. Intact FGF23 is the biologically active form of FGF23. The C-terminal FGF23 is a inactive form and results from furin-dependent degradation of intact FGF23 (207). In this study, both the C-terminal and intact FGF23 concentrations were measured by using ELISA kits. I found that both C-terminal and intact FGF23 is elevated in *gsk-3*^{KI} mice as compared to *gsk-3*^{WT} mice.

FGF23 binds to its receptor (FGFR) to exert its renal function. It requires α-klotho as a co-receptor. Klotho deficiency is associated with elevated serum FGF23 level (201). It suggests that klotho can act as a regulator of FGF23. Therefore, I determined renal klotho expression. However, I did not find any difference between the genotype. Thus, pointing that klotho is not relevant for enhanced serum FGF23 in *gsk-3*^{KI} mice.

The neurotransmitter epinephrine and norepinephrine are biomarkers for sympathetic nerve activity. G*sk-3*^{KI} mice exhibit higher sympathetic nerve activation than in *gsk-3*^{WT} mice. Consequently, the *gsk-3*^{KI} mice suffer from high blood pressure and they have higher heart rate as well than *gsk-3*^{WT} mice (172). In this study, I could confirm enhanced sympathetic activation of *gsk-3*^{KI} mice compared to *gsk-3*^{WT} by detecting urinary epinephrine and VMA as a measure of sympathetic nervous activation. I found significantly higher 24 h urinary excretion of epinephrine and VMA in *gsk-3*^{KI} mice than in *gsk-3*^{KI} mice. VMA is the main metabolite of epinephrine and norepinephrine. Since, it has recently been shown that sympathetic activation to induce FGF23 release; I aimed to define the role of the higher sympathetic activity for enhanced production of FGF23 in *gsk-3*^{KI} mice. Therefore, I treated *gsk-3*^{KI} mice and *gsk-3*^{WT} mice with the widely used β-blocker propranolol in drinking water for one week (500 mg/L) and analyzed the consequence for serum FGF23 and phosphate metabolism. One week treatment with propranolol markedly reduced serum FGF23 level of *gsk-3*^{KI} mice to the level of *gsk-3*^{WT} mice. Therefore, enhanced sympathetic activation accounted for the excess FGF23 formation of *gsk-3*^{KI} mice. Treatment with β-blocker propranolol reduced blood pressure in *gsk-3*^{KI} mice and abrogated the difference between the genotypes, suggesting that hypertension of *gsk-3*^{KI} mice was because of enhanced sympathetic nervous activity, since propranolol treatment significantly reduced blood pressure. Similarly, β-blocker propranolol treatment inhibits fgf23 mRNA expression in UMR 106 osteoblast-like cells.

As a consequence of reduced serum FGF23 level by propranolol treatment, both the hypophosphatemia and phosphaturia of *gsk-3*^{KI} mice were ameliorated by β-blocker treatment. The data presented here provide strong evidence that the renal phosphate wasting of *gsk-3*^{KI} mice was, at least in part, the direct effect of elevated serum FGF23 concentration. FGF23 and PTH are the main regulators of $1,25(OH)_2D_3$. Treatment with propranolol did not affect the low serum $1,25(OH)_2D_3$ level in *gsk-3*^{KI} mice despite its

profound effect on FGF23. However, the other main regulator of $1,25(OH)_2D_3$ is PTH which stimulates $1,25(OH)_2D_3$ formation, was also low in *gsk-3^{KI}* mice compared to *gsk-3^{WT}* mice and not significantly affected by propranolol, an effect likely to contribute to their low serum $1,25(OH)_2D_3$ concentration. Apart from this, GSK3 signaling may influence directly on vitamin D metabolism or VDR signalling. Recently, it has been published that mice treated with GSK3 inhibitor lithium chloride increased serum FGF23 concentration and decreased serum $1,25(OH)_2D_3$ concentration (208). To rule out the direct effects of GSK3 on vitamin D metabolism further research is needed.

In keeping with previous reports, I found that glomerular filtration rate (GFR) was significantly higher in *gsk-3^{KI}* mice compared to *gsk-3^{WT}* mice (171). β-blocker propranolol reduced blood pressure of *gsk-3^{KI}* mice. Blood pressure lowering effect of propranolol may affect glomerular filtration rate (GFR). Therefore, I measured GFR after treatment with propranolol. The effect of β-blocker to lower blood pressure was associated with decline GFR in *gsk-3^{KI}* mice too. This results indicating that mild reduction of phosphaturia and calciuria in *gsk-3^{KI}* mice following propranolol treatment was, at least in part, also because of decline of GFR.

β-blockers such as propranolol are a widely prescribed class of drugs all over the world. It is used for the treatment of hypertension, heart failure and atrial fibrillation (209-211). It is tempting to speculate that the FGF23-lowering effect of β-blockers observed in *gsk-3^{KI}* mice may have an implication for millions of patients treated with β-blockers. These drugs clearly reduce the cardiovascular mortality of high-risk patients. Interestingly, a high FGF23 level also positively correlates with cardiovascular mortality (103, 104, 107). Therefore, it appears possible that the benefit of β-blocker therapy is at least in part also related to its ability to lower the blood FGF23 concentration. Clearly, further studies are needed to test this hypothesis.

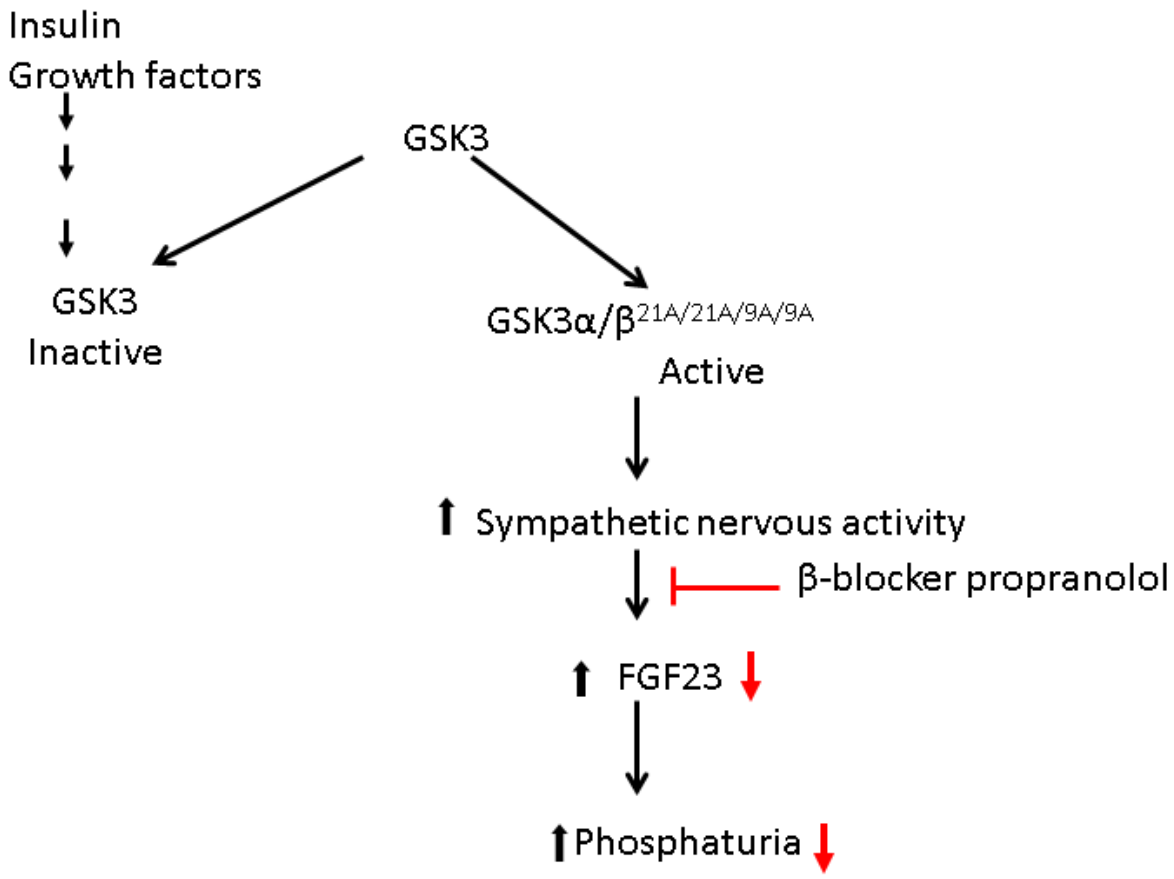

Fig. 26: GSK3-controlled sympathetic activity in FGF23 production: Insulin phosphorylates and inhibits GSK3. In gsk-3^{KI} mice serine residue was replaced by alanine. Thus, GSK3α/β$^{21A/21A/9A/9A}$ renders the kinase insensitive to the inhibitory effects of insulin. *Gsk-3^{KI}* mice have higher sympathetic nervous activity and elevated FGF23 levels which leads to phosphaturia. β-blocker propranolol normalized elevated FGF23 and reduced phosphaturia.

In second part of the study I investigated the role of reorganization of actin cytoskeleton in the regulation of FGF23 formation in UMR 106 osteoblast-like cells. The present observations reveal an impact of reorganization of actin cytoskeleton is required for the production of FGF23 as actin microfilament disrupting agent cytochalasin B blocked transcription of the Fgf23 gene. Here, this study demonstrated that stimulation of FGF23 formation with 1,25$(OH)_2D_3$ resulted in actin polymerization. Furthermore, the effect of 1,25$(OH)_2D_3$ on the reorganization of actin was blocked by NF-κB inhibitor wogonin (100

µM) that suppressed Fgf23 mRNA expression. During early and / or late actin reorganization, actin polymerization is induced by various signals including hormones (212), growth factors and cytokines (213, 214) and ions (215). $1,25(OH)_2D_3$ is a known trigger for Fgf23 expression. FGF23 is also stimulated by NF-κB signalling (203, 216). It has been shown previously that both $1,25(OH)_2D_3$ and NF-κB (217) plays a vital role in actin reorganization. $1,25(OH)_2D_3$ is implicated in osteoblast maturation, matrix calcification and overall bone metabolism (218). These results provide novel mechanistic evidence for $1,25(OH)_2D_3$-induced regulation of Fgf23 gene transcription by revealing the involvement of the actin specific Rac1 and PAK1 signaling. As NF-κB has an implication on actin polymerization, in a series of experiments, I used NF-κB inhibitor wogonin and observed, $1,25(OH)_2D_3$-induced actin polymerization was totally blocked. On the other hand, $1,25(OH)_2D_3$-induced up-regulation of Fgf23 was also abolished by actin depolymerising agent cytochalasin B. Hence, $1,25(OH)_2D_3$-stimulated Fgf23 expression is at least in part regulated by actin cytoskeleton reorganization. Rac1/PAK1 is a key regulatory molecule in the actin signalling pathway in various cell models (212, 219, 220). In this study, I used Rac1 and PAK1 specific inhibitor NCS 23766 (50 µM) and IPA3 (10 µM) respectively, to block actin cytoskeleton and questioned whether it affects gene transcription of Fgf23. Indeed, blocking of Rac1 and PAK1 activities lead to the suppression of Fgf23 mRNA transcription in UMR 106 cells. This study demonstrated that $1,25(OH)_2D_3$-induced actin cytoskeleton reorganization regulates Fgf23 gene expression by Rac1/PAK1 signaling.

Early or late actin cytoskeleton reorganization, following modification of actin polymerization is initiated by various signals and regulated by distinct signalling pathway which has been implicated in the control of gene transcription (221, 222). It has been shown that early actin polymerization by various signals may release transcription factor such as myocardin-related transcription factor (MRTF) (221, 223, 224), which may in turn regulate transcription of various genes (221). Likewise, early actin restructuring, following modification of actin polymerization regulates activation of RhoB-gene (225) as well as alpha smooth muscle actin (alpha SMA) gene (226) and guanine nucleotide exchanger factors (GEF's) including Net1/Net1A (227). These studies suggested that early actin restructuring may regulate transcription of genes encoding specific regulatory effectors

(221, 223) and may link early modification of actin cytoskeleton to late cell responses controlled by gene activation (221). These results further support this hypothesis. Indeed, actin polymerization induced by $1,25(OH)_2D_3$ in UMR 106 osteoblast-like cells seems to regulate $1,25(OH)_2D_3$-induced Fgf23 gene expression, as inhibition of actin polymerization either by actin microfilament disrupting agent cytochalasin B or by inhibition of specific actin signalling molecules (Rac1/PAK1) that govern actin cytoskeleton dynamics, blunted $1,25(OH)_2D_3$-induced up-regulation of Fgf23 mRNA expression.

Rac1 is a member of small GTPase Rho family. This is a major signalling molecule of actin cytoskeleton signalling pathway which act to the downstream of PI3K/FAK1 signaling pathway (228). Rac1 may itself stimulate actin polymerization, or it may associate with PAK1 that in turn is implicated in actin restructuring (200, 229). These results provide strong evidence that Rac1/and PAK1 are mainly involved in both, actin reorganization and Fgf23 gene transcription, which support previous studies describing an important role of Rac1 in osteoblastic cells (230). In fact, this study cannot exclude the involvement of other small Rho-GTPase signaling, may be these signaling involve as well. These findings strongly indicate that the Rac1/PAK1-stimulated actin redistribution is required for the $1,25(OH)_2D_3$-induced production of Fgf23 in UMR 106 osteoblast-like cells. Apart from this, as suppression of Fgf23 production by inhibiting NF-κB signaling with wogonin resulted in a blockade of actin network reorganization, these findings further support previous studies establishing the involvement of NF-κB signaling in actin cytoskeleton rearrangements (204, 205, 231).

Rac1/PAK1 signaling may turn out to be a potent regulator of renal phosphate and vitamin D metabolism by controlling FGF23 formation through reorganization of actin cytoskeleton. Further studies are needed to define its exact role.

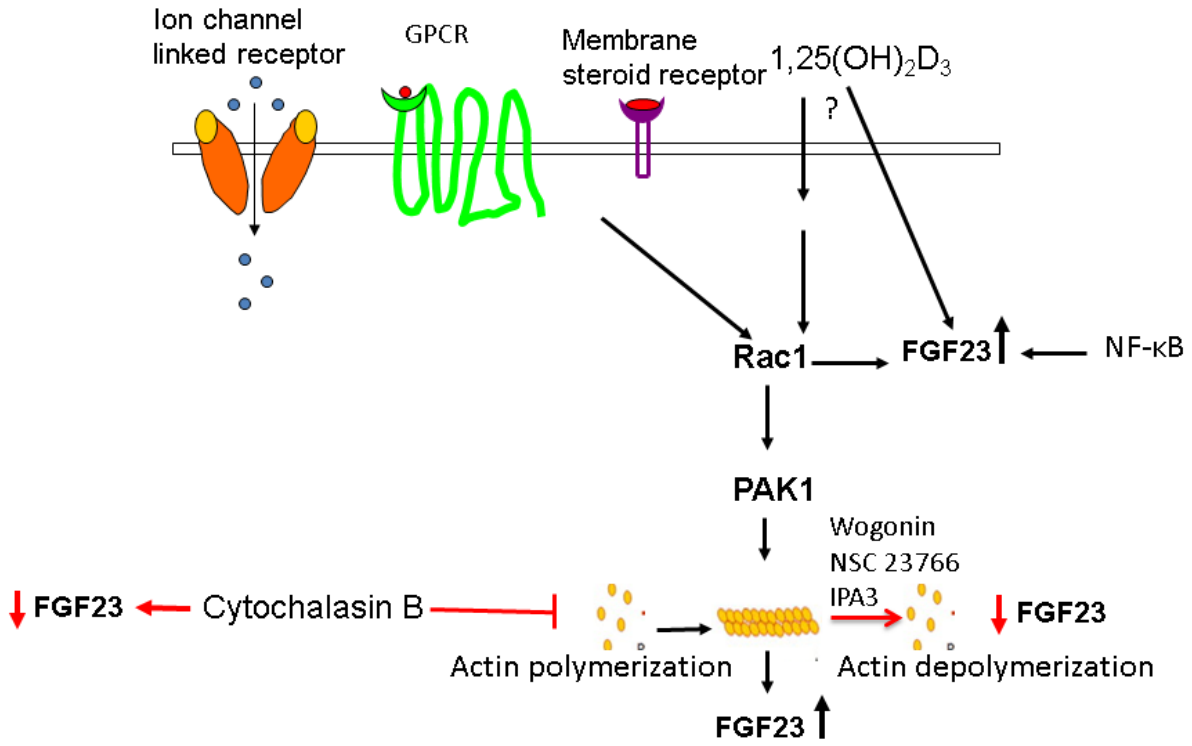

Fig. 27: Fgf23 gene regulation by actin cytoskeleton reorganization: 1,25$(OH)_2D_3$ stimulates Fgf23 gene transcription in UMR 106 cells. 1,25$(OH)_2D_3$-induced Fgf23 expression is inhibited by Rac1 inhibitor NSC 23766 and cytochalasin B. Rac1 inhibitor NSC 23766 and PAK inhibitor IPA3 also blocked 1,25$(OH)_2D_3$-induced actin polymerization. Black arrows indicate stimulation and red arrows indicate inhibition.

6. Conclusion

The present observations reveal that PKB/Akt-resistant GSK3 signaling controls sympathetic nervous activity which is an important regulator of FGF23 production and phosphate metabolism. Hence, *gsk-3*^{KI} mice exhibit a high serum FGF23 level, phosphaturia, and hypophosphatemia, normalized by β-blocker propranolol.

1,25$(OH)_2D_3$-induced Fgf23 gene transcription in UMR 106 cells requires reorganization of the actin network, govern by NF-κB and Rac1/PAK1 signaling. These observations reveal that actin cytoskeleton reorganization may be implicated in the control of gene transcription.

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Publications

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Contributions

The data presented in this thesis has been gathered from experiments that I carried out myself. Confocal microscopy was performed by Sabina Honisch. I received assistance in animal handling from Michael Föller, Anja T Umbach and Hong Chen.