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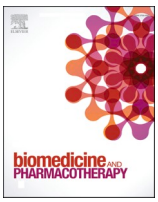

Prostaglandin E_2 signaling through prostaglandin E receptor subtype 2 and Nurr1 induces fibroblast growth factor 23 production

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ABSTRACT

Bone cells produce fibroblast growth factor 23 (FGF23), a hormone regulating renal phosphate and vitamin D homeostasis, and a paracrine factor produced in further tissues. Chronic kidney disease and cardiovascular disorders are associated with early elevations of plasma FGF23 levels associated with clinical outcomes. FGF23 production is dependent on many conditions including inflammation. Prostaglandin E_2 (PGE_2) is a major eicosanoid with a broad role in pain, inflammation, and fever. Moreover, it regulates renal blood flow, renin secretion, natriuresis as well as bone formation through prostaglandin E receptor 2 (EP2). Here, we studied the role of PGE_2 and its signaling for the production of FGF23. Osteoblast-like UMR-106 cells were exposed to EP receptor agonists, antagonists or RNAi. Wild type and EP2 knockout mice were treated with stable EP2 agonist misoprostol. Fgf23 or Nurr1 gene expression was determined by quantitative real-time PCR, hormone and further blood parameters by enzyme-linked immunosorbent assay and colorimetric methods. PGE_2 and EP2 agonists misoprostol and butaprost enhanced FGF23 production in UMR-106 cells, effects mediated by EP2 and transcription factor Nurr1. A single dose of misoprostol up-regulated bone *Fgf23* expression and FGF23 serum levels in wild type mice with subtle effects on parameters of mineral metabolism only. Compared to wild type mice, the FGF23 effect of misoprostol was significantly lower in EP2-deficient mice. To conclude, PGE_2 signaling through EP2 and Nurr1 induces FGF23 production. Given the broad physiological and pathophysiological implications of PGE_2 signaling, this effect is likely of clinical relevance.

1. Introduction

Fibroblast growth factor 23 (FGF23), along with FGF19 and FGF21, is a member of the endocrine family of FGFs and well established as an endocrine factor released by osteocytes/osteoblasts in bone [1]. Particularly the kidney is a target organ of FGF23 where it controls phosphate and vitamin D metabolism as a hormone [2]. In detail, FGF23 lowers expression of NaPi-IIa, the main Na^+-phosphate cotransporter in the apical membrane of proximal tubular epithelial cells [3]. Consequently, urinary phosphate excretion increases [3]. Moreover, FGF23 reduces expression of renal 25-hydroxyvitamin D-1 alpha-hydroxylase which produces active vitamin D ($1,25(OH)_2D_3$, calcitriol) in the kidney [4]. Hence, FGF23 lowers plasma $1,25(OH)_2D_3$ levels, an effect also due to the induction of 24-hydroxylase, a renal enzyme inactivating $1,25(OH)_2D_3$ [5].

In addition, FGF23 has versatile paracrine functions in other organs including heart [6] or liver [7]. Also, FGF23 production has been found to occur in further organs, e.g. bone marrow [8], thymus [9], heart [10], or kidney [11] at least under pathophysiological conditions.

αKlotho is a renal protein important for FGF23 effects [12,13]. As a membrane protein, αKlotho serves to increase affinity of FGF23 for its renal membrane FGF receptor to facilitate the aforementioned effects on phosphate and vitamin D homeostasis [12]. Soluble Klotho (sKL) is generated upon cleavage of membrane αKlotho by proteases ("shedding") [12].

In mice, FGF23 or αKlotho deficiency leads to diseases reminiscent of human aging, an effect due to severe derangement of renal phosphate and vitamin D metabolism [14–16]. Conversely, αKlotho overexpression confers longevity in mice [17].

FGF23 has been suggested as a disease biomarker. In particular, in chronic kidney disease (CKD) and cardiovascular disorders plasma FGF23 is positively correlated with outcomes and disease activity [2,18,

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19].

FGF23 production is subject to a complex network of extracellular and intracellular transcriptional and posttranslational regulators [20, 21]. Not surprisingly, factors affecting phosphate, vitamin D and calcium metabolism are important regulators, i.e. $1,25(OH)_2D_3$, parathyroid hormone (PTH) or dietary phosphate [20,22]. Notably, inflammation is a major driver of FGF23 production [23]. In line with this, pro-inflammatory cytokines and transcription factors (IL-1 [24], IL-6 [25], TNFα [26], NFκB [27]) control FGF23, and also CKD is an inflammatory condition elevating plasma FGF23 concentration [28]. Acute kidney injury results in the release of glycerol-3-phosphate, a metabolite of glycolysis, which in turn stimulates FGF23 production in bone [29]. Further hormones [30–32], and intracellular pathways [33, 34], control FGF23.

Prostaglandin E_2 (PGE_2) is a major eicosanoid with multiple effects in almost all organs and tissues [35,36]. It is of particularly high medical relevance due to its broad role in inflammation, pain, and fever [37]. The production of all prostaglandins and thromboxane from arachidonic acid depends on cyclooxygenase isozymes (COX-1 and COX-2) [35]. Adverse effects of renal COX inhibition through nonsteroidal anti-inflammatory drugs (NSAIDs) point to important renal effects of PGE_2, namely the regulation of blood flow through the induction of vasodilation, the regulation of renin secretion, and natriuresis [35].

The effects of PGE_2 are mediated by prostaglandin E receptors EP1–4 [38,39]. Importantly, PGE_2 is also essential for bone formation and resorption, effects mediated through EP2 and EP4 [40].

Due to the broad role of PGE_2 for inflammation and of inflammation for FGF23 production, we hypothesized that PGE_2 is a regulator of FGF23 synthesis. To verify this hypothesis and to elucidate the underlying signaling we performed *in vitro* and *in vivo* experiments.

2. Material and Methods

2.1. Cell culture and treatments

Experiments were performed in rat osteoblast-like UMR-106 cells (CRL-1661; ATCC, Manassas, VA, USA) that were cultured in Dulbecco's Modified Eagle Medium with high glucose (Gibco, Life Technologies, Darmstadt, Germany) supplemented with 10 % fetal bovine serum (Gibco, Life Technologies), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, Life Technologies) and seeded at a density of 200,000 cells per well (6-well plate). Twenty-four hours after seeding, the old medium was replaced with medium supplemented with 10 nM $1,25(OH)_2D_3$ (Tocris Bioscience, Bio-Techne, Bristol, UK). All treatments were performed in the presence of $1,25(OH)_2D_3$, as active vitamin D had been shown to significantly increase low basal *Fgf23* transcript levels in these cells [41]. UMR-106 cells were then treated with the indicated concentrations of PGE_2 (from Sigma-Aldrich, Schnelldorf, Germany or Tocris Bioscience, Bio-Techne; stock dissolved in ethanol), prostaglandin E receptor agonist misoprostol (stock dissolved in ethanol) or EP2 agonist butaprost (1 µM; stock dissolved in dimethyl sulfoxide (DMSO) (both chemicals from Tocris Bioscience, Bio-Techne) in the absence or presence of prostaglandin E receptor antagonists (stocks dissolved in DMSO) EP1 (SC-19220; 10 µM), EP2 (PF 04418948 or TG 4-155; 10 µM for both), EP3 (L-798,106; 10 µM), or EP4 (L-161,982; 10 µM) for 24 h. Control cells were incubated with the appropriate volume of vehicle.

For siRNA transfection experiments, UMR-106 cells were seeded at a density of 150,000 cells per well (6-well plate). Twenty-four hours after seeding, cells were transfected with 50 nM *Ptger2* siRNA (catalog no. L-095211-02), 50 nM *Nr4a2* siRNA (catalog no. L-091528-02) or 50 nM non-targeting control siRNA (catalog no. D-001810-10) using 5 µl DharmaFECT 1 transfection reagent (all from Dharmacon, Horizon Discovery, Cambridge, UK) under antibiotic-free conditions. After further 24 h, cells were additionally treated with or without 250 ng/ml misoprostol in the presence of 10 nM $1,25(OH)_2D_3$. Cells were harvested 30 h (*Nr4a2* silencing) or 48 h (*Ptger2* silencing) after transfection, and silencing efficiency was verified by quantitative real-time polymerase chain reaction (qRT-PCR).

2.2. FGF23 protein in the cell culture medium

UMR-106 cells were seeded as described above at a density of 150,000 cells per well and then treated with 250 ng/ml misoprostol or vehicle in the presence of 10 nM $1,25(OH)_2D_3$. After 48 h, cell culture supernatants were collected and stored at −70°C. The medium was concentrated by means of Vivaspin 2 centrifugal concentrators (Sartorius, Göttingen, Germany; original volume: app. 1.8 ml; final volume: app. 110 µl), and C-terminal FGF23 concentration was determined by enzyme-linked immunosorbent assay (ELISA) (mouse/rat FGF-23 (C-Term), Quidel, San Diego, CA, USA) according to the manufacturer's instructions. The FGF23 concentration was expressed in relation to the initial volume.

2.3. Animal experiments and tissue collection

All animal experiments were conducted in accordance with the federal law for the welfare of animals and were approved by the state government of Baden-Württemberg. The mice had free access to tap water and standard rodent chow (V1534; Ssniff, Soest, Germany) and were housed in a conventional animal facility.

Male (females were avoided due to potential uterine effects of misoprostol) 22–27-week-old wild type mice (C57Bl/6; Charles River, Sulzfeld, Germany) were randomized to subcutaneous (s.c.) injection with 670 µg/kg body weight misoprostol (Tocris Bioscience, Bio-Techne) dissolved in 0.9 % saline containing 0.17 % (*v/v*) ethanol [42] or control treatment, i.e. these animals received the appropriate volume of vehicle. After 6 h, blood was taken by retro-orbital puncture under isoflurane anesthesia. Animals were then sacrificed by cervical dislocation, kidneys were immediately harvested and snap-frozen in liquid nitrogen. Blood collection and cervical dislocation were carried out in an alternating manner to minimize potential confounders. Femora, tibiae and humeri were isolated from animals sacrificed 3 h after treatment and dissected and prepared by carefull scrubbing to remove muscle and connective tissue. Both epiphyses were then cut and the bone marrow was rinsed with ice-cold 0.9 % saline solution. Bones were immersed for 24 h in RNA*later* solution (Invitrogen, Life Technologies) and then stored at −70°C.

The sample size was calculated based on a power of 80 % and a minimal relevant difference of 1.5 standard deviation change in the FGF23 (C-terminal) concentration, yielding a sample size of $n = 9$. Interim analysis yielded significant difference in the FGF23 (C-terminal) concentration after inclusion of $n = 6$ wild type animals. According to the "3 R" principles, the remaining $n = 3$ animals were not included in the study. No mouse was excluded from the study. The experimentator was aware of the group allocation at all times. Age matching was the inclusion criteria. Exclusion criterium was obvious sickness of an animal.

At 10–11 weeks of age, blood was taken from male and female *Ptger2* knockout ($EP2^{-/-}$) and wild type ($EP2^{+/+}$) mice (JAX stock #004376; The Jackson Laboratory, Bar Harbor, ME, USA) as described above. After a period of 4 weeks to allow full recovery from the blood collection, the mice were treated with misoprostol (670 µg/kg body weight; s.c.) and blood was taken 6 h after the injection.

2.4. Blood chemistry

Serum phosphate and calcium concentrations were measured using a blood chemistry analyzer (Fuji DRI-CHEM NX500i, Fujifilm, Düsseldorf, Germany). ELISA kits were used to determine the serum concentrations of $1,25(OH)_2D$ (IDS, Boldon, UK), C-terminal and intact FGF23 and plasma concentration of intact PTH (1-84) (all three from Quidel). With

the exception of 1,25(OH)$_2$D (a lower sample volume was applied), the measurements were made according to the provided manuals.

2.5. Qualitative expression analysis

Total RNA was extracted from untreated UMR-106 cells using peqGOLD TriFast reagent (VWR, Darmstadt, Germany) followed by DNase treatment and purification using the NucleoSpin RNA Mini Kit (Macherey-Nagel, Düren, Germany). RNA (1.2 μg) was reverse-transcribed with the GoScript Reverse Transcription System and random primers (both from Promega, Mannheim, Germany).

For qualitative expression analysis of the four prostaglandin E receptor subtypes, *Ptger1–4*, polymerase chain reaction (PCR) was carried out on a Biometra TAdvanced thermocycler (Analytic Jena, Jena, Germany) using 7.5 μl GoTaq G2 Green Master Mix (Promega), 2 μl cDNA, forward and reverse primers (0.25 μM), and nuclease-free water up to 15 μl. A control without template (no template control, NTC) and a control without reverse transcriptase (no reverse transcriptase, NRT) were used to identify primer-dimer products and genomic DNA amplification. The PCR program started with an initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at primer-specific temperature for 30 s and extension at 72°C for 20 s, and a final elongation step at 72°C for 5 min.

The following rat primers (5'→3' orientation) and annealing temperatures were used for qualitative expression analysis:

Ptger1 (55°C): CTGGTATTGGTGGTGTTG, TAGGAGGCGAAGAAG TTG;

Ptger2 (57°C): CCATTATGACCATCACCTTC, AAGGATGACAAAAAC CCAAG;

Ptger3 (59°C): TGTGTGTACTGTCCGTCTGC, CGAACGGCGATTA GGAAGGA;

Ptger4 (57°C): ATCCAGATGGTCATCTTACTC, CACTTGGCTGAT ATAACTGG.

PCR products (10 μl) were separated on a 1.5 % agarose gel, stained with ethidium bromide and visualized using a Biometra BioDoc Analyze system (Analytic Jena).

2.6. Semi-quantitative expression analysis

Total RNA from UMR-106 cells and from mouse kidney was isolated using peqGOLD TriFast reagent. For cDNA synthesis, reverse transcription of 1.2 μg total RNA was performed with random primers and the GoScript Reverse Transcription System.

Bones were pulverized with a mortar and pestle under continuous addition of liquid nitrogen. Total RNA was extracted with TRIzol reagent (Invitrogen, Thermo fisher) and subjected to column-based DNase treatment (RNase-Free DNase SET and RNeasy Mini Kit from QIAGEN, Hilden, Germany). First-strand cDNA was synthesized using 300 ng of total bone RNA.

QRT-PCR was performed with the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Feldkirchen, Germany) using GoTaq qPCR Master Mix (Promega). The reaction mix contained 2 μl cDNA, 10 μl 2x GoTaq qPCR Master Mix, forward and reverse primers (0.25 or 0.5 μM), and nuclease-free water up to 20 μl. The qRT-PCR settings were: 95°C for 2 min; 40 cycles of denaturation at 95°C for 10 s, annealing at primer-specific temperature for 30 s, and extension at 72°C for 25 s followed by melting curve analysis.

The following primers (5'→3' orientation) and annealing temperatures were used for semi-quantitative expression analysis:

rat *Fgf23* (57°C): TAGAGCCTATTCAGACACTTC, CATCAGGGCA CTGTAGATAG;

rat *Nr4a2* (57°C): GTCAGCATTACGGTGTTCGC, AGTCAGGGTT TGCCTGGAAC;

rat *Ptger2* (57°C): CCATTATGACCATCACCTTC, AAGGATGACAAA AACCCAAG;

rat *Tbp* (57°C): ACTCCTGCCACACCAGCC, GGTCAAGTTTACAG CCAAGATTCA;

mouse *Cyp24a1* (58°C): CCCAAAGGAACAGTCTTAAC, GGTCTA AACTTGTCAGCATC;

mouse *Cyp27b1* (58°C): AGTGTTGAGATTGTACCCTG, CGTATCTT GGGGAATTACATAG;

mouse *Fgf23* (58°C): TCGAAGGTTCCTTTGTATGGA, AGTGATGCTT CTGCGACAAGT;

mouse *Gapdh* (58°C): GGTGAAGGTCGGTGTGAACG, CTCGCTCCTG GAAGATGGTG;

mouse *K1* (56°C): CCTTAAAAGCAATCAGACTGG, GAAAGCCAT TGTCCTCTATC;

mouse *Slc34a1* (58°C): AATGCAACCATATCTTCGTG, GGAAAGTC TGTGTTGATGAC.

Transcript levels in rat UMR-106 cells were normalized to the housekeeping gene *Tbp* using the $2^{-\Delta Ct}$ method. For animal experiments, relative mRNA expression compared to control group (fold change) was calculated by the $2^{-\Delta\Delta Ct}$ method using *Gapdh* as a housekeeping gene.

2.7. Statistics

Data are shown as bar graphs indicating arithmetic means (± SEM) with individual data points, and *n* denotes the number of independent cell culture experiments performed on different days or the number of mice per group, respectively. Normality was tested with Shapiro-Wilk test. Two groups were compared by paired *t* test or unpaired two-tailed *t* test. Statistical testing of more than two groups was performed by repeated measures one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey's multiple comparisons test, or Friedman test followed by Dunn's multiple comparisons test. A repeated measures two-way ANOVA was used in the *in vivo* experiment to compare the effects of genotype ($EP2^{+/+}$ *vs.* $EP2^{-/-}$) and treatment (basal *vs.* misoprostol) on serum concentration of C-terminal FGF23 and their interdependence. Only differences with $P < 0.05$ were considered statistically significant. IBM SPSS Statistics 29.0.1.0 (IBM, Armonk, NY, USA) or GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA) were used for statistical analysis.

2.8. Data availability

The authors confirm that the data supporting the findings of this study are available in the article and its supplementary materials.

3. Results

3.1. PGE_2 stimulates FGF23

Performing experiments in UMR-106 osteoblast-like cells, we started with a cell culture-based approach to investigate whether PGE_2 impacts the production of FGF23. As a first step, we analyzed the expression of *Ptger1–4* encoding EP1–4 in these cells by PCR in order to check whether they are capable of responding to PGE_2 stimulation. As illustrated in Fig. 1a, mRNA specific for all four subtypes *Ptger1–4* could be detected in UMR-106 cells with the strongest band for *Ptger2* encoding EP2. As a next step, we exposed the cells to increasing concentrations of PGE_2 and determined *Fgf23* expression by qRT-PCR. According to Fig. 1b, PGE_2 up-regulated *Fgf23* gene expression in a dose-dependent manner in UMR-106 cells. Since PGE_2 has a short life span in cellular systems, we performed another series of experiments with misoprostol, an EP2 and EP3 receptor agonist clinically used in the treatment of gastric ulcers and postpartum bleeding. Also, misoprostol dose-dependently enhanced *Fgf23* mRNA abundance (Fig. 1c). By ELISA, we verified that misoprostol-induced up-regulation of *Fgf23* gene expression also translates into enhanced FGF23 protein secretion into the cell culture supernatant of UMR-106 cells (Fig. 1d).

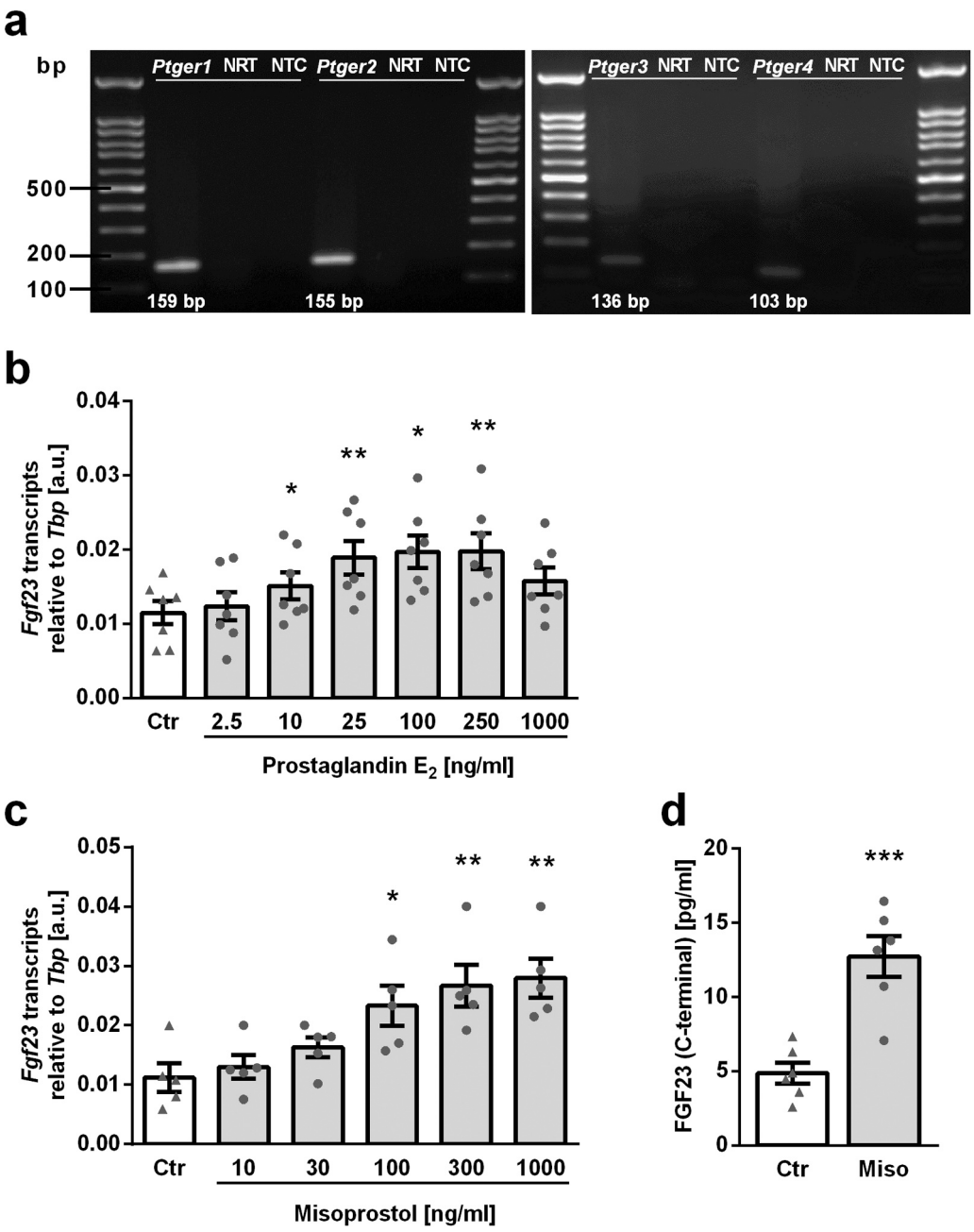

Fig. 1. Prostaglandin E_2 and prostaglandin E receptor agonist misoprostol stimulate fibroblast growth factor 23 production in osteoblast-like UMR-106 cells. (a) Original agarose gel photos illustrating amplified *Ptger1*-, *Ptger2*-, *Ptger3*- and *Ptger4*-specific cDNA in untreated UMR-106 cells. *Fgf23* transcripts in UMR-106 cells incubated with prostaglandin E_2 (b, n = 7) or misoprostol (c, n = 5) for 24 h in the presence of 10 nM $1,25(OH)_2D_3$. (d) C-terminal FGF23 concentration (n = 6) in the cell culture medium of UMR-106 cells treated with vehicle control (Ctr) or 250 ng/ml misoprostol (Miso) for 48 h in the presence of 10 nM $1,25(OH)_2D_3$. All data are shown as individual data points with bars indicating arithmetic mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ indicate statistically significant difference compared to vehicle-treated cells. a.u., arbitrary units; bp, base pairs; Fgf23, fibroblast growth factor 23; NRT, no reverse transcriptase; NTC, no template control; Ptger, prostaglandin E receptor; Tbp, TATA box binding protein.

3.2. EP2 receptor mediates the PGE_2 effect on FGF23

In order to identify the EP subtype accounting for the PGE_2 effect on FGF23, we carried out further experiments with specific EP antagonists. As demonstrated in Fig. 2, SC-19220, an EP1 receptor antagonist (Fig. 2a), L-798,106, an EP3 receptor antagonist (Fig. 2c), and L-161,982, an EP4 receptor antagonist (Fig. 2d) all failed to significantly affect the PGE_2 effect on *Fgf23* gene expression in UMR-106 cells. In contrast, two different and specific EP2 receptor antagonists, PF 04418948 and TG 4-155, significantly attenuated PGE_2-dependent up-regulation of *Fgf23* mRNA abundance (Fig. 2b).

Our experiments thus far indicate that EP2 agonism may stimulate FGF23 production in UMR-106 cells. In order to prove the relevance of EP2 for the regulation of FGF23, we performed experiments with butaprost, a selective EP2 agonist. Similar to PGE_2 and misoprostol, exposure of UMR-106 cells to butaprost resulted in a significant increase in *Fgf23* gene expression (Fig. 3a). We then employed siRNA specifically targeting *Ptger2* encoding the EP2 receptor. According to Fig. 3b, misoprostol failed to significantly enhance *Fgf23* transcript abundance in UMR-106 cells pretreated with siRNA targeting *Ptger2*. In UMR-106 cells exposed to non-targeting siRNA only, misoprostol, however, readily up-regulated *Fgf23* transcripts (Fig. 3b).

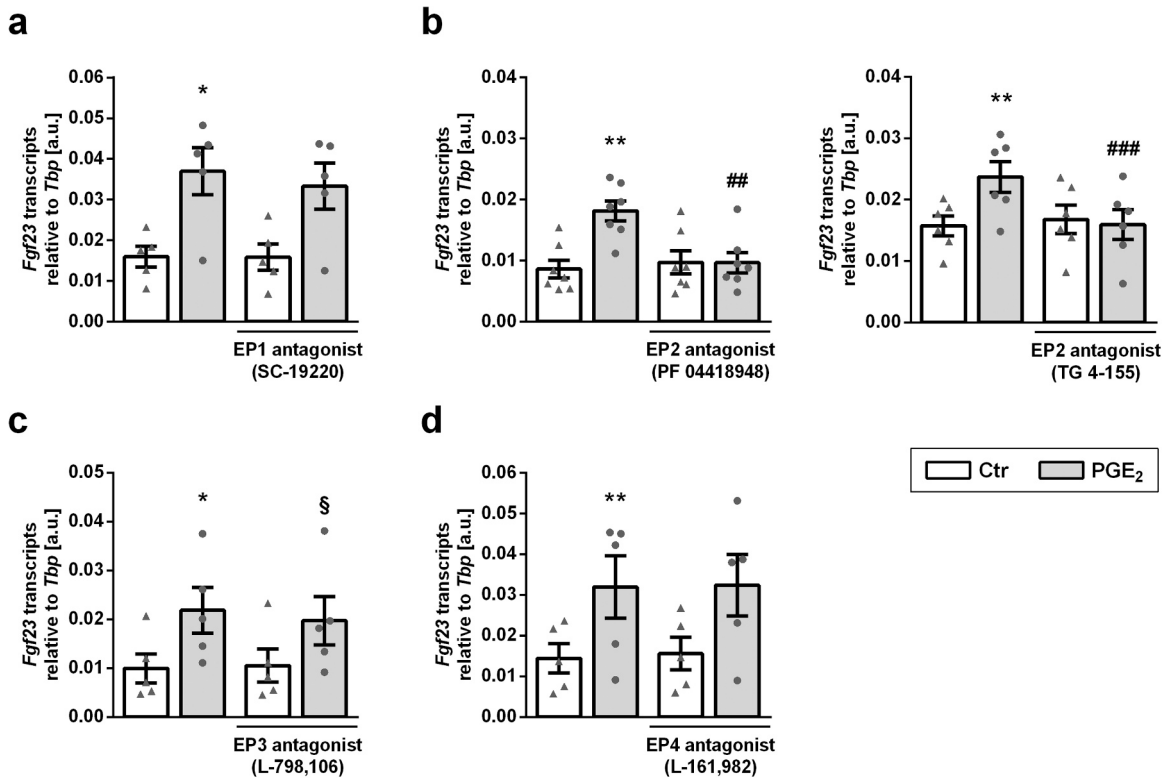

Fig. 2. Prostaglandin E receptor 2 antagonists suppress PGE_2-mediated FGF23 production in UMR-106 cells. *Fgf23* transcripts in UMR-106 cells treated with 250 ng/ml prostaglandin E_2 (PGE_2) or vehicle (Ctr) in the absence or presence of EP1 antagonist (SC-19220; 10 μM) (a, n = 5), EP2 antagonist (PF 04418948 (n = 7; left panel) or TG 4-155 (n = 6; right panel); 10 μM for each) (b), EP3 antagonist (L-798,106; 10 μM) (c, n = 5) or EP4 antagonist (L-161,982; 10 μM) (d, n = 5) and co-treatment with 10 nM $1,25(OH)_2D_3$ for 24 h. All data are displayed as individual data points with bars showing the arithmetic means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$ indicate statistically significant difference compared to vehicle-treated cells (1st bar). $\#\#P < 0.5$, $\#\#\#P < 0.001$ indicate statistically significant difference compared to the absence of EP antagonist (2nd bar *vs.* 4th bar). §$P < 0.05$ shows statistically significant difference compared to cells treated with the antagonist only (3rd bar *vs.* 4th bar). a.u., arbitrary units; Ctr, control; EP, prostaglandin E receptor; Fgf23, fibroblast growth factor 23; Tbp, TATA box binding protein.

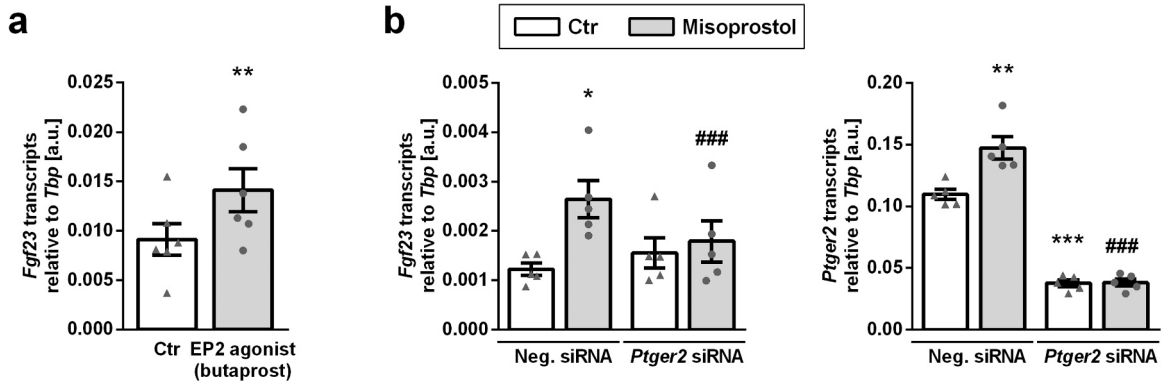

Fig. 3. EP2-mediated misoprostol-dependent FGF23 production. (a) *Fgf23* transcripts (n = 6) in UMR-106 cells incubated with 1 μM EP2 agonist butaprost or vehicle (Ctr) in the presence of 10 nM $1,25(OH)_2D_3$ for 24 h. (b) *Fgf23* (left panel) and *Ptger2* (right panel) transcript levels (n = 5) in UMR-106 cells transfected with 50 nM non-targeting siRNA (Neg. siRNA) or 50 nM *Ptger2* siRNA for 24 h, and then additionally treated with or without (Ctr) 250 ng/ml misoprostol in the presence of 10 nM $1,25(OH)_2D_3$ for further 24 h. All data are shown as individual data points with bars showing the arithmetic mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ indicate statistically significant difference compared to cells treated with vehicle or non-targeting siRNA only (1st bar). $\#\#\#P < 0.001$ indicates statistically significant difference from the absence of *Ptger2* siRNA (2nd bar *vs.* 4th bar). a.u., arbitrary units; Ctr, control; EP2, prostaglandin E receptor 2; Fgf23, fibroblast growth factor 23; Neg., negative; Tbp, TATA box binding protein.

3.3. Nurr1 is downstream of EP2

Nurr1, encoded by *Nr4a2,* is a transcription factor regulated by PGE_2 and has already been shown to be a regulator of FGF23 [22,43]. Therefore, our next series of experiments explored whether Nurr1 is also required for PGE_2 to affect *Fgf23* gene transcription. As illustrated in Fig. 4a, treatment of UMR-106 cells with PGE_2 up-regulated *Nr4a2* expression, an effect blunted by EP2 receptor antagonist PF 04418948. Importantly, misoprostol-induced up-regulation of *Fgf23* gene expression was significantly reduced in UMR-106 cells pre-treated with siRNA targeting *Nr4a2* compared to cells pretreated with non-targeting siRNA (Fig. 4b).

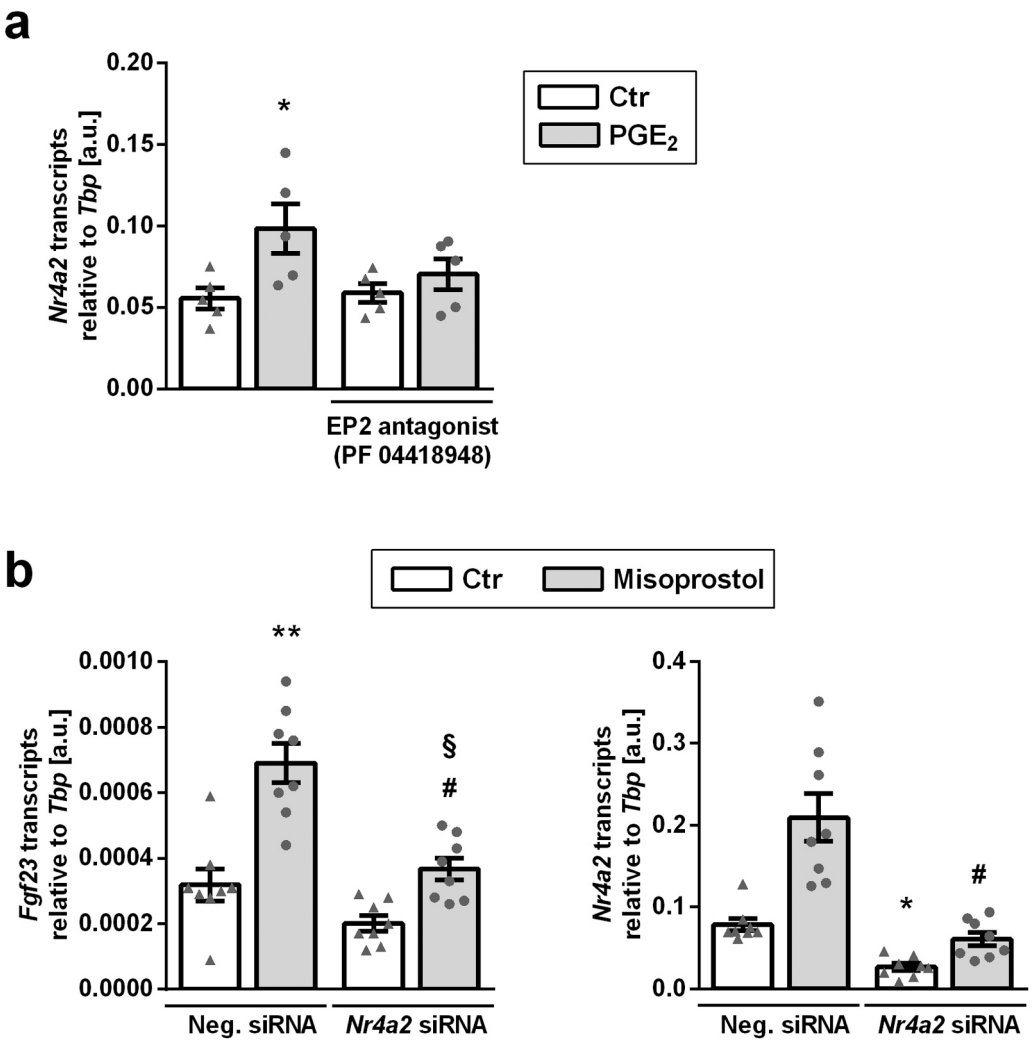

Fig. 4. Prostaglandin E_2- and misoprostol-induced up-regulation of fibroblast growth factor 23 expression is Nurr1-dependent. (a) *Nr4a2* (encoding Nurr1) transcripts (n = 5) in UMR-106 cells treated with 250 ng/ml prostaglandin E_2 (PGE_2) or vehicle (Ctr) in the presence or absence of 10 μM EP2 antagonist (PF 04418948) and co-treatment with 10 nM $1,25(OH)_2D_3$ for 24 h. (b) *Fgf23* (left panel) and *Nr4a2* (right panel) transcript levels (n = 8) in UMR-106 cells transfected with 50 nM non-targeting siRNA (Neg. siRNA) or 50 nM *Nr4a2* siRNA for 24 h, and then treated with or without (Ctr) 250 ng/ml misoprostol in the presence of 10 nM $1,25(OH)_2D_3$ for further 6 h. All data are shown as individual data points with bars showing the arithmetic mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$ indicate statistically significant difference compared to vehicle-treated cells (1st bar). $\#P < 0.05$ indicates statistically significant difference from the absence of *Nr4a2* siRNA (2nd bar *vs.* 4th bar). $\S P < 0.05$ shows statistically significant difference compared to *Nr4a2* silencing only (3rd bar *vs.* 4th bar). a.u., arbitrary units; Ctr, control; EP2, prostaglandin E receptor 2; Fgf23, fibroblast growth factor 23; Neg., negative; Nr4a2, nuclear receptor subfamily 4, group A, member 2; Tbp, TATA box binding protein.

3.4. PGE_2 signaling stimulates FGF23 in mice

According to our cell culture experiments in UMR-106 cells, PGE_2 or misoprostol activate Nurr1 through EP2, resulting in the up-regulation of FGF23 production. In order to investigate the relevance of this pathway *in vivo*, we performed animal experiments. First, we treated wild type mice with a single dose of misoprostol or with vehicle (control). Only 3 h later, *Fgf23* gene expression was strongly and significantly enhanced in bone from misoprostol-treated mice compared to vehicle-treated mice (Fig. 5a). Moreover, compared to control treatment, treatment of wild type mice with misoprostol resulted in a marked and significant surge in the serum C-terminal FGF23 (Fig. 5b) and intact FGF23 (Fig. 5c) concentration further 3 h later.

We performed additional analyses to uncover whether misoprostol-induced FGF23 formation is paralleled by changes in mineral metabolism. As shown in Fig. 6, neither $1,25(OH)_2D$ (Fig. 6a), nor PTH (Fig. 6b), or calcium (Fig. 6c) serum or plasma (PTH) concentration was significantly different between vehicle-treated and misoprostol-treated mice. Serum phosphate concentration (Fig. 6d) was, however, significantly higher in mice following misoprostol administration than in control-treated mice.

Renal expression of *Cyp27b1* encoding 1α-hydroxylase, the key enzyme for the synthesis of $1,25(OH)_2D$ (Fig. 6e), and *Cyp24a1* encoding 24-hydroxylase which inactivates $1,25(OH)_2D$ (Fig. 6f) was not significantly different between vehicle-treated and misoprostol-treated mice. In line with higher FGF23 serum concentrations in mice receiving misoprostol, renal expression of *Slc34a1* encoding NaPi-IIa, the main renal Na^+-phosphate cotransporter, was significantly lower in misoprostol-treated mice than in control mice (Fig. 6g). Also, renal αKlotho (*Kl*) expression was significantly reduced in mice following misoprostol administration compared to vehicle-treated mice (Fig. 6h).

3.5. Also in vivo, EP2 mediates the PGE_2 effect on FGF23

Our animal study thus far revealed that misoprostol induces FGF23 production *in vivo*, a finding in line with the results of our cell culture experiments. Lastly, we performed experiments in gene-targeted mice lacking EP2 receptor protein expression and in wild type mice in order to

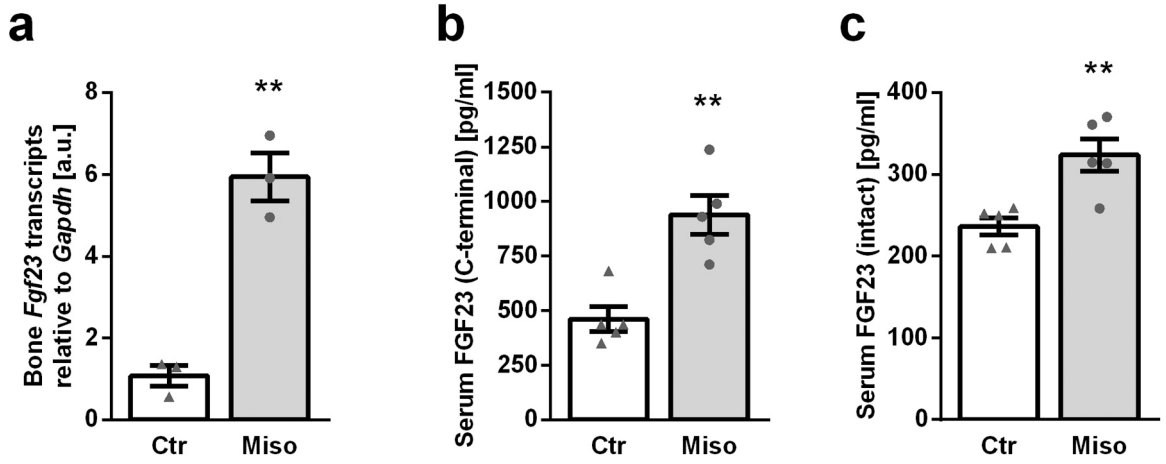

Fig. 5. Single-dose misoprostol stimulates fibroblast growth factor 23 production in wild type mice. Individual data points with bars showing the arithmetic mean ± SEM of bone *Fgf23* transcripts (a, n = 3), C-terminal FGF23 (b, n = 5) and intact FGF23 (c, n = 5) serum concentrations in wild type mice treated with vehicle (Ctr) or misoprostol (Miso; 670 μg/kg body weight). $^{**}P < 0.01$ indicates statistically significant difference compared to vehicle-treated wild type mice. a.u., arbitrary units; Ctr, control; Fgf23, fibroblast growth factor 23; Gapdh, glyceraldehyde-3-phosphate dehydrogenase.

investigate whether also *in vivo*, EP2 receptor is essential for misoprostol to regulate FGF23. *Per se*, $EP2^{+/+}$ and $EP2^{-/-}$ mice did not differ in their serum C-terminal FGF23 level (suppl. Fig. 1). Following treatment with misoprostol, the increase in the serum FGF23 concentration was, however, significantly smaller in $EP2^{-/-}$ mice compared to $EP2^{+/+}$ mice (Fig. 7).

4. Discussion

Our study uncovers a novel effect of PGE_2, i.e. stimulation of FGF23 production. Moreover, we identified the underlying signaling: The PGE_2 effect on FGF23 is mediated by membrane receptor EP2 and transcription factor Nurr1. Since FGF23 has already been demonstrated to affect PGE_2 production [44], our study suggests mutual influence of both factors.

In our cell culture experiments with UMR-106 osteoblast-like cells, three different prostaglandin E receptor agonists (PGE_2, misoprostol and EP2-specific butaprost) up-regulated *Fgf23* gene transcription and protein production. The use of specific EP1–4 receptor antagonists as well as siRNA-mediated EP2 silencing unequivocally demonstrated that the effect on FGF23 is dependent on EP2.

EP2 stimulation leads to enhanced cAMP formation [45] as does PTH

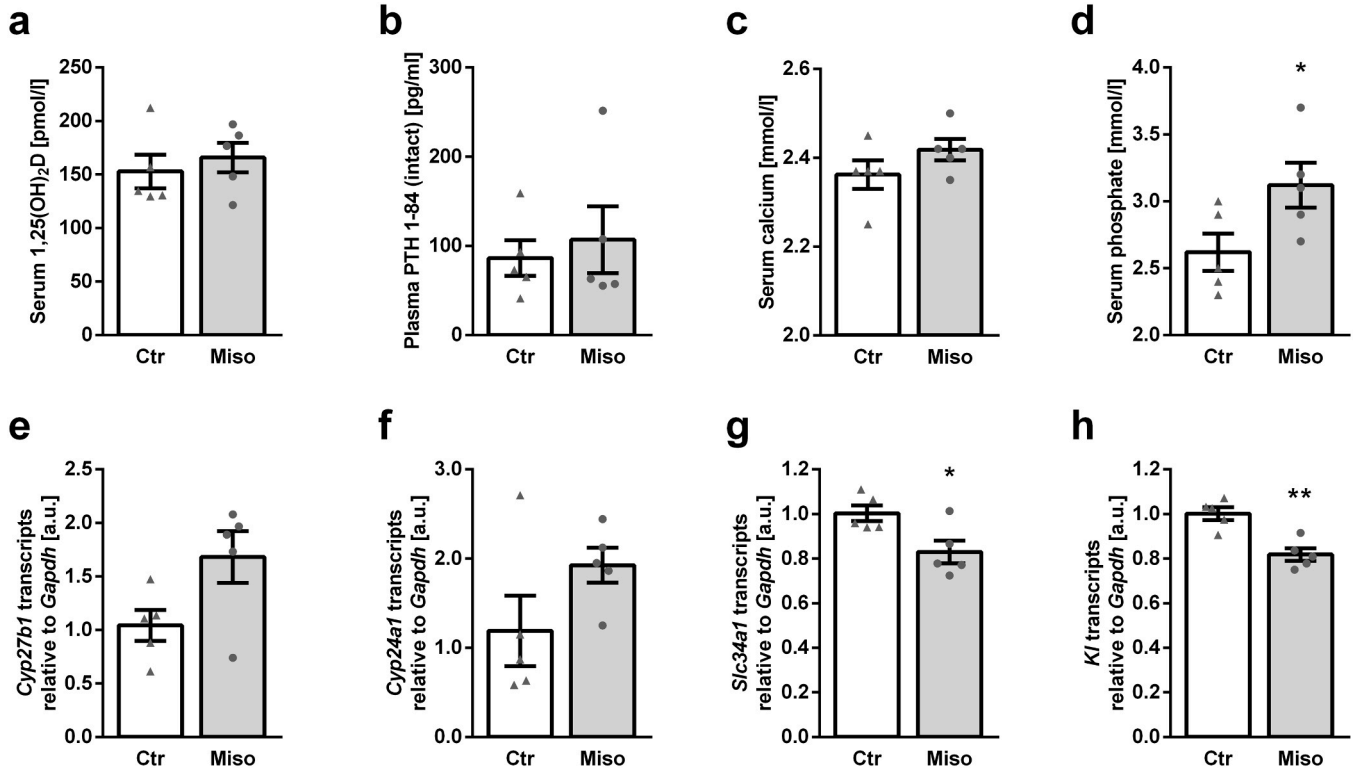

Fig. 6. Impact of misoprostol on mineral metabolism in wild type mice. Individual data points with bars showing the arithmetic mean ± SEM (n = 5) of $1,25(OH)_2D$ (a), intact PTH 1–84 (b), calcium (c), and phosphate (d) serum or plasma concentrations and *Cyp27b1* (e), *Cyp24a1* (f), *Slc34a1* (g), and *Kl* (h) transcripts in renal tissue in wild type mice treated with vehicle (Ctr) or misoprostol (Miso; 670 μg/kg body weight). $^{*}P < 0.05$, $^{**}P < 0.01$ indicate statistically significant difference compared to vehicle-injected wild type mice. a.u., arbitrary units; Ctr, control; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; PTH, parathyroid hormone.

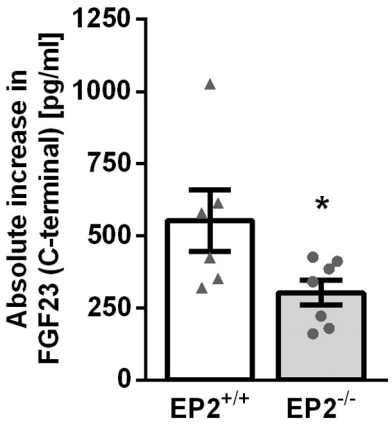

Fig. 7. Misoprostol-induced up-regulation of fibroblast growth factor 23 is attenuated in prostaglandin E receptor 2-deficient mice. Individual data points with bars showing the arithmetic mean ± SEM (n = 6–7) of the change in C-terminal FGF23 serum concentration (pg/ml) in matched male and female wild type ($EP2^{+/+}$) mice and prostaglandin E receptor 2 knockout ($EP2^{-/-}$) mice after treatment with 670 μg misoprostol per kg body weight. *$P < 0.05$ indicates statistically significant difference compared to wild type ($EP2^{+/+}$) mice. FGF23, fibroblast growth factor 23.

receptor stimulation, another major inducer of FGF23 production [22]. Importantly, EP2 and EP4 are expressed in bone with EP2 having particularly important functions [46]: EP2 knockout mice exhibit markedly reduced biomechanical bone strength and altered osteoclastogenesis [46]. Obviously, EP2 is also an important regulator of FGF23 formation by bone cells.

Our experiments further revealed that transcription factor Nurr1 is downstream of EP2 in the signaling mediating PGE_2-dependent FGF23 production. Notably, also PTH increases FGF23 production through Nurr1 [22]. Hence, PTH and PGE_2 signaling appear to converge to enhance cAMP production, Nurr1 activation and ultimately FGF23 synthesis. Importantly, it is a clear limitation of our study that we cannot rule out that signaling molecules others than Nurr1 are also required for PGE_2 to regulate FGF23.

Our animal experiments confirmed the pivotal role of PGE_2 signaling and EP2 receptor for FGF23: A single dose of misoprostol (PGE_2 itself has a very short half-life time, particularly *in vivo*) strongly up-regulated *Fgf23* transcripts in the bone of wild type mice within 3 h only. Three hours later, significantly higher C-terminal and intact FGF23 concentrations were found in the sera. This finding indicates the high relevance of PGE_2 signaling for FGF23 production. Since *Fgf23* transcripts went up more strongly in bone than did FGF23 serum levels, it appears to be possible that not all FGF23 was secreted or that FGF23 degradation was induced simultaneously. Our observation may have clinical relevance since misoprostol is a pharmacologically more stable EP2 receptor agonist that is used in obstetrics to stop postpartum bleeding or to induce uterine contractions [47]. Historically, it has also been used in internal medicine to confer the protective effects of PGE_2 on the gastric epithelium [48]. It therefore appears to be likely that this therapy affects plasma FGF23 levels of the patients. Definitely, clinical studies are needed to address this issue.

Our detailed analysis of parameters of mineral metabolism showed that misoprostol treatment elevates serum phosphate concentration despite reduced NaPi-IIa expression. The latter effect can be expected to occur in response to misoprostol-induced FGF23 production whereas the elevated phosphate concentration is in seeming contrast. However, it must be kept in mind that PGE_2 induces bone resorption, an effect that can be expected to enhance plasma phosphate levels [49]. The increase in C-terminal FGF23 was larger than the increase in intact FGF23 following misoprostol treatment. Given that C-terminal FGF23 may serve as an antagonist for intact FGF23 [50], this observation may also play a role for the higher phosphate serum level after exposure to misoprostol. Moreover, PGE_2 may have further direct and indirect effects on renal phosphate handling. In line with this, a direct stimulatory effect of PGE_2 on Na^+-dependent phosphate transport has been revealed before [51]. Whereas this effect can be expected to elevate plasma phosphate levels, PGE_2 has also been demonstrated to enhance renal calcium and phosphate excretion [52]. Obviously, the effects of PGE_2 on renal calcium and phosphate handling are complex. Moreover, it has to be kept in mind that our animal experiment reflected mineral metabolism 6 h after a single dose of misoprostol. It appears to be possible that longer exposure to PGE_2 agonists could impact mineral metabolism in a more sustained manner. PGE_2 signaling may not only affect FGF23, but has already been demonstrated to regulate vitamin D receptor expression [53]. Conversely, vitamin D metabolites control PGE_2 synthesis and degradation [54]. This interdependence of vitamin D and PGE_2 signaling may also be relevant for the PGE_2 effect on FGF23. Moreover, enhanced PGE_2 production in higher stages of CKD may also contribute to the derangement of vitamin D metabolism by stimulating FGF23.

Lower renal αKlotho expression in response to misoprostol may further contribute to enhanced FGF23 production.

Compared to wild type mice, EP2-deficient mice did not exhibit altered FGF23 serum levels *per se*. Following misoprostol administration, however, the increase in the serum FGF23 concentration was significantly lower in EP2-deficient mice compared to wild type mice. This observation suggests that EP2 signaling is not required for basal FGF23 production but mediates, at least in part, the PGE_2 effect on FGF23, presumably under conditions of enhanced PGE_2 signaling.

Our study may have clinical relevance: Most recently, urinary PGE_2 excretion has been demonstrated to be a CKD biomarker, suggesting enhanced PGE_2 production in the course of CKD [55]. Indeed, PGE_2 production by glomeruli is ramped up in streptozotocin-induced diabetes [56]. Also in 5/6 nephrectomy, renal PGE_2 production through microsomal prostaglandin E synthase 1 contributes to disease progression [57]. PGE_2-dependent FGF23 production may therefore be highly relevant in these and other conditions of renal failure. Also, our findings may be important for cardiac disease: In myocardial infarction, cardiac PGE_2 production is ramped up [58], protecting the heart from ischemia-reperfusion injury [58], a dangerous sequela requiring innovative therapies [59–61]. Notably, also FGF23 reduces ischemia-reperfusion injury at least in the kidney [62], and it is tempting to speculate whether cardiac effects of PGE_2 are, at least in part, dependent on FGF23. Moreover, worldwide, the most common approach to treat pain, inflammation, and fever is suppression of PGE_2 formation through COX inhibition [63]. Whereas conditions associated with enhanced PGE_2 formation can be expected to increase FGF23 production, COX inhibition with common drugs (e.g. aspirin, ibuprofen) may lower FGF23 levels, at least under conditions of enhanced PGE_2 production. In line with this, aspirin is widely used to treat cardiovascular disease, a condition associated with enhanced FGF23 production [64]. Furthermore, COX-2 inhibition has been demonstrated to blunt FGF23 production following acidosis [65]. Moreover, our study adds to the emerging knowledge of inflammation-triggered FGF23 production [23]. Future studies are needed to elucidate whether therapeutic strategies to lower PGE_2 production are effective, at least in part, by reducing FGF23 synthesis.

The regulation of FGF23 by PGE_2 signaling as uncovered by our study may suggest that current and future therapies targeting PGE_2 signaling may impact FGF23 production resulting in the induction of FGF23-dependent cellular responses. Conversely, future innovative therapeutic approaches antagonizing FGF23 may influence PGE_2 effects in different organs.

Our investigation and earlier reports demonstrating that many other pro-inflammatory molecules induce FGF23 production suggest that inflammation is a very important stimulator of FGF23 production and may explain why seemingly different diseases are associated with

elevated FGF23 levels that all have in common that they are inflammation-driven.

It is a clear limitation of our paper that it did not study the contribution of PGE_2 signaling and therapies inhibiting the production of PGE_2 to FGF23 in humans. Definitely, human studies are necessary to elucidate the role of PGE_2 for FGF23 in humans.

5. Conclusions

PGE_2-dependent EP2 receptor and Nurr1 signaling is a powerful stimulator of FGF23 production without significantly affecting serum phosphate levels. This pathway may have clinical relevance.

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CRediT authorship contribution statement

Michael Föller: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Jana Alber:** Investigation. **Martina Feger:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Katharina Hammerschmidt:** Investigation. **Ilona Liesche:** Investigation. **Steffen Rausch:** Investigation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. Martina Feger and Michael Föller report financial support was provided by German Research Foundation. Michael Föller reports a relationship with Kyowa Kirin Co Ltd that includes: speaking and lecture fees. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.117475.

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