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RESEARCH ARTICLE

The vitamin D_3 hormone, $1,25(OH)_2D_3$, regulates fibroblast growth factor 23 (FGF23) production in human skin cells

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Abstract

The bone hormone fibroblast growth factor 23 (FGF23) regulates renal phosphate reabsorption and the enzymatic production of active vitamin D_3 [$1,25(OH)_2D_3$]. Therefore, FGF23 production in bone cells is closely regulated by $1,25(OH)_2D_3$ acting via the vitamin D receptor (VDR). Skin cells can produce hydroxyvitamin D_3 metabolites from its precursor D_3 made through ultraviolet B light exposure. Interestingly, the expression of Fgf23 has been found in rodent skin, but its expression, regulation, and role in human skin are unclear. Therefore, we investigated whether hydroxyvitamin D_3 metabolites regulate FGF23 in human skin cells. Primary adult and neonatal epidermal keratinocytes (HEKn), melanocytes (HEMn), dermal fibroblasts (HDFn), as well as human melanoma cells, HaCaT, HaCaT VDR KO, and A431 epidermoid cells, were used to assess *FGF23* gene expression (quantitative reverse-transcription real-time PCR), cellular FGF23 protein (Western blot), or secreted FGF23 protein (ELISA) after treatment with hydroxyvitamin D_3 metabolites. HaCaT cells treated with recombinant FGF23 were used to explore its function in skin. Human skin cells can synthesize FGF23. Treatment with $1,25(OH)_2D_3$ significantly increased *FGF23* mRNA levels in HaCaT and HDFn cells, and moderately in HEKn cells, mediated in part by the VDR. It also moderately enhanced mRNA levels of the FGF23-processing enzyme *GALNT3* and stimulated secretion of hormonally active FGF23 from HaCaT cells. Treatment of HaCaT cells with FGF23 increased mRNA levels of the cholesterol- and vitamin D-metabolizing enzymes, *CYP11A1* and *CYP27A1*. In conclusion, human skin cells express and secrete FGF23, which is regulated by $1,25(OH)_2D_3$ acting in part by the VDR. FGF23 affects the expression of cutaneous sterol-metabolizing enzymes.

NEW & NOTEWORTHY This study shows for the first time the expression and secretion of the FGF23 hormone by human skin cells. In addition, we identified the active vitamin D_3 hormone, $1,25(OH)_2D_3$, to be a potent regulator of dermal FGF23 expression and protein secretion, partly involving the vitamin D receptor. Furthermore, we provide initial evidence demonstrating that FGF23 upregulates the gene expression of *CYP11A1* and *CYP27A1* in keratinocytes.

bone; cytochrome P450; CYP11A1; phosphate; vitamin D receptor

INTRODUCTION

Vitamin D_3, a secosteroid, is produced in human skin by a photochemical reaction where 7-dehydrocholesterol (7-DHC) absorbs ultraviolet B (UVB) energy from sunlight (1, 2). This process initially opens the B-ring of 7-DHC and forms previtamin D_3, which then undergoes thermal isomerization to form vitamin D_3 (1, 2). Once in the bloodstream, vitamin D_3 is metabolized in the liver by hydroxylation at C25 by the enzymes CYP2R1 or CYP27A1, resulting in the formation of 25-hydroxyvitamin D_3 ($25(OH)D_3$) (3). This major circulating form of vitamin D_3 then undergoes a second hydroxylation at C1 in the kidneys by the enzyme 25-hydroxyvitamin D-1α-hydroxylase, encoded by the *CYP27B1* gene, to produce the final active hormone, $1,25(OH)_2D_3$ (4, 5). The classical function of $1,25(OH)_2D_3$ in maintaining bone and calcium homeostasis and its mechanism of action by activating the vitamin D receptor (VDR) are well established (1, 5).

Interestingly, in addition to the liver and kidney (4), human skin also contains the complete enzymatic machinery

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necessary for the local production and degradation of 1,25(OH)$_2$D$_3$ (2). Notably, in addition to the classical enzymatic pathway in vitamin D$_3$ activation, skin cells also express CYP11A1, the rate-limiting enzyme of steroidogenesis (6, 7). Remarkably, it has been shown that CYP11A1 can also hydroxylate vitamin D$_3$ to produce biologically active metabolites such as 20-hydroxyvitamin D$_3$ [20(OH)D$_3$] and 20,23-dihydroxyvitamin D$_3$ [20,23(OH)$_2$D$_3$], which can be further hydroxylated by the classical CYP enzymes (CYP27A1, CYP2R1, or CYP27B1) to form 1,20,23-trihydroxyvitamin D$_3$ [1,20,23(OH)$_3$D$_3$] or 20,25-dihydroxyvitamin D$_3$ [20,25(OH)$_2$D$_3$] (8, 9). These novel vitamin D$_3$ metabolites have been detected in human serum and produced in various organs, including skin cells such as keratinocytes and fibroblasts (10–12). Recent studies have shown that these metabolites can activate nuclear receptors such as VDR, liver X receptor (LXR), aryl hydrocarbon receptor (AhR), RAR-related orphan receptors (RORs), or peroxisome proliferator-activated receptor gamma (PPARγ). This leads to inhibition of cell proliferation, promotion of keratinocyte differentiation, antiproliferative and antifibrotic effects in fibroblasts, suppression of proinflammatory cytokines, or anticancer activities in vitro and in vivo, and reduction of clinical signs of scleroderma arthritis and joint damage in vivo (8, 13–25). Their potential effects on bone, analogous to those of 1,25(OH)$_2$D$_3$, remain to be evaluated.

One main target gene regulated by 1,25(OH)$_2$D$_3$ in bone encodes the hormone fibroblast growth factor 23 (FGF23). As well as 1,25(OH)$_2$D$_3$ and the VDR (26), it was shown that the vitamin D photoderivative tachysterol$_2$ is capable of inducing osseous FGF23 production (27). In addition to vitamin D derivatives and phosphate as classical systemic regulators (28), proinflammatory cytokines such as interleukin-6 (29) and tumor necrosis factor alpha (30), regulators of cellular energy metabolism such as AMP-dependent protein kinase (31) and insulin (32), the hormones endothelin-1 (33) and erythropoietin (34), the myokines myostatin (35) and L-β-aminoisobutyric acid (36), and transcription factors such as NFκB (37) or PPARα (38) have also been shown to regulate FGF23 expression in bone cells.

In the body, FGF23 is produced primarily by osteocytes and osteoblasts, *O*-glycosylated by the catalytic enzyme UDP-GalNAc:Polypeptide *N*-Acetylgalactosaminyltransferase 3 (GALNT3) to protect it from cleavage into its C- and N-terminal fragments, and then secreted into the bloodstream as intact biologically active FGF23 (39). FGF23 interacts with its coreceptor αKlotho to maintain phosphate balance by suppressing renal phosphate reabsorption (40, 41). Furthermore, FGF23 is regulated by 1,25(OH)$_2$D$_3$ in a feedback manner, being one of the main regulators of 1,25(OH)$_2$D$_3$ levels via downregulating renal mRNA expression of *CYP27B1* and by upregulating mRNA of the 1,25(OH)$_2$D$_3$-degrading enzyme 25-hydroxyvitamin D-24-hydroxylase (*CYP24A1*) (41).

Besides its physiological function, FGF23 became of clinical significance following its identification in rare inherited disorders marked by hypophosphatemic rickets and phosphate wasting (42). In addition, supraphysiological plasma levels of FGF23, such as in conditions of chronic kidney disease, are also associated with deleterious effects such as heart failure, cardiac hypertrophy, fibrosis, and cancer (43, 44).

Interestingly, Fgf23 has been reported to be produced in the rodent skin being induced by wounding (45) and uremia (46). However, others have shown a lack of FGF23 expression in keratinocytes by immunocytochemical staining (47). Moreover, *Fgf23*$^{-/-}$ mice showed skin atrophy and reduced thickness (48). Interestingly, GALNT3, necessary for secreting hormonally active FGF23, and the responsible FGF receptors (FGFR) and αKlotho for FGF23 signaling, are also expressed in skin cells (45, 47, 49, 50). This suggests the skin as a potential site for the production of hormonally active FGF23 and indicates the presence of molecular machinery to initiate local FGF23-induced dermal effects. However, little is known about either.

Therefore, the aim of this study was to investigate whether FGF23 is expressed in different human skin cells and whether locally produced classical and novel vitamin D$_3$ metabolites also regulate cutaneous FGF23 synthesis.

MATERIALS AND METHODS

Human Tissue, Cell Culture, and Treatments

Human skin tissues from neonatal foreskin (n) or adults (a) (excess skin after surgery) of either black or white donors were used to isolate fresh fibroblasts (HDF), epidermal keratinocytes (HEK), or melanocytes (HEM). HDFn cells isolated from different skin tissues were combined for greater biological variability. Isolated HDFn and HDFa cells were cultured as described (20), using complete Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, L-glutamine, and sodium pyruvate (Corning Inc., Corning, NY) supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies, Waltham, MA), and 1% antibiotics (Cytiva, Marlborough, MA; 10,000 U/mL penicillin; 10,000 μg/mL streptomycin and 25 μg/L amphotericin). For treatments, passages (P) P1 through P11 were used and HDFn cells were plated in 100-mm dishes for proliferation to 80% confluence. The media were then changed, and HDFn cells were cultured in serum-deprived conditions overnight for synchronization. The next day, media were changed, and HDFn cells were treated with 100 nM of different vitamin D$_3$ metabolites (as described in the next section) or ethanol vehicle only in the presence of complete medium supplemented with 10% charcoal-stripped serum (chFBS; Gibco, Life technologies) instead of complete serum for 24 h. After 24 h of treatment, cell culture supernatant was collected for ELISA, and the cells were harvested for RNA-isolation. HEKa and HEKn were cultured in EpiGRO human epidermal keratinocyte media supplemented with the reagents of EpiGRO Human Epidermal Keratinocyte Complete Media Kit (Merck Millipore, Burlington, MA) as described elsewhere (51). HEKn cells were combined within the same ethnicity for greater biological variability and used between P0 and P4. For treatments, HEKn were grown in EpiGRO Human Epidermal Keratinocyte Complete Media in calf skin collagenated (Sigma-Aldrich, St. Louis, MO) 60-mm dishes and treated either with 100 nM 1,25(OH)$_2$D$_3$ or ethanol vehicle for 24 h before cells were harvested for RNA-isolation. For experiments with UVB exposure (UV transilluminator 2000 from Bio-Rad, Hercules, CA), HEKn P3 from black donors were used, and HEKn media were replaced by PBS before the treatment at a dose of 50 mJ/cm^2.

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After UVB treatment, HEKn cells were cultured in fresh EpiGRO Human Epidermal Keratinocyte Complete Media for an additional 24 h before supernatants were collected.

Human epidermal melanocytes from black and white donors were cultured in melanocyte growth medium supplemented with melanocyte growth factors (all from Lonza Walkersville Inc., Walkersville, MD) and harvested for RNA-isolation. Third passage HEMn cells were used for Western blotting experiments to determine cellular FGF23 protein.

For experiments with human epidermal HaCaT and HaCaT VDR KO, cells were seeded in T-75 cell culture flasks and cultured in DMEM (Corning Inc.) supplemented with 5% FBS (Gibco, Life Technologies), and 1% antibiotics (Cytiva). After 24 h, when cells reached 60% confluency, the complete media were replaced by FBS-free DMEM and HaCaT and HaCaT VDR KO cell lines were cultured in serum-deprived conditions overnight for synchronization. On the next day, HaCaT and HaCaT VDR KO cells were treated for 24 h with 100 nM of the indicated vitamin D$_3$ metabolites or ethanol only in a 5% chFBS-complete medium. In some experiments, HaCaT was treated after the same procedure with increasing (as indicated) concentrations of human recombinant FGF23 protein (R&D systems, Bio-techne, Minneapolis, MN, USA) or vehicle alone for 24 h in 5% chFBS-complete medium. After 24 h of incubation, supernatants of the cells were collected for ELISA and cells harvested for RNA-isolation or Western blotting.

UMR-106 rat osteoblastic-like cells (CRL-1661; ATCC, Manassas, VA) were cultured in DMEM (Corning Inc.) supplemented with 10% FBS (Gibco, Life Technologies), and 1% antibiotics (Cytiva), as previously described (52). For treatment experiments, 0.7×10^6 UMR-106 cells were plated in T-25 cell culture flasks. Twenty-four hours after seeding, the complete medium was replaced by DMEM supplemented with 10% chFBS (Gibco) and 1% antibiotics (Cytiva), and cells were treated with 100 nM of the indicated vitamin D$_3$ metabolites or ethanol only for 24 h for isolation of RNA.

Human squamous carcinoma cell line A431 (CRL-1555; ATCC) was cultured as described (22), using DMEM (Corning Inc.) supplemented with 10% FBS (Gibco, Life Technologies), and 1% antibiotics (Cytiva). For experimental treatment, chFBS complete medium was used supplemented with 100 nM of the indicated vitamin D$_3$ compounds or ethanol vehicle alone to incubate the A431 cells 24 h before analysis of *FGF23* mRNA expression or 12 h before the supernatants were collected for ELISA analysis.

Human SK Mel 188 melanoma cells were cultured, as described previously (53). Briefly, SK Mel 188 cells were cultured in Ham's F10 medium supplemented with glucose, L-glutamine, pyridoxine hydrochloride (Cellgrow, Manassas, VA, SA), 5% FBS (Gibco, Life Technologies), and 1% antibiotics (Cytiva) before cells were harvested to determine *FGF23* gene expression and protein secretion.

Human melanoma cells, designated by the initials YU, were cultured in Opti-Mem medium (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Fetal Bovine Serum, Atlanta Biologicals, Norcross, GA), as previously described (54), and cells were collected for determination of *FGF23* mRNA expression.

Vitamin D$_3$ Compounds and Chemical Synthesis

The following vitamin D$_3$ hydroxyderivatives (as indicated) were used in cell culture treatments to investigate their impact on FGF23 expression: 1α,25(OH)$_2$D$_3$ (Sigma-Aldrich), and the CYP11A1-derived 1α,20*S*,23*S*(OH)$_3$D$_3$, 20*S*(OH)D$_3$, 20*S*,23*S*(OH)$_2$D$_3$, and 20*S*,25(OH)$_2$D$_3$ hydroxyderivatives, which were synthesized and purified as described elsewhere (15, 55). The preparation of 1α,20*S*(OH)$_2$D$_3$ (Supplemental Fig. S1: https://doi.org/10.6084/m9.figshare.28360373) was accomplished by following the synthetic route developed by others (56). This procedure involved three main transformations: the formation of cyclovitamin D followed by C-1 α-hydroxylation, and subsequent solvolysis. As a starting material, 20*S*-hydroxyvitamin D$_3$ (1; Supplemental Fig. S1) was chosen (15), which was converted into tosylate (2; Supplemental Fig. S1). The latter was subjected to a cyclization with NaHCO$_3$, yielding cyclovitamin (3; Supplemental Fig. S1). Selective oxidation of 3 at C1 was achieved using tert-butyl alcohol peroxide and SeO$_2$, resulting in the formation of hydroxycyclovitamin (4; Supplemental Fig. S1). The obtained product was subsequently solvolyzed in the mixture of DMSO and acetic acid, providing a mixture of 5- and 5,6-trans-1α-hydroxyvitamin D$_3$ in a 2.5:1 ratio. The mixture was then treated with maleic anhydride in ethyl acetate, which allowed the removal of the 5,6-transisomer. After final HPLC purification, the desired 1α,20*S*(OH)$_2$D$_3$ (5; Supplemental Figs. S1 and S2) was obtained (Supplemental Figs. S3 and S4).

HaCaT VDR KO Transfection

To establish HaCaT cells without VDR expression, we used the genomic modification method clustered regularly interspaced short palindromic repeats (CRISPR)-technique to knock out VDR expression. This technique was used to specifically modify the cellular genome to knock out the expression of the *VDR* gene in HaCaT, as described previously (18). Briefly, HaCaT cells were plated in T-25 cell culture flasks and cultured in a complete medium as described above for 24 h. The next day, the complete medium was replaced with a medium containing polybrene (10 μg/mL) to stabilize the lentivirus, and the HaCaT cells were incubated with human *VDR* sgRNA CRISPR All-in-One Lentivirus or scrambled lentivirus (Applied Biological Materials Inc., Richmond, BC, Canada) for the next 24 h. The sgRNA used was designed to target all three isoforms of *VDR* (NM_000376, NM_001017535, NM_001017536). After 24 h of transfection, the transfection medium was replaced with a medium supplemented with 5 μg/mL puromycin to select lentivirally transduced cells. The resulting HaCaT cells with the *VDR* gene knocked out are designated HaCaT VDR KO, and the cells transfected with a nonspecific lentivirus served as a scramble control (HaCaT scramble). Western blotting was used to verify VDR protein expression in CRISPR-modified HaCaT VDR KO and scramble control cells prior to experiments.

RNA-Isolation and Quantitative Real-Time PCR

Total RNA was extracted from HaCaT, HDFn, A431, and UMR-106 cells by using an Agilent Absolutely RNA Miniprep Kit (Agilent Technologies, Cedar Creek, TX) and for HEKn

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using an RNAqueous-Micro Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's protocols. For the entire skin analysis, commercially available total RNA from normal adult skin pooled from five donors (female 21–83 yr old) was used (Biotrend, Köln, Germany). For cDNA synthesis, either 2 μg for HaCaT, HDFn, and adult skin, 1.5 μg for UMR-106, 1 μg for HEKn and HEM cells, or 0.5 μg for adult skin, A431, and melanoma cells of extracted total RNA were used with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems by Thermo Fisher Scientific Baltics, Vilnius, Lithuania) and the following program: 25°C for 10 min, 37°C for 2 h, and 85°C for 5 min. Quantitative reverse-transcription real-time PCR (qRT–PCR) was performed by using a QuantStudio 6 Flex cycler (Applied Biosystems, Waltham, MA) and Luminaris HiGreen Low ROX qPCR Master Mix (Thermo Fisher Scientific Baltics, Lithuania) to determine relative human and rat mRNA transcript level of the genes listed in Table 1 under the following conditions: 95°C for 5 min; 40 cycles of 95°C for 10 s, primer-specific annealing temperature (as indicated in Table 1) for 30 s, and 72°C for 30 s (for human *FGF23* 20 s). The *FGF23* and *GALNT3* qRT-PCR products of the HaCaT and HEKn cells were loaded on a 2% agarose gel and visualized by Midori Green. The calculated relative mRNA transcript level of the target gene was normalized to the expression level of *ACTB* (HDF, HEK, skin), *CYPB* (HaCaT and HaCat VDR KO), or *Tbp* (UMR-106) in the same cDNA sample. Quantification of relative target gene expression is presented as $2^{-\Delta CT}$ (ΔCT = CT [target gene] − CT [reference gene]) transformed data (57).

Western Blotting

The cells were grown and protein extraction was performed, as described previously. Briefly, after treatment, the cells were harvested and the total proteins were isolated by lysing cells in RIPA buffer (Thermo Fisher Scientific) on ice for 10 min and centrifugation at 15,000 *g* for 10 min. For Western blot analysis, 40 μg of proteins were separated on SDS-PAGE 4%–15% acrylamide gel (Bio-Rad Laboratories, Inc., Hercules, CA) followed by transfer to a PVDF membrane (Millipore Sigma, Burlington, MA) using Mini Trans-Blot cells (Bio-Rad). After blocking with 5% skim milk in Tris-buffered saline and incubation at 4°C overnight with the primary rabbit anti-FGF23 antibody (SAB4503210, Sigma, St. Louis, MO) 1:1000 diluted in 5% skim milk, the membranes were incubated with secondary anti-rabbit antibody conjugated to HRP (Santa Cruz Biotechnology, Dallas, TX) diluted 1:5,000 followed by incubation with SuperSignal WestPico Substrate (Thermo Fisher Scientific) for 5 min. The membranes were subsequently exposed to an autoradiography film (MidSci, St. Louis, MO) and developed with a film processor.

To prove the knockdown of VDR protein expression in HaCaT VDR KO cells, a Western blot was performed by loading 5 μg, 10 μg, and 20 μg of whole cell extracts on an SDS-PAGE gel followed by transferring to a PVDF membrane. VDR (D-6) primary antibody (1:200 diluted in 5% skim milk) and m-IgG2a BP-HRP secondary antibody (1:5,000 diluted in 5% skim milk) from Santa Cruz Biotechnology, Inc. (Dallas, TX) were used, respectively. HRP-conjugated GAPDH Mouse

Table 1. *qRT-PCR primers (5′→ 3′ orientation) and used annealing temperatures*

Gene	Species	Sequence F: 5′–3′ R: 5′–3′	Annealing, °C
FGF23	Human	GATGCTGGCTTTGTGGTGAT GAGGAGAGTGGTAGACGTCG	56
FGF23	Human	CAGCATGAGCGTCCTCAGAG GCCAGCATCCTCTGATCTGATC	55
ACTB	Human	AGTTCAACGGCACAGTCAAG TACTCAGCACCAGCATCAC	56
CYPB	Human	TTCATGTCTTCATTTTGGGC TCTCCAGGTCATATCTTACATC	56
CYP24A1	Human	CATCATGGCCATCAAAACAAT GCAGCTCGACTGGAGTGAC	60
CYP27B1	Human	CTTGCGGACTGCTCACTG CGCAGACTACGTTGTTCAGG	60
CYP2R1	Human	AGCCTCATCCGAGCTTCC CCACAGTTGATATGCCTCCA	60
CYP27A1	Human	CAGTACGGAACGACATGGAG GGTACCAGTGGTGTCCTTCC	60
CYP11A1	Human	CCAGACCTGTTCCGTCTGTT AAAATCACGTCCCATGCAG	60
GALNT3	Human	CTCTATGTCTGGATGTTGG TCATGTTGAGCAGAGTATTC	56
Fgf23	Rat	TAGAGCCTATTCAGACACTTC CATCAGGGCACTGTAGATAG	57
Tbp	Rat	ACTCCTGCCACACCAGCC GGTCAAGTTTACAGCCAAGATTCA	57
Galnt3	Rat	TAGGGGGAAATCAGTACTTTG CTTTATAGACACATGCCTTCAG	60
Cyp24a1	Rat	AAAGAATCCATGAGGCTTAC TTTTCTCCTTTTGAAGCCAG	60
Spp1	Rat	TGATGAACAGTATCCCGATG AACTGGGATGACCTTGATAG	60
Rankl	Rat	CTCATGCAGGAGAATGAAAC TTCCATCATAGCTGGAACTC	58
Alpl	Rat	ACCTCTTAGGTCTCTTTGAG CTTTGGGATTCTTTGTCAGG	56
Runx2	Rat	AGAGTCAGATTACAGATCCC TGTCATCATCTGAAATACGC	56
Vdr	Rat	CCTTTCTCCTGCCCAGCCTAACAC TCCCCCGGGTCAGAATAACACAG	60
Dmp1	Rat	ACTGTTATCCTCCTTACGTTC GGTCTATACTGGCTTCTGTC	58
Phex	Rat	ATGGCTGGATAAGCAATAAC GCTTTTTCAATCGCTTTCTC	58
Sost	Rat	ATGATGCCACAGAAATCATC CACGTCTTTGGTGTCATAAG	58
Ogn	Rat	CTTCCTCCAAAGCTTACTTTAC ATGCTGTTAAACTGAAGGTG	60
Bglap	Rat	CAACAATGGACTTGGAGC AGAGTAGCCAAAGCTGAAG	60
Cyp1a1	Rat	CCACAAGAGATACAAGTCTG CCGATGCACTTTCGCTTGC	60

ACTB, β-actin; Alpl, alkaline phosphatase; Bglap, osteocalcin; CYPB, cyclophilin B; Cyp1a1, cytochrome P450 family 1 subfamily A member 1; CYP24A1, 25-hydroxyvitamin D-24-hydroxylase; CYP27B1, 25-hydroxyvitamin D-1α-hydroxylase; CYP2R1, vitamin D 25-hydroxylase; CYP27A1, cytochrome P450 family 27 subfamily A member 1; CYP11A1; cytochrome P450 family 11 subfamily A member 1; Dmp1, dentin matrix acidic phosphoprotein 1; FGF23, fibroblast growth factor 23; GALNT3, UDP-GalNAc:Polypeptide *N*-Acetylgalactosaminyltransferase 3; Ogn, osteoglycin; Phex, phosphate regulating endopeptidase X-linked; Rankl, receptor activator of nuclear factor kappa B ligand; Runx2, RUNX family transcription factor 2; Sost, sclerostin; Spp1, osteopontin; Tbp, TATA-box binding protein; Vdr, vitamin D receptor.

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McAb (1:5,000 diluted in 5% skim milk) from Proteintech Group, Inc. (Rosemont, IL) was used as a housekeeping loading control. All blots were analyzed using Odyssey Fc Imager from LI-COR Corporate (Lincoln, NE).

Enzyme-Linked Immunosorbent Assay

HEKn, HDFn, A431, or SK Mel 188 cells were cultured as described above, and 100 μL of undiluted supernatant of treated and untreated cells were used to determine the secreted total FGF23 protein using the Human FGF23 enzyme-linked immunosorbent assay (ELISA) kit (LSBio, Lynnwood, WA), according to the manufacturer's protocol. For analysis of secreted intact or C-terminal FGF23 protein, HDFn and HaCaT cells were cultured as described above and cell culture supernatants were stored at −80°C. For quantification, cell culture supernatants were first concentrated using Vivaspin 6 centrifugal concentrators (Sartorius, Göttingen, Germany), and intact and C-terminal FGF23 protein levels were determined using a Human FGF23 (Intact) or (C-terminal) kit (Immutopics, San Clemente, CA) according to the manufacturer's protocol.

Statistics

The data are presented as arithmetic means ± SEM and n indicates the number of independent experiments conducted. Normal distribution of the data was assessed using the Shapiro–Wilk test. Two-group comparisons were performed using an unpaired Student's t test with Welch's correction applied when necessary, or a Mann–Whitney U test for non-normally distributed data. Comparisons involving more than two groups were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. Statistical significance was considered at $P < 0.05$.

RESULTS

FGF23 Is Expressed and Secreted by Human Primary and Carcinoma Skin Cells

The impact of vitamin D$_3$ metabolites on dermal FGF23 synthesis was investigated in this study in different primary and carcinoma skin cell lines. To test whether these in vitro models of human skin express FGF23, we first performed RT-PCR to examine the gene expression of *FGF23* in human keratinocytes. As illustrated in Fig. 1*A*, mRNA specific for *FGF23* could be detected in HaCaT and primary neonatal human epidermal keratinocytes (HEKn). Next, we determined the presence of *FGF23* mRNA in human skin samples and several cultured human epidermal skin and melanoma cells. Interestingly, *FGF23* expression was detectable in human skin and in all human skin cell lines tested, as listed in Table 2, indicating a ubiquitous expression of *FGF23* in different human skin cell types and melanoma cells. To investigate whether the detectable *FGF23* transcripts in skin cells are translated into FGF23 protein, we used Western blotting to determine cellular FGF23 expression in primary neonatal human epidermal melanocytes (HEMn) and HaCaT cells. As depicted in Fig. 1*B*, *FGF23* mRNA transcripts are translated into protein, as FGF23 protein was detectable in the whole cell extract of both HEMn and HaCaT cells. Next, we were interested in whether skin cells are also able to secret FGF23 protein. By using an ELISA kit, we detected secreted FGF23 protein in the supernatant of primary HEKn and neonatal human dermal fibroblasts (HDFn) as well as in the carcinoma cell lines A431 and SK Mel 188 (Fig. 1*C*), demonstrating dermal secretion of the hormone FGF23.

Dermal *FGF23* mRNA Abundance Is Increased by 1,25(OH)$_2$D$_3$

Having shown that FGF23 is produced and secreted by primary and carcinoma skin cells, we next investigated whether one of the major systemic regulators of FGF23 production in bone cells, 1,25(OH)$_2$D$_3$, is also capable of regulating and inducing dermal *FGF23* mRNA abundance. To investigate this, preliminary tests were first carried out to optimize the concentration and incubation time to be used. Therefore, HaCaT cells were treated with increasing concentrations of 1,25(OH)$_2$D$_3$ for 24 h and *FGF23* gene expression was measured by qRT-PCR. As shown in Supplemental Fig. S5, *A* and *B* (https://doi.org/10.6084/m9.figshare.28360469.v1), *FGF23* mRNA abundance increased with increasing concentrations of 1,25(OH)$_2$D$_3$ used. To investigate whether this increase might also be time-dependent, we used 100 nM as the most effective dose of 1,25(OH)$_2$D$_3$ to induce *FGF23* mRNA

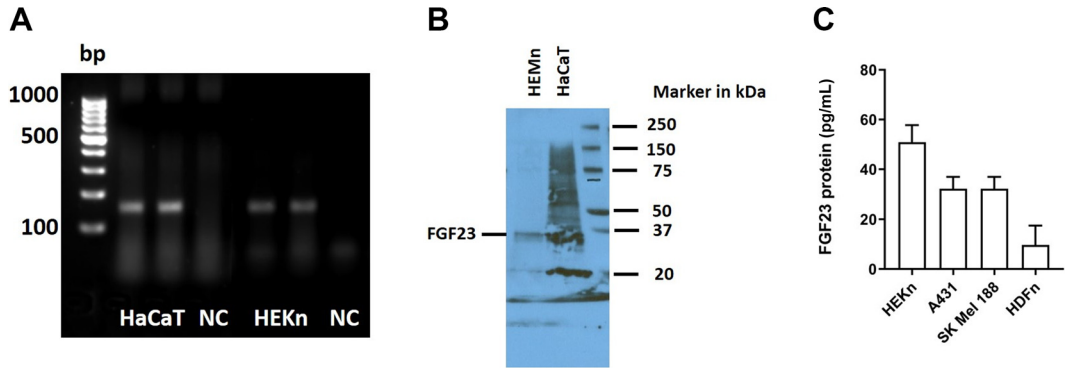

Figure 1. FGF23 is expressed and secreted by human primary and carcinoma skin cells. *A*: original agarose gel photo showing cDNA specific for *FGF23* in HaCaT and neonatal HEKn. *B*: original Western blot image demonstrating FGF23 protein expression in primary black neonatal HEMn and HaCaT cells. *C*: arithmetic means ± SEM of FGF23 protein in the supernatants of HEKn ($n = 2$), squamous cell carcinoma cells (A431; $n = 3$), SK Mel 188 melanoma cells ($n = 3$), and neonatal human epidermal fibroblasts (HDFn; $n = 3$) after 24 h of culturing. HEKn, human epidermal keratinocytes; HEMn, human epidermal melanocytes; NC, nontemplate control.

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Table 2. *Expression of FGF23 mRNA in human skin and cultured human epidermal skin and human melanoma cells*

Identification	Specimen Type	Mean ΔCt ± SD
Skin tissue	Skin tissue samples from white individuals	15.64 ± 1.22
HEKa	Human epidermal keratinocytes (adult)	10.73 ± 0.093
HEKn	Human epidermal keratinocytes (neonatal)	7.45 ± 0.136
HEFa	Human epidermal fibroblast (adult)	10.38 ± 0.017
HEFn	Human epidermal fibroblast (neonatal)	12.41 ± 0.102
HEMa	Human melanocytes (adult)	11.72 ± 0.204
HEMn	Human melanocytes (neonatal)	10.50 ± 0.204
HaCaT	Immortalized keratinocytes	9.49 ± 0.199
YUAME	Melanoma	13.91 ± 0.240
YUWERA	Melanoma	13.32 ± 0.110
YUTICA	Melanoma	14.46 ± 0.390
YUROB	Melanoma	13.74 ± 0.174
YUKSI	Melanoma	15.31 ± 0.141
YULAC	Melanoma	14.48 ± 0.262
YUCOT	Melanoma	13.23 ± 0.355
YUSIV	Melanoma	14.26 ± 0.314

The ΔCt values are inversely proportional to the amount of target cDNA in the sample. The lower the value, the greater the amount of *FGF23* mRNA.

synthesis and treated HaCaT cells in another experiment with increasing incubation times. Interestingly, in addition to the dose response, we observed a time-dependent increase in *FGF23* mRNA abundance after treatment with 1,25(OH)$_2$D$_3$ (Supplemental Fig. S5, *B* and *C*). The results of the observed time- and dose-dependent stimulation of *FGF23* mRNA abundance by 1,25(OH)$_2$D$_3$ served as the basis for the settings of the subsequent experiments. Next, we were interested in whether other hydroxyvitamin D$_3$ metabolites besides 1,25(OH)$_2$D$_3$, in particular CYP11A1-derived vitamin D$_3$ metabolites produced in the skin (8), were capable of stimulating *FGF23* mRNA expression. To investigate this, we treated HaCaT cells with 100 nM 1,25(OH)$_2$D$_3$ and other hydroxyvitamin D$_3$ metabolites for 24 h and measured *FGF23* gene expression. As shown in Fig. 2*A*, 1,25(OH)$_2$D$_3$ significantly induced *FGF23* mRNA abundance compared with control treatment. Interestingly, the CYP11A1-derived vitamin D$_3$ metabolites 20(OH)D$_3$ and 20,23(OH)$_2$D$_3$ were also able to significantly increase the amount of *FGF23* mRNA in HaCaT. Treatment with the 1α-hydroxylated CYP11A1-derivative 1,20(OH)$_2$D$_3$ or 1,20,23(OH)$_3$D$_3$ also resulted in a higher but not significantly increased *FGF23* mRNA abundance in HaCaT cells compared with control. Consistent with this, after 24 h of treatment with either 1,25(OH)$_2$D$_3$ or 20(OH)D$_3$, the amount of *FGF23* mRNA was also significantly higher in human squamous cell carcinoma A431 cells compared with the control (Fig. 2*B*), while treatment with 1,20(OH)$_2$D$_3$ also resulted in a higher, but not significantly increased *FGF23* mRNA in A431 cells compared with control (Fig. 2*B*). Next, HDFn cells were treated with the same regimen as HaCaT and *FGF23* gene expression was measured after 24 h.

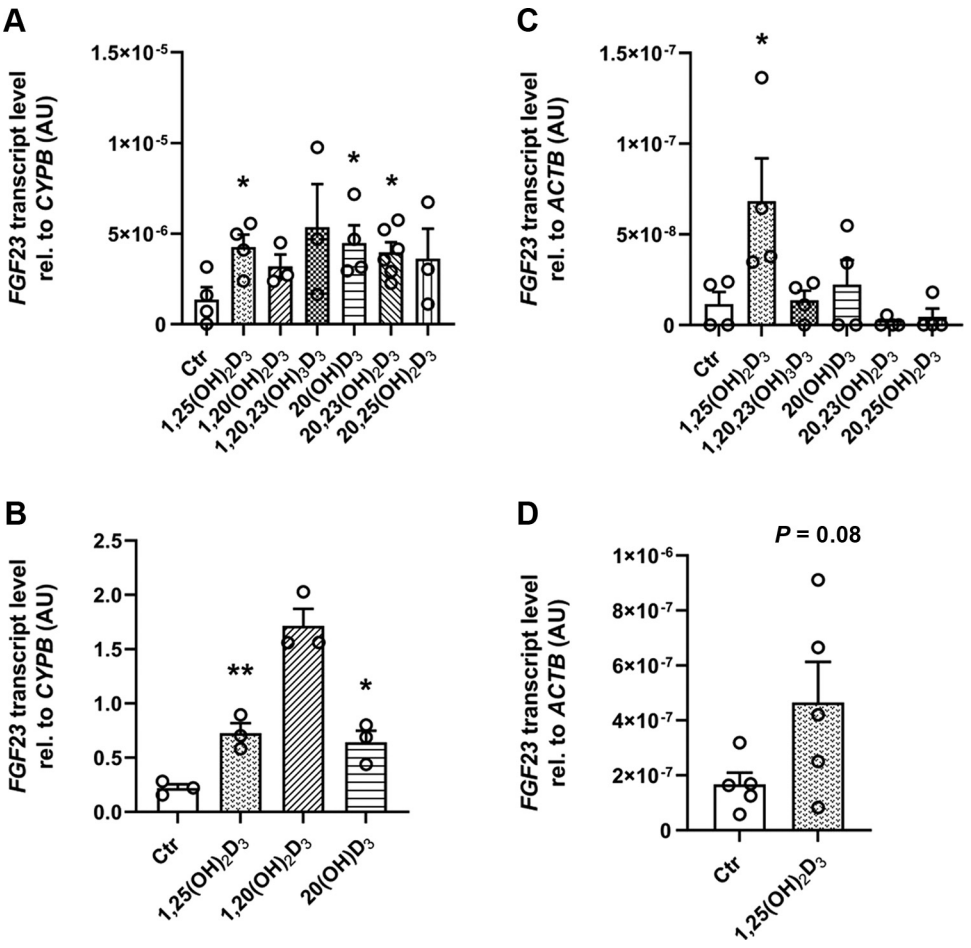

Figure 2. Dermal *FGF23* mRNA abundance is increased by 1,25(OH)$_2$D$_3$. Scatter dot plots and arithmetic means ± SEM of relative (rel.) *FGF23* mRNA abundance normalized to *CYPB* in HaCaT (*A*: $n = 3$–6) and A431 (*B*: $n = 3$) or *ACTB* in neonatal human epidermal fibroblasts (HDFn; *C*: $n = 4$) and neonatal human epidermal keratinocytes (HEKn; *D*: $n = 5$) treated with 100 nM of the indicated vitamin D$_3$ metabolite or ethanol vehicle, for 24 h. *$P < 0.05$; **$P < 0.01$: significant differences from the control (*A*–*D*: unpaired Student's *t* test and Mann–Whitney *U* test). ACTB, β-Actin; AU, arbitrary units; CYPB, cyclophilin B; Ctr, control; HEKn, human epidermal keratinocytes.

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Interestingly, in HDFn, only 1,25(OH)$_2$D$_3$ was able to significantly increase *FGF23* mRNA abundance compared with control (Fig. 2*C*). Since 1,25(OH)$_2$D$_3$ was the most potent vitamin D$_3$ metabolite, we also analyzed its effect on *FGF23* gene expression in HEKn cells from black and white donors after 24 h treatment. As shown in Fig. 2*D*, 1,25(OH)$_2$D$_3$ treatment also resulted in moderately higher *FGF23* mRNA abundance ($P = 0.08$) in primary HEKn compared with control.

Osteoblastic *Fgf23* Gene Expression Is Induced by 1,25(OH)$_2$D$_3$ and 1,20,23(OH)$_3$D$_3$

To demonstrate the ability of the novel CYP11A1-derived vitamin D$_3$ metabolites used to induce *FGF23* gene expression in bone, the major FGF23-synthesizing organ in the body, in a manner similar to 1,25(OH)$_2$D$_3$, we used rat UMR-106 osteoblast-like cells as an *Fgf23*-expressing in vitro cell line model (58). As expected and already known (58), 24 h treatment of UMR-106 cells with 100 nM 1,25(OH)$_2$D$_3$ significantly increased *Fgf23* mRNA abundance compared with control (Fig. 3*A*). Remarkably and in contrast to the results in HaCaT and HDFn cells, treatment with 1,20,23(OH)$_3$D$_3$ also resulted in a very strong and significant induction of the amount of *Fgf23* mRNA in UMR-106 osteoblast-like cells compared with control (Fig. 3*A*). Also, in contrast to the results from HaCaT cells, 20(OH)D$_3$, 20,23(OH)$_2$D$_3$, and 20,25(OH)$_2$D$_3$ did not stimulate *Fgf23* gene expression in UMR-106 osteoblasts compared with control. On one hand, this points to the importance of 1α-hydroxylation and thus the VDR-dependent signaling cascade for the induction of osseous *FGF23* expression, and on the other hand, it shows that the non-1α-hydroxylated vitamin D$_3$ metabolites in the skin can influence other FGF23-regulating signaling pathways and transcription factors that do not operate in bone. Since it has been shown elsewhere that 1,25(OH)$_2$D$_3$ increases not only *FGF23* gene expression but also its processing by regulating the expression of the catalytic enzyme GALNT3 (59), which is required for secretion of intact FGF23 protein (60), we investigated whether this enzyme is also regulated by the novel CYP11A1-derived vitamin D$_3$ metabolites. As shown in Fig. 3*B*, in addition to 1,25(OH)$_2$D$_3$ but in contrast to the non-1α-hydroxylated vitamin D$_3$ metabolites tested, 1,20,23(OH)$_3$D$_3$ significantly increased the mRNA abundance of *Galnt3* in UMR-106 cells compared with the control after 24 h treatment. This demonstrates the ability of 1,20,23(OH)$_3$D$_3$, like 1,25(OH)$_2$D$_3$, to not only impact *Fgf23* expression but also the post-transcriptional processing of Fgf23 protein for cellular secretion in these osteoblast-like cells.

Influence of 1,25(OH)$_2$D$_3$ and Novel CYP11A1-Derived Vitamin D$_3$ Metabolites on Osteoblastic Gene Expression

To further analyze whether the novel vitamin D$_3$ metabolites used are able to regulate other VDR-dependent or

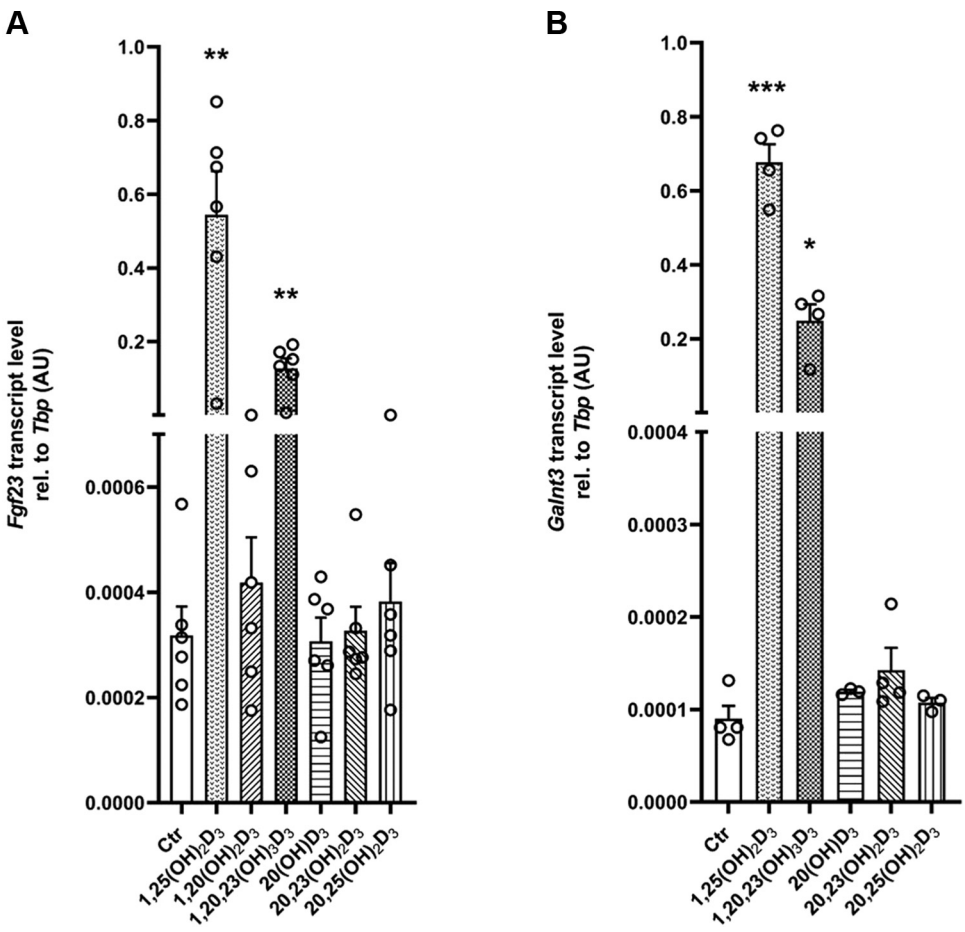

Figure 3. Osteoblastic *Fgf23* gene expression is induced by 1,25(OH)$_2$D$_3$ and 1,20,23(OH)$_3$D$_3$. Scatter dot plots and arithmetic means ± SEM of relative (rel.) *Fgf23* (*A*) or UDP-GalNAc:Polypeptide *N*-Acetylgalactosaminyltransferase 3 (*Galnt3*) (*B*) mRNA abundance normalized to *Tbp* in UMR-106 osteoblast-like cells (*A*: $n = 6$; *B*: $n = 4$) treated with 100 nM of the indicated vitamin D$_3$ metabolite or ethanol vehicle for 24 h. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ indicate significant differences from the control [*A* and *B*: unpaired Student's *t* test with Welch's correction (if necessary)]. AU, arbitrary units; Ctr, control; Tbp, TATA-box binding protein.

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-independent genes in contrast to FGF23 in bone cells, we determined their impact on the expression of several important genes involved in bone homeostasis and turnover, calcification processes, and osteoblastic marker genes. As shown in Fig. 4, both 1,25(OH)$_2$D$_3$ and 1,20,23(OH)$_3$D$_3$ were able to significantly increase the relative mRNA abundance of *Cyp24a1* (Fig. 4*A*), *Spp1* (Fig. 4*B*), *Rankl* (Fig. 4*C*), and *Phex* (Fig. 4*H*) and significantly decrease the amount of *Runx2* mRNA (Fig. 4*E*) after 24-h treatment in UMR-106 cells compared with control. Remarkably, the mRNA levels of these genes were not affected by treatment with either 20(OH)D$_3$, 20,23(OH)$_2$D$_3$, or 20,25(OH)$_2$D$_3$ compared with control. The mRNA abundance of *Bglap* showed a modest increase by 1,25(OH)$_2$D$_3$ treatment (Fig. 4*K*; $P = 0.06$) and the amount of *Vdr* mRNA was only significantly increased by 1,20,23(OH)$_3$D$_3$ treatment in UMR-106 cells compared with control (Fig. 4*F*). Interestingly, only 1,25(OH)$_2$D$_3$ was able to moderately stimulate *Dmp1* mRNA abundance in UMR-106 cells compared with control (Fig. 4*G*; $P = 0.09$). These data suggest that the 1,25(OH)$_2$D$_3$- and 1,20,23(OH)$_3$D$_3$-regulated genes are likely under control of VDR-signaling, and 1α-hydroxylation is apparently necessary for the regulation of these genes. In line with this hypothesis, treatment with 20(OH)D$_3$ moderately decreased the amount of *Dmp1* mRNA in UMR-106 cells compared with the control after 24 h (Fig. 4*G*; $P = 0.09$). This, due to the observed opposite effect, suggests the involvement of other signaling pathways than those of the 1α-hydroxylated metabolites. Notably and in line with this, the mRNA level of *Sost*, which encodes the bone-inhibiting protein sclerostin, was significantly decreased only by 20,25(OH)$_2$D$_3$ (Fig. 4*I*). In addition, treatment with 1,25(OH)$_2$D$_3$ significantly increased *Ogn* mRNA levels in UMR-106 cells (Fig. 4*J*). In contrast, *Ogn* mRNA levels were moderately decreased by 20(OH)D$_3$ ($P = 0.07$), 20,23(OH)$_2$D$_3$ ($P = 0.053$), or 20,25(OH)$_2$D$_3$ ($P = 0.057$) after 24 h compared with the control (Fig. 4*J*). *Cyp1a1* mRNA levels were significantly higher only by 20(OH)D$_3$ treatment and moderately by other CYP11A1-derived metabolites, but not by 1,25(OH)$_2$D$_3$ treatment, compared with control (Fig. 4*L*), indicating differential transcriptional effects of the classical and novel hydroxyvitamin D$_3$ metabolites in UMR-106 osteoblast-like cells based on the position of hydroxylation. None of

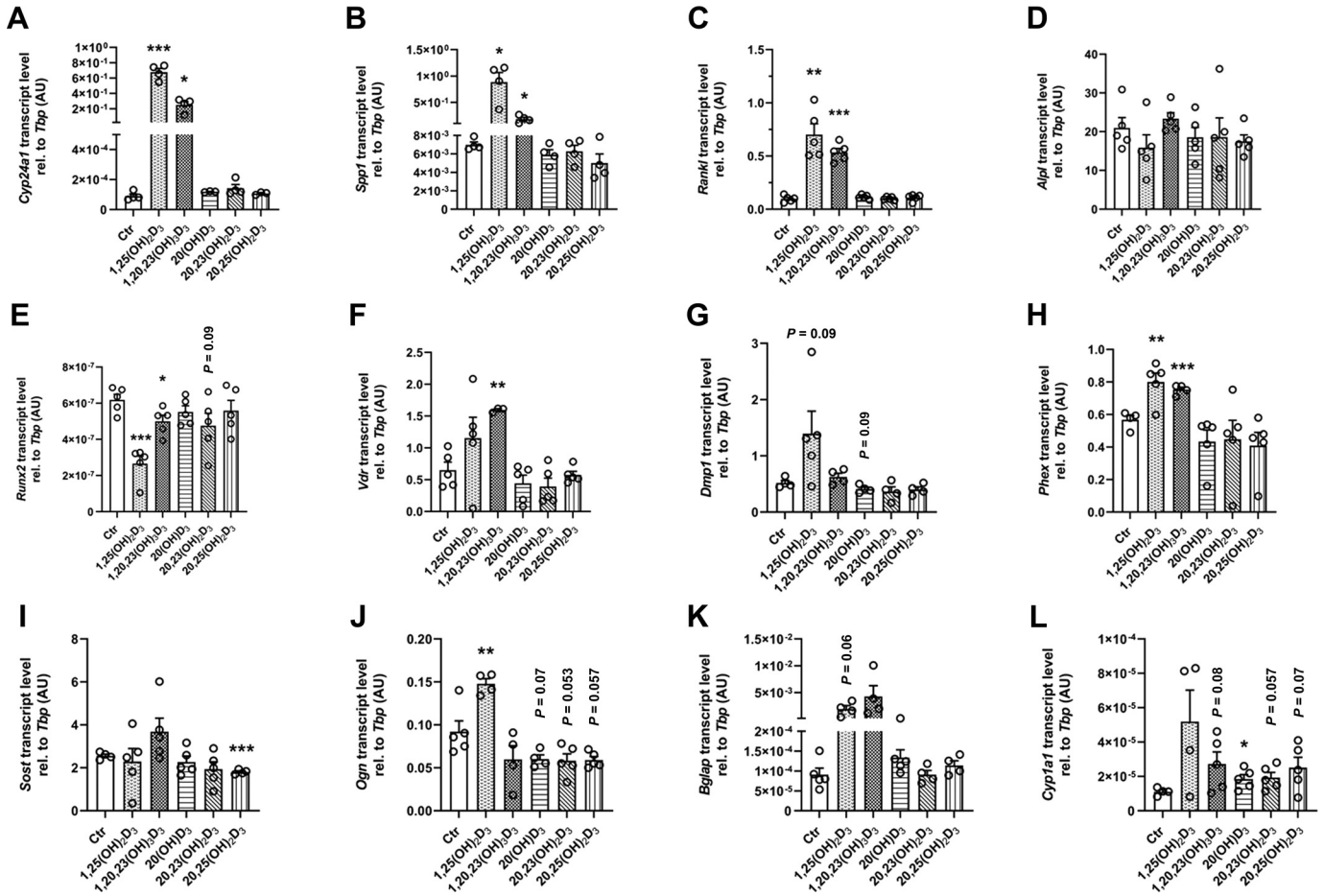

Figure 4. Influence of 1,25(OH)$_2$D$_3$ and novel CYP11A1-derived vitamin D$_3$ metabolites on osteoblastic gene expression. Scatter dot plots and arithmetic means ± SEM of relative (rel.) *Cyp24a1* (*A*), *Spp1* (*B*), *Rankl* (*C*), *Alpl* (*D*), *Runx2* (*E*), *Vdr* (*F*), *Dmp1* (*G*), *Phex* (*H*), *Sost* (*I*), *Ogn* (*J*), *Bglap* (*K*), or *Cyp1a1* (*L*) mRNA abundance normalized to *Tbp* in UMR-106 osteoblast-like cells (*A* and *B*: $n = 4$; *C*–*L*: $n = 5$) treated with 100 nM of the indicated vitamin D$_3$ metabolites or ethanol vehicle for 24 h. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ indicate significant differences from the control [*A*–*L*: unpaired Student's *t* test with Welch's correction (if necessary) or Mann–Whitney *U* test (for non-normally distributed data)]. Alpl, alkaline phosphatase; AU, arbitrary units; Bglap, osteocalcin; Ctr, control; Cyp1a1, cytochrome P450 family 1 subfamily A member 1; Cyp24a1, cytochrome P450 24A1; Dmp1, dentin matrix acidic phosphoprotein 1; Ogn, osteoglycin; Phex, phosphate regulating endopeptidase X-linked; Rankl, receptor activator of nuclear factor kappa B ligand; Runx2, RUNX family transcription factor 2; Sost, sclerostin; Spp1, osteopontin; Tbp, TATA-box binding protein; Vdr, vitamin D receptor.

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the hydroxyvitamin D$_3$ compounds tested regulated the mRNA abundance of *Alpl* (Fig. 4*D*).

Dermal Secretion of Intact FGF23 Protein Is Increased by 1,25(OH)$_2$D$_3$

In the next series of experiments, we were interested in the capability of skin cells to translate elevated *FGF23* gene expression also in a higher protein secretion. To test this, we choose the most potent vitamin D$_3$ metabolite for inducing dermal *FGF23* gene expression in this study, 1,25(OH)$_2$D$_3$, to investigate whether it also increases dermal FGF23 protein secretion. First, we used a total human FGF23 ELISA kit and analyzed the supernatant of A431 cells after 12 h treatment with 100 nM 1,25(OH)$_2$D$_3$. Interestingly, we observed a significantly increased amount of total FGF23 protein in the supernatant of A431 cells treated with 1,25(OH)$_2$D$_3$ compared with the ethanol control (Fig. 5*A*). FGF23 can be secreted from cells as cleaved inactive C- and N-terminal fragments or intact hormone and FGF23 is protected from cleavage by *O*-glycosylation by the enzyme GALNT3. Since the form of secretion of FGF23 as inactive C- and N-terminal fragments or as intact hormone depends on prior *O*-glycosylation by the responsible enzyme GALNT3, and since this enzyme appears to be expressed in dermal fibroblasts (49) in addition to FGF23 itself, we next investigated whether this enzyme is also expressed in the HaCaT cell model used in this study. As shown in Fig. 5*B*, mRNA specific for *GALNT3* was detected in HaCaT cells, demonstrating its ability to regulate dermal FGF23 protein processing. To test whether 1,25(OH)$_2$D$_3$ or 1,20,23(OH)$_3$D$_3$ treatment regulates *GALNT3* mRNA abundance not only in UMR-106 osteoblast-like cells but also in HaCaT and HDFn skin cells, we performed qRT-PCR analysis of *GALNT3* gene expression after 24 h treatment with 100 nM of these secosteroids. Interestingly, as shown in Fig. 5*C*, 1,25(OH)$_2$D$_3$ moderately increased *GALNT3* mRNA abundance in HaCaT cells compared with control ($P = 0.057$), whereas 1,20,23(OH)$_3$D$_3$ raised but did not significantly increase *GALNT3* expression. In HDFn cells, a 24 h treatment with 1,25(OH)$_2$D$_3$ resulted in a relative *GALNT3* expression of $3.73 \times 10^{-6} \pm 6.11 \times 10^{-6}$ and $7.11 \times 10^{-6} \pm 1.18 \times 10^{-6}$ for treatment with 1,20,23(OH)$_3$D$_3$, levels not significantly different from that in control cells ($4.37 \times 10^{-6} \pm 1.32 \times 10^{-6}$; $n = 4$; unpaired Student's *t* test). Having shown that 1,25(OH)$_2$D$_3$ treatment also regulates gene expression of the catalytic enzyme required for secretion of intact FGF23, we were finally interested in whether HaCaT cells were capable of secreting hormonally active FGF23 protein after treatment with 1,25(OH)$_2$D$_3$. Remarkably, using a human intact FGF23 ELISA kit, we detected significantly higher levels of intact FGF23 protein in 1,25(OH)$_2$D$_3$-treated HaCaT cells compared with control cells after 24 h (Fig. 5*D*), demonstrating both that 1,25(OH)$_2$D$_3$ induces cutaneous FGF23 protein production and secretion and that keratinocytes are capable of secreting not only the FGF23 protein but especially its hormonally active form. Interestingly, using a human C-terminal FGF23 ELISA kit, we detected significantly lower levels of C-terminal FGF23 protein in 1,25(OH)$_2$D$_3$-treated HaCaT cells (0.38 ± 0.07 pg/mL) compared with control cells (0.53 ± 0.05 pg/mL; $n = 5$; $^{**}P < 0.01$ unpaired Student's *t* test) after 24 h. Surprisingly, but in line with the unaffected *GALNT3* expression in HDFn, ELISA quantification of the supernatant of these cells after 24 h treatment with 1,25(OH)$_2$D$_3$ (0.28 pg/mL ± 0.04) did not show any differences for the secreted intact FGF23 protein amount compared with control cells (0.25 pg/mL ± 0.05; $n = 5$; unpaired Student's *t* test), whereas C-terminal FGF23 protein was significantly higher in the supernatants of 1,25(OH)$_2$D$_3$-treated HDFn (0.67 ± 0.16 pg/mL) compared with control-treated HDFn (0.40 pg/mL ± 0.04; $n = 5$; $^{*}P < 0.05$ unpaired Student's *t* test). These results show the importance of the GALNT3 enzyme for the processing of FGF23 and the resulting levels of secreted intact or C-terminal FGF23 as well as differences in the relevance of different skin cell types for the 1,25(OH)$_2$D$_3$-mediated secretion of hormonally active FGF23.

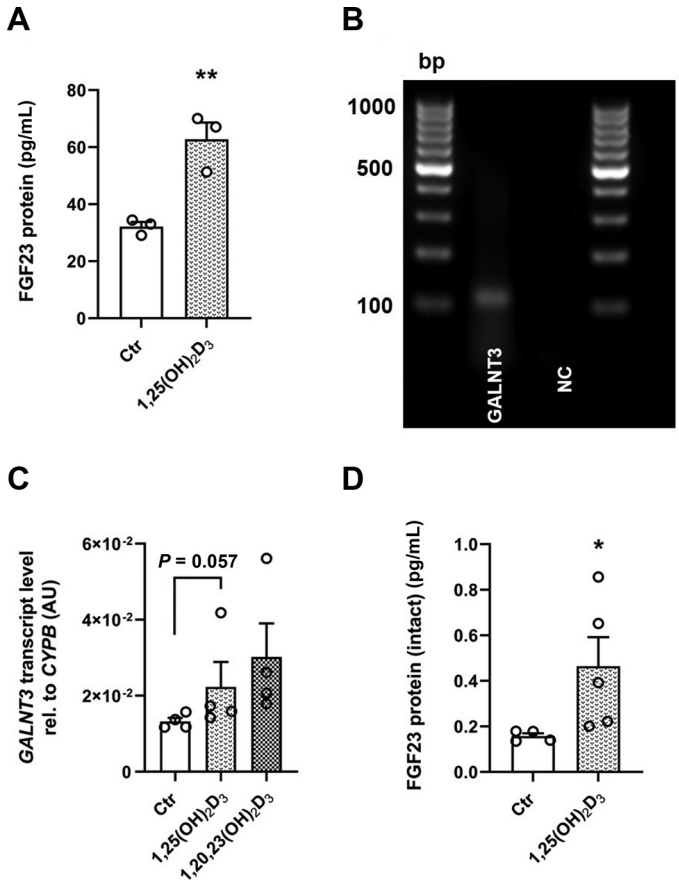

Figure 5. Dermal secretion of intact FGF23 protein is increased by 1,25(OH)$_2$D$_3$. *A*: scatter dot plots and arithmetic means ± SEM of FGF23 protein in the supernatant of A431 cells (*A*: $n = 3$) or (*D*: $n = 4$–5) intact FGF23 protein in the supernatant of HaCaT cells or (*C*: $n = 5$) relative (rel.) *GALNT3* mRNA abundance normalized to *CYPB* in HaCaT cells treated with 1,25(OH)$_2$D$_3$ or ethanol vehicle (*A, C,* and *D*: 100 nM; *A*: 12 h; *C* and *D*: 24 h). *B*: expression of UDP-GalNAc:Polypeptide *N*-Acetylgalactosaminyltransferase 3 (*GALNT3*) in HaCaT cells. Original agarose gel photo showing cDNA specific for *GALNT3*. $^{*}P < 0.05$; $^{**}P < 0.01$: significant differences from the control (*A* and *D*: unpaired Student's *t* test; *C*: Mann–Whitney *U* test). AU, arbitrary units; Ctr, control; CYPB, cyclophilin B; NC, non-template control.

UVB-Treatment Does Not Affect Dermal FGF23 Protein Secretion

Since the production of the vitamin D$_3$ metabolites used in this study is initiated in the skin by exposure to UVB, we

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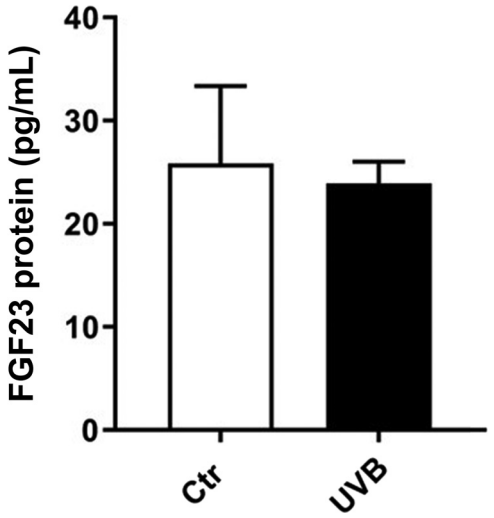

Figure 6. UVB treatment does not affect dermal FGF23 protein secretion. Arithmetic means ± SEM of FGF23 protein in the supernatants of neonatal HEKn treated with or without UVB-light (50 mJ/cm^2; $n = 3$) (unpaired Student's *t* test). Ctr, control; HEKn, human epidermal keratinocytes; UVB, ultraviolet B.

were interested in whether UVB-light could directly regulate FGF23 secretion in keratinocytes. To test this, we exposed primary HEKn cells to 50 mJ/cm^2 and measured the amount of secreted FGF23 protein in the supernatant of the cells after 24 h using ELISA. As shown in Fig. 6, UVB-treatment did not affect FGF23 protein secretion from HEKn cells.

Dermal *FGF23* Gene Expression Is Partially Dependent on VDR

Having shown that the active vitamin D$_3$ hormone 1,25(OH)$_2$D$_3$ is the most potent metabolite to regulate FGF23 production in skin cells and that 1α-hydroxylation of CYP11A1-derived vitamin D$_3$ metabolites was also necessary to induce osseous *Fgf23* expression, we hypothesized that the VDR, as in bone cells, might be responsible for the transcriptional regulation of *FGF23* gene expression through its activation by binding of 1,25(OH)$_2$D$_3$. To test this, we used CRISPR-modified HaCaT cells with a knockout specific for the *VDR* (Fig. 7*A*). To verify the efficient knockout of VDR protein, we used Western blotting to determine VDR protein expression after CRISPR-modification of the cells. As shown in Fig. 7*B*, VDR protein expression was largely eliminated in HaCaT VDR KO cells compared with scramble HaCaT cells as control. Next, we used qRT-PCR to examine the relative abundance of *FGF23* mRNA in HaCaT VDR KO cells lacking functional VDR after 24 h of culturing. Here we detected a significantly reduced, but not fully abolished amount of *FGF23* mRNA in HaCaT VDR KO cells compared with scramble HaCaT cells (Fig. 7*C*), demonstrating that the VDR is a crucial transcription factor involved in the regulation of cutaneous *FGF23* gene expression.

FGF23 Increases mRNA Abundance of Sterol-Metabolizing CYP Enzymes *CYP27A1* and *CYP11A1* in HaCaT Cells

Our data identified FGF23 as a novel regulatory target of classical and novel CYP11A1-derived hydroxyvitamin D$_3$ metabolites in skin cells. Since the dermal expression of the FGFRs and its co-receptor αKlotho is already known (47), we were interested in whether the production of FGF23 only leads to endocrine effects after entering the circulation, or whether FGF23 could also mediate local effects in the surrounding skin cells in a paracrine manner after its secretion. Given that FGF23 is a major regulator of systemic vitamin D$_3$ metabolism by regulating renal *CYP24A1* and *CYP27B1* to reduce 1,25(OH)$_2$D$_3$ levels (41), we hypothesized that FGF23 may also regulate dermal 1,25(OH)$_2$D$_3$ metabolism through the regulation of the expression of these necessary enzymes as a local feedback mechanism of dermal 1,25(OH)$_2$D$_3$ production. To test this hypothesis, we used recombinant

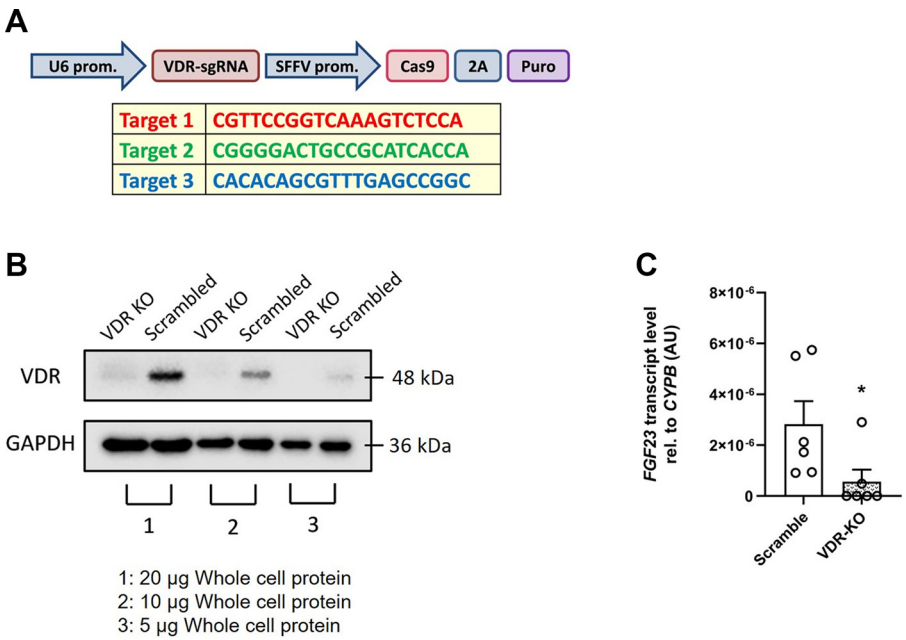

Figure 7. Dermal *FGF23* gene expression is largely dependent on VDR. Design of the human *VDR* sgRNA CRISPR construct (*top*) and the three target sequences (*bottom*) used for VDR knockout (*A*). Original Western blot showing representative VDR protein expression in HaCaT VDR KO cells (*B*). Scatter dot plot and arithmetic means ± SEM of relative (rel.) *FGF23* mRNA abundance normalized to *CYPB* in HaCaT VDR KO cells ($n = 6$) after 24 h of culturing (*C*). *$P < 0.05$: significant differences from the control (*B*: Mann–Whitney *U* test). AU, arbitrary units; Ctr, control; CYPB, cyclophilin B.

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human FGF23 protein and treated HaCaT cells with increasing concentrations for 24 h and subsequently analyzed *CYP* gene expression. Surprisingly, there was no difference in the amount of *CYP24A1* (Fig. 8*A*) or *CYP27B1* (Fig. 8*B*) mRNA in the FGF23-treated cells compared with the vehicle control. Similarly, the mRNA level of the 25-hydroxylating enzyme *CYP2R1* was unaffected after 24 h of treatment with recombinant FGF23 (Fig. 8*C*). In contrast and unexpected, we detected a significantly higher mRNA abundance of the gene *CYP27A1*, encoding a cholesterol- and vitamin D-hydroxylating enzyme, after treatment for 24 h with 100 ng/mL FGF23 protein compared with control (Fig. 8*D*). Notably, FGF23 treatment also resulted in a dose-dependent and significant increase in the mRNA abundance of another cholesterol- and vitamin D-metabolizing enzyme, *CYP11A1*, after 24 h in HaCaT cells compared with control (Fig. 8*E*), suggesting a potentially unknown new role of FGF23 in affecting sterol and secosteroid metabolism in human skin cells.

DISCUSSION

The FGF23 hormone, the negative regulator of 1,25(OH)$_2$D$_3$ synthesis and downstream target of 1,25(OH)$_2$D$_3$-VDR-signaling, is mainly produced and secreted in the body by bone cells (39). Here, we show at mRNA and protein levels that human skin and human epidermal and melanoma skin cells also express *FGF23* mRNA and produce and secrete FGF23 protein. Thus, FGF23 mRNA, cellular, and secreted proteins were detectable in intact skin, cells, and supernatants of primary HEK, HEM, and HDF cells as well as carcinoma HaCaT, SK Mel 188, A431 cells, and several melanoma skin cell lines. Consistent with our results, Fgf23 mRNA and cellular protein have been detected in the skin of mice and rats (45, 46). Our data show for the first time the capability of human skin cells to secrete FGF23 protein. Only wounding and uremia are presently known to regulate cutaneous FGF23 synthesis (45, 46), and a major regulator for FGF23 in bone is 1,25(OH)$_2$D$_3$ (58). Our study has identified vitamin D$_3$ metabolites as regulators of dermal FGF23 synthesis, with 1,25(OH)$_2$D$_3$ being the most effective of the hydroxyvitamin D$_3$ compounds we tested to increase FGF23 expression in HaCaT, A431, HDFn, and HEKn cells. Remarkably, CYP11A1-derived 20,23(OH)D$_3$ also increased *FGF23* expression in HaCaT and 20(OH)D$_3$ in HaCaT and A431, an effect not observed in UMR-106 osteoblast-like cells, the model used for the classical FGF23-producing bone tissue. Interestingly, the stimulatory effect of 1,20,23(OH)$_2$D$_3$ on *FGF23* mRNA abundance was much more pronounced in UMR-106 osteoblast-like cells than in human epidermal HaCaT or HDFn cells. 1,20,23(OH)$_3$D$_3$, similar to 1,25(OH)$_2$D$_3$, was also able to strongly increase the mRNA abundance of the catalytic

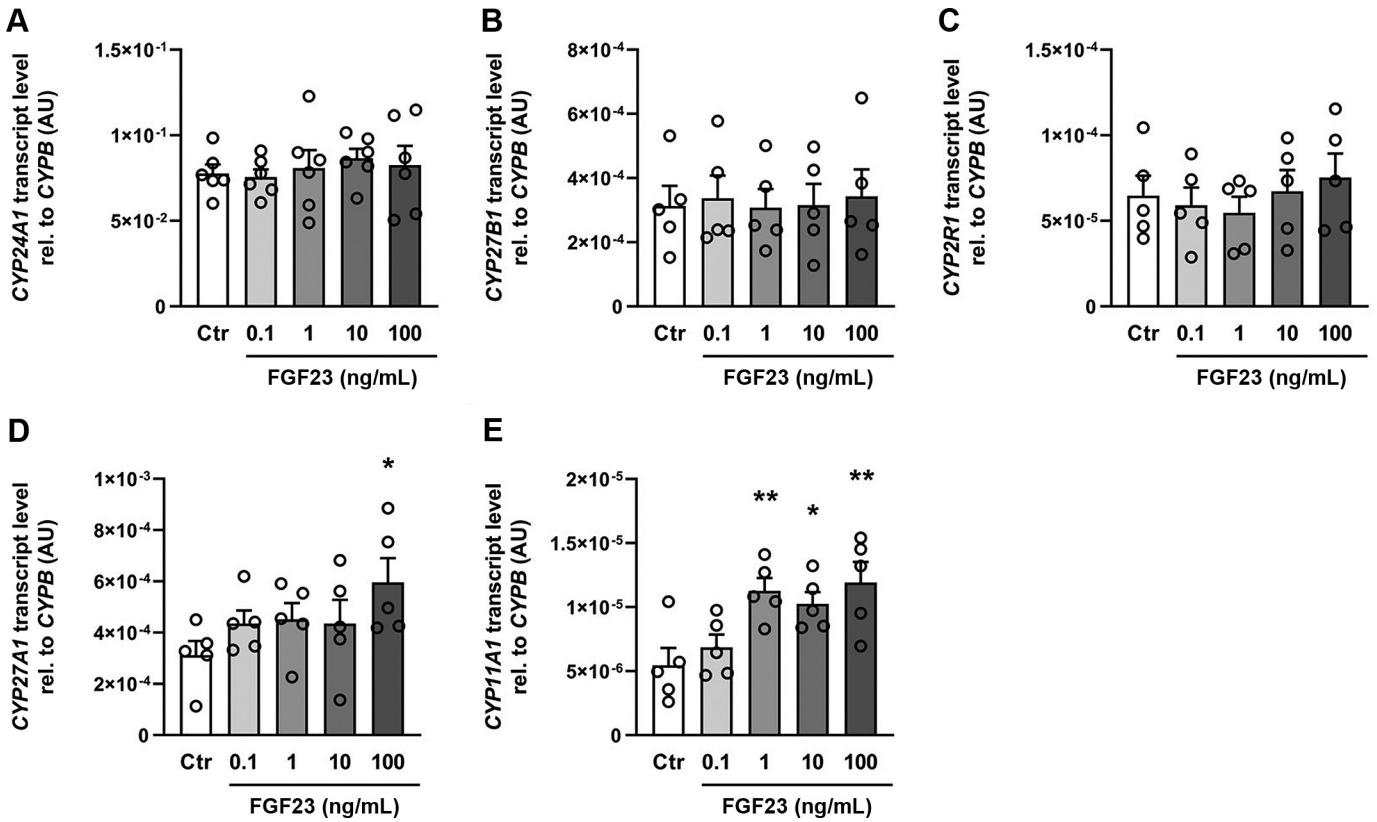

Figure 8. FGF23 increases mRNA abundance of sterol-metabolizing CYP enzymes *CYP27A1* and *CYP11A1* in HaCaT cells. Scatter dot plots and arithmetic means ± SEM of relative (rel.) *CYP24A1* (*A*), *CYP27B1* (*B*), *CYP2R1* (*C*), *CYP27A1* (*D*), or *CYP11A1* (*E*) mRNA abundance normalized to *CYPB* in HaCaT cells (*A*: $n = 6$; *B*–*E*: $n = 5$) treated with recombinant human FGF23 protein or vehicle for 24 h. *$P < 0.05$; **$P < 0.01$: significant differences from the control (*A*–*E*: one-way ANOVA with Dunnett's post hoc test). AU, arbitrary units; Ctr, control; CYPB, cyclophilin B; CYP24A1, cytochrome P450 24A1; CYP27B1, 25 hydroxyvitamin D3-1-alpha hydroxylase; CYP2R1, vitamin D 25-hydroxylase; CYP27A1, cytochrome P450 family 27 subfamily A member 1; CYP11A1, cytochrome P450 family 11 subfamily A member 1.

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enzyme for intact FGF23 secretion, *Galnt3*, in UMR-106 cells. This demonstrates that 1α-hydroxylation of the CYP11A1-derived vitamin D$_3$ metabolite 20,23(OH)$_2$D$_3$ to form 1,20,23(OH)$_3$D$_3$, increases its ability to regulate *Fgf23* gene expression and protein processing in bone cells. Although it is already known that GALNT3 is expressed by dermal fibroblasts (49), we were able to show that epidermal HaCaT keratinocytes also express mRNA for *GALNT3*, which is necessary for intact FGF23 hormone secretion. In addition, we found that its mRNA abundance, as in bone cells (59), is moderately stimulated by 1,25(OH)$_2$D$_3$. Thus, our data demonstrate that active vitamin D$_3$ hormone not only stimulates *FGF23* gene expression but also its cellular protein processing in skin cells, similar to that described in bone cells (58, 59). Furthermore, it could be speculated that 1,25(OH)$_2$D$_3$ influences FGF23 processing not only by regulating *GALNT3* but also by inhibiting the activity of furin, which interestingly is also expressed in skin cells (61) and cleaves intact FGF23 as a protease (62). Consistent with this, our data show for the first time that 1,25(OH)$_2$D$_3$ stimulates not only FGF23 protein secretion in A431 cells but, more importantly, the secretion of hormonally active intact FGF23 during 1,25(OH)$_2$D$_3$ treatment by HaCaT cells. In contrast to the observed induction of *FGF23* transcription by 1,25(OH)$_2$D$_3$ and the increased secretion of intact FGF23 protein in this study, the amount of C-terminal FGF23 in the supernatants of 1,25(OH)$_2$D$_3$-treated HaCaT cells was lower than in controls. Unlike conditions such as iron deficiency or inflammation, which link elevated FGF23 production to enhanced cleavage, 1,25(OH)$_2$D$_3$ seems to disrupt this connection, leading to an overall rise in intact FGF23 levels (62, 63). This is consistent with observations by us and others demonstrating reduced FGF23 cleavage and a vitamin D-mediated reduction in C-terminal FGF23 (62). However, the effect of 1,25(OH)$_2$D$_3$ on *GALNT3* gene expression and intact FGF23 secretion in HaCaT was not observed in the HDFn cells used. In addition, we also observed differences in secreted levels of C-terminal FGF23 between HDFn and HaCaT, with supernatants of 1,25(OH)$_2$D$_3$-treated HDFn cells having higher C-terminal FGF23 levels than control cells, in contrast to HaCaT cells. This indicates that 1,25(OH)$_2$D$_3$ not only affects FGF23 mRNA expression and protein synthesis but also post-transcriptional processing of FGF23 protein, which appears to be different between fibroblasts and keratinocytes, suggesting functional differences in FGF23 processing between skin cell types. Therefore, it could be speculated that keratinocytes may have stronger processing capacities for the GALNT3 and furin enzymes compared with fibroblasts. Alternatively, keratinocytes may respond differently to 1,25(OH)$_2$D$_3$-regulated processing of FGF23 compared with fibroblasts due to their different physiological functions, which may partially explain the differential secretion of C-terminal or intact FGF23 fractions between these cell types. However, these hypotheses require further studies. These differences between cell types in the secretion of C-terminal or intact FGF23 fractions into the circulation highlight the importance of routinely using both assays to study FGF23, especially in large clinical studies (63, 64). Interestingly, in contrast to the UVB-light-derived hydroxylated vitamin D$_3$ metabolites, we found no direct effect of UVB-light exposure on FGF23 secretion in primary HEKn. Thus, UVB-light only indirectly affects cutaneous FGF23 production through synthesis of the precursor vitamin D$_3$, which gives rise to the hydroxyvitamin D$_3$ metabolites, we tested. Interestingly, however, it has been reported that UVB-irradiation can induce gene expression of *FURIN* in HaCaT cells (61), which encodes the protease responsible for cleaving intact FGF23, suggesting a possible influence of UVB-light on FGF23 processing. Since the VDR is the top canonical signaling pathway for 1,25(OH)$_2$D$_3$ in keratinocytes (65) and also involved in osseous FGF23 expression (26), we hypothesized that the VDR might also be involved in FGF23 regulation in HaCaT. Consistent with this hypothesis, *FGF23* mRNA was largely reduced in the presence of VDR KO compared with scramble HaCaT, suggesting that VDR is an important regulator of *FGF23* expression in these cells. This is consistent with other previous data showing numerous VDR-response elements in the human *FGF23* gene capable of binding the VDR and participating in 1,25(OH)$_2$D$_3$-stimulated *FGF23* transcription (58). Since the non-1α-hydroxylated metabolites 20(OH)D$_3$ and 20,23(OH)$_2$D$_3$ were also able to regulate *FGF23* in HaCaT but not in UMR-106 cells, the incomplete abrogation of *FGF23* expression in the presence of VDR KO also suggests the involvement of other transcription factors and signaling pathways. 1,25(OH)$_2$D$_3$, and even more so the CYP11A1-derived metabolites 20(OH)D$_3$ and 20,23(OH)$_2$D$_3$, have been shown to act as agonists for AhR and LXR in keratinocytes and dermal fibroblasts (65, 66). Since the agonistic activation of these two transcription factors is associated with higher FGF23 expression (67, 68), it is tempting to speculate that 1,25(OH)$_2$D$_3$, 20(OH)D$_3$, and 20,23(OH)$_2$D$_3$ are also capable of stimulating *FGF23* expression in HaCaT in a VDR-independent manner by activating AhR and LXR. This hypothesis is further supported by the observed stimulating effect of 20(OH)D$_3$ and 20,23(OH)$_2$D$_3$ on the gene expression of *Cyp1a1* in UMR-106 cells, a classical downstream target gene of AhR (65). In line with previous data (69, 70), we found differences between the hydroxyvitamin D$_3$ metabolites tested, to regulate the mRNA abundance of the osteoblastic differentiation and mineralization genes studied in UMR-106, as well as partial differences of the non-1α-hydroxylated secosteroids to the effects of 1,25(OH)$_2$D$_3$ and 1,20,23(OH)$_3$D$_3$. Notably, 1,25(OH)$_2$D$_3$ and 1,20,23(OH)$_3$D$_3$ mediated similar effects by increasing mRNA abundance of *Cyp24a1*, *Spp1*, *Rankl*, *Phex*, and *Bglap* and decreasing mRNA abundance of *Runx2* in UMR-106 cells. This suggests that 1α-hydroxylation leads to the canonical signaling, which is most likely the VDR pathway, which is already known to transcriptionally regulate these genes (71, 72). Consistent with the hypotheses that non-1α-hydroxylated metabolites used in this study act more through VDR-independent pathways, we found opposite effects through inhibition of the expression of *Dmp1*, *Sost*, and *Ogn* genes by 20(OH)D$_3$, 20,23(OH)$_2$D$_3$, or 20,25(OH)$_2$D$_3$, as well as a stronger stimulation of *Cyp1a1* by these compounds compared to the 1α-hydroxylated metabolites tested, 1,25(OH)$_2$D$_3$ and 1,20,23(OH)$_3$D$_3$. Considering that CYP11A1-derived 20(OH)D$_3$ and 20,23(OH)$_2$D$_3$ have also been shown to act as inverse agonists for RORα and γ in skin cells (73), and since siRNA-mediated suppression of this transcription factor is associated with decreased *DMP1* expression in a human osteoblast model (74), it is tempting to speculate that the

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observed 20(OH)D$_3$-mediated inhibition of *Dmp1* expression in UMR-106 osteoblast-like cells is mediated by acting on RORs. Furthermore, 20(OH)D$_3$ and possibly its derivative 20,25(OH)$_2$D$_3$ or 20,23(OH)$_2$D$_3$ inhibit NFκB activity (75, 76), which is not only required for *Sost* expression in bone (77) but also for *Ogn* (78), leading to the speculation that the observed 20,25(OH)$_2$D$_3$-mediated decrease of *Sost* expression and 20(OH)D$_3$-, 20,23(OH)$_2$D$_3$-, and 20,25(OH)$_2$D$_3$-mediated reduction of *Ogn* expression in UMR-106 may be due to suppressed NFκB activity. The cutaneous secretion of hormonally active FGF23 raises the question whether this also leads to paracrine effects of the hormone on the surrounding skin cells since the necessary (co-)receptors for the induction of FGF23 signaling are expressed in the skin (45, 47, 50). Surprisingly, we found a strong and dose-dependent increase of the mRNA of cholesterol side-chain cleavage enzyme *CYP11A1* in HaCaT after treatment with FGF23, which, as far as we know, is an unknown target gene of FGF23. Interestingly, *Fgf23* KO mice have been shown to have lower levels of testosterone and androstenedione than wild-type mice (79), sex hormones whose precursor pregnenolone results from the cleavage of cholesterol by CYP11A1 (80). In addition, glucocorticoids, which also result from the metabolism of the CYP11A1 product pregnenolone, were shown to inhibit FGF23 production (52), suggesting a potential negative feedback mechanism following FGF23 upregulation of *CYP11A1* expression. In this context, it could be speculated that the FGF23-mediated transcriptional regulation of the catalytic enzyme *CYP11A1* shown here for the first time could influence the local cutaneous and extra-adrenal synthesis of steroidogenic compounds such as sex hormones or glucocorticoids and will therefore be the subject of our future investigations. This feedback could also influence the synthesis of CYP11A1-derived hydroxyvitamin D$_3$ compounds such as 20(OH)D$_3$ and 20,23(OH)$_2$D$_3$. Surprisingly, our data also reveal that FGF23 treatment results in the upregulation of *CYP27A1* gene transcription, which encodes another sterol-metabolizing CYP enzyme producing 27-hydroxycholesterol (27-HC), in epidermal HaCaT cells, which has not been described for other tissues (81). Interestingly, 27-HC plays an important role in promoting the proliferation of prostate cancer cells (82), which is also described for FGF23 (83), adding new data to the growing concept of a potential role of FGF23 in cancer development (44) through regulation of *CYP27A1* expression. Furthermore, CYP27A1 also acts as one of the vitamin D$_3$-25-hydroxylases and its upregulation may affect 1,25(OH)$_2$D$_3$ concentrations, at least at the local level.

Unexpectedly, our data did not show the expected classical effects of 1,25(OH)$_2$D$_3$-mediated feedback to induce FGF23 release causing inhibition of *CYP27B1* expression and stimulation of *CYP24A1* expression and hence downregulation of local 1,25(OH)$_2$D$_3$ levels as occurs in the kidney as the classical target organ of this cascade (41). We observed that the expression of these two enzymes remained unchanged by FGF23 treatment in epidermal HaCaT cells. Consistent with this, a previous study also found no altered FGF23-mediated expression of CYP24A1 in HaCaT (84), similar to findings in bone cells where FGF23 did not regulate *Cyp24a1* and *Cyp27b1* mRNA abundance (85). However, another study clearly showed an FGF23-mediated decrease in CYP27B1 and an increase in CYP24A1 protein after 24 h in HaCaT cells (47), suggesting the extra-renal potential of skin cells to locally regulate 1,25(OH)$_2$D$_3$ metabolism. This supports our findings of 1,25(OH)$_2$D$_3$-mediated dermal induction of FGF23 secretion as a negative-feedback regulator of CYP enzyme-mediated inhibition of local 1,25(OH)$_2$D$_3$ production and its degradation. Interestingly, Wu et al. (47) showed that the FGF23-mediated MAPK/ERK pathway is involved in the FGF23-dependent regulation of CYP27B1 and CYP24A1. Therefore, it is reasonable to speculate that the FGF23-dependent stimulation of *CYP11A1* observed in this study is also mediated by the induction of the MAPK/ERK cascade, as this pathway has been shown elsewhere to be involved in the transcriptional regulation of *CYP11A1* gene expression (86, 87). We must comment that Wu et al. (47) did not find FGF23 expression in keratinocytes and human epidermis by immunostaining in their study. However, immunocytochemistry is known for low sensitivity and specificity depending on primary and secondary antibodies, in comparison with analytical methods of protein or peptide detection. Also, the authors did not investigate secretion of the protein or its detection by other biochemical methods as listed in this study.

In line with previous data for other tissues (81), our data show that FGF23 does not alter dermal *CYP2R1* mRNA abundance. Given that FGF23 maintains phosphate balance in the body (40, 41) and that phosphate itself is a systemic regulator of FGF23 production (28), our data on cutaneous FGF23 production fit very well with the reports by others that phosphate induces GALNT3 expression in dermal fibroblasts (49), similar to bone cells (59). This suggests that the skin, through its ability to secrete hormonally active FGF23, may be able to register high plasma phosphate levels and, in support of or in addition to osseous FGF23 production, secrete FGF23 itself and thus potentially contribute to the homeostatic regulation of plasma phosphate. However, more clinical data correlating FGF23 and 1,25(OH)$_2$D$_3$ levels in skin samples are needed to validate our in vitro findings and assess their physiological relevance. Future studies could explore the effects of topically applied vitamin D$_3$ on dermal FGF23 production to confirm these in vitro results in vivo.

In conclusion, this study shows that human skin, as well as primary and carcinoma skin cells, express and secrete FGF23 and that 1,25(OH)$_2$D$_3$ acts as a regulator to induce dermal FGF23 synthesis and secretion. This is mediated, at least in part, via the VDR resulting in cutaneous secretion of bioactive FGF23 hormone, which can stimulate gene expression of the cholesterol- and vitamin D-metabolizing enzymes *CYP11A1* and *CYP27A1* in keratinocytes.

DATA AVAILABILITY

Data will be made available upon reasonable request.

SUPPLEMENTAL MATERIAL

Supplemental Figs. S1–S4: https://doi.org/10.6084/m9.figshare.28360373.

Supplemental Fig. S5: https://doi.org/10.6084/m9.figshare.28360469.v1.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

F.E. and A.T.S. conceived and designed research; F.E., Z.J., T.-K.K., A.A.M., A.A.B., S.R., A.F., P.B., and R.C.T. performed experiments; F.E., Z.J., T.-K.K., A.A.M., A.A.B., S.R., A.F., P.B., R.R.S., R.C.T., and A.T.S. analyzed data; F.E., Z.J., T.-K.K., and A.T.S. interpreted results of experiments; F.E. prepared figures; F.E., G.I.S., and A.T.S. drafted manuscript; F.E., G.I.S., R.C.T., and A.T.S. edited and revised manuscript; F.E., Z.J., T.-K.K., A.A.M., A.A.B., S.R., A.F., P.B., R.R.S., G.I.S., R.C.T., and A.T.S. approved final version of manuscript.

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