Published in final edited form as:

Bone. 2016 March ; 84: 120–130. doi:10.1016/j.bone.2015.12.055.

Posttranslational processing of FGF23 in osteocytes during the osteoblast to osteocyte transition

Hiroyuki Yamamoto^{1,3,@}, Bruno Ramos-Molina^{1,4,@}, Adam N. Lick¹, Matthew Prideaux², Valeria Albornoz¹, Lynda Bonewald², and Iris Lindberg^{1,*}

¹Department of Anatomy and Neurobiology, University of Maryland School of Medicine, Baltimore, MD 21201

²School of Dentistry, Department of Oral and Craniofacial Sciences, University of Missouri-Kansas City, Kansas City, MO 64108

Abstract

FGF23 is an O-glycosylated circulating peptide hormone with a critical role in phosphate homeostasis; it is inactivated by cellular proprotein convertases in a pre-release degradative pathway. We have here examined the metabolism of FGF23 in a model bone cell line, IDG-SW3, prior to and following differentiation, as well as in regulated secretory cells. Labeling experiments showed that the majority of ³⁵S-labeled FGF23 was cleaved to smaller fragments which were constitutively secreted by all cell types. Intact FGF23 was much more efficiently stored in differentiated than in undifferentiated IDG-SW3 cells. The prohormone convertase PC2 has recently been implicated in FGF23 degradation; however, FGF23 was not targeted to forskolin-stimulatable secretory vesicles in a regulated cell line, suggesting that it lacks a targeting signal to PC2-containing compartments. *In vitro*, PC1/3 and PC2, but not furin, efficiently cleaved glycosylated FGF23; surprisingly, PC5/6 accomplished a small amount of conversion. FGF23 has recently been shown to be phosphorylated by the kinase FAM20C, a process which was shown to reduce FGF23 glycosylation and promote its cleavage; our *in vitro* data, however, show that phosphorylation does not directly impact cleavage, as both PC5/6 and furin were able to efficiently cleave unglycosylated, phosphorylated FGF23. Using qPCR, we found that the expression of FGF23 and PC5/6, but not PC2 or furin, increased substantially following osteoblast to osteocyte differentiation. Western blotting confirmed the large increase in PC5/6 expression

*To whom correspondence should be addressed: Iris Lindberg, Ph.D., Department of Anatomy and Neurobiology, University of Maryland School of Medicine, 20 Penn St., HSF II, Room S251, Baltimore, MD 21201, USA, Tel: 410-706-4778, ilind001@umaryland.edu.

³Present address: Nihon Pharmaceutical University, Komuro, Saitama, Japan

⁴Present address: Department of Human Genetics, Catholic University of Leuven, Leuven, Belgium

@These two authors contributed equally to this manuscript

Disclosures: All authors state they have no conflict of interest.

Author's roles: Study concept and design, IL, LB, HY, AL, VA, BRM, MP; statistical analysis, MP; data interpretation and critical revision of the manuscript for important intellectual content, IL, BRM, AL, LB; writing of the report and approval of the final version, IL. IL takes responsibility for the integrity of the data analyses.

Disclosure Statement: The authors declare they have nothing to disclose.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

upon differentiation. FGF23 has been linked to a variety of bone disorders ranging from autosomal dominant hypophosphatemic rickets to chronic kidney disease. A better understanding of the biosynthetic pathway of this hormone may lead to new treatments for these diseases.

Keywords

FGF23; posttranslational processing; proprotein convertase; 7B2; furin; osteocyte; PC5/6; osteocyte; differentiation

1. Introduction

The circulating peptide hormone FGF23, a known regulator of bone mineralization and serum phosphate levels, was first described in brain [1] but is most highly expressed and released from immature and mature osteocytes within the bone matrix [2, 3]. While a great deal of information is available on the physiology of this important peptide hormone and its effects on the kidney, heart, and other organs [4], detailed information as to the biosynthesis and storage of osteocyte FGF23 is still lacking. Many studies (performed in HEK and undifferentiated osteoblast cell lines) have shown that the release of FGF23 as intact bioactive entity is controlled by intracellular proteolytic degradative processing (reviewed in [5]). Cleavage destroys the ability of FGF23 to act on the kidney FGFR1 receptor (reviewed in [6]), and the cellular cleavage process is closely controlled to ensure the release of appropriate amounts of intact FGF23. Human diseases in which intact circulating FGF23 is raised result in hypophosphatemia, while diseases in which intact FGF23 is lowered result in hyperphosphatemia (reviewed in [4, 5]). O-glycosylation of FGF23 by the enzyme GalNT3 is required for the secretion of intact protein by blocking its degradation [7–9]. More recently, FGF23 has been shown to be the target of phosphorylation by the new secretory kinase FAM20C [10, 11]; while phosphorylation and O-glycosylation represent competitive processes [10], the intracellular fate of phosphorylated FGF23 is not yet clear.

The degradative cleavage of FGF23 is thought to be accomplished by a member of the proprotein convertase family, since it is blocked by general convertase inhibitors such as decanoyl RVKR-chloromethyl ketone [12, 13], and known human mutations within the convertase cleavage site which remove the convertase consensus site decrease cellular degradative cleavage and enhance release of intact FGF23, resulting in severe bone disease [8, 14]; reviewed in [4]. The mammalian eukaryotic proprotein convertases (reviewed in [15, 16]) constitute a family of serine proteases usually associated with the biosynthesis of secreted proteins; the degradative cleavage of FGF23 is unusual in this regard. Precisely which convertases are involved in FGF23 cleavage is not yet clear, and recent work has implicated both furin [10] and the prohormone convertase PC2 (together with its obligate binding partner 7B2; [17]) in FGF23 degradation [18]. However, whereas furin activity is associated with the constitutive secretory pathway, both proPC2 maturation as well as PC2 enzymatic activity require the acidic environment present within the regulated secretory pathway (pH 5; reviewed in [19]). While regulated secretory granules within endocrine cells undergo acidification upon maturation, providing the appropriately acidic compartment, it is not clear whether osteocytes contain an analogous secretory compartment and/or whether

FGF23 itself contains granule sorting information which would direct it to such a compartment. We address this latter question in the study below.

A major problem in studying the osteocyte as an endocrine cell is the fact that osteocytes are quite difficult to access. A model system has been developed to study osteocyte differentiation in which cell proliferation is induced by a thermolabile SV40 large-T antigen regulated by γ -interferon [20]. When these cells, termed IDG-SW3, are cultured at 37°C in the absence of γ -interferon, T-antigen expression is dramatically reduced within 24h and the cells differentiate, faithfully reproducing primary osteoblast to osteocyte differentiation [20]. Differentiated IDG-SW3 cells produce and mineralize an extracellular matrix, and display many of the hallmark proteins of differentiated osteocytes, such as E11/gp38, Dmp1, Phex, Mepe, and sclerostin, in addition to increased FGF23.

In the report presented below, we have investigated the biochemistry and cell biology of FGF23 cleavage, and the potential contribution of various convertases as well as of the secretory chaperone 7B2, in both undifferentiated and differentiated osteocyte model cells. In order to compare osteocyte FGF23 processing to that occurring in known secretory processing systems containing PC2, FGF23 synthesis was also examined in an established regulated secretory model cell line, AtT-20/PC2 [21].

2. Materials and methods

2.1 Materials

The preparation of recombinant mouse PC1/3, PC2, and soluble human furin from Chinese hamster ovary cell-conditioned medium has been described previously [22–24]. The purity of these recombinant enzymes was estimated at greater than 95% using SDS-PAGE stained with Coomassie blue. Recombinant human PC5/6A, produced in S2 cells, was obtained from Robert Day, University of Sherbrooke [25], and soluble human PC7 (Leu38-Thr667, His-tagged at the amino terminus) was purchased from R&D Systems, Minneapolis, MN (2984-SE-010). The Flag-tagged human FGF23 vector and the GalNT3 vector were generous gifts of Shoji Ichikawa and Michael Econs, University of Indiana [8], while the pCAGEN vector used for subcloning human FGF23 was obtained from Joseph Stains (University of Maryland-Baltimore). The Flag-tagged vectors encoding FAM20C and its inactive D478A variant were obtained from Vincent Tagliabracci and Jack Dixon [26]. Recombinant bacterial hFGF23 was obtained from PeproTech (Rocky Hill, NJ), while the glycosylated hFGF23 (prepared via eukaryotic expression and lectin purification and C-terminally His-tagged) was obtained from R&D Systems. AtT-20 cells stably expressing mouse PC2 were obtained from Richard Mains, University of Connecticut Health Sciences Center [21].

2.2 Methods

In vitro proteolysis reactions using recombinant proprotein convertases—

Recombinant C-terminally His-tagged glycosylated human FGF23 (1 μ g of lectin-purified material, purchased from R&D Systems, Minneapolis, MN, 2604-FG-025/CF) was incubated with 5 units of either furin, PC1/3, PC2, PC5/6, or PC7 in 50 μ l reactions containing appropriate enzyme assay buffers (100 mM sodium acetate, 5 mM CaCl₂, 0.05%

Brij-35 pH 5.5 for PC1/3; 100 mM sodium acetate, 5 mM CaCl₂, 0.05% Brij-35 pH 5.0 for PC2; 100 mM HEPES, 5 mM CaCl₂, 0.05% Brij-35 pH 7.0 for furin; 20 mM bis-Tris, 1 mM CaCl₂, 0.1% Triton pH 6.5 for PC5/6; 50 mM Tris, 10 mM CaCl₂, 150 mM NaCl, 0.05% Brij-35 pH 7.5 for PC7) at 37°C for 100 minutes. The reactions were then subjected to SDS-PAGE using 15% Tris-HCl acrylamide gels. Cleavage patterns were analyzed by Western blotting using the R&D polyclonal FGF23 antibody (AF2604, 1:1000). One unit of PC activity is equal to the amount of the enzyme required to cleave 1 pmol/min of pRTKR-aminomethyl coumarin fluorogenic substrate (Peptides International, Louisville, KY).

In other experiments, 2 µg of recombinant human FGF23 lacking all posttranslational modifications (generated in bacteria) were incubated with either Flag-tagged FAM20C^{WT} or FAM20C^{D478A} immunopurified from transiently-transfected HEK cell medium using anti-Flag resin together with 5 µCi of γ-labeled ATP (10 Ci/mmol, Perkin Elmer) in kinase assay buffer (100 mM HEPES pH 7.0, 10 mM MnCl₂, 100 mM NaCl, 1 mg/ml BSA) at 30°C for 1 hour prior to cleavage with 200 ng recombinant human soluble furin or PC5/6 in its specific assay buffer (see above). Reactions were separated on 18% SDS-PAGE gels and analyzed both by Western blotting using the polyclonal R&D FGF23 antiserum (AF2604, 1:1000) as well as by phosphoimaging to assess transfer of radioactive phosphate. These Western blots were cross-linked in 3% glutaraldehyde in PBS for 30 minutes prior to blocking with milk in order to better retain the C-terminal peptide.

IDG-SW3 cell culture—The details of IDG-SW3 cell culture have already been described [20]. Tissue culture medium (α-MEM), fetal bovine serum, and recombinant mouse γ-interferon (IFN-γ, PMC4031) were purchased from Gibco Life Technologies (Grand Island, NY). Rat tail collagen type 1, 99% pure, was purchased from BD Biosciences (Bedford, MA, USA). Cells were expanded under growth conditions (33°C) in α-MEM with 10% heat-treated FBS and 50 U/mL of gamma interferon (IFN-γ) on rat tail type 1 collagen-coated flasks. To induce osteogenesis, confluent SW3-FGF23 cells were exposed to osteogenic conditions (37°C, in α-MEM medium containing 10% heat-treated FBS and 50 µg/mL of ascorbic acid and 4 mM β-glycerophosphate (Sigma, St. Louis, MO) but lacking IFN-γ; “osteogenic medium”). Both undifferentiated and differentiated cells were plated into 6-well collagen-coated plates for transfection, blotting, and radioimmunoassay experiments. Cells were differentiated to the desired time points, replacing osteogenic medium every 3–4 days.

Generation of an FGF23-expressing cell line (SW3-FGF23)—SW3 cells maintained under growth conditions were split from a confluent T-25 flask to three 10-cm collagen-coated dishes and two dishes were transfected with 10 µg each of N-terminal Flag-tagged human FGF23 [27] using Fugene HD overnight at 33°C. The medium was replaced twice a week with medium containing 250 µg/mL G418 until colonies began to form. Clones were screened for FGF23 secretion by Western blotting and the highest expressing clone chosen. However, expression of FGF23 was greatly diminished after two to three passages, indicating rapid loss of the transgene. For this reason, these cells were always additionally transiently transfected with FGF23 cDNA prior to experimentation. On some occasions, SW3-FGF23 cells were used for transient transfection with FGF23 cDNA. No differences

were detected between cells which were transiently transfected and SW3-FGF23 cells, supporting the rapid loss of transgene expression.

Transient transfection—As described above, in order to obtain increased expression of FGF23, we transiently transfected SW3 cells with a Flag-tagged FGF23 vector prior to labeling. The Flag-tagged insert from pcDNA3-FGF23 [8] was first subcloned into the pCAGEN vector at the *EcoRI* and *XhoI* sites. SW3 and SW3-FGF23 cells grown as described above were transfected with 2 µg per well of pCAGEN-FGF23 cDNA; transfections were also performed using 1 µg of FGF23 cDNA in the presence or absence of 1 µg of vectors encoding rat 27 kDa 7B2 [17] and/or murine proPC2 [28] using Fugene HD. Total cDNA was kept constant with the addition of empty pcDNA3.1 vector. Cells were incubated overnight at 33°C and radiolabelled with ³⁵S-methionine in methionine-free medium the next day (0.5 mCi/well) for 4 h, following a 1 h starvation period in DMEM or RPMI lacking methionine. Cells were then cultured in collection medium (Opti-MEM containing 1% aprotinin, or DMEM containing 1% dialyzed FBS) for 2.5 hours to collect secreted materials, then cells were extracted using radioimmunoprecipitation buffer containing protease inhibitors (Roche “Halt”). Following a pre-clear step with 100 µl of 20% protein A-Sepharose (or protein G for Flag immunoprecipitations), immunoprecipitation of cell lysate and culture media was carried out according to previously described procedures [28] using 4 µl of either R&D polyclonal antiserum or 5 µl of Sigma M2 Flag-tag antiserum. Antigen-antibody complexes were precipitated using either protein A- or protein G-Sepharose (the latter for Flag immunoprecipitates). Immunoprecipitates were electrophoresed on 15–18% gels, as indicated in figure legends; dried; and exposed to a phosphorimager screen for 1–3 days. Each labeling experiment was repeated at least twice.

AtT-20/PC2 cells, also in 6-well plates, were transfected with FGF23 cDNA the day before labeling; cells were starved in methionine-free medium for 20 min, labeled for 20 min, and then chased for successive 20 minute periods in OptiMem containing 100 µg/ml aprotinin with and without the secretagogue forskolin (10 µM; Roche). Cells were extracted with 500 µl of 5 N acetic acid containing 1 mg/ml BSA and the clarified, dried extracts were resuspended in radioimmunoprecipitation buffer for immunoprecipitation, as described above.

Immunofluorescence and confocal microscopy—Undifferentiated and differentiated FGF23-transfected SW3-FGF23 cells were grown in 24-well plates on collagen coverslips as described above. Cells were rinsed with PBS, treated with 4% paraformaldehyde for 10 min and incubated with blocking solution containing 3% bovine serum albumin (BSA) in 0.1 % Triton X-100 in PBS for 1h to permeabilize the cells and block non-specific protein-protein interactions. Cells were stained with two of the following primary antibodies in blocking solution overnight (4 °C). Antisera were then applied as follows: affinity-purified rat anti-mouse FGF23 (R&D Systems MAB26291, 1:100); M2 Flag antibody (Sigma F3165, 1:50); sheep anti-rat TGN38 (Serotec AHP499G, 1:100); affinity-purified rabbit polyclonal furin antibody (Affinity Bioreagents PA1-062, 1:100); affinity-purified sheep anti-human/mouse PC2 (R&D Systems AF6018, 1:100); and/or rabbit anti-human PC5/6 serum at 1:40 (R. Day, U. Sherbrooke). After washing, the cells

were incubated for 1 hour at room temperature with one or more of the following fluorescence-conjugated secondary antibodies at 1:800 to 1:1000: anti-rat IgG-Cy3, anti-rabbit IgG-Cy3, anti-sheep IgG-Cy5, or anti-rat IgG-Cy5 (Jackson ImmunoResearch, West Grove, PA) in blocking solution containing DAPI nuclear/DNA stain reagent (1:10,000, ALX-620-050, Axxora LLC, San Diego, CA). Cells were rinsed in PBS and mounted on object slides with Fluoromount G (Electron Microscopy Sciences, Hatfield, PA). Images were collected and analyzed on an Olympus BX61 confocal microscope (Olympus, Tokyo, Japan) using a 60× objective.

RNA extraction, reverse transcription and RT-PCR—IDG-SW3 were cultured for 0–28 days in the presence of 50 µg/ml ascorbic acid and 4 mM beta-glycerophosphate. RNA was harvested from triplicate cultures on days 0, 7, 14, 21 and 28 by scraping the cells in 1 ml Trizol (Ambion). RNA was isolated from the Trizol extract using chloroform and isopropanol extraction as described in the manufacturer's instructions, washed in 75% ethanol and resuspended in nuclease-free water. Purified RNA was treated with DNase I (DNA-free kit, Ambion) for 1 hour at 37°C to remove any genomic DNA. RNA concentrations were measured by spectrophotometry and the quality/purity was checked to ensure that the 260/280 absorbance ratio of all samples was between 1.9 and 2.0. Five µg of RNA were then reverse-transcribed into cDNA using a MultiScribe™ Reverse Transcriptase (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems) according to the manufacturer's instructions.

Real-time PCR was performed using a Step One Plus cycler (Applied Biosystems) with each reaction containing 50 ng cDNA template, 1 µl 20X Taqman Gene Expression Assay (Applied Biosystems), 10 µl 2X Taqman Gene Expression Master Mix (Applied Biosystems) and made up to a volume of 20 µl with nuclease-free water. The samples were cycled using the following conditions: 1 cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The Ct values for the samples were normalized to that of β-actin and the relative expression was calculated using the Ct method [29].

7B2 radioimmunoassay—SW3-FGF23 cells, grown in 6-well plates, were washed with Opti-MEM and incubated overnight with 1 ml of Opti-MEM containing 0.1 mg/ml aprotinin. After the conditioned media were collected, cells were scraped into 1 ml of ice-cold 0.1 N HCl and transferred to Eppendorf tubes on ice. Cells were frozen, thawed, and centrifuged at 4°C. The supernatant was then lyophilized and resuspended in 500 µl of RIA buffer (50 mM sodium phosphate, pH 7.6, 150 mM NaCl, 0.1 mg/ml heat-treated BSA and 0.02% NaN₃) and centrifuged before use. One hundred µl of cell extracts or conditioned medium were assayed in triplicate. The ¹²⁵I-7B2 peptide (residues 23-39) was prepared by the chloramine-T method; RIAs were carried out according to protocols and antiserum described previously [17]. Cross-reactivity with the native antigen, intact 21 kDa 7B2, is difficult to accurately determine due to the propensity of 7B2 to aggregate [30], which renders cross-reaction curves non-parallel.

3. Results

3.1 Convertase and 7B2 expression during osteocyte differentiation

In order to examine the expression of proteins related to FGF23 processing during differentiation, mRNA samples from SW3 cells were subjected to qPCR. The results are shown in Figure 1. **Panels A–E** depict the message levels of GalNT3, furin, 7B2, PC2, and PC5/6 as a function of differentiation time in weeks. Interestingly, the relative increase in PC5/6 during differentiation greatly exceeded that of these other convertases (note axis units). While we were not able to reproducibly obtain Western blots of either proPC2/PC2 or furin in either undifferentiated or in differentiated cells, suggesting low protein expression, PC5/6 expression was readily detectable by Western blotting in differentiated cells (**panels E and H**); the lower molecular weight species likely represent cleaved enzyme forms, which have been previously observed [31]. After an initial drop, 7B2 mRNA expression also increased as a function of differentiation time (**panel C**); radioimmunoassay confirmed increased synthesis of this protein (which was predominantly secreted) in differentiated vs. undifferentiated cells (**panel G**). Of note, we were unable to measure any PC2-specific enzyme activity [32] in either cell extracts or conditioned medium, using either differentiated or undifferentiated SW3-FGF23 cells (data not shown). **Panel F** shows the expected increase in FGF23 mRNA [20] with differentiation time, and Western blotting confirmed the presence of greatly increased intact FGF23 within 28-day differentiated SW3 cells, along with a lack of basal secretion (**panel I**). It should be noted that while PC5/6 expression increased most highly as a consequence of differentiation, furin was still the most highly expressed convertase message at each time point (cycle numbers are given in the figure legend).

3.2 Immunocytochemical localization of FGF23 and convertases

We investigated whether the proprotein convertases furin, PC2, and/or PC5/6 were specifically colocalized with FGF23 immunoreactivity in transfected SW3-FGF23 cells. Figure 2, **panel A**, shows transfected furin immunoreactivity and colocalization with FGF23 in a perinuclear compartment, likely the trans-Golgi network (TGN) (see below). Similar results were obtained for PC5/6 (**panel B**). In endocrine cells, a large proportion of cellular proPC2 is located within the endoplasmic reticulum (reviewed in [19]). In agreement, transfected PC2/proPC2 expression was observed both in the perinuclear area as well as throughout the cytoplasm (consistent with ER localization); colocalization with FGF23 only occurred in the perinuclear compartment (**panel C**). High resolution images of merged images from panels A–C are shown in **panel D**, while the identification of the FGF23-containing compartment as the TGN is shown in **panel E**. These data depict colocalization of FGF23 with all convertases studied in the same compartment, the TGN. Unfortunately, we were not able to image convertase localization in differentiated SW3 cells due to their low transfection efficiency and the contribution of the collagen matrix to background staining.

3.2 Cleavage of recombinant human FGF23 in vitro

While furin was the first proprotein convertase linked to the degradative cleavage of FGF23 [12, 33], the prohormone convertase PC2 has also been implicated in this cleavage event [18]. In order to identify convertases with the enzymatic capacity to cleave glycosylated FGF23, recombinant human glycosylated FGF23, tagged with a C-terminal His-tag (Figure 3, **panel A**) (prepared by R&D using a eukaryotic expression method), was incubated with various recombinant convertases, normalized to achieve similar activities against a synthetic fluorogenic substrate. Because this FGF23 preparation is purified by lectin chromatography, it contains only glycosylated forms; the three bands of 30–33 kDa FGF23 in the starting material can therefore be attributed to variable numbers of attached O-linked sugar chains. The reactions were subjected to Western blotting using an affinity-purified goat polyclonal antibody directed against the entire FGF23 protein (R&D); while this antiserum clearly recognizes both the N and the C-terminal portions of FGF23, the C-terminal portion of FGF23 was not detected in these experiments, and His-tag blotting confirmed that these C-terminal fragments were not retained on blots. Figure 3, **panel B** shows that recombinant PC1/3 and PC2 efficiently cleaved this glycosylated precursor while furin and PC7 were ineffective, most likely due to the presence of sterically-hindering O-linked sugars [9]. Interestingly, PC5/6 was also able to slightly cleave the glycosylated precursor, though very inefficiently as compared to PC1/3 and PC2.

FGF23 has recently been shown to be phosphorylated at Ser180 by the secretory kinase FAM20C [10] as well as at two other C-terminal serine FAM20C consensus sequences [11]. In order to determine whether phosphorylation impacts convertase cleavage, we incubated unglycosylated, bacterially-derived FGF23 (Figure 4, **panel A**) with immunopurified active FAM20C or with an inactive FAM20C mutant [26] prior to cleavage with furin or PC5/6 (Figure 4, **panels B and C**). As expected, wild-type but not mutant FAM20C was able to phosphorylate FGF23, as evidenced by ³²P-phosphate labeling (**panels C and D**). However, there was no difference in the extent of furin-mediated cleavage of FGF23 following phosphorylation (**panel C**). The pan-convertase inhibitor decanoyl-RVRR-chloromethyl ketone (CMK) was able to block both furin and PC5/6-mediated cleavage (**panel D**), confirming that cleavage resulted from convertase action. These results clearly show that both furin and PC5/6 are able to cleave both phosphorylated and unphosphorylated forms of FGF23; thus, phosphorylation *per se* neither impedes nor favors convertase cleavage of FGF23. (Within cells, phosphorylation and glycosylation represent competing reactions, and glycosylation –but not phosphorylation –blocks FGF23 cleavage [10].)

3.3 Cleavage of FGF23 in SW3 cells prior to and following differentiation

We next examined the posttranslational processing of radiolabeled FGF23 in undifferentiated and differentiated SW3 cells transiently expressing Flag-tagged FGF23. Because FGF23 was not abundantly synthesized, a long labeling time with ³⁵S-methionine was required; this was then followed by a 2.5h chase period in methionine-containing medium. Cells and medium were then collected and immunoprecipitated with the R&D polyclonal antiserum, which is directed against the entire recombinant protein. Figure 5, **panel A** shows that levels of intact 30 kDa cellular FGF23 are greatly increased in T28-differentiated FGF23-transfected SW3 cells; this increase appears to be mostly attributable to

increased storage of endogenously expressed FGF23, as a much weaker relative increase in cellular FGF23 was observed following differentiation using the Flag-tag antiserum, which detects only transfected FGF23 (*i.e.* not endogenously expressed FGF23) (Figure 5, **panel B**). The major anti-FGF23 immunoprecipitable bands in T0 cells were the intact FGF23 protein and two cleavage products of about 17 and 12–14 kDa. The major secreted product from T0 and T28 cells was the N-terminal 17 kDa peptide, indicating efficient cleavage of secreted FGF23 both time points. Exposure of cells to the secretagogue forskolin during the chase period did not result in increased secretion of FGF23 or products, in either undifferentiated or differentiated cells (data not shown). It is possible that secretagogues other than forskolin would be more effective in stimulation of FGF23 secretion from bone cells; however, at the present time, it is not clear what an effective bone secretagogue might be.

3.4 Effect of 7B2 expression on FGF23 expression and cleavage in SW3 cells

The small acidic protein 7B2 is an obligate cellular cofactor for the expression of PC2 activity [17]. Previous experiments carried out in transiently-transfected t-MOB cells provided evidence for the idea that cellular FGF23 can undergo a 7B2- and PC2-dependent cleavage event [18]. In the experiment shown in Figure 6, we attempted to replicate this finding in FGF23-transfected SW3 cells; however, our results did not confirm a PC2-dependent 7B2 effect on FGF23 cleavage. Figure 6, **panel A**, shows immunoprecipitation results using the M2 Flag antiserum in undifferentiated (T0) cells, and **panel B** depicts results with the same antiserum in differentiated (T28) cells. We detected increased expression of secreted 30 kDa FGF23 and the 17 kDa N-terminal fragment in the presence of co-transfected 7B2; this experiment was repeated with similar results. We conclude that 7B2 overexpression increases FGF23 expression or stability, which is manifested in the T0 cells as increased secretion of the N-terminal cleavage product, and in the T28 cells as increased secretion of intact FGF23. PC2 was not required for this effect.

3.5 Cleavage of FGF23 in AtT-20/PC2 cells

In order to compare the metabolism and storage of FGF23 in bone cells with its trafficking in known regulated secretory pathway-containing cells, Flag-tagged human FGF23 cDNAs were transiently transfected into AtT-20/PC2 cells, an anterior pituitary cell line that stably expresses PC1/3 and PC2 [21] which is frequently used as a model system for PC2-mediated cleavage [34]. Labeled FGF23-immunoreactive peptides were immunoprecipitated from cell and media extracts using the R&D polyclonal antiserum. Figure 7 shows that transfected wild-type FGF23 is efficiently cleaved in AtT-20 cells, with major products of 17 kDa (N-terminal fragment) and 12–14 kDa (C-terminal peptides). As in SW3 cells, the secretagogue forskolin was not able to increase the secretion of FGF23 or its immunoreactive products from AtT-20 cells, indicating its probable lack of storage in stimutable secretory granules. The endogenously expressed convertase PC1/3 was used as a positive control, and its secretion was efficiently forskolin-stimulated (not shown). Similar results were seen in FGF23-transfected α -TC6 cells. We conclude that PC2 does not contribute to FGF23 cleavage in AtT-20 cells, most likely because FGF23 is constitutively secreted rather than directed to, and stored within, PC2-containing secretory granules.

4. Discussion

The pre-release cleavage of cellular FGF23 is a regulatory mechanism in the secretion of this important bioactive factor from osteocytes, yet the biochemistry and cell biology of this degradative cleavage event is not yet clear. We have here investigated the cleavage of FGF23 in differentiated and undifferentiated osteocytes, and compared FGF23 processing in these cells to processing in commonly used models of constitutive and regulated secretion.

4.1 FGF23 processing in differentiated vs. undifferentiated SW3 cells

We first confirmed previously published results [12] that HEK cells transfected with FGF23 cDNA secreted an about-30 kDa precursor protein, a 17 kDa N-terminal product, and 12–14 kDa C-terminal products (data not shown). The present data show that similar products are observed in undifferentiated transfected SW3 cells. It is interesting to note that both HEK and undifferentiated SW3 cells secrete the vast majority of FGF23 cleavage fragments they synthesize, suggesting that a convertase-mediated cleavage event occurs either just prior to or during secretion in a poorly understood coupled proteolysis/secretion process. Similar cleavage timing occurs for other constitutively-trafficked precursors in other cell systems, for example pro-atrial natriuretic factor [35], proaugurin [36], and bone morphogenetic protein 4 (BMP4) [37].

In SW3 cells, proliferation is halted and differentiation is induced following removal of γ -interferon and placement in osteogenic medium [20]. Metabolic labeling of undifferentiated, FGF23- transfected SW3 cells shows that FGF23 was largely cleaved to a major secreted peptide of 17 kDa; lesser amounts of intact precursor and 12–14 kDa C-terminal products were present in cells. Supporting the qPCR results, a large increase in the amount of cellular FGF23 precursor is detected both in radiolabeling experiments and in Western blots (Figures 1 and 5) following differentiation; this result likely reflects highly increased storage of endogenous FGF23, known to be upregulated by differentiation [20]. The cellular compartment(s) in which this additional FGF23 is stored does not resemble typical regulated secretory granules (the only compartment in which PC2 cleavage is known to occur, due to the low pH requirement for activation of proPC2 [38]), as no stimulated release of FGF23 was obtained following the application of a general secretagogue. It may represent a TGN-associated compartment, or the TGN itself; imaging of differentiated cells- technically quite difficult due to interference from the collagen matrix - will be required to answer this question.

4.2 7B2 increases FGF23 expression, but not PC2-dependent cleavage

Previous experiments in t-Mob cells had shown that expression of the prohormone convertase PC2 together with its chaperone 7B2 increases FGF23 cleavage to smaller forms [18]. Using both SW3 cells and MC3T3 cells (not shown), we could not confirm 7B2-dependent, PC2-mediated cleavage of FGF23 to smaller forms, but instead found 7B2-mediated upregulation of FGF23 secretion of cleaved forms in undifferentiated cells and intact FGF23 in differentiated cells, an effect which was not PC2-dependent. Yuan *et al.* observed an increase in total FGF23 protein (precursor + cleaved forms) within t-Mob cells following co-transfection of 7B2, PC2 and FGF23 cDNAs, though not with 7B2 alone.

(Figure 1, panel A in [18] and associated erratum figure). Paradoxically, application of 7B2 RNAi also increased the secretion of intact FGF23, with reduced secretion of the C-terminal product (Figure 1, panel C in [18]). 7B2 overexpression may impact the trafficking, storage, or degradation of FGF23 and/or other secretory products. This phenomenon has previously been demonstrated for the secretory proteins chromogranins A [39–41] and B [42, 43], proteins which have been proposed to assist in granule formation and efficient hormone storage. While these two secretory granule proteins do not structurally resemble 7B2, all have been termed “granins” due to the presence of a specific acidic amino acid motif [44], and 7B2 has known secretory chaperone effects [45, 46]. However, in our radiolabeling experiments of 7B2- and FGF23-transfected SW3 cells, we did not detect increased cellular FGF23 storage. It is possible that higher levels of 7B2 expression must be achieved to observe these effects, or that a different bone cell system is required. Alternatively, 7B2 may exert its effect on FGF23 secretion by other means, such as protein stabilization.

4.2 Convertase-mediated cleavage of FGF23

While previous work [18] as well as our *in vitro* cleavage data seem to implicate PC2 as a potential FGF23-cleaving enzyme, as discussed above, PC2 requires a low pH compartment both for proenzyme activation as well as enzymatic activity. Whether bone-derived cells contain such a low pH compartment, and whether FGF23 would be targeted to such a compartment, is unclear. We examined the latter question using a standard neuroendocrine cell line which contains well-regulated, acidic-pH secretory granules and is commonly employed to examine prohormone processing and targeting: the neuroendocrine cell line AtT-20 [34]. AtT-20/PC2 cells produced similarly-sized FGF23-derived products as those detected in bone cell lines. We could obtain no evidence for storage of FGF23 or its cleavage products in regulated secretory granules, and did not detect secretagogue-mediated stimulated release. These experiments show that the cellular transport of FGF23 bypasses the regulated secretory pathway in which PC2 operates. We conclude that despite its efficient cleavage by PC2 *in vitro*, FGF23 does not possess an endogenous routing signal for conventional secretory granule storage- and thus will never naturally encounter PC2. A similar conclusion was reached for the constitutively-secreted peptide augurin [36].

The relative expression of the various proprotein convertases in osteocytes is controversial. Benet-Pages *et al.* found strong expression of only furin, PACE4 and PC7 in primary undifferentiated mouse osteoblasts with only slight expression of PC1/3 and PC2, and no expression of PC5/6 [12]. In agreement, we found strong expression of furin in undifferentiated cells, but also found strong relative expression of both furin and PC5/6 in differentiated cells, with negligible expression of PC2 at both time points. Our qPCR experiments showed that following differentiation, the expression of FGF23, PC5/6, PC2, and 7B2, but not of furin and GalNT3, increases; surprisingly, the expression of PC5/6 rather than furin message increased most radically following differentiation. Interestingly, only PC5/6- but not furin- could be detected in Western blots of either undifferentiated or differentiated cells. While Benet-Pages *et al.* failed to detect PC5/6 expression in mouse osteoblasts [12], two other groups have both identified PC5/6 message in bone osteocytes, and have shown reduced expression of this convertase in X-linked hypophosphatemia [47, 48], supporting our findings and further implicating PC5/6 in FGF23 cleavage.

While Bhattacharyya *et al.* [47] have demonstrated colocalization of FGF23 with furin in the Golgi of bone marrow stromal cells, colocalization of other convertases with FGF23 has not yet been performed in bone cells. Our confocal data demonstrate clear colocalization of FGF23 with furin, PC2, and PC5/6 in the TGN of SW3 cells. From these data, it is not possible to determine which convertase has the requisite localization to accomplish cellular FGF23 inactivation. However, it should be noted that PC2 would not be expected to be active in the TGN due to its strict requirement for an acidic compartment for activation and cleavage (reviewed in [19]). Convertase knockout studies using siRNA will be most informative in determining the relative convertase contributions to FGF23 inactivation, but our data showing large changes in convertase expression with differentiation indicate that differentiated cells must be examined in such studies. In this regard, it is interesting to mention that PC2 null mice do not show bone defects [49], while PC5/6 null mice are known to exhibit severe skeletal defects [50, 51]. (The global furin knockout has an embryonic lethal phenotype and thus cannot be examined for bone defects [52]). Crispr/Cas9-mediated knockout of furin in the osteoblast-like cell line U2OS abrogated FGF23 cleavage [10]; these data indicate a primary role for furin in FGF23 degradation in this undifferentiated cell line, but do not address the possible participation of other convertases in differentiated cells and bone osteocytes. Our finding that both furin and PC5/6 are able to efficiently cleave phosphorylated FGF23 is in accord with recent data showing furin cleavage of the short Ser180-phosphorylated peptide FGF23₁₇₂₋₁₉₀ [10], and indicate that the phosphate moiety neither impedes nor favors convertase cleavage.

In summary, our Western blotting, qPCR, immunoprecipitation, and confocal data all show robust PC5/6 expression in differentiated osteocytes and strongly implicate this convertase in the processing of osteocyte protein precursors. Since PC5/6 exhibits a substrate specificity quite similar to furin (reviewed in [53]), the possibility must be entertained that this convertase may also contribute to FGF23 cleavage within differentiated bone cells. PC5/6 has been shown to participate in the physiological processing of several different growth factors during developmental processes (including BMPs; [54]); PC5/6 is itself upregulated during embryo implantation and endometrial stromal cell decidualization [55] and is involved in collagen deposition [56]. Consistent with the idea that PC5/6 plays a role in bone protein maturation, PC5/6 knockout mice exhibit defects in ossification [50, 51], while knockouts of the PC5/6 inhibitor latent TGF β -binding protein 3 (LTBP3) [57] exhibit osteopetrosis [58].

5. Conclusions

We conclude that in undifferentiated bone cell lines FGF23 exhibits a degradative pathway very similar to that previously described in HEK cells. However, following bone cell differentiation, we observe great improvement in cellular storage of this peptide hormone. We show that FGF23 represents a constitutively-trafficked protein in bone and neuroendocrine cells, and is thus unlikely to naturally encounter the prohormone convertase PC2, as previously proposed. We confirm that the small secretory chaperone 7B2 increases FGF23 secretion, but show that this effect is not PC2-dependent. Lastly, we find that the proprotein convertase which undergoes the greatest amplification as a consequence of

differentiation is PC5/6, which efficiently cleaves phosphorylated FGF23. We speculate that PC5/6 may play an important role in precursor processing in differentiated osteocytes.

Acknowledgments

This work was partially supported by NIH grant DK49703-16 to I.L. and PO1AR046798 to L.B. We are grateful to J.P. Stains for advice and the gift of the pCAGEN vector; R. Day for recombinant PC5/6 protein and antiserum; S. Ichikawa and M. Econs for the gifts of FGF23-encoding and GalNT3 vectors; V. Tagliabracci and J. Dixon for FAM20C-encoding vectors; and N. Rajpurohit for performing the PC5/6 Western blot. We also thank K. Z. Li, H.W. Pang, and C.W. Patch for additional assistance with figure preparation.

ABBREVIATIONS

ER	endoplasmic reticulum
CMK	decanoyl RVRR-chloromethyl ketone
PC2	prohormone convertase 2
PC5/6	proprotein convertase 5/6
TGN	trans-Golgi network

Reference List

1. Yamashita T, Yoshioka M, Itoh N. Identification of a novel fibroblast growth factor, FGF-23, preferentially expressed in the ventrolateral thalamic nucleus of the brain. *Biochem Biophys Res Commun.* 2000; 277:494–8. [PubMed: 11032749]
2. Riminucci M, Collins MT, Fedarko NS, Cherman N, Corsi A, White KE, Waguespack S, Gupta A, Hannon T, Econs MJ, Bianco P, Gehron Robey P. FGF-23 in fibrous dysplasia of bone and its relationship to renal phosphate wasting. *J Clin Invest.* 2003; 112:683–92. [PubMed: 12952917]
3. Feng JQ, Ward LM, Liu S, Lu Y, Xie Y, Yuan B, Yu X, Rauch F, Davis SI, Zhang S, Rios H, Drezner MK, Quarles LD, Bonewald LF, White KE. Loss of DMP1 causes rickets and osteomalacia and identifies a role for osteocytes in mineral metabolism. *Nat Genet.* 2006; 38:1310–5. [PubMed: 17033621]
4. Liu S, Quarles LD. How fibroblast growth factor 23 works. *J Am Soc Nephrol.* 2007; 18:1637–47. [PubMed: 17494882]
5. Bhattacharyya N, Chong WH, Gafni RI, Collins MT. Fibroblast growth factor 23: state of the field and future directions. *Trends Endocrinol Metab.* 2012; 23:610–8. [PubMed: 22921867]
6. Gram Schjoldager KT, Vester-Christensen MB, Goth CK, Petersen TN, Brunak S, Bennett EP, Levery SB, Clausen H. A systematic study of site-specific GalNAc-type O-glycosylation modulating proprotein convertase processing. *J Biol Chem.* 2011; 286:40122–32. [PubMed: 21937429]
7. Bergwitz C, Banerjee S, Abu-Zahra H, Kaji H, Miyauchi A, Sugimoto T, Juppner H. Defective O-glycosylation due to a novel homozygous S129P mutation is associated with lack of fibroblast growth factor 23 secretion and tumoral calcinosis. *J Clin Endocrinol Metab.* 2009; 94:4267–74. [PubMed: 19837926]
8. Ichikawa S, Lyles KW, Econs MJ. A novel GALNT3 mutation in a pseudoautosomal dominant form of tumoral calcinosis: evidence that the disorder is autosomal recessive. *J Clin Endocrinol Metab.* 2005; 90:2420–3. [PubMed: 15687324]
9. Kato K, Jeanneau C, Tarp MA, Benet-Pages A, Lorenz-Depiereux B, Bennett EP, Mandel U, Strom TM, Clausen H. Polypeptide GalNAc-transferase T3 and familial tumoral calcinosis. Secretion of fibroblast growth factor 23 requires O-glycosylation. *J Biol Chem.* 2006; 281:18370–7. [PubMed: 16638743]

10. Tagliabracci VS, Engel JL, Wiley SE, Xiao J, Gonzalez DJ, Nidumanda Appaiah H, Koller A, Nizet V, White KE, Dixon JE. Dynamic regulation of FGF23 by Fam20C phosphorylation, GalNAc-T3 glycosylation, and furin proteolysis. *Proc Natl Acad Sci U S A*. 2014; 111:5520–5. [PubMed: 24706917]
11. Lindberg I, Pang HW, Stains JP, Clark D, Yang AJ, Bonewald L, Li KZ. FGF23 is endogenously phosphorylated in bone cells. *J Bone Miner Res*. 2014
12. Benet-Pages A, Lorenz-Depiereux B, Zischka H, White KE, Econs MJ, Strom TM. FGF23 is processed by proprotein convertases but not by PHEX. *Bone*. 2004; 35:455–62. [PubMed: 15268897]
13. Anglikar H. Synthesis of tight binding inhibitors and their action on the proprotein-processing enzyme furin. *J Med Chem*. 1995; 38:4014–4018. [PubMed: 7562936]
14. Larsson T, Davis SI, Garringer HJ, Mooney SD, Draman MS, Cullen MJ, White KE. Fibroblast growth factor-23 mutants causing familial tumoral calcinosis are differentially processed. *Endocrinology*. 2005; 146:3883–91. [PubMed: 15961556]
15. Zhou A, Webb G, Zhu X, Steiner DF. Proteolytic processing in the secretory pathway. *J Biol Chem*. 1999; 274:20745–20748. [PubMed: 10409610]
16. Seidah NG, Sadr MS, Chrtien M, Mbikay M. The multifaceted proprotein convertases: their unique, redundant, complementary, and opposite functions. *J Biol Chem*. 2013; 288:21473–81. [PubMed: 23775089]
17. Zhu X, Lindberg I. 7B2 facilitates the maturation of proPC2 in neuroendocrine cells and is required for the expression of enzymatic activity. *J Cell Biol*. 1995; 129:1641–50. [PubMed: 7790360]
18. Yuan B, Feng JQ, Bowman S, Liu Y, Blank RD, Lindberg I, Drezner MK. Hexa-D-arginine treatment increases 7B2*PC2 activity in hyp-mouse osteoblasts and rescues the HYP phenotype. *J Bone Miner Res*. 2013; 28:56–72. [PubMed: 22886699]
19. Hoshino, A.; Lindberg, I. *Peptide Biosynthesis: Prohormone Convertases 1/3 and 2*. Fricker, LD.; Devi, L., editors. Morgan & Claypool Life Sciences Publishers; 2012.
20. Woo SM, Rosser J, Dusevich V, Kalajzic I, Bonewald LF. Cell line IDG-SW3 replicates osteoblast-to-late-osteocyte differentiation in vitro and accelerates bone formation in vivo. *J Bone Miner Res*. 2011; 26:2634–46. [PubMed: 21735478]
21. Zhou A, Mains RE. Endoproteolytic processing of proopiomelanocortin and prohormone convertases 1 and 2 in neuroendocrine cells overexpressing prohormone convertases 1 or 2. *J Biol Chem*. 1994; 269:17440–7. [PubMed: 8021247]
22. Zhou Y, Lindberg I. Purification and characterization of the prohormone convertase PC1(PC3). *J Biol Chem*. 1993; 268:5615–23. [PubMed: 8449925]
23. Lamango NS, Zhu X, Lindberg I. Purification and enzymatic characterization of recombinant prohormone convertase 2: stabilization of activity by 21 kDa 7B2. *Arch Biochem Biophys*. 1996; 330:238–50. [PubMed: 8660652]
24. Kacprzak MM, Peinado JR, Than ME, Appel J, Henrich S, Lipkind G, Houghten RA, Bode W, Lindberg I. Inhibition of furin by polyarginine-containing peptides: nanomolar inhibition by nona-D-arginine. *J Biol Chem*. 2004; 279:36788–94. [PubMed: 15197180]
25. Fugere M, Limperis PC, Beaulieu-Audy V, Gagnon F, Lavigne P, Klarskov K, Leduc R, Day R. Inhibitory potency and specificity of subtilase-like pro-protein convertase (SPC) prodomains. *J Biol Chem*. 2002; 277:7648–56. [PubMed: 11723118]
26. Tagliabracci VS, Engel JL, Wen J, Wiley SE, Worby CA, Kinch LN, Xiao J, Grishin NV, Dixon JE. Secreted kinase phosphorylates extracellular proteins that regulate biomineralization. *Science*. 2012; 336:1150–3. [PubMed: 22582013]
27. White KE, Carn G, Lorenz-Depiereux B, Benet-Pages A, Strom TM, Econs MJ. Autosomal-dominant hypophosphatemic rickets (ADHR) mutations stabilize FGF-23. *Kidney Int*. 2001; 60:2079–86. [PubMed: 11737582]
28. Muller L, Zhu X, Lindberg I. Mechanism of the facilitation of PC2 maturation by 7B2: involvement in ProPC2 transport and activation but not folding. *J Cell Biol*. 1997; 139:625–38. [PubMed: 9348280]

29. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001; 25:402–8. [PubMed: 11846609]
30. Dasgupta I, Sanglas L, Enghild JJ, Lindberg I. The neuroendocrine protein 7B2 is intrinsically disordered. *Biochemistry*. 2012; 51:7456–64. [PubMed: 22947085]
31. De Bie I, Marcinkiewicz M, Malide D, Lazure C, Nakayama K, Bendayan M, Seidah NG. The isoforms of proprotein convertase PC5 are sorted to different subcellular compartments. *J Cell Biol*. 1996; 135:1261–75. [PubMed: 8947550]
32. Li QL, Naqvi S, Shen X, Liu YJ, Lindberg I, Friedman TC. Prohormone convertase 2 enzymatic activity and its regulation in neuro-endocrine cells and tissues. *Regul Pept*. 2003; 110:197–205. [PubMed: 12573800]
33. Liu S, Guo R, Simpson LG, Xiao ZS, Burnham CE, Quarles LD. Regulation of fibroblastic growth factor 23 expression but not degradation by PHEX. *J Biol Chem*. 2003; 278:37419–26. [PubMed: 12874285]
34. Zhou A, Bloomquist BT, Mains RE. The prohormone convertases PC1 and PC2 mediate distinct endoproteolytic cleavages in a strict temporal order during proopiomelanocortin biosynthetic processing. *J Biol Chem*. 1993; 268:1763–9. [PubMed: 8380577]
35. Sei CA, Hand GL, Murray SF, Glembotski CC. The cosecretional maturation of atrial natriuretic factor by primary atrial myocytes. *Mol Endocrinol*. 1992; 6:309–19. [PubMed: 1533896]
36. Ozawa A, Lick AN, Lindberg I. Processing of proaugin is required to suppress proliferation of tumor cell lines. *Mol Endocrinol*. 2011; 25:776–84. [PubMed: 21436262]
37. Tilak A, Nelsen SM, Kim H-S, Donley N, McKnite A, Lee H, Christian JL. Simultaneous rather than ordered cleavage of two sites within the BMP4 prodomain leads to loss of ligand in mice. *Development (Cambridge, England)*. 2014; 141:3062–3071.
38. Lamango NS, Apletalina E, Liu J, Lindberg I. The proteolytic maturation of prohormone convertase 2 (PC2) is a pH- driven process. *Arch Biochem Biophys*. 1999; 362:275–82. [PubMed: 9989936]
39. Kim T, Tao-Cheng JH, Eiden LE, Loh YP. Chromogranin A, an “on/off” switch controlling dense-core secretory granule biogenesis. *Cell*. 2001; 106:499–509. [PubMed: 11525735]
40. Jain RK, Joyce PB, Gorr SU. Aggregation chaperones enhance aggregation and storage of secretory proteins in endocrine cells. *J Biol Chem*. 2000; 275:27032–6. [PubMed: 10862758]
41. Fasciotto BH, Kuhn U, Cohn DV, Gorr SU. Secretory cargo composition affects polarized secretion in MDCK epithelial cells. *Mol Cell Biochem*. 2008; 310:67–75. [PubMed: 18049865]
42. Stettler H, Beuret N, Prescianotto-Baschong C, Fayard B, Taupenot L, Spiess M. Determinants for chromogranin A sorting into the regulated secretory pathway are also sufficient to generate granule-like structures in non-endocrine cells. *Biochem J*. 2009; 418:81–91. [PubMed: 18973469]
43. Beuret N, Stettler H, Renold A, Rutishauser J, Spiess M. Expression of regulated secretory proteins is sufficient to generate granule-like structures in constitutively secreting cells. *J Biol Chem*. 2004; 279:20242–9. [PubMed: 14996840]
44. Huttner WB, Gerdes HH, Rosa P. The granin (chromogranin/secretogranin) family. *Trends Biochem Sci*. 1991; 16:27–30. [PubMed: 2053134]
45. Helwig M, Hoshino A, Berridge C, Lee SN, Lorenzen N, Otzen DE, Eriksen JL, Lindberg I. The neuroendocrine protein 7B2 suppresses the aggregation of neurodegenerative disease-related proteins. *J Biol Chem*. 2013; 288:1114–24. [PubMed: 23172224]
46. Peinado JR, Sami F, Rajpurohit N, Lindberg I. Blockade of islet amyloid polypeptide fibrillation and cytotoxicity by the secretory chaperones 7B2 and proSAAS. *FEBS Lett*. 2013
47. Bhattacharyya N, Wiench M, Dumitrescu C, Connolly BM, Bugge TH, Patel HV, Gafni RI, Cherman N, Cho M, Hager GL, Collins MT. Mechanism of FGF23 processing in fibrous dysplasia. *J Bone Miner Res*. 2012; 27:1132–41. [PubMed: 22247037]
48. Liu S, Tang W, Fang J, Ren J, Li H, Xiao Z, Quarles LD. Novel regulators of Fgf23 expression and mineralization in Hyp bone. *Mol Endocrinol*. 2009; 23:1505–18. [PubMed: 19556340]
49. Furuta M, Carroll R, Martin S, Swift HH, Ravazzola M, Orci L, Steiner DF. Incomplete processing of proinsulin to insulin accompanied by elevation of Des-31,32 proinsulin intermediates in islets of mice lacking active PC2. *J Biol Chem*. 1998; 273:1–7. [PubMed: 9417035]

50. Szumska D, Pielek G, Essalmani R, Bilski M, Mesnard D, Kaur K, Franklyn A, El Omari K, Jefferis J, Bentham J, Taylor JM, Schneider JE, Arnold SJ, Johnson P, Tymowska-Lalanne Z, Stammers D, Clarke K, Neubauer S, Morris A, Brown SD, Shaw-Smith C, Cama A, Capra V, Ragoussis J, Constam D, Seidah NG, Prat A, Bhattacharya S. VACTERL/caudal regression/Currarino syndrome-like malformations in mice with mutation in the proprotein convertase Pcsk5. *Genes Dev.* 2008; 22:1465–77. [PubMed: 18519639]
51. Essalmani R, Zaid A, Marcinkiewicz J, Chamberland A, Pasquato A, Seidah NG, Prat A. In vivo functions of the proprotein convertase PC5/6 during mouse development: Gdf11 is a likely substrate. *Proc Natl Acad Sci U S A.* 2008; 105:5750–5. [PubMed: 18378898]
52. Roebroek AJ, Umans L, Pauli IG, Robertson EJ, van Leuven F, Van de Ven WJ, Constam DB. Failure of ventral closure and axial rotation in embryos lacking the proprotein convertase Furin. *Development.* 1998; 125:4863–4876. [PubMed: 9811571]
53. Seidah NG. What lies ahead for the proprotein convertases? *Ann N Y Acad Sci.* 2011; 1220:149–61. [PubMed: 21388412]
54. Cui Y, Jean F, Thomas G, Christian JL. BMP-4 is proteolytically activated by furin and/or PC6 during vertebrate embryonic development. *EMBO J.* 1998; 17:4735–43. [PubMed: 9707432]
55. Paule S, Aljofan M, Simon C, Rombauts LJ, Nie G. Cleavage of endometrial alpha-integrins into their functional forms is mediated by proprotein convertase 5/6. *Hum Reprod.* 2012; 27:2766–74. [PubMed: 22740495]
56. Marchesi C, Essalmani R, Lemarie CA, Leibovitz E, Ebrahimian T, Paradis P, Seidah NG, Schiffrin EL, Prat A. Inactivation of endothelial proprotein convertase 5/6 decreases collagen deposition in the cardiovascular system: role of fibroblast autophagy. *J Mol Med (Berl).* 2011; 89:1103–11. [PubMed: 21681612]
57. Sun X, Essalmani R, Susan-Resiga D, Prat A, Seidah NG. Latent transforming growth factor beta-binding proteins-2 and -3 inhibit the proprotein convertase 5/6A. *J Biol Chem.* 2011; 286:29063–73. [PubMed: 21700711]
58. Dabovic B, Levasseur R, Zambuto L, Chen Y, Karsenty G, Rifkin DB. Osteopetrosis-like phenotype in latent TGF-beta binding protein 3 deficient mice. *Bone.* 2005; 37:25–31. [PubMed: 15878314]
59. Ramos-Molina B, Lindberg I. Phosphorylation and alternative splicing of 7B2 reduce prohormone convertase 2 activation. *Mol Endocrinol.* 2015:me20141394.

Highlights

In undifferentiated bone cells FGF23 exhibits a degradative pathway similar to that previously described in HEK cells.

Following bone cell differentiation, cellular storage of FGF23 is enhanced.

FGF23 represents a constitutively-trafficked protein which does not encounter secretory granule PC2

The convertase which undergoes the greatest amplification during differentiation is PC5/6, which efficiently cleaves phosphorylated FGF23.

PC5/6 may play an important role in precursor processing in differentiated osteocytes.

FIGURE 1. Expression levels of PC2, 7B2, furin, PC5/6, GalNT3, and FGF23, prior to and following differentiation of SW3 cells

Panels A–F: mRNA expression, determined by qPCR, was normalized to 18S mRNA in differentiating SW3 cells for the proteins shown. As cells approach day 28 (“T28”), mRNA levels of PC2, FGF23 and PC5/6, but not GalNT3 or furin, increase significantly as compared to T0. Note the differences in the scale of the Y axes depicting the relative expression levels of each protein. **Panel G:** 7B2 protein levels increase with differentiation (radioimmunoassay). **Panel H:** PC5/6 protein expression increases greatly following differentiation (Western blot). **Panel I:** FGF23 protein expression increases following differentiation (Western blot of a single gel with irrelevant lanes removed). Data depict the mean + SE of four biological replicates. **Asterisks:** $p < 0.05$ as compared to T0 samples, as assessed using one-way ANOVA with a *post-hoc* Bonferroni correction. Ct values showed that furin (Ct 21) and GalNT3 (Ct 22) were the most highly expressed messages, with PC5 rising from a Ct of 34 to 29 over the course of differentiation. PC2 was the least abundant message (Ct=34).

FIGURE 2. Distribution of FGF23, PC2, furin and PC5/6 in transfected SW3 cells: colocalization in the trans-Golgi network

Panel A: Colocalization of FGF23 with furin. **Panel B:** Colocalization of FGF23 with PC5/6. **Panel C:** Colocalization of FGF23 with PC2. **Panel D:** High magnification image showing the merged images in **Panels A, B, and C**, respectively. **Panel E:** Colocalization of FGF23 immunoreactivity with the TGN marker TGN38. The bars indicate 20 μ m.

FIGURE 3. Glycosylated FGF23 is efficiently cleaved *in vitro* by PC1/3 and PC2, slightly cleaved by PC5/6, but is not cleaved by furin

Panel A: Schematic of cleavage of eukaryotic FGF23 recombinant protein (the His-tag is not drawn to scale). **Panel B:** Western blot of recombinant glycosylated human FGF23 showing intact FGF23 and N-terminal FGF23-immunoreactive products in cleavage reactions (R&D polyclonal antiserum); the unidentified 15 kDa band seen in all samples is present in the starting material and is thus not a reaction product. No lower molecular mass immunoreactive bands were detected using either FGF23 or His-tag antisera. Note that FGF23 was prepared by lectin chromatography; therefore all substrates and N-terminal products necessarily contain sugars.

FIGURE 4. Phosphorylated FGF23 is efficiently cleaved *in vitro* by both furin and PC5/6
Panel A: FGF23 schematic showing bacterial protein cleavage and known phosphorylation sites. (Note that although the C-terminal fragment has a molecular mass of only 7.5 kDa, it consistently migrates as a larger peptide [10–12]). **Panel B:** Cleavage of FAM20C-phosphorylated and mock-phosphorylated (FAM20C-D478A) recombinant FGF23 by furin (Western blot); **Panel C:** Phosphoimaging of a furin cleavage reaction of FAM20C-phosphorylated ³²P-labeled FGF23, confirming that the intact substrate and the C-terminal peptide product are phosphorylated; **Panel D:** Phosphoimaging of furin and PC5/6 cleavage reactions, showing the production of similar levels of C-terminal radiolabeled fragments in both reactions. The convertase inhibitor CMK (decanoyl-RVRR-chloromethyl ketone) was used to show that cleavages are convertase-mediated.

FIGURE 5. Differentiation of SW3 cells results in increased storage of intact FGF23

Panel A: ^{35}S -methionine labeling and immunoprecipitation of both endogenously-expressed and transfected FGF23 (R&D antiserum). **Panel B:** Immunoprecipitation of the same reactions using Flag-tag antiserum, showing increased storage of transfected FGF23. Cells were labeled for 4 h with radioactive methionine, and then chased for 2.5h in medium containing cold methionine. Both cells and media were then subjected to immunoprecipitation.

FIGURE 6. Transfection of 7B2 cDNA together with FGF23 cDNA into SW3 cells increases FGF23 secretion; lack of effect of PC2
Panel A: Metabolic labeling of undifferentiated SW3 cells following transfection of FGF23 together with PC2 and/or 7B2 cDNAs; immunoprecipitation was performed using Flag-tag antiserum. **Panel B:** Metabolic labeling of differentiated SW3 cells following transfection of FGF23, PC2 and/or 7B2 cDNAs; immunoprecipitation with Flag-tag antiserum.

FIGURE 7. Processing of transfected FGF23 in AtT-20/PC2 cells: lack of stimulated secretion

Cells were transfected with FGF23-encoding cDNAs, pulsed for 20 min in medium containing radioactive methionine, and then chased in methionine-containing medium for the indicated periods of time with and without the secretagogue forskolin. Immunoprecipitation was performed using the R&D polyclonal antiserum. FAM20C is not expressed by AtT-20 cells [59]; thus, the lower molecular weight C-terminal peptide likely represents a cleavage product rather than a differentially modified peptide.