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Adamts1 is highly induced in rachitic bones of FGF23 transgenic mice and participates in degradation of non-mineralized bone matrix collagen

Lijuan Hu [a], Göran Andersson [b], Kenneth B. Jonsson [c], Håkan Melhus [a], Thomas Lind [a,*]

[a] Department of Medical Sciences, Uppsala University, SE-75185 Uppsala, Sweden
[b] Division of Pathology, Department of Laboratory Medicine, Karolinska Institute, Karolinska University Hospital, SE-14152 Huddinge, Sweden
[c] Department of Surgical Sciences, Uppsala University, SE-75185 Uppsala, Sweden

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ABSTRACT

Transgenic mice overexpressing fibroblast growth factor 23 (FGF23) in osteoblasts have a rachitic bone phenotype. These mice display hypomineralized bones, increased expression of osteoblast markers, but osteoclast numbers are unaltered or slightly reduced. Paradoxically, they show increased serum levels of the bone resorption marker CTX, a type I collagen degradation fragment. Here we analyzed a matrix metalloproteinase- (MMP-) like secreted protease, Adamts1, that has previously been associated with osteoblastic type I collagen breakdown *in vitro*. Bones from FGF23 transgenic (tg) mice displayed increased Adamts1 protein upon both immunohistological staining and Western blotting. We further found Adamts1 protein together with excessively degraded type I collagen in the non-mineralized bone fraction of FGF23 tg mice. A similar degradation pattern of type I collagen was noticed upon forced expression of Adamts1 in osteoblastic cells *in vitro*. Importantly, these Adamts1-expressing osteoblastic cells exhibited increased release of CTX fragments when cultured on demineralized bone discs. Together, these results demonstrate for the first time that Adamts1 can be highly induced in bone tissue and that this MMP-like protease can increase osteoblastic release of CTX fragments from non-mineralized bone. Thus, Adamts1 potentially contributes to the increased serum levels of CTX in rickets/osteomalacia.

1. Introduction

Fibroblast growth factor 23 (FGF23) is a hormone that plays an important role in mineral ion homeostasis. The major functions of FGF23 are to inhibit renal phosphate reabsorption and suppress circulating $1,25(OH)_2D$ levels. FGF23 is mainly secreted by osteoblasts/osteocytes and serum levels are elevated in oncogenic osteomalacia, X-linked hypophosphatemia, chronic kidney disease and fibrous dysplasia. Transgenic overexpression of FGF23 in mice induces phenotypic changes similar to those of patients with increased FGF23 levels [1,2]. These mice have reduced bone mineral density, but they display increased trabecular bone volume together with increased expression of markers for the bone building osteoblasts. In contrast, bone resorbing osteoclast number/volume appears unchanged or slightly decreased. FGF23 transgenic (tg) mice thus exhibit a rachitic/osteomalacia bone phenotype, hypophosphatemia and reduced $1,25(OH)_2D_3$ levels. In addition, they also have secondary hyperparathyroidism.

Adamts1 is a secreted matrix metalloproteinase with thrombospondin motifs first described as highly induced in inflammation [3]. It appears to be expressed in most tissues although at a lower level in normal adult tissue [3–6]. Generally, the multifunctional Adamts1 participates in tissue remodeling, wound healing and angiogenesis by cleaving itself, membrane bound growth factors and extracellular proteins. In rat bone Adamts1 has been shown to be induced by osteotropic agents in osteoblasts but not osteoclasts [7,8]. Moreover, global gene expression analyses of bone biopsies from human primary hyperparathyroidism patients have shown that parathyroidectomy reverses disease parameters and reduces Adamts1 expression in bone [9]. In line with this, we have demonstrated that the bone phenotype of mild Adamts1 overexpression in mice mimics the human bone phenotype of primary hyperparathyroidism [10]. We have further shown that the Adamts1 protein locates to collagen fibrils in bone tissue and provides osteoblastic cells with increased ability to degrade type I collagen [11].

Our aim with this study was to explore if osteoblastic Adamts1 may contribute to the paradoxal increased type I collagen degradation, in the form of CTX fragments, observed in the FGF23 tg mice. Thus, we analyzed Adamts1 expression by quantitative real time-PCR, Western blotting and immunohistochemistry in the rachitic bones of FGF23 tg animals. We analyzed α1(I) collagen protein present in FGF23 tg bone extracts. In addition, we have also performed cell-based assays culturing osteoblastic cells, forced to

* Corresponding author. Address: Department of Medical Sciences, Section of Clinical Pharmacology, Uppsala University, Entrance 70 3rd floor, Lab 22, SE-75185 Uppsala, Sweden. Fax: +46 18 553601.
E-mail addresses: lijuan.hu@ki.se (L. Hu), goran.andersson@ki.se (G. Andersson), kenneth.jonsson@surgsci.uu.se (K.B. Jonsson), hakan.melhus@medsci.uu.se (H. Melhus), thomas.lind@medsci.uu.se (T. Lind).

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express Adamts1, on bone discs to study its effect on the generation CTX fragments from bone matrix.

2. Materials and methods

2.1. Animals

The generation, breading and maintenance of transgenic mice overexpressing human fibroblast growth factor-23 (FGF23), under the control of a 2.3-kb fragment of the mouse α1(I) collagen promoter, in bone have been previously described [2]. The genotypes were confirmed by measuring serum levels of FGF23 using an ELISA (Immunotopics, San Clemente, CA). Long bones, i.e. femora and tibiae, as well as serum from wt and FGF23 tg mice of mixed genders were collected at the age of 5 weeks. The project was approved by the Local Animal Ethical Committee in Uppsala (approval number C211/3).

2.2. Total RNA purification and quantitative real-time PCR

After homogenization of long bones with a Polytron homogenizer, RNAwas extracted using RNeasy® Lipid Tissue Mini Kit (QIAGEN, Hilden, Germany) and treated with 3U DNase I (Invitrogen, Carlsbad, CA). Quantification was carried out using RiboGreen (Molecular Probes, Eugene, OR, USA). Total RNA (400 ng) was transcribed to cDNA using the TaqMan system (Applied Biosystems). Quantitative PCR was performed using inventoried TaqMan Gene Expression Assays for Adamts1 (Mm00477355), Ext1 (Mm00468769), cathepsin L (Mm00515597) and PPARγ (Mm00440945) according to the manufacturer's protocol, on a TaqMan 7000 apparatus. Cycling protocol was as follows: 50 °C for 2 min followed by 95 °C for 10 min and then 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min. Expression levels were divided by β-actin (Mm00607939) levels for standardization. $n = 4$ in each group.

2.3. Immunohistochemistry

The metaphyses of distal femora were used and the immunohistochemistry has previously been described in detail [12]. The bones from all animals were sectioned in the same orientation in order to make comparable sections. For protein localization, sections were incubated with a rabbit anti-mouse Adamts1 (1:400, sc-5468, Santa Cruz, CA, USA). Visualization was achieved by incubation with secondary biotinylated antibody at a dilution of 1:200 in 10% serum and phosphate-buffered saline followed by an avidin–biotin–peroxidase complex incubation using the VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA, USA) and the substrate diaminobenzidine tetrahydrochloride (DAB) (Dako, Glostrup, Denmark).

2.4. Bone protein extraction

The bone protein was extracted into a non-mineralized (Gua) and mineralized (EDTA) fraction essentially as described before [13]. The extraction solution contained a proteinase inhibitor cocktail (P8340, Sigma–Aldrich, St. Louis, MO, USA) with inhibitors of serine-, cysteine-, aspartic- and amino-peptidases. The EDTA extract was clarified by centrifugation at 10,000×g for 40 min. The supernatant of the extract was concentrated and transferred into 7 M urea, 0.1 M sodium acetate, 10 mM Tris/HCl buffer, pH 6.0, at 4 °C by ultrafiltration (PM-10 filter; Amicon Corp., Easton, TX, USA). Protein concentration was determined using BCA protein reagent (Pierce, Rockford, IL, USA) with bovine serum albumin as standard.

2.5. CTX release experiment

Saos-2 human osteosarcoma cells were forced to express full-length wild-type human ADAMTS1 (Ats) in mammalian expression vector pcDNA3/V5-His (Invitrogen) or were transfected with empty vector (mock). Stable expression of these constructs was generated by transfection of Saos-2 cells using FuGENE 6 (Roche) followed by antibiotic selection (500 μg/ml Zeocin, Invitrogen) according to the manufacturer's instructions. Surviving cells were pooled and maintained in a medium containing 25 μg/ml Zeocin. Cells were maintained in α-minimal essential medium containing 10% fetal bovine serum and 100 units/ml penicillin/streptomycin. To generate de-mineralized bone, bone slices (Immunodiagnostic Systems Nordic, Denmark) were treated in 0.5 M EDTA for 7 days as described [14]. For the CTX release experiment, 5000 cells/bone slice in a 96-well plate were cultured for 24 h, with or without a cysteine proteinase inhibitor (10 μM, E-64, Sigma–Aldrich) or the matrix metalloproteinase (MMP) inhibitor (1 μM, GM6001, Merck) before supernatants were collected. C-terminal telopeptide of collagen I (CTX) concentrations in the supernatants were determined using CrossLaps for Cellculture ELISA (Nordic Bioscience Diagnostics, Herlev, Denmark) according to the manufacturer's protocol. Data represents three samples/group and is presented as mean ± SD. $n = 3$ per treatment.

2.6. Immunoblotting

Ten micrograms of extracted bone protein or Saos-2 cell lysate was precipitated with acetone, mixed with equal volumes of reduced sample buffer (Bio-Rad, Hercules, CA, USA) and separated on a 4–15% gradient SDS–PAGE mini-gel (Bio-Rad). The separated proteins were transferred to a nitrocellulose membrane using a wet transfer, according to the Bio-Rad manufacturer's manual. After transfer, membranes were incubated in Tris-buffered saline, pH 7.5 and 4% (w/v) non-fat milk, with an antibody recognizing the metalloproteinase domain of Adamts1 (11) or antisera LF-67 for the collagen pro-α1(I) C-telopeptide [15], with shaking overnight at 4 °C. After washing in Tris-buffered saline, pH 7.5, followed by incubation with HRP conjugated secondary antibody (Dako) diluted 1:20,000 in the same buffers as for respective primary antibody, the membranes were then processed for chemiluminescence with ECL reagents (GE Healthcare, Buckinghamshire, UK).

2.7. Gelatin zymography

Forty-eight hours serum-free supernatants, containing 50 μg/ml heparin (H4784, Sigma–Aldrich) were collected from Saos-2 cells expressing either ADAMTS1 or empty vector. The gelatin zymogram separation and detection was performed as described previously [16].

2.8. Statistical analyses

Excel software (Microsoft) was used for all statistical analyses. The data was evaluated by t-test. In every case, tests were two-tailed and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Adamts1 immunohistochemistry in FGF23 transgenic bone tissue

FGF23 tg animals exhibit disorganized growth plates, especially affecting the hypertrophic zone. Immunostaining of FGF23 tg bones showed Adamts1 staining in hypertrophic chondrocytes, whereas in wt animals the chondrocytes were negative for

Adamts1 (Fig. 1A). In trabecular bone tissue of wt mice, Adamts1 stained lining and marrow cells whereas cells embedded in osteoid/bone were not labeled (Fig. 1B). In contrast, trabecular bone matrix from FGF23 tg mice showed clear Adamts1 staining together with some Adamts1 positive osteoid/bone embedded cells. Diaphyseal cortical bone matrix and osteocytes were Adamts1 negative in wt animals (Fig. 1C). However, in diaphyseal cortical bone from FGF23 tg animals apparent staining was observed in the bone matrix and in osteocytes mainly restricted to the endosteal side along side staining around large cavities (Fig. 1C).

3.2. Adamts1 expression in FGF23 tg bone tissue

Quantitative real time-PCR (RT-qPCR) analysis of the bone tissue showed approximately a 3-fold increase of *Adamts1* transcript in FGF23 tg bones compared to wt bones (Fig. 2A). Western blot analysis detected Adamts1 positive fragments (37 kDa) clearly only in bone tissue extracts of FGF23 tg mice and only in the fraction of proteins associated with non-mineralized bone (Gua) (Fig. 2B). Quantification of Adamts1 protein from the osteoid extract revealed almost a 250-fold excess in FGF23 tg bone tissue (Fig. 2C).

3.3. Collagen type I in FGF23 tg bone tissue

Western blot analysis of the Adamts1 positive, non-mineralized bone matrix extracts from FGF23 tg mice demonstrated reduced amounts of high molecular weight, partially processed precursor forms (140–210 kDa) of collagen type I α1 chains, compared to wt mice (Fig. 3A). Similarly, forced expression of ADAMTS1 in Saos-2 cells (Ats), induced a reduction in the amounts of incompletely processed collagen type I α1 chains (Fig. 3A). Mock transfected Saos-2 cells does not produce detectable ADAMTS1 levels whereas Ats cells show presence of the expected molecular sizes of ADAMTS1, 115 kDa for full-length, 90 kDa for the pro-domain processed form and 65 kDa for the pro-domain and C-terminal processed mature form. Notably, addition of heparin, which is known to inhibit ADAMTS1 autocatalytic activity, not only preserves the larger forms of ADAMTS1, but also induces accumulation of smaller ADAMTS1 fragments around 37 kDa, similar to what is found *in vivo*. Then we tested if ADAMTS1 from Saos-2 cells could function in gelatin zymography, a commonly used method to assay for MMP activity. We found that serum-free conditioned media from Ats cells have two distinct clear zones of collagen type I gelatinolytic activity corresponding to the molecular size of mature ADAMTS1 (90 kDa) and MMP2 (68 kDa) (Fig. 3B). In contrast, conditioned media from empty vector transfected (mock) cells displayed only the 68 kDa gelatinolytic activity (Fig. 3B). Next we tested if ADAMTS1 could contribute to release of CTX fragments from bone tissue. Thus Ats cells or mock cells were grown on bone discs. ADAMTS1 expressing Ats cells released more CTX fragments from demineralized bone discs compared to mock cells (Fig. 3B). Adding inhibitors to either cysteine proteases (E64) or MMPs, including ADAMTS1, (GM, GM6001) reduced CTX release from Ats cells to a similar extent (Fig. 3C). CTX release by ADAMTS1 was inefficient on intact mineralized bone discs (data not shown).

3.4. Transcript levels of genes associated with Adamts1

To begin to try to understand why high amounts of the unstable autocatalytic Adamts1 protein are present in FGF23 tg bones we analyzed a gene necessary for the synthesis of heparan sulfate, *Exostosin 1* (*Ext1*). The transcript level of *Ext1* was increased in FGF23 tg bones (Fig 4A). We also analyzed the expression level of

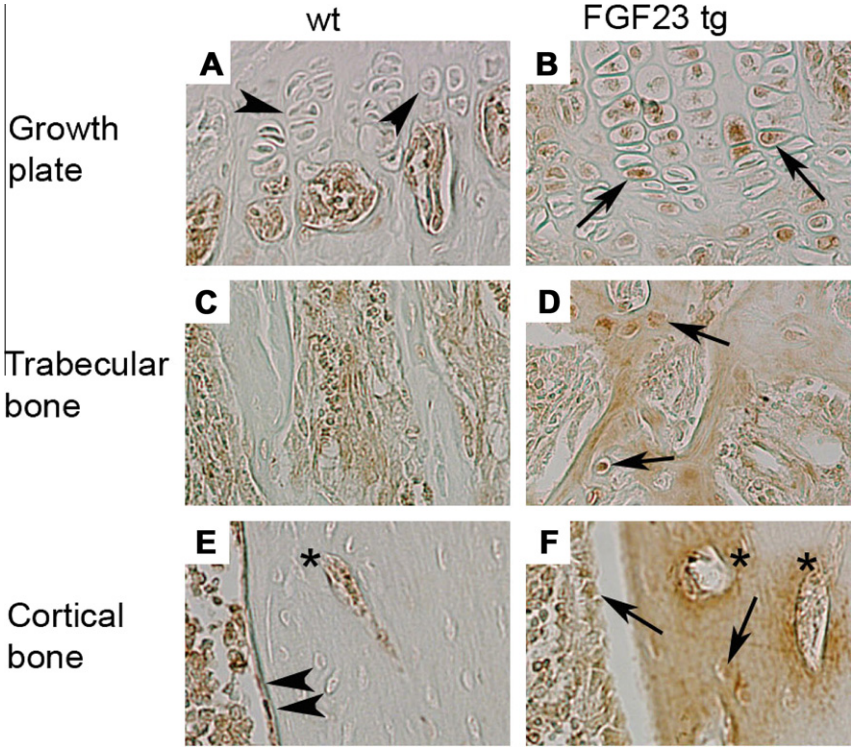

Fig. 1. Adamts1 immunohistochemical staining of paraffin-embedded bone sections from wt and FGF23 tg mice. Arrowheads show hypertrophic chondrocytes negative for Adamts1 in wt bone (A) and arrows show Adamts1 positive hypertrophic chondrocytes in FGF23 tg bone (B). Bone lining and marrow cells are Adamts1 positive, whereas trabecular bone is Adamts1 negative in wt bone (C). FGF23 tg trabecular bone shows staining for Adamts1. Arrows indicate Adamts1 positive hypertrophic chondrocyte-like cells embedded in bone matrix (D). wt cortical bone is negative for Adamts1, but bone lining cells are positive (arrowhead, E). FGF23 tg cortical bone matrix, lining cells (arrow) and bone matrix close to vascular lacunas (*) are Adamts1 positive (F).

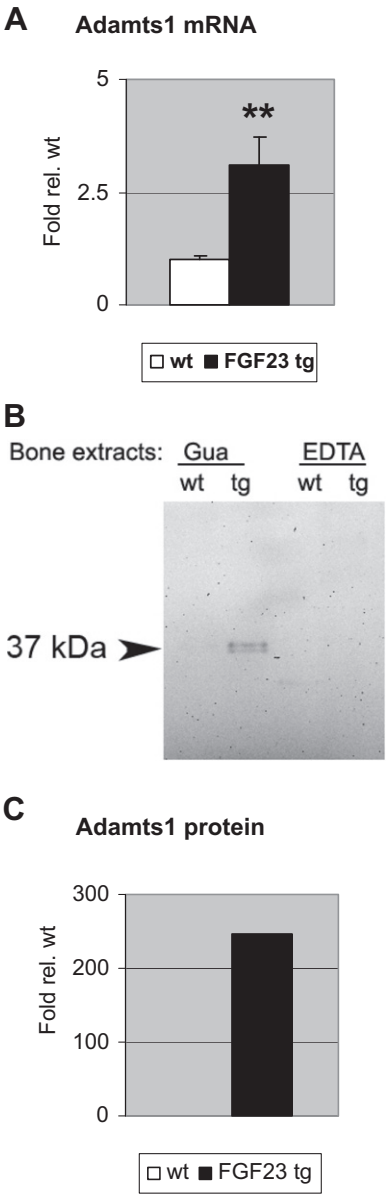

Fig. 2. Analysis of bone Adamts1 RNA and protein expression. (A) RT-qPCR analysis of bone tissue RNA from wt and FGF23 tg mice (wt is set to 1). (B) Western blot of non-mineralized bone (Gua) and mineralized bone (EDTA) extracted bone proteins (10 μg/lane) from wild type (wt) and FGF23 tg (tg) animals probed with an antibody recognizing the metalloproteinase domain of Adamts1. (C) Quantification of the Adamts1 Western blot in (B). $^{**}p < 0.01$.

cathepsin L, a PTH-inducible collagenolytic cysteine protease not unlike the most efficient generator of CTX fragments, cathepsin K. RT-qPCR analysis showed that *cathepsin L* was slightly overexpressed in FGF23 tg bones (Fig. 4A). As an unrelated control we also disclosed that the adipocyte marker gene *PPARγ*, was not altered by FGF23 tg overexpression in bone.

4. Discussion

We here show that the secreted metalloproteinase Adamts1 is highly induced in bones of rachitic FGF23 tg mice. Moreover, Adamts1 increased the ability of osteoblastic cells to release CTX fragments from demineralized bone discs. Thus, our data support the notion that osteoblastic Adamts1 is a contributing factor to the increased type I collagen degradation and suggests that it is a significant factor contributing to the bone phenotype of the rachitic FGF23 tg mice.

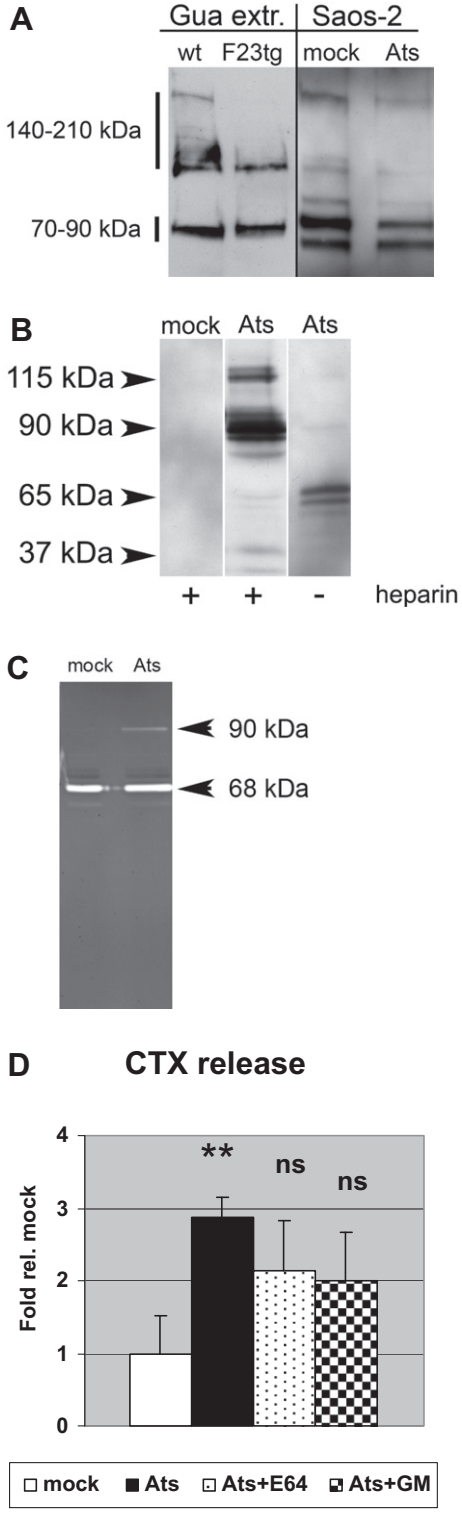

Fig. 3. Type I collagen degradation. (A) Western blot analysis of type I collagen from non-mineralized bone tissue extracts (Gua extr.) of wt or FGF23 tg mice and from Saos-2 cells (Saos-2) either forced to express ADAMTS1 (Ats) or empty vector transfected (mock). (B) Western analysis, showing ADAMTS1, of conditioned media from Ats cells or mock cells with or without heparin addition. (C) Gelatin type I zymogram of Saos-2 cell supernatants from Ats cells or mock cells. (D) CTX release from mock cells or Ats cells cultured on demineralized bone slices. CTX generation was also measured in the presence of either a cysteine proteinase inhibitor, 10 μM E64 (Ats + E64) or a matrix metalloproteinase inhibitor, 1 μM GM6001 (Ats + GM). $^{**}p < 0.01$, ns = not significant from mock or Ats.

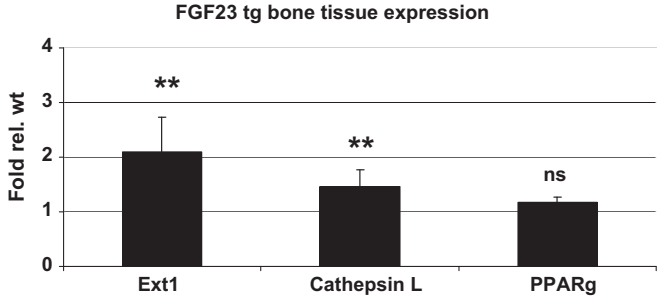

Fig. 4. Adamts1 associated gene analysis. RT-qPCR analysis of bone tissue RNA from wt and FGF23 tg mice. $^{**}p < 0.01$.

For the first time we show that the Adamts1 protein can be induced in bone tissue *in vivo*. Previously Adamts1 transcripts were found to be induced by osteotropic hormones in osteoblasts but not in osteoclasts [7]. Here we find that the Adamts1 protein is present in cells lining trabecular and endosteal bone in wt mice, similarly to observations in normal rat bone [17,18]. However, in FGF23 tg mice Adamts1 is additionally found in hypertrophic chondrocytes, in trabecular bone matrix, in the bone matrix around vascular lacunas and in osteocytes. Adamts1 has been described to be expressed in hypertrophic chondrocytes and although Adamts1 knock-out mice are smaller, they have no growth plate- or bone phenotypes [19,20]. Thus it appears that Adamts1 is redundant during normal growth plate formation. This is in agreement with observations that show that Adamts1 is expressed at low levels in normal adult tissues [3–6]. However, Adamts1 was discovered as an inflammation-associated gene [3] and we now show that it can be induced in bone and may contribute to pathological bone remodeling. In line with this, mild overexpression of Adamts1 in mice has been shown to induce a bone phenotype mimicking hyperparathyroidism [10]. These mice did not show increased serum CTX levels. We believe this may be due to a combination of the mild Adamts1 overexpression and the smaller amount of non-mineralized bone matrix in these mice.

In earlier observations of direct bone resorption by non-osteoclastic cells, it has been noticed that proteases derived from osteoblasts can be involved in pathological bone degradation [21]. We found here that Adamts1 appeared in the non-mineralized rachitic bone matrix together with increased type I collagen degradation. Notably the increased collagen degradation found in the FGF23 tg appeared similar to the increased collagen degradation found in osteoblasts overexpressing Adamts1, suggesting that excess Adamst1 in FGF23 tg bone might be involved in the type I collagen degradation. In conformity with increased collagen type I degradation in FGF23 tg bone tissue, it was previously shown that serum CTX levels are increased in FGF23 tg animals [22]. Osteoclastic cathepsin K has been shown to be the most efficient generator of CTX fragments. Interestingly, although the FGF23 tg mice show increased serum CTX, their bone cathepsin K expression and osteoclast number was not increased compared to controls [22]. This indicates that other proteases might be involved in the increased CTX generation. Along these lines, mice lacking cathepsin K had increased serum levels of CTX compared to controls and they had increased expression of cathepsin L, MMP9, MMP13 and MMP14 [23]. Cathepsin L is a collagenolytic cysteine protease which is similar to cathepsin K and has been shown to be co-expressed with Adamts1 during ovulation [24]. In addition, cathepsin L has been shown to be inducible by PTH in calvarial organ cultures [25] and has been shown to be expressed in osteoblast and hypertrophic chondrocytes during fracture healing [26]. Here we find that cathepsin L transcripts are significantly increased in FGF23 tg bones. We were unable to detect cathepsin L protein in either FGF23 tg or control bone extracts. However we clearly observed cathepsin L in Adamts1 expressing Saos-2 cells when cathepsin K expression was undetectable (data not shown). The Saos-2 cells, forced to express Adamts1, increase release of CTX fragments from demineralized bone discs *in vitro*. This is in line with our previous observation that osteoblastic Adamts1 generated several small collagen type I fragments [11]. The CTX generating activity could be inhibited equally efficiently by MMP- and cysteine protease inhibitors. It is known that MMPs can be activated by certain cysteine proteinases [27] and that cathepsin L functions together with MMP activity in calvarial bone resorption [28]. In accordance with cooperation's between proteases, Adamts1 has also been shown to synergize with MMP1 in the osteolytic signaling cascade for bone metastasis [29]. The metalloproteinase domain of Adamts1 is similar to metalloproteinase domains of MMPs, and Adamts1 has been shown, here and before, to function in gelatin zymography, a commonly used method to detect MMP activity [16]. In addition, Adamts1 can be inhibited by MMP inhibitors like Timps and GM6001. Thus, it will be interesting to further investigate the relationship between Adamts1, cathepsin L and MMPs in type I collagen degradation, CTX formation and bone remodeling.

Adamts1 is a secreted protease that binds to heparan sulfate in the extracellular matrix and protects it from degradation [30,31]. In its purified form, Adamts1 is autocatalytic and is degraded to several distinct active fragments down to 27 kDa [16]. Here we found that the size of Adamts1 extracted from bone tissue was mainly around 37 kDa. This is in agreement with the size of Adamts1 extracted from bones of an Adamts1 tg mouse [10]. Heparan sulfate is synthesized by the gene products of *Ext1* and *Ext2*, which when mutated cause exostosis [32]. Here we find that *Ext1* expression is increased in FGF23 tg bones suggesting that there may be increased production of heparan sulfate in FGF23 tg bone. Along these lines, preliminary results from our laboratory show that PTH treatment of osteoblastic cells increases their heparan sulfate content. Thus, an explanation to the high concentration of the Adamts1 protein in the FGF23 tg bone might, in part, be due to excess heparan sulfate which protect Adamts1 from autocatalytic degradation.

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