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Parathyroid hormone induces expression and proteolytic processing of Rankl in primary murine osteoblasts

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ABSTRACT

Rankl, the major pro-osteoclastogenic cytokine, is synthesized as a transmembrane protein that can be cleaved by specific endopeptidases to release a soluble form (sRankl). We have previously reported that interleukin-33 (IL-33) induces expression of *Tnfsf11*, the Rankl-encoding gene, in primary osteoblasts, but we failed to detect sRankl in the medium. Since we also found that PTH treatment caused sRankl release in a similar experimental setting, we directly compared the influence of the two molecules. Here we show that treatment of primary murine osteoblasts with PTH causes sRankl release into the medium, whereas IL-33 only induces *Tnfsf11* expression. This difference was not explainable by alternative splicing or by PTH-specific induction of endopeptidases previously shown to facilitate Rankl processing. Since sRankl release after PTH administration was blocked in the presence a broad-spectrum matrix metalloprotease inhibitor, we applied genome-wide expression analyses to identify transcriptional targets of PTH in osteoblasts. We thereby confirmed some of the effects of PTH established in other systems, but additionally identified few PTH-induced genes encoding metalloproteases. By comparing expression of these genes following administration of IL-33, PTH and various other *Tnfsf11*-inducing molecules, we observed that PTH was the only molecule simultaneously inducing sRankl release and *Adamts1* expression. The functional relevance of the putative influence of PTH on Rankl processing was further confirmed *in vivo*, as we found that daily injection of PTH into wildtype mice did not only increase bone formation, but also osteoclastogenesis and sRankl concentrations in the serum. Taken together, our findings demonstrate that transcriptional effects on *Tnfsf11* expression do not generally trigger sRankl release and that PTH has a unique activity to promote the proteolytic processing of Rankl.

1. Introduction

One of the most important regulatory systems controlling bone remodeling involves receptor activator of nuclear factor κB (Rank), its ligand (Rankl) and the Rankl decoy receptor osteoprotegerin (Opg) [1]. More specifically, Rankl expressed by cells of the osteoblast lineage is known to activate osteoclastogenesis from hematopoietic progenitors after binding to Rank in a Traf6-dependent manner [2]. The physiological relevance of the Rankl-Rank interaction is probably best underscored by the fact that mutations in either gene causes osteoclast-poor osteopetrosis in humans [3]. Likewise, the therapeutic relevance of the system is highlighted by the fact that Denosumab, a monoclonal antibody neutralizing human RANKl, has been successfully introduced as an *anti*-resorptive drug for the treatment of individuals with osteoporosis or osteolytic bone destruction [4]. Molecularly, the Rankl-Rank-Opg-system has been extensively studied through a combination of mouse genetics and tissue culture experiments. Interestingly however, one aspect of Rankl biology is still not fully understood, as Rankl is synthesized as a type II transmembrane protein that can be proteolytically processed to generate a soluble form (sRankl) [5]. In fact, since the majority of published studies either analyzed expression of the Rankl-encoding gene (*i.e. Tnsfsf11*) or the extracellular presence of soluble Rankl (sRankl), the question about its proteolytical processing was not generally addressed. Moreover, since the previously identified

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cleavage sites for putative Rankl sheddases (Adam10, Adam17 or Mmp14) are all located within a region of the murine Rankl protein that is not conserved in human RANKl (Fig. S1), it is at least debatable, if the findings obtained by forced expression studies are physiologically relevant [6–8].

Our interest in this particular question was triggered by a previous study, where we analyzed the molecular effects of the cytokine IL-33 on bone remodeling. More specifically we found that IL-33 acts as a potent inhibitor of osteoclastogenesis, both *in vitro* and *in vivo* [9,10]. The physiological relevance of this finding was supported by the analysis of mice lacking the IL-33 receptor, which displayed increased osteoclast indices, assessed by cellular histomorphometry on bone sections [9]. In the context of this study we additionally analyzed the influence of IL-33 on primary osteoblasts. Whereas chronic IL-33 administration during the course of differentiation did not interfere with matrix mineralization, a short-term treatment of primary osteoblasts with IL-33 induced the expression of several genes potentially affecting bone remodeling, including *Tnfsf11*. Interestingly however, and potentially explaining the absence of a pro-osteoclastogenic effect of IL-33, we failed to detect sRankl in the medium of osteoblasts after treatment with IL-33. In contrast, in the context of analyzing a mouse model with mucolipidosis-II, we found that sRankl was detectable in the medium of primary osteoblasts following treatment with PTH for 6 h [11]. This apparent inconsistency led us to directly compare the effects of IL-33 and PTH on *Tnfsf11* expression and sRankl release.

Here we report that PTH and IL-33 induce *Tnfsf11* expression to a similar extent, whereas sRankl release into the medium is specifically induced by PTH. Since this unexpected observation suggested that understanding this remarkable difference could lead to the identification of Rankl-specific endopeptidases, we performed additional experiments, including genome-wide expression analysis of primary osteoblasts after short-term treatment with PTH. Most importantly however, by analyzing the response of primary osteoblasts towards several *Tnfsf11*-inducing molecules, we identified a unique property of PTH, which was the only molecule causing sRankl release after short-term administration. Taken together, our data provide novel insights into the regulation of bone remodeling by PTH and the ectodomain shedding of Rankl.

2. Materials and methods

2.1. Primary murine osteoblasts

Primary osteoblasts were obtained from the calvariae of 5 days old male C57BL/6 wildtype mice. The key finding regarding the differential effect of IL-33 and PTH on Rankl processing was also confirmed in cell cultures obtained from female mice (Fig. S2). Cells were isolated by sequential digestion with collagenase/dispase and plated in α-MEM (Sigma-Aldrich) including 10% fetal bovine serum at a density of 130 cells per mm^2. After three days the cells were cultured in medium supplemented with ascorbic acid (50 μg/ml, Sigma-Aldrich) and β-glycerophosphate (10 mM, Sigma-Aldrich) for 10 days. Treatment with IL-33 (100 ng/ml, R&D Systems), hPTH [1–34] (10 nM, Bachem Inc.), IL-1β (100 ng/ml, Peprotech Inc.), IL-6 (100 ng/ml, Peprotech Inc.), soluble IL-6 receptor (100 ng/ml, R&D Systems), IL-17 (10 ng/ml, Peprotech Inc.), 1,25$(OH)_2$-vitamin-D3 (10 nM, Sigma-Aldrich), TNFα (10 ng/ml, Peprotech Inc.), IFN-γ (100 IU/ml, Peprotech Inc.), all-trans retinoic acid (1 μM, Sigma-Aldrich), prostaglandin-E2 (10 nM, Sigma Aldrich) or isoproterenol (10 μM, Sigma Aldrich) was performed in serum-free medium for 6 h after serum starvation of the cultures over night. To demonstrate that the release of sRankl induced by PTH was dependent on a metalloprotease activity, we added marimastat (Sigma-Aldrich) at a final concentration of 10 μM together with PTH. While RNA was isolated for expression analysis, the medium was collected for sRankl detection.

2.2. Human osteosarcoma cell lines

Human osteosarcoma cell lines (SaOS-2 and U2-OS) were obtained from ATCC (#HTB-85 and #HTB-96). The cells were plated at a density of 210 cells per mm^2 and differentiated for 5 or 10 days using commercially available osteoblast mineralization medium (PromoCell #C27020). Treatment with hPTH [1–34] was performed in serum-free medium for 6 h after serum starvation of the cultures over night. While RNA was isolated for expression analysis, the medium was collected for sRANKL detection by ELISA.

2.3. Expression analysis

RNA was isolated using the RNeasyMini kit (Qiagen), and DNase digestion was performed according to manufacturer's instructions. Concentration and quality of RNA were measured using a NanoDrop ND-1000 system (NanoDrop Technology). For qRT-PCR expression analysis, 1 μg of RNA was reversed transcribed using Verso cDNA Synthesis Kit (Thermo Fisher Scientific) according to manufacturer's instructions. The quantitative expression analysis was performed using a StepOnePlus system and predesigned TaqMan gene expression assays (Applied Biosystems). *Gapdh* expression was used as an internal control. Relative quantification was performed according to the $\Delta\Delta C_T$ method, and results were expressed in the linear form using the formula $2^{-\Delta\Delta CT}$. For genome-wide expression analysis, 5 μg of RNA were used for first strand cDNA synthesis. Synthesis of biotinylated cRNA was carried out using the IVT Labeling Kit (Affymetrix). For Gene Chip hybridization, the fragmented cRNA was incubated in hybridization solution at 45 °C for 16 h, before the Gene Chips (Affymetrix MG 430 2.0) were washed using the Affymetrix Fluidics Station 450. Microarrays were scanned with the Affymetrix Gene Chip Scanner 7G, and the signals were processed using GCOS (Affymetrix). To compare samples, the trimmed mean signal of each array was scaled to a target intensity of 300. Absolute and comparison analysis was performed using the Affymetrix MAS algorithm. Annotations were further analyzed with interactive query analysis at www.affymetrix.com.

2.4. sRankl detection

To quantify medium or serum concentrations of sRankl we utilized commercially available ELISA systems (R&D Systems, #MTR00 and #DY626 for mouse and human Rankl, respectively) following the manufacturer's instructions. For Western Blotting 10 μl medium or 20 μg of cell lysate was subjected to SDS-PAGE and then transferred to PVDF membranes (GE Healthcare) using 25 mM Tris-HCl (incl. 192 mM Glycin, 20% Methanol, pH 8.3) as a transfer buffer. After blocking the membranes with 5% skim milk powder in 1× TBST they were incubated with antibodies against murine Rankl (R&D Systems, AF462) and secondary antibodies (Polyclonal Rabbit Anti-Goat, Dako, #0449). Bound antibodies were detecting by chemilumiscence after incubation with luminol (500 mM, Sigma-Aldrich) and p-coumaric acid (80 mM, Sigma-Aldrich) in buffer (0,1 M Tris pH 8.5).

2.5. Osteoclastogenesis assays

Osteoclast precursor cells were isolated from the bone marrow of 12 weeks old mice and plated in α-MEM (Sigma-Aldrich) including 10% fetal bovine serum at a density of 2600 cells per mm^2. After one day the cells were cultured in medium supplemented either with 1,25$(OH)_2$-vitamin-D3 or hPTH [1–34]. On day 7 the osteoclast number was determined, RNA was isolated for expression analysis and medium was collected for sRankl detection.

2.6. Animal models

hPTH [1–34] (100 μg/kg, Bachem Inc.) was daily injected intraperitoneally into 12 weeks old C57BL/6 wildtype female mice for 7 seven days. *Adamts16*-deficient rats [12] and respective wildtype controls (Dahl Salt–sensitive (S) rats) were analyzed at 24 weeks of age. *Adam17*-mutant mice have been described previously [13] and were analyzed at 10 weeks of age. All animals received two intraperitoneal injections of calcein (30 mg/kg, Sigma-Aldrich) 9 and 2 day before sacrifice. Animal experiments were approved by the animal facility of the University Medical Center Hamburg-Eppendorf and by the "Amt für Gesundheit und Verbraucherschutz" (60/09, Org529).

2.7. Skeletal analysis

Animals were sacrificed, and the dissected skeletons were fixed in 3.7% PBS-buffered formaldehyde for 18 h at 4 °C, before they were stored in 80% ethanol. All skeletons were analyzed by contact radiography using a Faxitron X-ray cabinet (Faxitron X-ray Corp., USA) to measure the length of the lumbar spine and femora. For histology, the lumbar vertebral bodies L3 to L6 and one tibia of each mouse were dehydrated in ascending alcohol concentrations and then embedded in methylmetacrylate as described previously [14]. Sections of 5 μm thickness were cut in the sagittal plane on a Microtec rotation microtome (Techno-Med GmbH, Germany). These were stained by toluidine blue and von Kossa/van Gieson staining procedures as described [14]. Histomorphometry was performed according to the ASBMR guidelines [15] using the OsteoMeasure histomorphometry system (Osteometrics Inc., USA). To determine the rate of bone resorption we measured bone-specific collagen degradation products (Crosslaps) in the serum by ELISA (Immunodiagnosticsystems, #AC-06F1).

2.8. Statistical analysis

All data presented in the manuscript are presented as means ± standard deviations. Statistical analysis was performed using unpaired, two-tailed Student's *t*-test, and p-values below 0.05 were considered statistically significant.

3. Results

3.1. Differential effects of IL-33 and PTH on Tnfsf11 expression and sRankl release

Based on our previous conflicting findings [9,11] we first addressed the question, if IL-33 and PTH would differentially influence *Tnfsf11* expression and sRankl release by osteoblasts. We therefore isolated primary murine calvarial osteoblasts and treated them with the two molecules at day 10 of osteogenic differentiation. More specifically, cells were serum-starved over night before IL-33 or PTH was added for 6 h or 24 h in serum-free medium. We then isolated RNA and medium for further analyses. Using qRT-PCR to monitor *Tnfsf11* expression we found that both molecules significantly induced *Tnfsf11* expression to a similar extent (Fig. 1A). Importantly however, sRankl was only detectable by ELISA in the medium of cells treated by PTH, but not in medium of cells treated by IL-33 for 6 or 24 h (Fig. 1B).

Since our previous findings also revealed that PTH treatment of primary osteoblasts simultaneously reduces Opg production [11], we next measured the medium concentrations of Opg in cultures treated with IL-33 or PTH for 6 h (Fig. 1C). As we found that IL-33 administration did not significantly reduce Opg production, we applied Western Blotting with a Rankl-specific antibody to rule out the possibility that Opg interference with sRankl detection by ELISA could explain the observed differences. Here we found that sRankl was specifically detectable in the medium of PTH-treated cultures, thereby confirming that PTH does not only induce *Tnfsf11* expression, but also sRankl release (Fig. 1D). To rule out the possibility that the differential effect of IL-33 and PTH is explained by alternative splicing [16–18] we again treated primary osteoblasts with the two molecules for 6 and 24 h and performed qRT-PCR with primer pairs amplifying different regions of the *Tnfsf11* transcript (Fig. 1E). Here we did not observe significant differences between cells treated with IL-33 or PTH, thus suggesting that the PTH-specific induction of sRankl is due to proteolytic processing of the Rankl protein.

3.2. Identification of PTH-regulated genes encoding metalloproteases in primary osteoblasts

To analyze if the PTH-induced proteolytic processing of Rankl requires the activity of a metalloprotease, we treated primary osteoblasts with PTH in the absence or presence of marimastat, a broad-spectrum matrix metalloprotease inhibitor [19]. By measuring sRankl in the medium after 6 h of treatment we observed a full blockade of the PTH-induced sRankl release in the presence of marimastat (Fig. 2A). To address the question if PTH rather affects expression or activity of a putative Rankl ectodomain sheddase, we next administered PTH for <1 h, but failed to detect sRankl in the medium of the treated cells (Fig. 2B). Similarly, when we administered PTH for 15 and 30 min to cultures previously incubated with IL-33 for 6 h, there was no detectable release of sRankl into the medium.

Since these findings suggested that the effect of PTH on sRankl processing involves transcriptional activation of specific genes encoding metalloproteases, we next addressed the question if PTH would specifically induce the expression of *Adam10, Adam17* or *Mmp14*. Although all of the three endopeptidases were previously shown to facilitate ectodomain shedding of Rankl [5–8], we observed that none of the corresponding genes was significantly regulated by PTH treatment (Fig. 2C). Moreover, since *Adam17* and *Mmp14* expression was significantly increased by 6-hour administration of IL-33, these findings could not explain the differential effect of PTH and IL-33 on sRankl release.

To identify other potential regulators of sRankl processing in response to PTH we performed Affymetrix Gene Chip hybridization in primary murine osteoblasts treated with PTH for 6 h at day 10 of osteogenic differentiation. By sorting all genes according to their logarithmic ratio of signal intensities (SLR, signal log ratio) between treated and untreated cultures, we found that 14 genes, including *Tnfsf11*, were significantly induced by PTH with a mean SLR higher than 4.0 (Fig. 3A). Whereas PTH did not immediately affect transcription of several genes encoding osteoblast differentiation markers (such as *Bglap*, *Col1a1*, *Alpl* or *Ibsp*), it was interesting to observe that *Dkk1* and *Smpd3,* two genes with proven relevance in bone biology [20,21], were significantly repressed by PTH with a mean SLR lower than −4.0. To validate these results in independently isolated cultures we applied qRT-PCR and consistently observed the same effects as found by Gene Chip hybridization (Fig. S3). We finally analyzed the genome-wide expression data for genes encoding endopeptidases of the Adam, Adamts and Mmp family, respectively. Here we found that only three genes (*Adamts4*, *Adamts16* and *Adamts1*) were significantly induced by PTH treatment of primary osteoblasts (Fig. 3B).

3.3. PTH is the most potent inducer of sRankl release by cultured osteoblasts

Given the potential relevance of our findings to understand the process of Rankl ectodomain shedding, we next addressed the question, which other molecules with a known positive influence on *Tnfsf11* expression would simultaneously cause sRankl release by primary osteoblasts. For that purpose we treated cultures for 6 h with PTH, IL-1ß, IL-6 (+IL-6R), IL-17, 1,25$(OH)_2$-vitamin-D3, TNFα, IFNγ, all-trans retinoic acid, isoproterenol or prostaglandin-E2 and monitored gene expression and sRankl concentrations in the medium. Whereas all molecules (except IL-6 and IFNγ) caused a significant induction of *Tnfsf11* expression (Fig. 4A), sRankl was only detectable in two cases, PTH and IL-1ß (Fig. 4B). While the latter observation is possibly explained by the finding

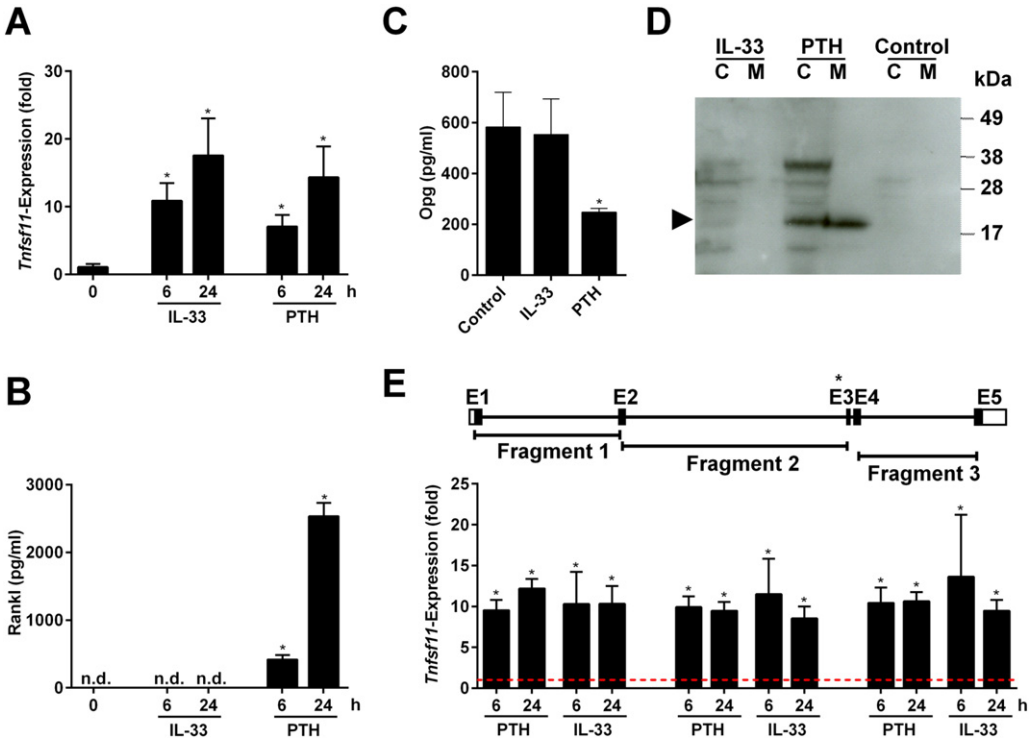


Fig. 1. Differential effects of IL-33 and PTH on *Tnfsf11* expression and sRankl release into the medium. (A) qRT-PCR monitoring *Tnfsf11* expression in primary murine osteoblasts following administration of IL-33 or PTH for 6 or 24 h. Bars represent mean ± SD (n = 4 per group). Asterisks indicate statistically significant differences compared to untreated cells ($p < 0.05$). (B) Medium concentrations of sRankl in the same cultures. Bars represent mean ± SD (n = 4 per group). Asterisks indicate statistically significant difference compared to untreated cells ($p < 0.05$). (C) Medium concentrations of Opg in primary murine osteoblasts following administration of IL-33 or PTH for 6 h. Bars represent mean ± SD (n = 4 per group). The asterisk indicates a statistically significant difference compared to untreated cells ($p < 0.05$). (D) Western Blot with an antibody against sRankl using cell lysates (C) or medium (m) from primary murine osteoblasts following administration of IL-33 or PTH for 6 h. The arrowhead indicates the position of sRankl in the medium of PTH-treated cells. (E) qRT-PCR monitoring expression of *Tnfsf11* in primary murine osteoblasts following administration of IL-33 or PTH for 6 or 24 h. The different primer combinations resulting in the amplification of three different mRNA fragments are indicated below the genomic structure of the *Tnfsf11* gene (E1-5, exon 1-5). The dotted red line indicates expression in untreated cells. Bars represent mean ± SD (n = 4 per group). Asterisks indicate statistically significant differences compared to untreated cells ($p < 0.05$).

that IL-1ß was the strongest inducer of *Tnfsf11* expression amongst all of the tested molecules, these results fully confirmed our hypothesis that PTH has a unique influence on Rankl ectodomain shedding. In an attempt to identify a specific transcriptional regulation explaining the PTH effect, we again applied qRT-PCR to monitor expression of endopeptidases. Here we first analyzed expression of *Adam10, Adam17* or *Mmp14*, yet again there was no transcriptional regulation of these genes that correlated with sRankl release (Fig. 4C).

We next analyzed expression of the three PTH-induced endopeptidase-encoding genes, *Adamts4*, *Adamts16* and *Adamts1,* identified by our genome-wide expression analysis, here again including IL-33. We found that all three genes were immediately induced by PTH, again confirming the results obtained by our genome-wide expression analysis. Most importantly however, whereas *Adamts4* was similarly induced by several other molecules, including IL-33, the induction of *Adamts16* and *Adamts1* appeared more specific (Fig. 4D). In fact, *Adamts16* expression was not affected by the *Tnfsf11*-inducing molecules IL-33, IL-1ß, IL-17, 1,25$(OH)_2$-vitamin-D3, and TNFα, yet is was induced by all-trans retinoic acid, isoproterenol or prostaglandin-E2 to a similar extent as by PTH. In contrast, *Adamts1* was only induced by PTH and IL-6, the latter cytokine however without detectable *Tnfsf11*-inducing activity in our experimental setting. Taken together, these data demonstrate that transcriptional effects on *Tnfsf11* expression do not generally affect sRankl release, and they provide the basis for additional experiments regarding the molecular mechanisms involved in Rankl ectodomain shedding.

3.4. PTH induces sRankl release in vivo

To assess the potential relevance of the observed PTH effect on Rankl processing we first monitored osteoclastogenesis in bone marrow cultures. Here we compared the influence towards 1,25$(OH)_2$-vitamin-D3, which is known to stimulate osteoclastogenic differentiation by inducing *Tnfsf11* expression [22,23]. After 7 days of PTH or 1,25$(OH)_2$-vitamin-D3 administration we first determined *Tnfsf11* expression and sRankl release into the medium (Fig. 5A). Here we found, consistent with the results in calvarial osteoblasts, that PTH treatment resulted in 10-fold higher sRankl levels compared to 1,25$(OH)_2$-vitamin-D3 treatment. Importantly however, this differential effect on Rankl processing did not translate into a major influence on osteoclastogenesis, as determined by quantification of TRAP-positive multinucleated cells or qRT-PCR for the osteoclastogenesis marker *Acp5* (Fig. 5B).

We finally addressed the question, if PTH would also induce sRankl release *in vivo*. Therefore we injected PTH daily into 12 weeks old C57/Bl6 mice and analyzed their skeletal phenotype after one week of treatment (Fig. 5C). Histomorphometric quantification revealed that PTH treatment significantly increased the trabecular bone volume and the bone formation rate, as expected (Fig. 5D). Importantly however, it also increased the osteoclast number as well as the serum concentrations of sRankl without affecting the serum concentrations of Opg (Fig. 5E). Taken together, our collective findings demonstrate that PTH has a unique activity,

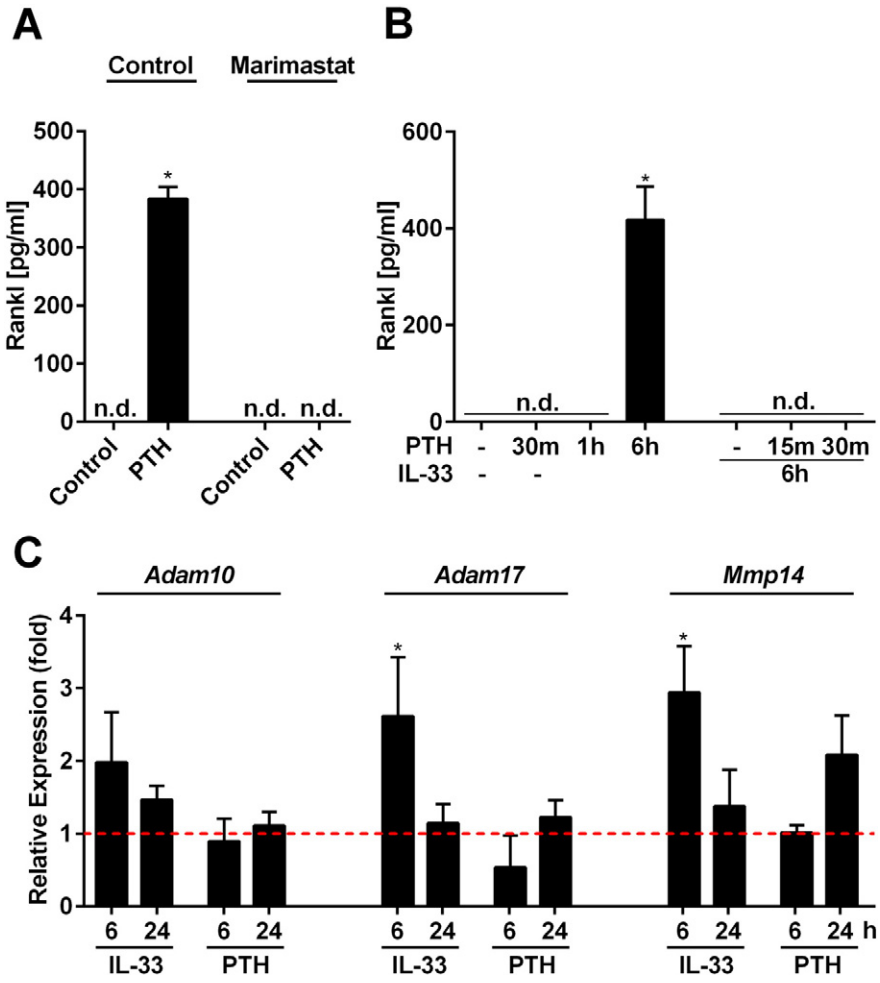


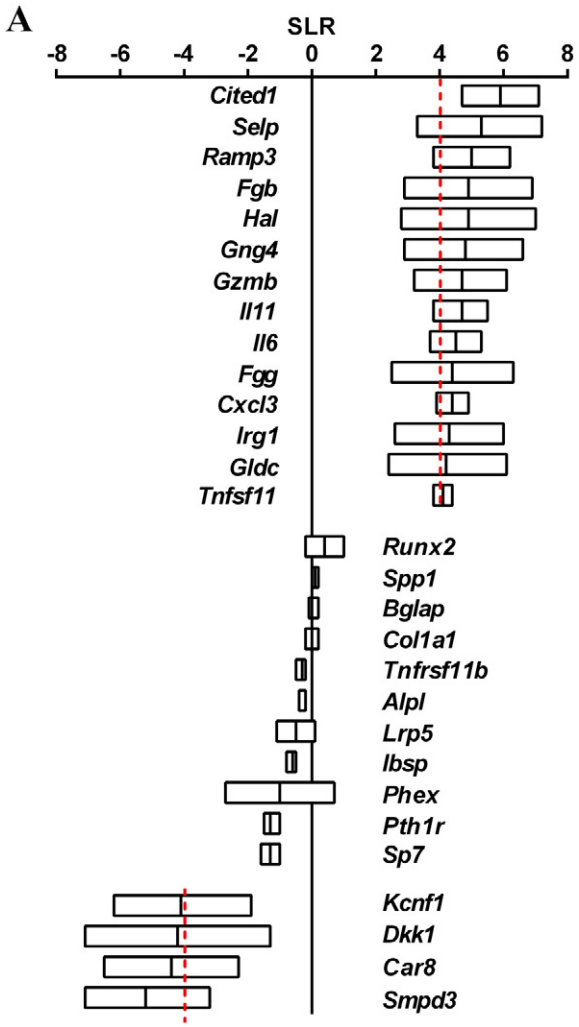
Fig. 2. The PTH-induced sRankl release is blocked by a metalloprotease inhibitor, yet PTH does not affect expression of genes encoding Rankl-sheddases. (A) Medium concentrations of sRankl in primary murine osteoblasts following administration of PTH and/or marimastat for 6 h. Bars represent mean ± SD (n = 4 per group). The asterisk indicates a statistically significant difference compared to untreated cells ($p < 0.05$). (B) Medium concentrations of sRankl in primary murine osteoblasts following administration of PTH and/or IL-33 for the indicated times. Bars represent mean ± SD (n = 4 per group). The asterisk indicates a statistically significant difference compared to untreated cells ($p < 0.05$). (C) qRT-PCR monitoring expression of *Adam10*, *Adam17* or *Mmp14* in primary murine osteoblasts following administration of IL-33 or PTH for 6 or 24 h. The dotted red line indicates expression in untreated cells. Bars represent mean ± SD (n = 4 per group). Asterisks indicate statistically significant differences compared to untreated cells ($p < 0.05$).

as it does not only induce *Tnfsf11* expression, but also the proteolytic processing of Rankl.

4. Discussion

Whereas the key role of Rankl in promoting osteoclastogenesis and bone resorption is undoubted, there is still a remarkable uncertainty about the relevance and mechanisms of Rankl ectodomain shedding. It is well established that Rankl exists in (at least) two forms, *i.e.* as a full-length transmembrane protein or as a circulating form (sRankl) lacking the N-terminal part including the transmembrane domain [24]. It is also evident that sRankl has pro-osteoclastogenic activity, not only because it is commonly utilized to induce osteoclastogenic differentiation of cultured hematopoietic progenitor cells, but also since transgenic mice over-expressing sRankl display severe osteoporosis [25,26]. On the other hand, several *in vitro* studies came to the conclusion that membrane-bound Rankl is more effective than sRankl in promoting osteoclastogenesis, thus suggesting that the activity of Rankl is actually inhibited by ectodomain shedding [7,24,27]. In any case, given the central importance of the Rankl/Rank/Opg system for bone biology, it is quite important to obtain a more profound understanding of the molecular mechanisms controlling the release of sRankl from osteoblasts.

Until now there are few enzymes, all of them metalloproteases, that were identified as putative Rankl-sheddases [5–8]. These enzymes were shown to proteolytically process the murine Rankl protein within the so-called stalk region containing a specific protein sequence (GPQRFSGAPAMMEGSW). However, since this sequence (including the identified cleavage sites for Adam10, Adam17 or Mmp14) is not conserved in the human RANKL protein (Fig. S1), it remains to be addressed, if the findings obtained by forced expression studies are physiologically relevant. With respect to Adam17 (also known as TACE, *i.e.* TNF alpha-converting enzyme) [28,29] we can additionally state that we have analyzed a mouse model with severely impaired Adam17 activity [13], but failed to detect any difference towards wild type controls in terms of bone mass, osteoclast number or sRankl concentration (Fig. S4). So far, the most solid *in vivo* evidence for the requirement of a specific enzyme for Rankl shedding came from the analysis of *Mmp14*-deficient mice, which were found to display osteopenia due to enhanced osteoclastogenesis [7]. Here it was also reported that the medium concentration of sRANKL in primary osteoblasts stimulated with $1,25(OH)_2$-vitamin-D3 and prostaglandin-E for 72 h was significantly decreased in cultures derived from *Mmp14*-deficient mice. Only in the



B

Gene	PTH -	PTH +	SLR
Adamts4	320 ± 30	1311 ± 301	1,92 ± 0,1
Adamts16	22 ± 8	64 ± 5	1,47 ± 0,4
Adamts1	808 ± 152	1615 ± 213	1,07 ± 0,2

Fig. 3. PTH administration for 6 h affects expression of specific genes in primary murine osteoblasts. (A) Shown are PTH-induced genes (top) with a mean Affymetrix signal log ratio (SLR) above 4.0, known osteoblast markers (middle), and PTH-repressed genes (bottom) with a mean Affymetrix signal log ratio (SLR) below −4.0. Bars represent the range of SLR found in three independent experiments. (B) Given are the Affymetrix signal intensities and signal log ratios for three PTH-induced genes encoding putative endopeptidases. Values represent mean ± SD (n = 3 per group).

Discussion section of the respective manuscript it was mentioned that sRankl was undetectable in the serum of *Mmp14*-deficient mice, thereby providing evidence for a physiological role of Mmp14 as a Rankl-specific sheddase, at least in mice [7].

Our own approach to address this question was triggered by findings obtained in the context of experiments with IL-33, a cytokine inhibiting osteoclastogenesis *in vitro* and *in vivo* [9,10]. Since we found that IL-33 significantly induced *Tnfsf11* expression in primary murine osteoblasts without causing sRankl release into the medium, in contrast to PTH [11], we asked the question, how this differential influence might be explained. In these experiments we found, unexpectedly and reproducibly, that only PTH treatment of primary osteoblasts resulted in detectable medium concentrations of sRankl after 6 h. Since we additionally observed that PTH administration for up to 1 h did not result in the presence of sRankl in the medium, we hypothesized that the effect of PTH on Rankl shedding involves transcriptional activation of specific genes, which led us to perform genome-wide expression analyses. By doing so we confirmed that *Tnfsf11* is one of the immediate target genes of PTH in primary murine osteoblasts and we also confirmed the previously reported induction of other genes, such as *Cited1* [30], *Ramp3* [31], or *Il6* [32]. Although many of the observed transcriptional effects are potentially relevant to bone remodeling, our major focus was to identify PTH-induced genes encoding metalloproteases, as we did not observe specific induction of *Adam10*, *Adam17* or *Mmp14* by PTH administration *via* qRT-PCR. Since we found only three such genes, *Adamts4*, *Adamts1* and *Adamts16*, significantly induced by PTH, we addressed the question, how their expression is affected by PTH, IL-33 and other known *Tnfsf11*-inducing molecules. Here we found that *Adamts1* expression was most specifically induced by PTH, whereas *Adamts16* expression was induced by PTH, all-trans retinoic acid, isoproterenol and prostaglandin-E2, but not by IL-33, in contrast to *Adamts4*. In order to identify a physiologically relevant Rankl-processing enzyme, we additionally applied shRNA-mediated knockdown of Adamts4, Adamts1 and Adamts16 in primary murine osteoblasts, before we treated the respective cells with PTH. Unfortunately however, we observed that the transfection itself fully impaired the response the PTH, because sRANKL was repeatedly undetectable also in the medium in control-transfected cells.

There are 19 known members of the Adamts family, representing secreted disintegrin-like metalloproteinases with thrombonspondin motifs [33]. The potential role of Adamts16 in blood pressure regulation, as suggested by a positional cloning approach, was previously confirmed through the generation of a mutant rat model displaying reduced systolic and diastolic blood pressure [12,34]. Another function of Adamts16 is linked to the male genitourinary system, since *Adamts16*-deficient rats additionally displayed cryptorchidism and infertility [35]. With respect to skeletal biology, it was reported that *ADAMTS16* expression is increased in articular cartilage from individuals with osteoarthritis and that over-expression of *ADAMTS16* in SW1353 chondrosarcoma cells reduces their proliferative and migratory capacity [36–38]. In the context of bone remodeling however, the specific role of Adamts16 has not been analyzed so far. Therefore, we studied the skeletal phenotype of 24 weeks old female *Adamts16*-deficient rats and observed a significant reduction of the osteoclast number per bone surface, together with a non-significant decrease of serum crosslaps and sRankl concentrations (Fig. S5). Most importantly however, in the light of unaffected osteoblastogenesis and bone formation, these subtle differences did not cause a change in trabecular bone parameters, thereby ruling out a major physiological function of Adamts16 in bone remodeling and Rankl processing.

In contrast to Adamts16, Adamts1 has been formerly analyzed in the context of bone remodeling, and its transcriptional activation by PTH has already been established [39]. More specifically, it was reported that *Adamts1* is expressed by osteoblasts, that *Adamts1*-deficient mice are growth-retarded, and that transgenic over-expression of *Adamts1* causes reduced bone mineral density in female mice [40–42]. At a functional level it was reported that Adamts1 enhances degradation of type I collagen and CTX-release from demineralized bone slices [43]. That Adamts1 can principally act by ectodomain shedding has been demonstrated in the context of cancer metastases [44,45]. Here it was found that Adamts1, in synergy with Mmp1, promotes the release of EGF-like growth factors by breast cancer cells, which in turn favors osteoclastogenesis by increasing the Rankl-Opg ratio [45]. Since there was no evidence for a direct effect of the two enzymes on sRANKL production by MC3T3-E1 osteoblasts however, it remains to be addressed if Adamts1, alone or in combination with other metalloproteases, is required for Rankl processing *in vitro* and *in vivo*. At that point we would like to state that we also performed one series of experiments, where we added recombinant Adamts1 to primary osteoblasts, in the presence or absence of IL-33. Here we failed to detect sRankl in the medium,

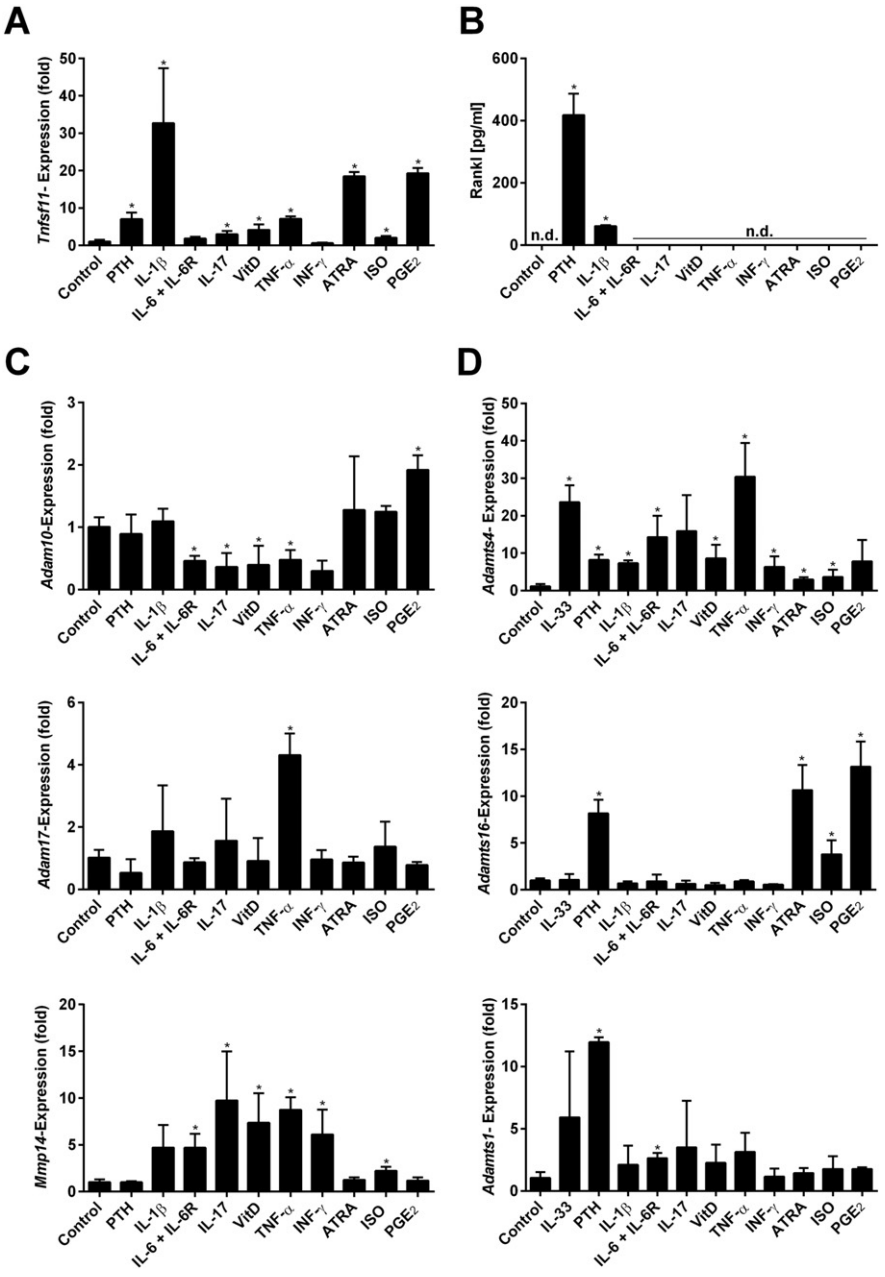


Fig. 4. PTH is the most potent inducer of sRankl release and *Adamts1* expression. (A) qRT-PCR monitoring *Tnfsf11* expression in primary murine osteoblasts following administration of the indicated molecules for 6 h. Bars represent mean ± SD (n = 4 per group). Asterisks indicate statistically significant differences compared to untreated cells ($p < 0.05$). (B) Medium concentrations of sRankl in primary murine osteoblasts following administration of the indicated molecules for 6 h. Bars represent mean ± SD (n = 4 per group). Asterisks indicate statistically significant differences compared to untreated cells ($p < 0.05$). (C) qRT-PCR monitoring expression of *Adam10*, *Adam17,* and *Mmp14* in primary murine osteoblasts following administration of the indicated molecules for 6 h. Bars represent mean ± SD (n = 4 per group). Asterisks indicate statistically significant differences compared to untreated cells ($p < 0.05$). (D) qRT-PCR monitoring expression of *Adamts4*, *Adamts1,* and *Adamts16* in the same samples. Bars represent mean ± SD (n = 4 per group). Asterisks indicate statistically significant differences compared to untreated cells ($p < 0.05$).

thus suggesting that Adamts1 alone is not sufficient to mediate the proteolytic processing of Rankl in response to PTH.

Despite the fact that we were unable so far to identify one specific enzyme being physiologically required for Rankl ectodomain shedding, our findings provide the basis for several additional experiments. For instance, it would be very informative to determine serum levels of sRankl in mice lacking specific metalloproteases, in particular Adamts1, either alone or in combination. Moreover, since we found that daily PTH injections into wildtype mice did not only increase osteoclastogenesis, but also sRankl concentrations, in line with a study in patients [46], it would be highly important to analyze, if the production of sRankl is required for proper bone resorption. Ideally, such a question about the physiological relevance of Rankl ectodomain shedding should be addressed by generating a mouse model carrying a mutation in the relevant cleavage site. This however requires a precise mapping of this site, which should not be based on forced expression studies, but possibly on sRankl purification from mouse and/or human serum with subsequent N-terminal sequencing. Another issue to be addressed is the question regarding the human relevance of our findings. In fact, when we treated the two human osteosarcoma cell lines SaOS-2 and U2-OS with PTH, we did not detect sRANKL in the medium of these cells and we also failed to detect robust induction of *TNFSF11* expression (Fig. S6). In our opinion, these results imply that the use of osteosarcoma cell lines is not sufficient to address this question, and that primary cells are required for future studies. Although these collective experiments are beyond the scope of the present manuscript, we truly believe that our findings are

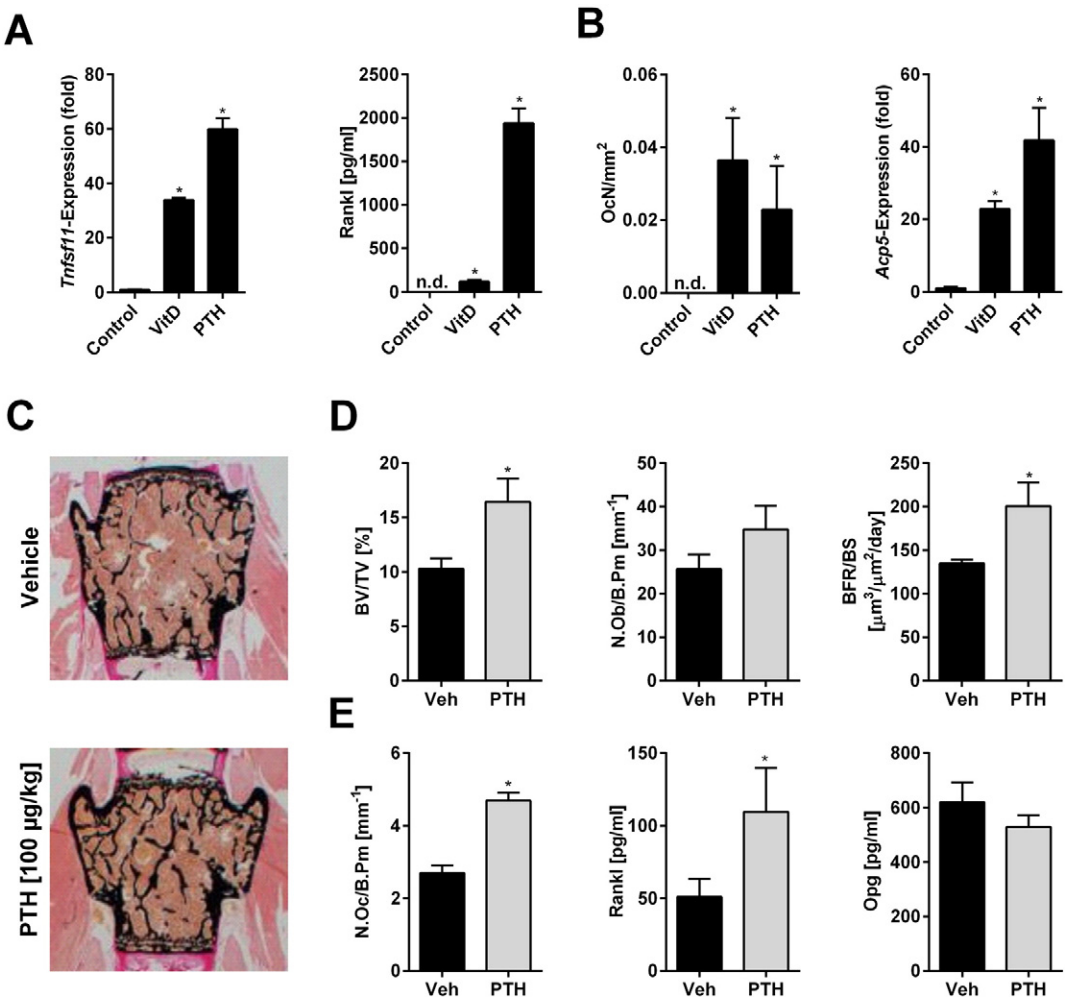


Fig. 5. PTH induces sRankl release in primary bone marrow cells and *in vivo*. (A) qRT-PCR monitoring *Tnfsf11* expression (left) and medium concentrations of sRankl (right) in primary bone marrow cells differentiated for 7 days in the presence of $1,25(OH)_2$-vitamin-D3 (VitD) or PTH. Bars represent mean ± SD (n = 4 per group). Asterisks indicate statistically significant differences compared to untreated cells ($p < 0.05$). (B) qRT-PCR monitoring osteoclast numbers (left) and *Acp5* expression (right) and in the same cultures. Bars represent mean ± SD (n = 4 per group). Asterisks indicate statistically significant differences compared to untreated cells ($p < 0.05$). (C) Von Kossa/van Gieson staining of spine sections from wildtype mice having received daily injections of vehicle or PTH for one week. (D) Quantification of the trabecular bone volume per tissue volume (BV/TV), the osteoblast number per bone perimeter (N·Ob./B·Pm) and the bone formation rate per bone surface (BFR/BS). All bars represent mean ± SD (n = 5 per group). Asterisks indicate statistically significant differences between the two groups ($p < 0.05$). (E) Quantification of the osteoclast number per bone perimeter (N·Oc./B·Pm) and the serum levels of Rankl and Opg. All bars represent mean ± SD (n = 5 per group). Asterisks indicate statistically significant differences between the two groups ($p < 0.05$).

highly relevant, as they demonstrate that transcriptional activation of *Tnfsf11* does not generally result in sRankl production and that PTH has a unique activity, as it was the only tested molecule causing rapid and robust sRankl release into the medium.

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Disclosures

All authors state that they have no conflicts of interest.

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