Original Full Length Article

Thrombin receptor deficiency leads to a high bone mass phenotype by decreasing the RANKL/OPG ratio

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A B S T R A C T

Thrombin and its receptor (TR) are, respectively, expressed in osteoclasts and osteoblasts. However, their physiological roles on bone metabolism have not been fully elucidated. Here we investigated the bone microarchitecture by micro-computed tomography (μCT) and demonstrated increased trabecular and cortical bone mass in femurs of TR KO mice compared to WT littermates. Trabecular thickness and connectivity were significantly enhanced. The physiological role of TR on both inorganic and organic phases of bone is illustrated by a significant increase in BMD and a decrease in urinary deoxyypyridinoline (DPD) crosslink concentration in TR KO mice. Moreover, TR KO cortical bone expanded and had a higher polar moment of inertia (J), implying stronger bone. Bone histomorphometry illustrated unaltered osteoblast and osteoclast number and surface in femoral metaphyses, indicating that thrombin/TR regulates osteoblasts and osteoclasts at functional levels. Serum analysis showed a decrease in RANKL and an increase in osteoprotegerin (OPG) levels and reflected a reduced RANKL/OPG ratio in the TR KO group. In vitro experiments using MC3T3 pre-osteoblasts demonstrated a TR-dependent stimulatory effect of thrombin on the RANKL/OPG ratio. This effect was blocked by TR antagonist and p42/p44-ERK inhibitor. In addition, thrombin also intensified p42/p44-ERK expression and phosphorylation. In conclusion, the thrombin/TR system maintains normal bone remodeling by activating RANKL and limiting OPG synthesis by osteoblasts through the p42/44-ERK signaling pathway. Consequently, TR deficiency inhibits osteoclastogenesis, resulting in a high bone mass phenotype.

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Introduction

Osteoblasts together with osteoclasts are responsible for bone remodeling. The bone-forming osteoblasts produce an organic collagenous matrix and mineralize it with an inorganic calcium-phosphate hydroxyapatite [Ca10(PO4)6(OH)2]. The bone-resorbing osteoclasts degrade the mineralized matrix by two means—creating acidic milieu to resorb the inorganic mineralized phase and secreting proteases to degrade organic collagen I-based extracellular matrix (ECM) [1,2]. Bone remodeling is regulated by the coupling mechanism of osteoblast and osteoclast activities [3]. On the one hand, osteoclast development requires a stimulation of its surface receptor activator of nuclear factor-κB ligand [B (RANK) by RANK ligand (RANKL) secreted from osteoblasts [4]. On the other hand, osteoblasts secrete a decoy receptor osteoprotegerin (OPG) that specifically binds and neutralizes RANKL [1,4]. Therefore, a decrease in the RANKL/OPG ratio determines domination of bone resorption over bone formation and vice versa. Disturbances of RANKL/OPG causes skeletal abnormalities represented by reduced (osteoporosis) and increased (osteopetrosis) bone mass [5].

The serine protease thrombin (coagulation factor II, EC 3.4.21.5), with known function to convert fibrinogen to fibrin in the final step of the coagulation cascade, is also synthesized and secreted by osteoclasts [6,7]. Heterozygous thrombin-deficient mice are lethal in the embryonic phase, while surviving neonates died after a few days due to severe hemorrhage [8]. Among other proteases, thrombin has been shown to play a role in bone remodeling including direct cleavage of the bone protein osteopontin, which is important for anchoring osteoclasts to the mineralized matrix [9]. Recently, thrombin has been found to inhibit osteoclast differentiation and down-regulate RANK expression in isolated pre-osteoclast cultures [10]. In addition, thrombin can also exert its biological effect via protease-activated receptors (PARs)-1, -3, and -4 [11]. PAR-1 (thrombin receptor, TR) is expressed in osteoblasts, but not in osteoclasts [12]. PAR-3 is not expressed in murine osteoblasts, whereas PAR-4 has been reported only in osteoblast-like calvarial cells [6]. Therefore, TR is responsible for almost, if not all, thrombin signaling
in mouse osteoblast cells [13]. TR is a transmembrane G protein-coupled receptor (GPCR) of which the extracellular domain can be activated upon proteolytic cleavage by thrombin [6]. In vitro experiments showed that activation of TR increased the proliferation rate of osteoblasts and stimulated the activity of alkaline phosphatase (ALP), an osteoblast differentiation marker [14]. TR has been reported to convey its intracellular signal via the PLC-β, p42/44-ERK, and PKC pathways [15,16]. By using in vitro calvarial and long bone cultures, thrombin induced bone resorption, while TR knockout mice (TR KO) manifested decreased migration and proliferation of mesenchymal cells into the bone fracture area of bone-drilled model [11,17,18]. These data suggest a potentially physiological importance of TR. However, the role of the thrombin/TR system in bone homeostasis has not been investigated. The purpose of the present study was, therefore, to investigate the function of thrombin/TR in bone metabolism using in vivo TR KO mouse model and in vitro osteoblast cell cultures. We hypothesize that the thrombin/TR system is necessary for the normal bone remodeling process.

Materials and methods

Animal experiments and collection of samples

TR KO mice and WT littersmates (75% C57Bl/6 × 25% 129/Sv background, 10–12 weeks old) purchased, respectively, from The Jackson Laboratory (Bar Harbor, ME, USA) and Charles River (Maastricht, The Netherlands) were genotyped. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Amsterdam. Four male mice of WT and the other four male TR KO mice were placed in metabolic cages. Twenty-four-hour food and water consumption; and fecal and urine excretions were collected for weight, volume, electrolyte, and DPD determinations. The mice were anesthetized under 2% (v/v) isoflurane. Blood was collected for electrolyte, RANKL, and OPG determinations before termination. Bones were removed, rinsed in iced-cold PBS and kept in 75% (v/v) ethanol at 4 °C for further μCT analyses. Kidney and duodenal tissues were removed, rinsed in iced-cold PBS and kept in — 80 °C and/or PLP for further RNA and protein extraction, and cryosection, respectively as described previously [19].

Serum, urine, and fecal analyses

Serum and urine Ca²⁺ concentrations were determined by a colorimetric assay as described previously [20]. Fecal samples were desiccated at 50 °C overnight and stored at — 80 °C until analysis. Each 24-h fecal sample was dissolved in 20 ml nitric acid (1.5 M) at room temperature for 30 h. A solution was prepared to a total volume of 20 ml with deionized water, and Ca²⁺ was determined with the same methods for serum and urine Ca²⁺ analyses. A flame spectrophotometer FCM6343 (Eppendorf) was used to measure serum and urine Na⁺ concentrations and urine K⁺ concentrations. Urine osmolality was obtained using a Halbmikro-Osmometer K-7400 (Knauer).

μCT measurements

Femurs from male WT and TR KO mice (n = 4) were scanned at a resolution of 0.9 μm, using a SkyScan 1172 system (SkyScan). According to guidelines recently published [21], the following settings were used: X-ray power and tube current were 40 kV and 0.25 mA, respectively. Beam hardening (20%) was reduced using a 1 mm aluminium filter, ring- and artifact were reduced at a slice of 5 μm, exposure time was 5.9 s and an average of three pictures were taken at each angle (0.9°) to generate final images. Using different software packages from SkyScan (NRecon, CtAn and Dataviewer), bone microarchitectural parameters were assessed in trabecular and cortical bones of all mice. The trabecular bone parameters: trabecular tissue volume, bone volume, volume fraction (BV/TV), thickness, number and patterning factor (connectivity of trabecular) were determined in the distal metaphysis of the femur (scan area of 1.3 mm from distal growth plate towards femoral center). In the mid-diaphysis (scan area of 0.43 mm in the femoral center), cortical volume, thickness, J (proxy for bone strength) and perimeter were analyzed. For image processing, trabecular bone was manually selected and cortical bone was automatically selected. In addition, trabecular and cortical BMD was measured on the basis of calibration scanning, using two phantoms with known density (0.25 mg/cm² and 0.75 mg/cm²; SkyScan) under identical conditions as for the femurs (method note from SkyScan provided on http://www.skyscan.be). All terms used in μCT experiments comply with the standard nomenclature, symbols, and units previously described [21].

Bone histomorphometry

After excision, femurs were routinely embedded in methyl-metacrylate as described before [22]. Sections of 6 μm were subjected to tartrate-resistant acid phosphatase (TRAP) staining. Sections were deacrylated, hydrated and rinsed in 0.2 M sodium acetate/50 mM tartaric acid for 5 min. Naphthol AS-MX (0.5 mg/ml) and 1.1 mg/ml Fast red TR salt (both from Sigma) were added and incubated for 120 min at 37 °C. Counterstaining was performed with haematoxylin for 5 s and after air-drying; the sections were embedded in Permount (Thermo Fisher Scientific). For osteoblast and osteocyte measurements, sections were stained with a Goldner staining as described before [23]. Images were taken from the TRAP and Goldner stainings with a Nikon Eclipse E400 system (Nikon). Measurements were performed, using the software package Bioquant (Version 7.20; Bioquant Image Analysis Corporation).

Preparation of cultured pre-osteoblastic cells

MC3T3 pre-osteoblastic cells were purchased from ATCC and grown in α-minimal essential medium (αMEM) (Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco), 50 μg/ml penicillin/streptomycin (Gibco), 50 μg/ml vitamin C (Sigma), and 10 mM β-glycerophosphate (Sigma) in 5% (v/v) CO₂ at 37 °C. The medium was refreshed twice a week. The cells were seeded at 2.5 × 10⁴ cells/cm² into 6-well plates and left in the incubator for 24 h prior to exposure to different treatments for 16 h.

qPCR expression analyses

To evaluate mRNA expression of RANKL and OPG, total RNA was extracted from thrombin-treated (16 h) MC3T3 cells using TriZol Total RNA Isolation Reagent (Life Technologies BRL) according to the manufacturer’s protocol. The obtained RNA was subjected to DNase treatment (Promega) to prevent genomic DNA contamination. Thereafter, 2 μg of RNA was reverse transcribed by Moloney-Murine Leukemia Virus-Reverse Transcriptase (Invitrogen). The cDNA was used to determine mRNA expression levels by SYBR green PCR both of the target genes of interest and of the housekeeping gene Gapdh as an endogenous control. Primers targeting the genes of interest were designed using the computer program Primer3 (v0.4.0) and are listed in Table 1.

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>Tnfsf11</td>
<td>CATTCGCCCACTCACACAACCTTGAAC</td>
<td>CAGTTTGCCACGGCACAATCTAAC</td>
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<tr>
<td>Tnfsf11b</td>
<td>CAATGGCCTGTTCTGCTTCTTAGC</td>
<td>CTGAAACACCACATGGAATGAC</td>
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<tr>
<td>Gapdh</td>
<td>TAACCATCAGGTCGTGCGAG</td>
<td>GTCTCCACCATCACAAC</td>
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</table>

Tnfsf1, receptor activator of nuclear factor-κB ligand gene; Tnfsf11b, osteoprotegerin gene; Gapdh, glyceraldehyde–3-phosphate dehydrogenase gene.
Western blot analysis

MC3T3 cell lysates were added to sample buffer and incubated for 30 min at 37 °C. Homogenates of mouse duodenal and kidney tissues; and cells were prepared and analyzed as previously described [24,25]. Protein concentrations were determined by a BCA protein assay kit (Bio-Rad) and volumes of samples were normalized accordingly. Subsequently, 30-μg samples were loaded and separated on 8% SDS-PAGE gels for p42/44 ERK. Protein fractions were transferred to polyvinylidene difluoride–nitrocellulose membranes (Immobilon-P, Millipore). Proteins on the membranes were detected using rabbit monoclonal anti-phospho-p42/44 ERK and anti-p42/44 ERK antibodies (Cell Signaling Technology). Mouse polyclonal anti-β-actin Ab was from Santa Cruz.

Statistical analysis

If not specified otherwise, the data are expressed as mean ± SEM. Multiple sets of data were compared by ANOVA. The significant differences between the means of the two groups were analyzed by paired Student’s t-test, while multiple comparisons between groups were performed by Tukey’s post hoc multi analysis. The level of statistical significance is P < 0.05. All data were analyzed by GraphPad Prism (version 4.0c for Mac OS X; GraphPad Software).

Results

TR-deficient mice have increased bone mass

Gross observation revealed that TR KO mice have an increased body weight compared to WT littermates (27.2 ± 1.9 vs 30.1 ± 0.3 g, P < 0.05). TR KO mice consumed similar amounts of food and water and excreted the same extent of feces compared to WT mice (3.0 ± 1.1 vs 3.6 ± 1.8 g, 4.8 ± 1.5 vs 4.8 ± 0.5 ml, and 2.3 ± 0.6 vs 2.8 ± 0.7 g, respectively, P > 0.05). Subsequently, we analyzed the bone tissue of TR KO mice. First, we compared the bone microarchitectural structure of the femoral metaphysis and diaphysis between WT and TR KO mice. Data in Table 2 showed that, in the WT and TR KO groups respectively, trabecular bone volume (BV), femoral head volume (Fh.V), and trabecular number (Tb.N) were significantly increased (0.47 ± 0.01 vs 0.81 ± 0.06 mm³, 4.15 ± 0.09 vs 5.06 ± 0.18 mm³, and 3.13 ± 0.04 vs 3.98 ± 0.19 mm⁻¹, respectively, P < 0.05) whereas trabecular separation (Th.Sp) was decreased (145 ± 0.3 vs 129 ± 4.2 μm, P < 0.05) (Table 2). Diaphyseal endocortical volume (Ec.V) was also increased (0.46 ± 0.02 vs 0.54 ± 0.01 mm³). The mice lacking TR had a small, albeit significant, increase in femur length (15.79 ± 0.08 vs 16.26 ± 0.03 mm, P < 0.05).

TR deficiency increased cortical bone size, strength proxy, and bone mineral density

At the level of the cortical bone, as measured in the distal metaphysis of the femur, endocortical and cortical volumes were significantly increased (3.13 ± 0.07 vs 3.87 ± 0.17 mm³ and 1.01 ± 0.02 vs 1.20 ± 0.02 mm³ respectively, P < 0.05, Figs. 1A–C), while diaphyseal thickness was reduced (142 ± 1.9 vs 123 ± 2.2 μm, P < 0.05, Fig. 1D). Also a significant increase in diaphyseal bone circumference (cortical perimeter, 10.40 ± 0.02 vs 12.85 ± 0.23 mm, P < 0.05, Fig. 1E) was observed. The bone expansion led to a higher J (0.42 ± 0.01 vs 0.60 ± 0.05).

Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT (mean ± SD)</th>
<th>TR KO (mean ± SD)</th>
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<tr>
<td>Metaphysis</td>
<td></td>
<td></td>
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<tr>
<td>BV (mm³)</td>
<td>0.47 ± 0.01</td>
<td>0.81 ± 0.06⁣</td>
</tr>
<tr>
<td>Fr.V (mm³)</td>
<td>4.15 ± 0.09</td>
<td>5.06 ± 0.18⁣</td>
</tr>
<tr>
<td>Tb.Sp (μm)</td>
<td>145 ± 0.3</td>
<td>129 ± 4.2</td>
</tr>
<tr>
<td>Diaphysis</td>
<td></td>
<td></td>
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<tr>
<td>Ec.V (mm³)</td>
<td>0.46 ± 0.02</td>
<td>0.54 ± 0.01⁣</td>
</tr>
<tr>
<td>Whole femur</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femur length (mm)</td>
<td>15.79 ± 0.08</td>
<td>16.26 ± 0.03⁣</td>
</tr>
</tbody>
</table>

Femurs from wild-type (WT) and thrombin receptor-deficient (TR KO) mice (n = 4) were analyzed in detail by μCT. Parameters indicated are femoral head volume (Fr.V), bone volume, metaphyseal bone volume (BV), trabecular separation (Th.Sp), trabecular number (Tb.N), diaphyseal endocortical volume (Ec.V) and femoral length. Data are presented as mean ± SD.⁣ P < 0.05 versus wild-type. P < 0.01 versus wild-type.
0.01 mm², P < 0.05, Fig. 1F), BMD was also higher in TR KO mice (1.21 ± 0.004 vs 1.25 ± 0.01 g/cm³, P < 0.05, Fig. 1G).

TR deficiency increased trabecular bone thickness and connectivity

Detailed structural analysis of the femurs revealed that trabecular bone volume fraction in the distal metaphyseal bone compartment of TR KO mice was elevated (15.14 ± 0.15 vs 20.94 ± 0.01%, P < 0.05, Figs. 2A and B), which is explained by increased trabecular thickness (48.37 ± 0.12 vs 52.53 ± 0.41 μm, P < 0.05) and trabecular number (3.13 ± 0.04 vs 3.98 ± 0.19 mm⁻¹, P < 0.05) (Fig. 2C and Table 2, respectively). This led to reduced trabecular separation (145 ± 0.3 vs 129 ± 4.2 μm, P < 0.01) and more trabecular connectivity (indicated by a decrease in trabecular patterning factor, 23.27 ± 0.75 vs 15.65 ± 0.76 mm⁻¹, P < 0.01) as shown in Table 2 and Fig. 2D, respectively.

The structure model index estimating whether a bone structure is plate-like (value of 0) or rod-like (value of 3) revealed that TR deficiency leads to a more plate-like microarchitecture (1.99 ± 0.04 vs 1.70 ± 0.03, P < 0.01, Fig. 2E).

Histomorphometrical parameters of WT and TR KO mice

In order to investigate bone turnover, histological analyses of femoral metaphyseal bone sections from WT and TR KO mice were performed with Goldner's trichrome (Figs. 3A and B) and TRAP (Figs. 3C and D) staining for osteoblasts and osteoclasts, respectively. Osteoblast number and surface were not significantly different between the WT and TR KO groups (0.61 ± 0.05 vs 0.91 ± 0.25 no./mm and 1.78 ± 0.17 vs 2.96 ± 0.85%, P > 0.05, Figs. 3E and F, respectively). Similarly, Figs. 3G and H also showed no changes in osteoclast number and surface.
Electrolyte analyses revealed that urinary Ca^2+ contents when corrected for bone surface (1.69 ± 0.11 vs 1.49 ± 0.21 no./mm^2 and 15.94 ± 1.40 vs 15.12 ± 2.77%, P > 0.05).

TR deficiency decreased urinary bone resorption marker protein, but did not affect serum and urinary Ca^2+ contents

Because bone proteases are involved in ECM degradation [5] and bone mass was increased, urinary DPD crosslinks (degradation products of collagen I); and serum and urinary Ca^2+ were measured. Urinary DPD concentration in TR KO mice was reduced compared to WT (141 ± 27 vs 79 ± 3 nM in WT and TR KO, respectively, P < 0.05, Fig. 4), indicating that TR is required for proper bone resorption. Electrolyte analyses revealed that urinary Ca^2+ excretion was unchanged, but polyuria (0.8 ± 0.2 vs 2.1 ± 0.3 ml, P < 0.05) and alkaline urine (pH 6.7 ± 0.1 vs 7.1 ± 0.1, P < 0.05) were observed (Table 3). Serum Ca^2+ concentration was not affected (2.23 ± 0.03 vs 2.16 ± 0.15, P > 0.05). Additionally, urinary Na+ excretion was enhanced whereas serum Na+ and K+ values appeared normal (see Table 3).

TR deficiency decreased serum RANKL/OPG ratio

Based on the fact that TR is expressed in osteoblasts and the finding of an abnormal increase in bone mass, we hypothesized that TR deficiency might alter osteoblast function to secrete RANKL and OPG. Fig. 5A shows that the serum RANKL level was significantly decreased from 1419 ± 576 pg/ml to 355 ± 34 pg/ml in WT and TR KO mice, respectively, P < 0.05, while the OPG level was increased (992 ± 29 pg/ml vs 1189 ± 57 pg/ml in WT and TR KO mice, respectively, P < 0.05) (Fig. 5B). As a consequence, the RANKL/OPG ratio was significantly reduced (1.00 ± 0.35 vs 0.22 ± 0.03, P < 0.05, Fig. 5C).

Thrombin upregulated RANKL/OPG ratio in osteoblast-culture medium via TR-dependent and p42/44-ERK-mediated pathway

In order to elucidate the mechanism of thrombin action, TR-expressing MC3T3 pre-osteoblastic cells [13] were cultured with thrombin-containing medium (0.1–10 nM) for 16 h. The RANKL/OPG ratio in the cultured medium was significantly upregulated after incubation with 10-nM thrombin (1.00 ± 0.06 vs 1.47 ± 0.13 in control and 10-nM thrombin-treated respectively, P < 0.05, Fig. 6A), RANKL and OPG mRNA expression was analyzed in the cells treated with a maximal dose of thrombin (10 nM). Interestingly, only RANKL mRNA was increased whereas the OPG mRNA and RANKL/OPG mRNA ratio were unchanged by thrombin incubation (Figs. 6B–D). We further investigated if thrombin action was dependent on TR and if so, which signaling pathway was responsible for this stimulatory effect. Fig. 6E shows that pre-incubation (30 min prior to thrombin) with TR antagonist, 20 μM SCH79797, prevents an upregulation of the RANKL/OPG ratio by thrombin (1.00 ± 0.14, 1.47 ± 0.27, and 0.84 ± 0.11 in control, 10-nM thrombin-treated, and 10-nM thrombin-treated + SCH79797, respectively). Moreover, the inhibitors for p42/44-ERK (25 μM U0126) and PLC-β (10 μM U73122) blocked the effect of thrombin, whereas PKC inhibitors (10 μM Chelerythrine) failed to reverse the effect of thrombin (1.87 ± 0.31, P < 0.05 compared to control, Fig. 6E). Furthermore, immunoblotting analysis showed that both phosphorylated and total p42/44-ERK expression is stimulated by thrombin (100 ± 23 vs 170 ± 21 and 100 ± 14 vs 177 ± 2, respectively, P < 0.05 vs WT).
To investigate the origin of the increment of BMD in TR KO mice, fecal Ca\(^{2+}\) content was measured in both mouse strains, but no significant difference was observed between the groups (105 ± 25 vs 145 ± 56 μmol/g/day, Fig. 7A). Furthermore, 10-nM thrombin had no effect on transepithelial Ca\(^{2+}\) transport in Caco-2 cells that endogenously express TR, TRPV6, and CasBP-D [27,28] (Fig. 7B). Moreover, a 2-h incubation with 10-nM thrombin did not affect PD and TEER, suggesting that thrombin had also no effect on the paracellular transport (Fig. 7C).

Discussion

Serine proteases and their receptors have been characterized as important players in bone metabolism as exemplified by tissue- and urokinase-type plasminogen activators and plasminogen/plasmin [29–31]. The present study provides evidence that thrombin/TR system is required for a normal bone resorption process mainly by stimulating RANKL secretion from osteoblasts, thereby indirectly triggering osteoclastogenesis. Furthermore, the system seems to be crucial for proper degradation of both inorganic and organic bone compartments.

These conclusions are drawn from the following observations in TR KO mice and osteoblast cells. First, TR KO mice manifested increments in both cortical and trabecular bone mass, increased BMD, and changes in bone microarchitecture. Second, urine analyses showed a reduction of DPD crosslink concentration, an organic-phase bone resorption marker. Third, TR deficiency decreased the serum RANKL/OPG ratio. Fourth, in vitro experiments revealed that thrombin upregulated the RANKL/OPG protein ratio in osteoblast-culturing medium via the TR and p42/44-ERK-mediated pathway. Finally, thrombin had no direct effect on intestinal Ca\(^{2+}\) transport.

The physical appearance of the TR KO mice was normal, but their body weights were higher compared to WT in spite of normal food and water intakes. These observations can be explained by the increases in bone size and mass as well as possibly adipocyte hypertrophy since thrombin was reported to control the size of adipocytes [10]. Indeed, femur length was increased in TR KO mice, as were various parameters of cortical and trabecular bone mass. In addition, a transformation of rod-like into plate-like microarchitecture indicated that TR-deficient mice gained bone [32]. As a consequence of the microstructural changes in cortical and trabecular bones, a higher \(J\) ensued. This finding confirms the osteoporotic phenotype since the combined measurement of BMD and \(J\) has been used as important indicators for bone strength and stiffness [33]. It is unlikely that the observed increase in bone mass and strength in TR KO mice was due to the enhanced bone-forming activity of osteoblasts, since bone histomorphometry showed that osteoblast number and surface (Ob.N/BS and Ob.S/BS, respectively) were not altered. Moreover, osteoclast number (Oc.N/BS) was also not changed. These findings are in line with the study by Song and colleagues who reported that Ob.N/BS and Oc.N/BS were not different between WT and TR KO mice [11]. Therefore, the observed increase in bone mass likely resulted from a decrease in the cytokine-producing activity of osteoblasts as signified by decreases in the serum RANKL/OPG ratio and urinary DPD concentration in the present study. However, how the change in RANKL did not affect osteoclast histology is complex and needs to be clarified in further studies.

RANKL and OPG can be considered as central players harmonizing bone formation and resorption [34]. RANKL-deficient mice completely...
lack osteoclasts and have severe osteopetrosis whereas OPG deficiency essentially causes the opposite traits i.e., osteoporosis with severe cortical and trabecular bone thinning and porosity [35,36]. Various factors can positively or negatively affect RANKL and OPG synthesis. For example, 1,25(OH)2D3, PTH, interleukin-11 (IL-11), and prostaglandin E2 (PGE2) stimulate RANKL synthesis whereas estrogen and transforming growth factor-β (TGF-β) are inhibitors of RANKL production [5,35,37]. RANKL and osteoprotegrins are bone regulators that work in close interrelationship. For instance, a cysteine protease cathepsin K localized in lysosomes in the ruffled border of osteoclasts has been identified as a major osteoclastic protease and plays a principal role in degrading the ECM [5]. RANKL, upon its binding to RANK, has been reported to stimulate cathepsin K mRNA and protein expression [38]. Besides, ADAM (a disintegrin and metalloproteinase domain), a member of metalloproteinases, also indirectly facilitates RANKL-induced osteoclastogenesis by shedding a RANKL enhancer TNF-α [5]. Importantly, MMP and ADAM family members have been shown to cleave RANKL in vitro, increasing the RANKL concentration in the culture medium [23,39]. In the present study; however, the possibility that thrombin cleaves RANKL was excluded by the observation that an increase in the RANKL/OPG ratio after thrombin treatment was blocked by TR antagonist. Altogether, the thrombin/TR signaling cascade seems essential for RANKL-dependent osteoclastogenesis.

Osteoclast cells are derived from hematopoietic stem cells (HSCs) that reside in the endosteal region i.e., the active bone modeling and remodeling interface region between bone and bone marrow [40]. Recently, Mansour and colleagues demonstrated that the proper osteoclast function is imperative for HSC niches and osteoblast differentiation [40]. Even though TR is not expressed in osteoclasts, it was detected in HSC and found to be important for cell repopulation [12,41]. In addition, Aronovich and co-workers reported that TR ablation reduced bone mass in adult female, but not newborn mice [41]. Taken together with our finding of a high bone mass phenotype in TR-KO adult male mice, the role of TR may be dependent on gender and developmental stage. Of note, a functional androgen response element is located in the promoter region of TR, which is ubiquitously expressed in bone-derived cancer cells [42,43]. In fact, our preliminary study revealed that TR deficiency had no effect on both trabecular and cortical μCT parameters in female mice (data not shown). The roles of sex hormones on TR-associated bone remodeling still need to be elucidated in future studies. Interestingly, thrombin/TR was found to be involved with osteoblast differentiation, which is comprised of three overlapping phases: collagenous phase (synthesis of collagen type 1, a major component of ECM), non-collagenous phase (osteonectin and ALP expression), and mineralizing phase (osteocalcin expression) [14,44]. In vitro experiment showed that thrombin inhibited ALP activity in primary osteoblast-like cells through TR, indicating the negative effect of thrombin on the non-collagenous phase [14]. Accordingly, the results in the present study suggested that lower concentration of urinary DPD crosslinks and increased BMD might be due to the enhanced collagenous and mineralizing phases in the absence of TR. In other words, it could be extrapolated that thrombin/TR negatively influences all steps of bone formation by osteoblasts. On the other hand, RANKL synthesis and OPG synthesis were independent of the osteoblast differentiation stages, but rather relied on the presence of an intact TR/p42/44-ERK signaling system [44].

The serine proteases can exert their physiological effects via various signaling pathways including p42/44-ERK, p38-MAPK, PKC, and Rac1 [15,16,31,45,46]. Upon stimulation by thrombin; however, TR conveys its signal via either PKC through G91/G92 or p42/44-ERK through G13/G11 [16,47–50]. Based on our finding that thrombin stimulated RANKL expression through p42/44 ERK signaling, it was thus presumed in the present study that TR mediated its effect via G13/G11. An activation of p42/44-ERK signaling by other serine proteases demonstrated differential effects on osteoblast activity. Urokinase, through urokinase receptor/p42/44-ERK, inhibited bone-forming osteoblast activity whereas plasmin induced OPG expression and preserved normal BMD [30,31]. Of note, thrombin has been reported to stimulate IL-6 synthesis in osteoblasts through the p42/44-ERK pathway [15]. IL-6 was a potent stimulator of RANKL production and surface membrane expression [29,51]. Therefore, in the present study, thrombin/TR might also indirectly raise the RANKL/OPG ratio in osteoblasts through IL-6.

Many proteases including thrombin are synthesized as inactive precursors. Prothrombin mainly produced in parenchymal cells of the liver is converted to active thrombin by coagulation factor Xa (FXa) [52]. Recently, Karlstrom and colleagues reported that prothrombin was also expressed in osteoclasts as well as in the metaphyseal trabecular bone and to a lesser extent, in cortical bone [7]. The highest expression of prothrombin in the subepiphysis, the bone-growing area, might explain our observation of longer femurs in TR KO mice in such a way that TR served as bone growth suppressor [7]. This research group also demonstrated interplay between RANKL and FXa in osteoclasts that, in a co-culture of the monocyte/macrophage cells, RANKL induced the precursor differentiation into mature osteoclast phenotypes and the prothrombinase activity of FXa [53]. This finding suggested a positive feedback of thrombin synthesis from osteoclasts, which in turn exerted its stimulatory effect on osteoblasts as observed in the present study. In addition to the effect on osteoclast maturation presented in the current study, thrombin has been found to recruit non-adherent osteoclast precursors and inhibit osteoclast differentiation, but these effects were TR-independent [10,11]. Moreover, thrombin could not directly inhibit osteoclast differentiation unless bone marrow cells were primed with PTH and 1,25(OH)2D3 [10]. Therefore, we postulate here that thrombin activates osteoclastogenesis by majorly inducing RANKL synthesis and inhibiting OPG production from osteoblasts in a TR-dependent manner.

Apart from bone, thrombin and TR are also expressed in the intestine [27,54]. Therefore, an excessive BMD in TR-deficient bone might be taken up from Ca2+ hyperabsorption in the gastrointestinal tract. However, intestinal Ca2+ absorption signifies with fecal Ca2+ excretion appeared normal in TR KO mice. Moreover, in vitro data indicates that thrombin had no effect on Ca2+ transport as well as electrical parameters across Caco-2 cells, suggesting that both transcellular Ca2+ absorption and paracellular Ca2+ absorption were unaffected. These data are
in line with a previous finding that an activation of PAR-2, but not PAR-1 (TR) changed intestinal paracellular permeability and ion transport [55]. Further study is needed to define the source of Ca^{2+} deposited in TR-deficient bone.

In conclusion, the thrombin/TR system regulates bone remodeling by stimulating RANKL synthesis in osteoblasts via a p42/44 ERK signaling pathway. Consequently, RANKL/RANK binding induces osteoclast maturation and concomitant bone resorption. In TR KO mice, the lack of TR causes a decrease in RANKL and partially increases OPG secretion from osteoblasts, thereby retarding proper osteoclastogenesis, which results in a high bone mass phenotype (see Fig. 8). We suggest that TR is a kind of ‘gate keeper’ for RANKL production during osteoblast differentiation.

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References


Fig. 8. Cartoon postulating the molecular mechanism of thrombin/TR action on bone metabolism. In wild-type (WT) mice, TR is expressed on osteoblasts, of which precursors secrete receptor activator of nuclear factor-κB ligand (RANKL), an osteoclast-activating factor (green line); and by osteoblasts themselves osteoprotegerin (OPG), an anti-maturation factor of osteoclasts (red line). In WT mice, RANKL/OPG secretion ratio was in balance. In TR-deficient mice, the lack of TR causes a reduction in RANKL and an upregulation of OPG secretion from osteoblasts and impairs osteoclastogenesis and bone resorative capacity. This eventually results in an increase in bone mass. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)


