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Enhanced mineralization potential of vascular cells from *SM22 α -Rankl^{tg}* mice

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Abstract

Vascular calcification, prevalent in diabetes and chronic kidney disease, contributes to morbidity and mortality. To investigate the effect of receptor activator of NF- κ B ligand (RANKL) on vascular calcification in vivo, transgenic mice, where RANKL expression was targeted to vascular smooth muscle cells using SM22 α promoter (*SM22 α -Rankl^{tg}*), was created. Sixteen-month-old male *SM22 α -Rankl^{tg}* mice had higher body weight and higher serum calcium levels but lower lumbar bone mineral density (BMD) compared with age- and gender-matched WT littermates. BMD of long bones, body fat (% of weight) of the leg, and serum levels of phosphate and RANKL were not significantly different. No significant differences in these parameters were observed in the female mice. Histological analysis did not reveal calcium deposits in the aortic roots of the *SM22 α -Rankl^{tg}* mice. To analyze the osteoblastic differentiation and mineralization potentials of vascular cells, aortic smooth muscle cells (SMCs) were isolated and cultured. Results showed that *SM22 α -Rankl^{tg}* SMCs had higher baseline alkaline phosphatase (ALP) activity, but not the baseline matrix calcification. When induced by the PKA agonist, forskolin, ALP activity was greater in *SM22 α -Rankl^{tg}* than in WT SMCs. Realtime RT-qPCR revealed higher baseline expression of ALP and ankylosis genes, but lower osteoprotegerin gene, in *SM22 α -Rankl^{tg}* SMCs. Matrix mineralization induced by inorganic phosphate or forskolin was greater in *SM22 α -Rankl^{tg}* than in WT SMCs. Treatment of these cells with the ALP inhibitor, levamisole, abolished forskolin-induced matrix mineralization but not Pi-induced matrix mineralization. These findings suggest that RANKL overexpression in the vasculature may promote mineralization potential.

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Keywords

RANKL; smooth muscle cells; vascular; calcification; SM22 alpha

INTRODUCTION

Vascular calcification is present in half of middle-aged individuals and in 90% of those over age 70 [1]. It particularly affects patients with atherosclerosis, diabetes, end-stage renal disease, and other metabolic disorders [2]. It dramatically alters vascular physiology, and key molecular regulators need to be identified to develop therapeutic approaches. We previously developed an in vitro model in which aortic cells undergo osteogenic differentiation with matrix mineral formation [3]. Using both in vitro cell culture and in vivo studies, we and others have demonstrated that many of the same factors that modulate bone formation and resorption, including cytokines and hormones, also modulate vascular calcification [4–9].

Two cytokines, receptor activator of NF- κ B ligand (RANKL) and its soluble decoy receptor, osteoprotegerin (OPG), have a central role in controlling differentiation of osteoclasts [10]. Their potential role in vascular calcification is evidenced by the unexpected finding of extensive aortic mineralization in mice deficient in OPG [11]. Since the absence of OPG results in unopposed activity of RANKL, it suggests that RANKL may promote vascular calcification. In support of this, calcified arteries are known to have higher levels of RANKL expression and lower levels of OPG [12, 13]. However, other findings suggest a more complex picture. Epidemiological studies show vascular calcification is inversely related to serum RANKL and positively related to serum OPG [14–18]. Moreover, some in vitro studies showed that RANKL treatment induced vascular calcification [19, 20], whereas we and others found no effect [21, 22], albeit in different species and culture conditions.

In previous studies with RANKL transgenic mice, the overexpression was global or the distribution systemic. Mice with ubiquitous RANKL overexpression die at the late fetal stage [23]. Mice with overexpression of soluble RANKL, released from the liver, have osteoporosis [23]. These models would not be suitable for studying the role of RANKL on vascular calcification, since bone and vascular mineralization share many endocrinological mechanisms, and since excess resorption in bone may cause spontaneous mineral deposition in other tissues, including arteries. Thus, in the present study, we targeted the expression of RANKL to vascular smooth muscle cells (*SM22 α -Rankl^{f/f}*). Results showed no effect of vascular-specific RANKL overexpression on basal levels of calcification but enhancement of osteoblastic differentiation and matrix mineralization in vascular cells treated with inorganic phosphate and a PKA agonist, forskolin.

METHODS

Generation of Rankl^{tg} mouse

The promoter sequence for the SM22alpha gene was PCR amplified from pSM1343-luciferase vector (1344 bps of the 5' promoter and 62 base pairs of intron 1; kindly provided by Dr. Eric Olson, UT Southwestern) into pCR-Script vector, kindly provided by Dr. Scott Simonet (Amgen Inc.). The first coding sequence (CDS1) of the full-length (membrane-bound) murine RANKL cDNA (951 bp) was then sub-cloned into the newly constructed SM22alpha promoter transgenic expression vector [24]. The sequences of the sub-cloned SM22 α and *Rankl* gene were confirmed by direct and reverse strand sequencing and compared with the published GeneBank entries (SM22 α promoter U36589; RANKL AF053713). Gel-purified linearized transgenic constructs were microinjected into the male

pronucleus of fertilized C57BL/6 one-cell oocytes. Surviving oocytes were subsequently transplanted into the oviducts of pseudopregnant foster mothers. Transgenic founder mice (on a C57BL/6 background) were identified by PCR analysis of genomic DNA isolated from tail biopsies. To minimize variability, we used a line with high penetrance for subsequent studies. The relative copy number of the transgene was determined to be approximately 14–16 (Charles River, New York). Animals were fed a normal chow diet ad lib. At necropsy, blood, heart, aorta, and long bones were isolated. The experimental protocols were reviewed and approved by the Office of Animal Research Oversight of the University of California at Los Angeles and the Institutional Animal Care and Use Committee at Amgen Inc.

Bone mineral density

Bone mineral density (BMD) was determined in vivo using dual energy x-ray absorptiometry (DXA; PIXImus II, GE Lunar, Madison, WI, USA), with lumbar vertebrae (LV1–LV5) and whole leg subregion analyses.

Histological analysis

Aortic root specimens were embedded in OCT and cryosectioned at 10 μ m. The sections were stained for mineralization by the von Kossa silver nitrate method and counterstained with eosin. Immunohistochemical staining was performed using anti-RANKL antibody (R&D Systems).

Serum biochemical assays

Serum RANKL was measured by ELISA (R&D Systems) following the manufacturer's protocol. Serum calcium and phosphate levels were determined using a Hitachi 717 auto-analyzer (Roche Diagnostics, Indianapolis IN).

Cell culture

Murine aortic cells (passages 6–10) were isolated from the aortas of C57BL6 (WT) and *SM22 α -Rankl^{tg}* mice as described previously [25]. Cells were maintained in DMEM containing 20% fetal bovine serum (FBS). One day after plating, cells were treated with forskolin (25 μ M) or inorganic phosphate (3 mM) in MEM media supplemented with 10% FBS. β -glycerophosphate (5 mM) was supplemented in control media and in media with forskolin.

Matrix calcium quantitation

After the indicated periods, matrix calcium levels were analyzed by the α -cresolphthalein complexone method (Teco Diagnostics, Anaheim, CA). Each condition was assayed in quintuplicate and normalized to total protein using the Bradford method [25].

Alkaline phosphatase activity

After the indicated periods, alkaline phosphatase activity was assessed colorimetrically using Sigma 104 Phosphatase Substrate (Sigma St. Louis, MO). Each condition was assayed in quintuplicate and normalized to total protein using the Bradford method [25].

Gene expression

Total RNA was isolated from cells using TRIzol reagent (Invitrogen Carlsbad, CA). Realtime RT-qPCR was performed using 1-step qRT-PCR (Biochain Institute, Inc. Hayward, CA) in the Mx3005P (Stratagene La Jolla, CA). β -actin was used for normalization.

Data analysis

Each experiment was performed in at least quadruplicate wells and repeated at least three times ($n = 3$). Data are expressed as mean \pm SEM. Student's *t*-test was used for comparison between two groups. For more than two groups, mean values were compared using one-way ANOVA, with comparison of different groups by Fisher's protected least significant difference test. A value of $p < 0.05$ was considered significant.

RESULTS

SM22 α -Rankl^{tg} mice

To test the effects of RANKL on vascular calcification specifically, we created RANKL transgenic mice under the control of the *SM22 α* promoter (*SM22 α -Rankl^{tg}*). Li et al., showed that *SM22* expression in vivo is highly restricted to vascular SMCs in adult tissues [24], and Lepore et al. showed that expression via this promoter is in arterial SMCs as well as in cardiac, but not in skeletal, myocytes [26]. Western blot analysis showed a band with the expected molecular weight of 35 kDa only in the aortic lysates from the *SM22 α -Rankl^{tg}* mice (lower band, Fig 1A). Consistent with the findings of Lepore et al. [26], transgene expression was observed in SMCs and cardiomyocytes (Fig. 1A). There were no obvious phenotypes in the founders, and, in the male and female mice of the F1 generation, no vascular calcification was detected by DXA analysis (data not shown). Histological analyses by von Kossa staining of aortic sections from 16-month-old *SM22 α -Rankl^{tg}* mice also showed no calcium deposits (Fig 1B).

To test whether vascular-specific overexpression of RANKL affected circulating levels, serum levels of RANKL were assessed. Results showed that serum RANKL levels were not increased in the *SM22 α -Rankl^{tg}* compared with WT mice (Fig. 1C). As observed in humans, female mice had higher serum RANKL levels than male mice [27]. In male *SM22 α -Rankl^{tg}* mice, serum RANKL was found to be lower than in WT littermates (Fig. 1C).

We also assessed the effects of vascular-specific RANKL expression on body weight, percent fat, and bone mineral density of long bones and vertebrae. Although no weight difference was detected in female mice, male *SM22 α -Rankl^{tg}* weighed more than WT mice at 16 months of age (Fig 2A). Body fat of the leg was not changed in either male or female mice (Fig 2B). In female mice, serum calcium, phosphorus levels and BMD were not altered compared with WT mice (Fig 2C, Fig 3). In male *SM22 α -Rankl^{tg}* mice, although serum RANKL levels were lower, serum calcium levels were higher than in WT mice (Fig. 2C). BMD of lumbar vertebrae, but not of long bones or tibial metaphyses, were less in male *SM22 α -Rankl^{tg}* than in WT mice (Fig 3).

SM22 α -Rankl^{tg} SMCs

We further evaluated osteogenic activity of aortic SMCs from *SM22 α -Rankl^{tg}* mice. Results showed that aortic SMCs from *SM22 α -Rankl^{tg}* mice had substantially higher baseline alkaline phosphatase activity than WT cells at 2 and 4 days of culture (1.2 ± 0.04 vs. 0.09 ± 0.02 , $p < 0.001$ on day 2; 2.02 ± 0.05 vs. 0.16 ± 0.03 , $p < 0.001$ on day 4; Fig 4A). When induced using the PKA agonist, forskolin, ALP activity was induced in both *SM22 α -Rankl^{tg}* and WT SMCs, with a much greater induction in *SM22 α -Rankl^{tg}* than in WT SMCs (14 ± 4 to 103 ± 9 vs. 2 ± 0.3 to 46 ± 2 , $p < 0.05$; Fig 4B). Realtime RT-qPCR revealed that baseline expression levels of ALP and ankylosis genes were approximately 5-fold and 2-fold greater in *SM22 α -Rankl^{tg}* compared with WT SMCs; whereas that of OPG was lower (0.4-fold; Fig 4C). Expression levels of Enpp1, *SM22 α* , and Pit-1 were not significantly different between the groups (data not shown).

When induced by inorganic phosphate or forskolin, matrix calcium levels were greater in *SM22 α -Rankl^{f/g}* than in WT SMCs (Fig 5A). Treatment with an alkaline phosphatase inhibitor, levamisole, abolished forskolin-induced matrix mineralization but not Pi-induced matrix mineralization (Fig 5B–C).

DISCUSSION

The RANKL/RANK/OPG axis regulates differentiation of resorptive osteoclasts in bone. RANKL has been implicated in vascular calcification in some studies [19, 20, 28] but not others [21, 22, 29]. These differences may relate to different model systems and cell types. Previous efforts to elucidate its role in vascular calcification in vivo used a transgenic mouse model overexpressing soluble RANKL released from the liver [24]. However, this model was confounded by RANKL induction of skeletal resorption, which led to levels of serum calcium and phosphate capable of inducing spontaneous soft tissue calcification. To overcome this confounding effect, in the present study, we generated transgenic mice with vascular-specific expression of RANKL driven by the SM22 promoter. As evidence that this model provided adequate specificity, there was no significant difference in serum RANKL levels, suggesting that vascular-specific overexpression of the membrane-bound RANKL overcame the confounding effect. We found that these *SM22 α -Rankl^{f/g}* mice did not develop spontaneous vascular calcification.

In cell culture, *SM22 α -Rankl^{f/g}* SMCs showed significantly greater baseline alkaline phosphatase gene expression and activity than WT SMCs. However, this increase was not sufficient to cause matrix calcification. Possible explanations include that the levels of ALP activity were below the threshold required for calcification, and/or that the increase in ALP activity was overcome by even greater increase in pyrophosphate, an inhibitor of mineralization which is 1,000-fold more potent an inhibitor than Pi is an activator. The latter possibility is supported by our finding of a simultaneous increase in expression of Ank, the membrane-bound channel responsible for pyrophosphate transport, which may compensate for the increase in ALP. *SM22 α -Rankl^{f/g}* SMCs did produce more matrix mineral in response to the procalcific factors, inorganic phosphate and forskolin, and these findings probably have distinct mechanisms. The response to inorganic phosphate is considered independent of ALP activity, whereas the response to forskolin is considered dependent on ALP activity (Fig 6). The mechanism for reduced expression of OPG is not clear. However, given that *Opg^{-/-}* mice develop substantial vascular calcification, this fall in OPG may have a role in our observed potentiation of forskolin- and Pi-induced mineralization.

Since, in our previous studies, exogenous (soluble) RANKL showed no induction of mineralization in cultured SMCs, there may be a different mechanism for the action of endogenously expressed RANKL in *SM22 α -Rankl^{f/g}* SMCs. Another possible explanation for the different effects of exogenous vs. transgenic RANKL is that intracellular RANKL may have a novel function in osteoblastic differentiation. Such a phenomenon was reported for intracellular Enpp1, which when altered by overexpression or shRNA, regulated osteoblastic differentiation of calvarial osteoblastic cell line, in a manner independent of its catalytic activity [30].

Interestingly, we found effects on serum RANKL levels, body weight, and bone density of lumbar vertebrae that appeared to be gender specific. The gender differences may be due to effects of estrogen, which has been shown to inhibit RANKL signaling [28]. Gender differences in serum RANKL in our mice are also consistent with the gender differences reported in human studies, with levels almost twice as high in women as in men [27, 31]. Serum RANKL levels are also influenced by female hormonal cycling, with levels higher in the follicular vs. luteal phases [32]. Estrogen is also known to regulate RANKL expression

in osteoblasts [33] and in peripheral blood mononuclear cells in human [34]. The increased body weight observed in male mice does not appear to be due to skeletal effects, since BMD was decreased rather than increased. It also does not appear to be due to circulating RANKL levels since the levels were reduced. However, the body fat showed a non-significant trend toward an increase, which suggests a role of RANKL interactions with neuro-endocrine activation [35].

Although *SM22a-Rankl^{tg}* mice do not develop spontaneous vascular calcification, it is possible that these mice may develop greater vascular calcification than WT mice when exposed to pro-calcific interventions, such as uremia, renal insufficiency, and/or hyperlipidemia, especially in light of our in vitro finding that transgenic SMCs produce more calcium mineral than WT SMCs in the presence of pro-calcific factors.

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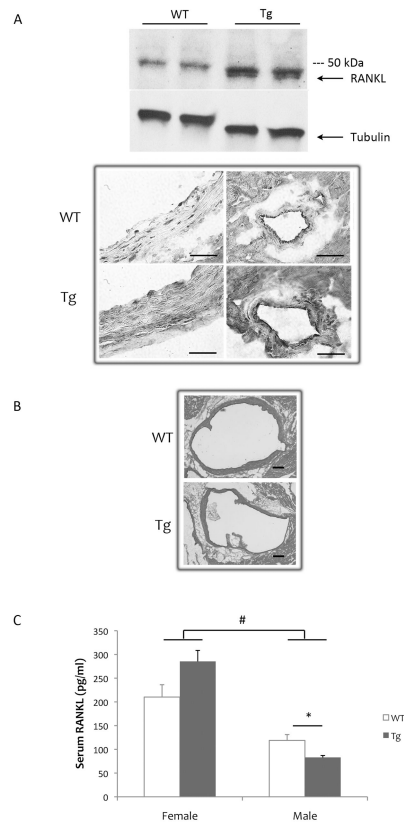


Figure 1. RANKL expression in *SM22 α -Rankl^{tg}* mice

(A) Upper panel: Western analysis of aortas from 2.5-month-old male mice with tubulin as a loading control. Lower panel: RANKL immunohistochemistry of aorta (left) and intramyocardial coronary arteries (right) of WT and Tg mice. As expected for this promoter, cardiomyocytes also express the transgene. Scale bar 50 μ . (B) Von Kossa histochemistry staining of aortic root sections for calcium mineral. Scale bar: 200 μ (C) Levels of serum RANKL assessed by ELISA (n = 6/group). * p < 0.05; # p < 0.001.

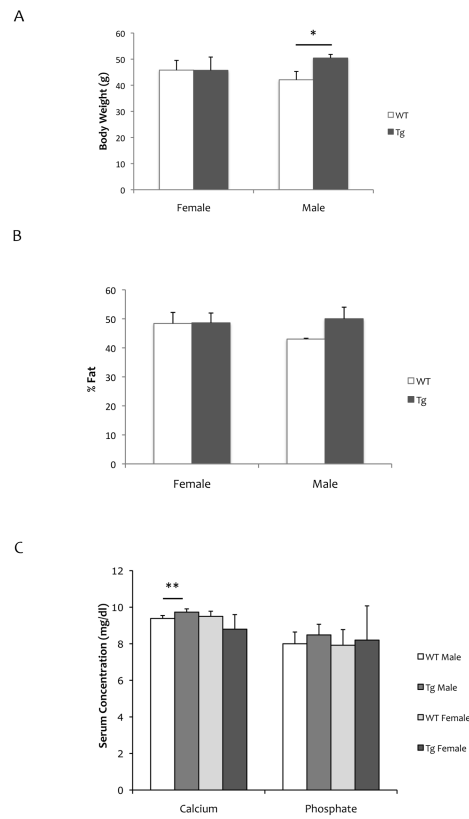


Figure 2. Physical and biochemical properties of *SM22 α -Rank1^g* mice (A) Body weight, (B) percent body fat of the whole leg, and (C) serum calcium and phosphate levels in 16-month old mice (n = 6/group). * p = 0.05; ** p < 0.01.

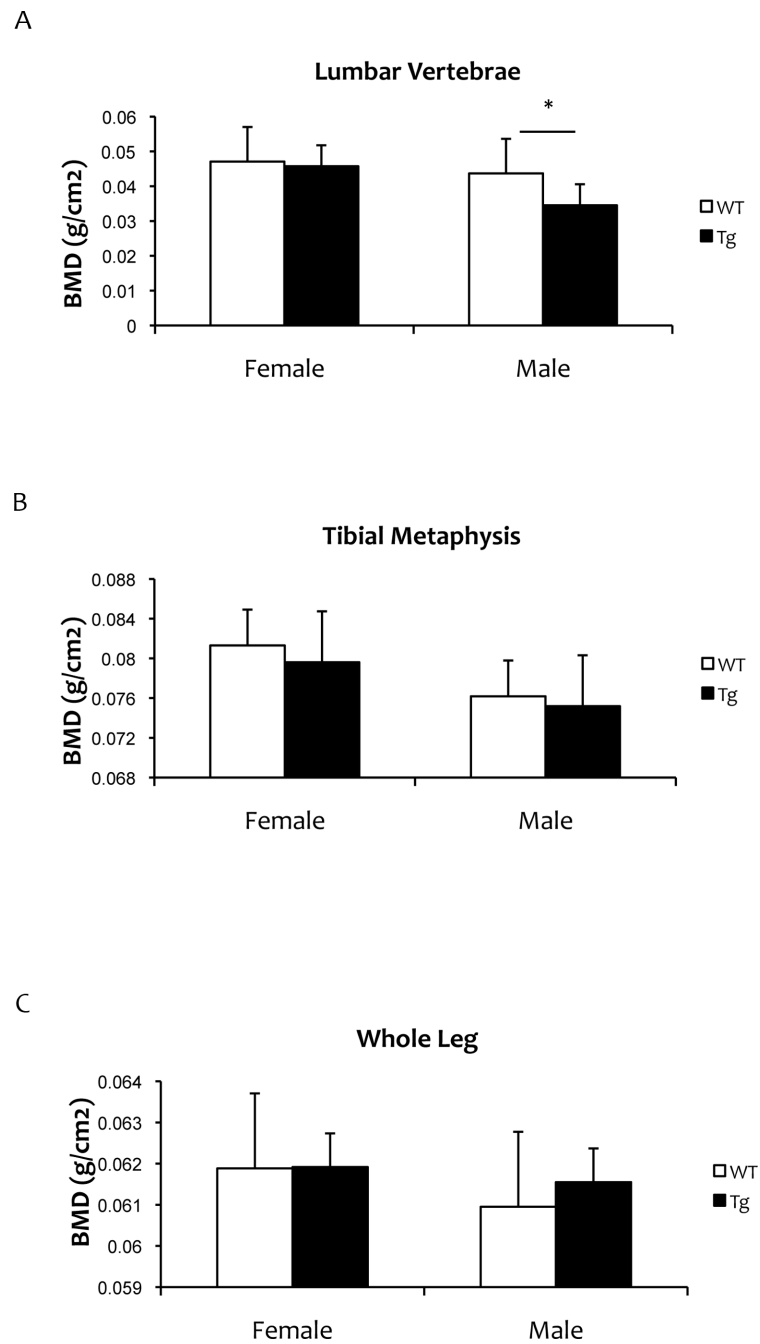


Figure 3. Bone mineral density of *SM22 α -Rank1^{tg}* mice
 Dual energy x-ray absorptiometry analysis of 12-month-old mice (n = 6/group). (A) Lumbar vertebrae, (B) tibial metaphyses, and (C) whole leg. *p < 0.05.

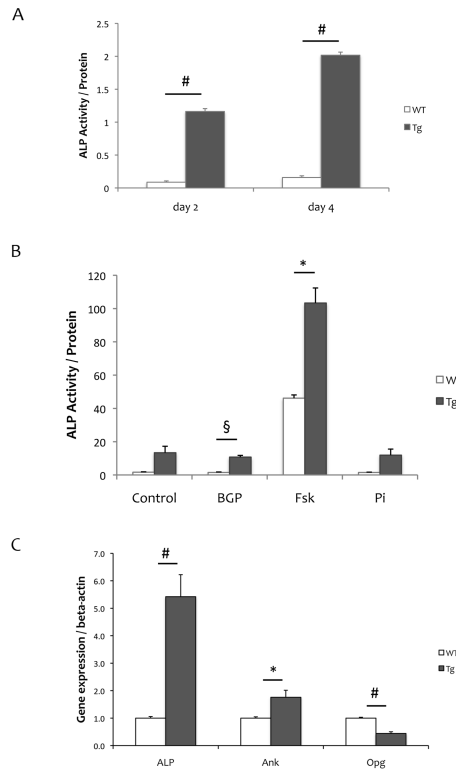


Figure 4. Osteoblastic differentiation of SM22 α -*Rankl*^{tg} SMCs

(A) Alkaline phosphatase activity, normalized for total protein, in cell lysates from *Rankl*^{tg} and WT SMCs cultured for 2 and 4 days. (B) ALP activity, normalized for total protein, in *Rankl*^{tg} and WT SMCs treated with control vehicle, beta-glycerophosphate (BGP, 5 mM), forskolin (Fsk, 25 μ M), or inorganic phosphate (Pi, 3 mM) for 4 days. (C) Realtime RT-qPCR analysis of RNA isolated from *Rankl*^{tg} and WT SMCs cultured for 7 days. *p < 0.05; §p < 0.01; #p < 0.001.

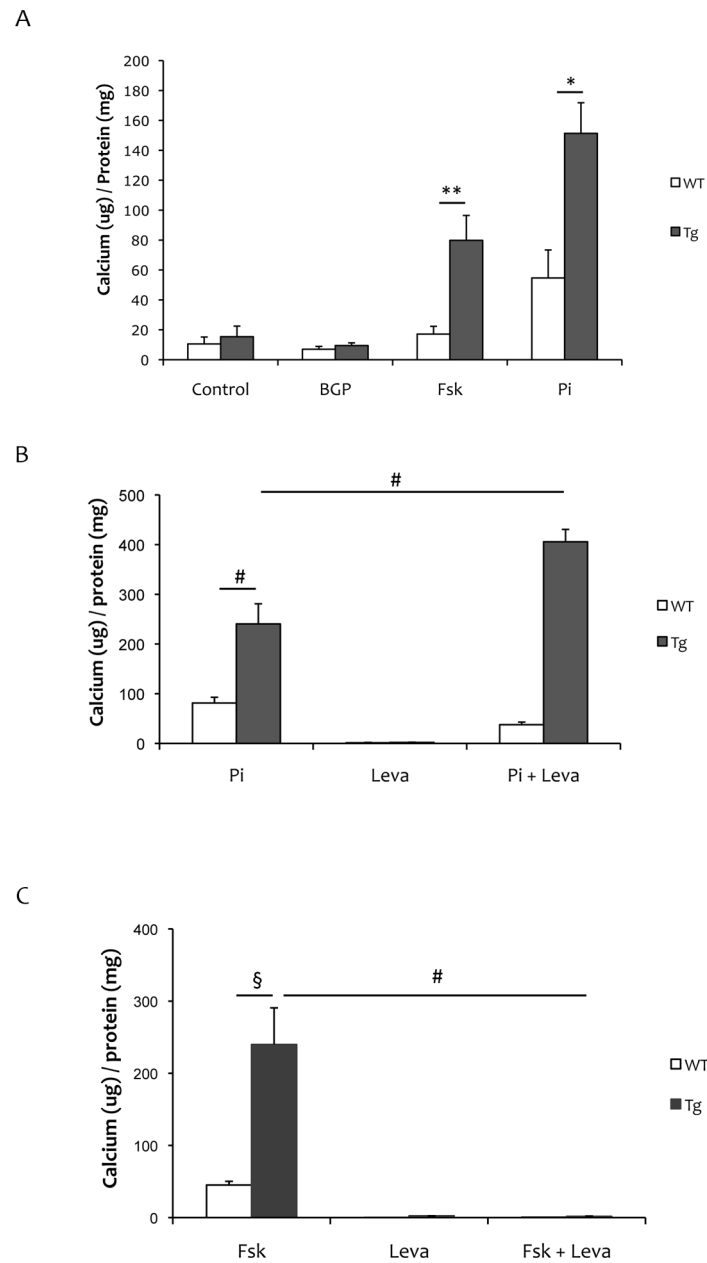


Figure 5. Matrix mineralization of SM22 α -*Rank1*^{tg} SMCs

(A) Matrix calcium, normalized for total protein, in *Rank1*^{tg} and WT SMCs treated with control vehicle, beta-glycerophosphate (BGP, 5 mM), forskolin (Fsk, 25 μ M) or inorganic phosphate (Pi, 3 mM) for 7 days. (B) Matrix calcium, normalized for total protein, in *Rank1*^{tg} and WT SMCs treated with inorganic phosphate (Pi, 3 mM), and/or levamisole (Leva, 100 μ M) for 10 days. (C) Matrix calcium, normalized for total protein, in *Rank1*^{tg} and WT SMCs treated with forskolin (Fsk, 25 μ M) and/or levamisole (Lev, 100 μ M) for 13 days. ** $p = 0.05$; * $p < 0.05$; § $p < 0.01$; # $p < 0.001$.