ORIGINAL RESEARCH

Enhanced Mineralization Potential of Vascular Cells from $SM22\alpha$-$Rankl^{tg}$ 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-kB ligand (RANKL) on vascular calcification in vivo, transgenic mice, where RANKL expression was targeted to vascular smooth muscle cells using the SM22α promoter ($SM22\alpha$-$Rankl^{tg}$), were created. Sixteen-month-old male $SM22\alpha$-$Rankl^{tg}$ mice had higher body weight and higher serum calcium levels but lower lumbar bone mineral density (BMD) compared with age- and gender-matched wild-type (WT) littermates. BMD of long bones, body fat (percent 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 female mice. Histological analysis did not reveal calcium deposits in the aortic roots of $SM22\alpha$-$Rankl^{tg}$ mice. To analyze the osteoblastic differentiation and mineralization potentials of vascular cells, aortic smooth muscle cells (SMCs) were isolated and cultured. Results showed that $SM22\alpha$-$Rankl^{tg}$ SMCs had higher baseline alkaline phosphatase (ALP) activity but not baseline matrix calcification. When induced by the PKA agonist forskolin, ALP activity was greater in $SM22\alpha$-$Rankl^{tg}$ than in WT SMCs. Real-time RT-qPCR revealed higher baseline expression of ALP and ankylosis genes but lower osteoprotegerin gene in $SM22\alpha$-$Rankl^{tg}$ SMCs. Matrix mineralization induced by inorganic phosphate or forskolin was greater in $SM22\alpha$-$Rankl^{tg}$ than in WT SMCs. Treatment of these cells with the ALP inhibitor levamisole abolished forskolin-induced matrix mineralization but not inorganic phosphate-induced matrix mineralization. These findings suggest that RANKL overexpression in the vasculature may promote mineralization potential.

Keywords RANKL · Smooth muscle cell · Vascular · Calcification · SM22α

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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-κB ligand (RANKL) and its soluble decoy receptor osteoprotegerin (OPG), have a central role in controlling the 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, 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 that 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 in vascular calcification since bone and vascular mineralization share many endocrinological mechanisms and 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 (SMCs) in mice (SM22α-Rankl^{tg}). The results showed no effect of vascular cell-specific RANKL overexpression on basal levels of calcification but enhancement of osteoblastic differentiation and matrix mineralization in vascular cells treated with inorganic phosphate (Pi) and the PKA agonist forskolin.

Methods

Generation of the Rankl^{tg} Mouse

The promoter sequence for the SM22α gene was PCR-amplified from pSM1343-luciferase vector (1,344 bp of the 5′ promoter and 62 bp of intron 1; kindly provided by Dr. Eric Olson, University of Texas Southwestern Medical Center, Dallas, TX) into pCR-Script vector (kindly provided by Dr. Scott Simonet, Amgen). The first coding sequence (CDS1) of the full-length (membrane-bound) murine RANKL cDNA (951 bp) was then subcloned into the newly constructed SM22α promoter transgenic expression vector [24]. The sequences of the subcloned SM22α and Rankl gene were confirmed by direct and reverse-strand sequencing and compared with the published GeneBank entries (SM22α 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, Troy, NY). 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.

Bone Mineral Density

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

Histological Analysis

Aortic root specimens were embedded in OCT and cryosectioned at 10 μm. 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, Minneapolis, MN).

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 autoanalyzer (Roche Diagnostics, Indianapolis, IN).

Cell Culture

Murine aortic cells (passages 6–10) were isolated from the aortas of C57BL6 [wild-type (WT)] and SM22α-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 μM) or Pi (3 mM) in αMEM supplemented with 10 % FBS. β-Glycerophosphate (5 mM) was supplemented in control medium and in medium with forskolin.

Matrix Calcium Quantitation

After the indicated periods, matrix calcium levels were analyzed by the *o*-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 (ALP) 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). Real-time RT-qPCR was performed using a one-step method (Biochain Institute, 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. 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. $p \leq 0.05$ was considered significant.

Results

SM22α-Rankl^{tg} Mice

To test the effects of RANKL on vascular calcification specifically, we created RANKL transgenic mice under the control of the SM22α promoter (*SM22α -Rankl^{tg}*). Li et al. [24] showed that SM22α expression in vivo is highly restricted to vascular SMCs in adult tissues, and Lepore et al. [26] showed that expression via this promoter is in arterial SMCs as well as in cardiac, but not in skeletal, myocytes. Western blot analysis showed a band with the expected molecular weight of 35 kDa only in the aortic lysates from *SM22α-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 male and female mice of the F_1 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 *SM22α-Rankl^{tg}* mice also showed no calcium deposits (Fig. 1b).

To test whether vascular cell-specific overexpression of RANKL affected circulating levels, serum levels of RANKL were assessed. Results showed that serum RANKL levels were not increased in *SM22α-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 *SM22α-Rankl^{tg}* mice, serum RANKL was found to be lower than in WT littermates (Fig. 1c).

We also assessed the effects of vascular cell-specific RANKL expression on body weight, percent fat, and BMD of long bones and vertebrae. Although no weight difference was detected in female mice, male *SM22α-Rankl^{tg}* mice 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 and phosphorus levels and BMD were not altered compared with WT mice (Figs. 2c, 3). In male *SM22α-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, was less in male *SM22α-Rankl^{tg}* than in WT mice (Fig. 3).

SM22α-Rankl^{tg} SMCs

We further evaluated the osteogenic activity of aortic SMCs from *SM22α-Rankl^{tg}* mice. The results showed that aortic SMCs from *SM22α-Rankl^{tg}* mice had substantially higher baseline ALP 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 *SM22α-Rankl^{tg}* and WT SMCs, to a higher level in *SM22α-Rankl^{tg}* than in WT SMCs (14 ± 4–103 ± 9 vs. 2 ± 0.3–46 ± 2, $p < 0.05$; Fig 4b). Real-time RT-qPCR revealed that baseline expression levels of ALP and ankylosis genes were approximately fivefold and twofold greater in *SM22α-Rankl^{tg}* compared with WT SMCs, respectively, whereas that of OPG was lower (0.4-fold) (Fig. 4c). Expression levels of Enpp1, *SM22α*, and Pit-1 were not significantly different between the groups (data not shown).

Fig. 1 RANKL expression in *SM22α-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 wild-type (*WT*) and transgenic (*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). $*p < 0.05$, $^{\#}p < 0.001$

When induced by Pi or forskolin, matrix calcium levels were greater in *SM22α-Rankl^tg* than in WT SMCs (Fig. 5a). Treatment with an ALP inhibitor, levamisole, abolished forskolin-induced, 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 [23]. However, this model was confounded by RANKL induction of skeletal resorption, which may lead to levels of serum 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 SM22α promoter. As evidence that this model provided adequate specificity, there was no significant difference in serum RANKL levels, suggesting that vascular cell-specific overexpression of membrane-bound RANKL overcame the confounding effect. We found that these *SM22α-Rankl^tg* mice did not develop spontaneous vascular calcification.

In cell culture *SM22α-Rankl^tg* SMCs showed significantly greater baseline ALP 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 may have been below the threshold required for calcification and/or that the increase in ALP activity may have been overcome by an 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. *SM22α-Rankl^tg* SMCs did produce more matrix mineral in response to the procalcific factors Pi and forskolin, and these findings probably have distinct
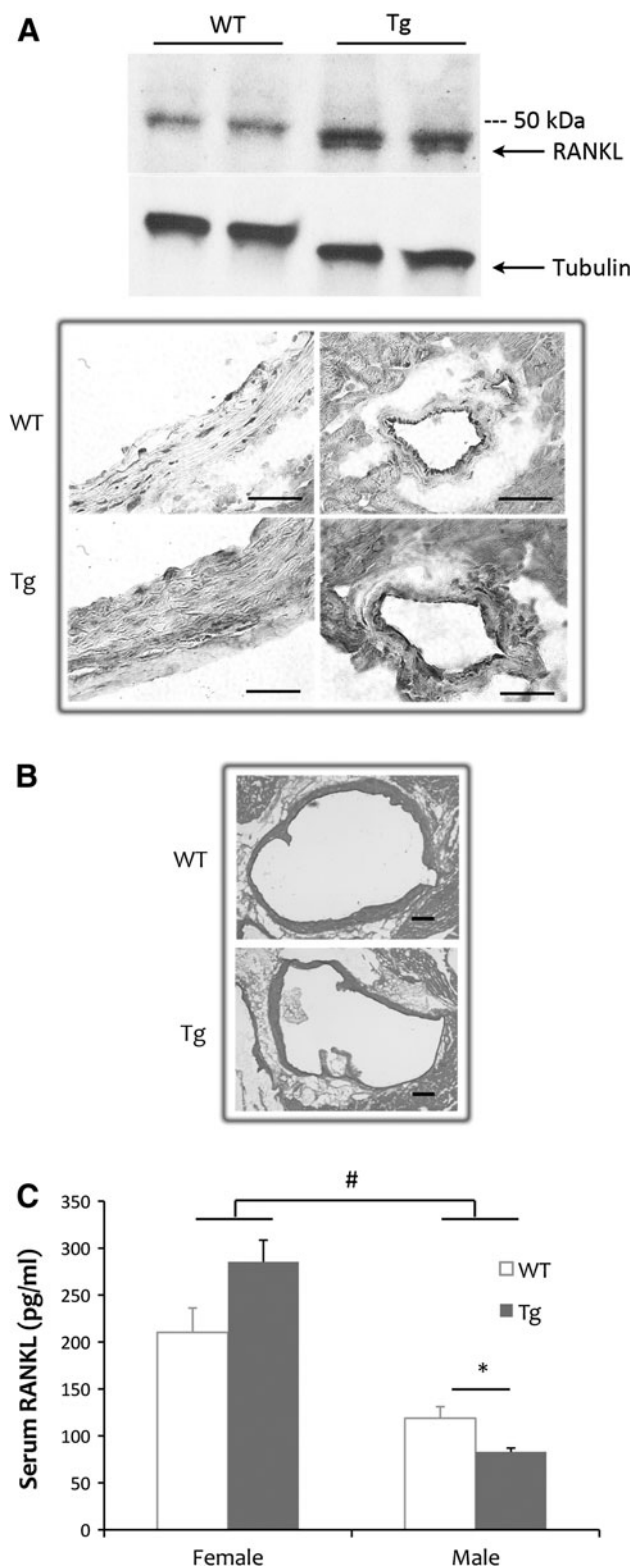
mechanisms. The response to Pi is considered independent of ALP activity, whereas the response to forskolin is considered dependent on ALP activity. The mechanism for reduced expression of OPG is not clear. However, given that *Opg^{-/-}* mice develop substantial vascular
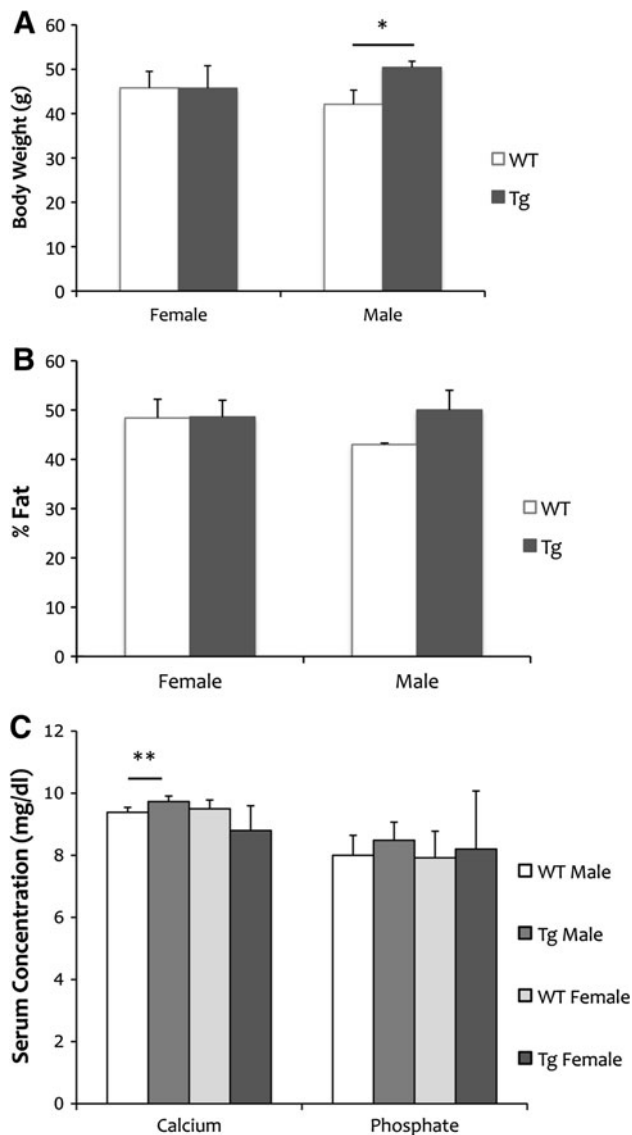
Fig. 2 Physical and biochemical properties of *SM22α-Rankl^tg* mice. Body weight (**a**), percent body fat of the whole leg (**b**), and serum calcium and phosphate levels (**c**) in 16-month-old mice (*n* = 6/ group). *p = 0.05, **p < 0.01

Fig. 3 Bone mineral density of *SM22α-Rankl^tg* mice. Dual-energy X-ray absorptiometric analysis of 12-month-old mice (*n* = 6/group). Lumbar vertebrae (**a**), tibial metaphyses (**b**), and whole leg (**c**). *p < 0.05

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 *SM22α-Rankl^tg* SMCs. Another possible explanation for the different effects of exogenous versus transgenic RANKL is that intracellular RANKL may have a novel function in osteoblastic differentiation. Such a phenomenon was reported for intracel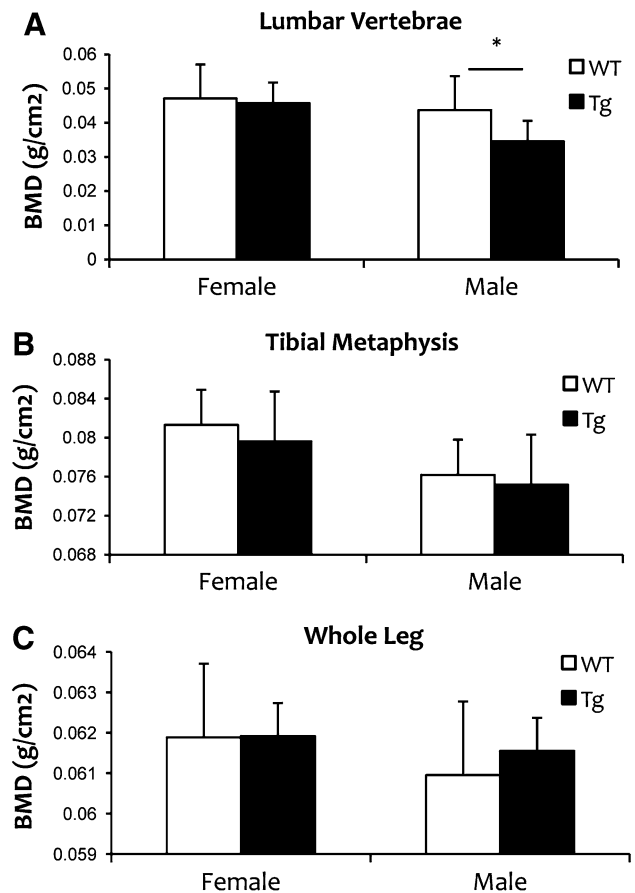lular Enpp1, which when altered by overexpression or shRNA, regulated osteoblastic differentiation of a calvarial osteoblastic cell line, in a manner independent of its catalytic activity [30].

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 phase [32]. Estrogen is also known to regulate RANKL expression in osteoblasts [33] and in peripheral blood mononuclear cells in humans [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, body fat showed a
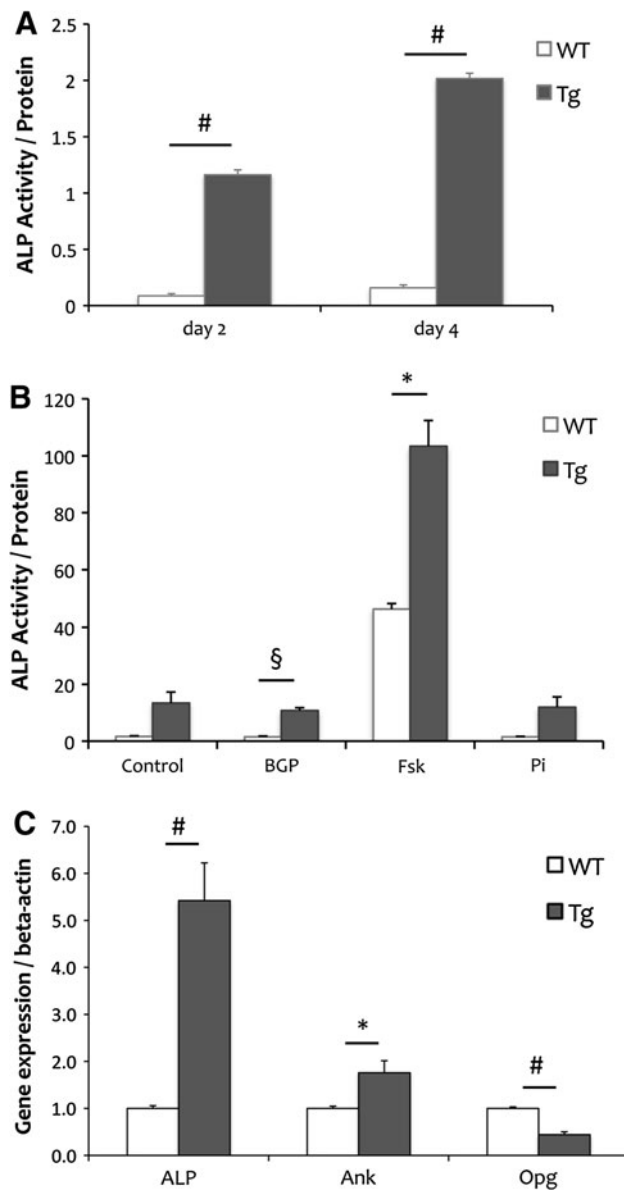
Fig. 4 Osteoblastic differentiation of *SM22α-Rankl^tg* SMCs. **a** Alkaline phosphatase (*ALP*) activity, normalized for total protein, in cell lysates from *Rankl^tg* and wild-type (*WT*) SMCs cultured for 2 and 4 days. **b** ALP activity, normalized for total protein, in *Rankl^tg* and WT SMCs treated with control vehicle, beta-glycerophosphate (*BGP*, 5 mM), forskolin (*Fsk*, 25 μM), or inorganic phosphate (*Pi*, 3 mM) for 4 days. **c** Real-time RT-qPCR analysis of RNA isolated from *Rankl^tg* and WT SMCs cultured for 7 days. $*p < 0.05$, $^§p < 0.01$, $^#p < 0.001$

Fig. 5 Matrix mineralization of *SM22α-Rankl^tg* SMCs. **a** Matrix calcium, normalized for total protein, in *Rankl^tg* and wild-type (*WT*) SMCs treated with control vehicle, beta-glycerophosphate (*BGP*, 5 mM), forskolin (*Fsk*, 25 μM), or inorganic phosphate (*Pi*, 3 mM) for 7 days. **b** Matrix calcium, normalized for total protein, in *Rankl^tg* and WT SMCs treated with Pi (3 mM) and/or levamisole (*Leva*, 100 μM) for 10 days. **c** Matrix calcium, normalized for total protein, in *Rankl^tg* and WT SMCs treated with Fsk (25 μM) and/or Leva (100 μM) for 13 days. $**p = 0.05$, $*p < 0.05$, $^§p < 0.01$, $^#p < 0.001$

nonsignificant trend toward an increase, which suggests a role of RANKL interactions with neuroendocrine activation [35].

Although *SM22α-Rankl^tg* mice do not develop spontaneous vascular calcification, it is possible that these mice develop greater vascular calcification than WT mice when exposed 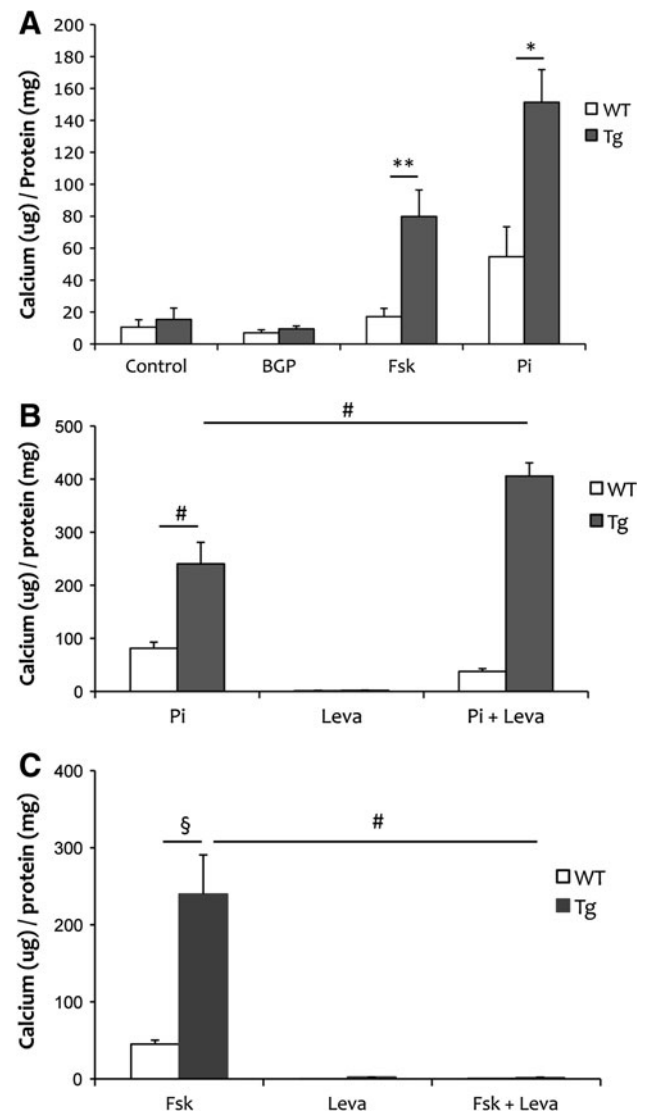to procalcific interventions, such as uremia, renal insufficiency, and/or hyperlipidemia, especially in light of our in vitro finding that transgenic SMCs produce more calcium mineral than WT SMCs in the presence of procalcific factors.

References

1. Newman AB, Naydeck BL, Sutton-Tyrrell K, Feldman A, Edmundowicz D, Kuller LH (2001) Coronary artery calcification in older adults to age 99: prevalence and risk factors. Circulation 104:2679–2684

2. Moe SM, Chen NX (2004) Pathophysiology of vascular calcification in chronic kidney disease. Circ Res 95:560–567

3. Tintut Y, Parhami F, Bostrom K, Jackson SM, Demer LL (1998) cAMP stimulates osteoblast-like differentiation of calcifying vascular cells. Potential signaling pathway for vascular calcification. J Biol Chem 273:7547–7553

4. Tintut Y, Patel J, Parhami F, Demer LL (2000) Tumor necrosis factor-alpha promotes in vitro calcification of vascular cells via the cAMP pathway. Circulation 102:2636–2642

5. Cheng SL, Shao JS, Halstead LR, Distelhorst K, Sierra O, Towler DA (2010) Activation of vascular smooth muscle parathyroid hormone receptor inhibits Wnt/beta-catenin signaling and aortic fibrosis in diabetic arteriosclerosis. Circ Res 107:271–282

6. Li X, Yang HY, Giachelli CM (2008) BMP-2 promotes phosphate uptake, phenotypic modulation, and calcification of human vascular smooth muscle cells. Atherosclerosis 199:271–277

7. Johnson K, Polewski M, van Etten D, Terkeltaub R (2005) Chondrogenesis mediated by PPi depletion promotes spontaneous aortic calcification in NPP1$^{-/-}$ mice. Arterioscler Thromb Vasc Biol 25:686–691

8. Ewence AE, Bootman M, Roderick HL, Skepper JN, McCarthy G, Epple M, Neumann M, Shanahan CM, Proudfoot D (2008) Calcium phosphate crystals induce cell death in human vascular smooth muscle cells: a potential mechanism in atherosclerotic plaque destabilization. Circ Res 103:e28–e34

9. Chen NX, O'Neill KD, Duan D, Moe SM (2002) Phosphorus and uremic serum up-regulate osteopontin expression in vascular smooth muscle cells. Kidney Int 62:1724–1731

10. Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Boyle WJ, Riggs BL (2000) The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. J Bone Miner Res 15:2–12

11. Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, Scully S, Tan HL, Xu W, Lacey DL, Boyle WJ, Simonet WS (1998) Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. Genes Dev 12:1260–1268

12. Min H, Morony S, Sarosi I, Dunstan CR, Capparelli C, Scully S, Van G, Kaufman S, Kostenuik PJ, Lacey DL, Boyle WJ, Simonet WS (2000) Osteoprotegerin reverses osteoporosis by inhibiting endosteal osteoclasts and prevents vascular calcification by blocking a process resembling osteoclastogenesis. J Exp Med 192:463–474

13. Schoppet M, Al-Fakhri N, Franke FE, Katz N, Barth PJ, Maisch B, Preissner KT, Hofbauer LC (2004) Localization of osteoprotegerin, tumor necrosis factor-related apoptosis-inducing ligand, and receptor activator of nuclear factor-kappaB ligand in Monckeberg's sclerosis and atherosclerosis. J Clin Endocrinol Metab 89:4104–4112

14. Browner WS, Lui LY, Cummings SR (2001) Associations of serum osteoprotegerin levels with diabetes, stroke, bone density, fractures, and mortality in elderly women. J Clin Endocrinol Metab 86:631–637

15. Jono S, Ikari Y, Shioi A, Mori K, Miki T, Hara K, Nishizawa Y (2002) Serum osteoprotegerin levels are associated with the presence and severity of coronary artery disease. Circulation 106:1192–1194

16. Kiechl S, Schett G, Wenning G, Redlich K, Oberhollenzer M, Mayr A, Santer P, Smolen J, Poewe W, Willeit J (2004) Osteoprotegerin is a risk factor for progressive atherosclerosis and cardiovascular disease. Circulation 109:2175–2180

17. Schoppet M, Sattler AM, Schaefer JR, Herzum M, Maisch B, Hofbauer LC (2003) Increased osteoprotegerin serum levels in men with coronary artery disease. J Clin Endocrinol Metab 88:1024–1028

18. Schoppet M, Schaefer JR, Hofbauer LC (2003) Low serum levels of soluble RANK ligand are associated with the presence of coronary artery disease in men. Circulation 107:e76

19. Kaden JJ, Bickelhaupt S, Grobholz R, Haase KK, Sarikoc A, Kilic R, Brueckmann M, Lang S, Zahn I, Vahl C, Hagl S, Dempfle CE, Borggrefe M (2004) Receptor activator of nuclear factor kappaB ligand and osteoprotegerin regulate aortic valve calcification. J Mol Cell Cardiol 36:57–66

20. Panizo S, Cardus A, Encinas M, Parisi E, Valcheva P, Lopez-Ongil S, Coll B, Fernandez E, Valdivielso JM (2009) RANKL increases vascular smooth muscle cell calcification through a RANK-BMP4-dependent pathway. Circ Res 104:1041–1048

21. Tseng W, Graham LS, Geng Y, Reddy A, Lu J, Effros RB, Demer L, Tintut Y (2010) PKA-induced receptor activator of NF-kappaB ligand (RANKL) expression in vascular cells mediates osteoclastogenesis but not matrix calcification. J Biol Chem 285:29925–29931

22. Byon CH, Sun Y, Chen J, Yuan K, Mao X, Heath JM, Anderson PG, Tintut Y, Demer LL, Wang D, Chen Y (2011) Runx2-upregulated receptor activator of nuclear factor kappaB ligand in calcifying smooth muscle cells promotes migration and osteoclastic differentiation of macrophages. Arterioscler Thromb Vasc Biol 31:1387–1396

23. Mizuno A, Kanno T, Hoshi M, Shibata O, Yano K, Fujise N, Kinosaki M, Yamaguchi K, Tsuda E, Murakami A, Yasuda H, Higashio K (2002) Transgenic mice overexpressing soluble osteoclast differentiation factor (sODF) exhibit severe osteoporosis. J Bone Miner Metab 20:337–344

24. Li L, Miano JM, Mercer B, Olson EN (1996) Expression of the SM22alpha promoter in transgenic mice provides evidence for distinct transcriptional regulatory programs in vascular and visceral smooth muscle cells. J Cell Biol 132:849–859

25. Huang MS, Sage AP, Lu J, Demer LL, Tintut Y (2008) Phosphate and pyrophosphate mediate PKA-induced vascular cell calcification. Biochem Biophys Res Commun 374:553–558

26. Lepore JJ, Cheng L, Min LuM, Mericko PA, Morrisey EE, Parmacek MS (2005) High-efficiency somatic mutagenesis in smooth muscle cells and cardiac myocytes in SM22alpha-Cre transgenic mice. Genesis 41:179–184

27. Jung K, Lein M, Hosslin K, Grosse A, Roth S, Possinger K, Luftner D (2002) Osteoprotegerin and receptor activator of nuclear factor-kappaB ligand (RANKL) in the serum of healthy adults. Int J Biol Markers 17:177–181

28. Osako MK, Nakagami H, Koibuchi N, Shimizu H, Nakagami F, Koriyama H, Shimamura M, Miyake T, Rakugi H, Morishita R (2010) Estrogen inhibits vascular calcification via vascular RANKL system: common mechanism of osteoporosis and vascular calcification. Circ Res 107:466–475

29. Olesen M, Skov V, Mechta M, Mumm BH, Rasmussen LM (2012) No influence of OPG and its ligands, RANKL and TRAIL, on proliferation and regulation of the calcification process in primary human vascular smooth muscle cells. Mol Cell Endocrinol 362:149–156

30. Nam HK, Liu J, Li Y, Kragor A, Hatch NE (2011) Ectonucleotide pyrophosphatase/phosphodiesterase-1 (ENPP1) protein regulates osteoblast differentiation. J Biol Chem 286:39059–39071

31. Kerschan-Schindl K, Wendlova J, Kudlacek S, Gleiss A, Woloszczuk W, Pietschmann P (2008) Serum levels of receptor activator of nuclear factor kappaB ligand (RANKL) in healthy women and men. Exp Clin Endocrinol Diabetes 116:491–495

32. Hofbauer LC, Schoppet M, Schuller P, Viereck V, Christ M (2004) Effects of oral contraceptives on circulating osteoprotegerin and

soluble RANK ligand serum levels in healthy young women. Clin Endocrinol (Oxf) 60:214–219

33. Bord S, Ireland DC, Beavan SR, Compston JE (2003) The effects of estrogen on osteoprotegerin, RANKL, and estrogen receptor expression in human osteoblasts. Bone 32:136–141

34. Bashir A, Mak YT, Sankaralingam S, Cheung J, McGowan NW, Grigoriadis AE, Fogelman I, Hampson G (2005) Changes in RANKL/OPG/RANK gene expression in peripheral mononuclear cells following treatment with estrogen or raloxifene. Steroids 70:847–855

35. Loncar G, Bozic B, Cvorovic V, Radojicic Z, Dimkovic S, Markovic N, Prodanovic N, Lepic T, Putnikovic B, Popovic-Brkic V (2010) Relationship between RANKL and neuroendocrine activation in elderly males with heart failure. Endocrine 37:148–156