Cardiovascular calcification is a common consequence of diabetes. High fat diets induce diabetes and arterial calcification in male low density lipoprotein receptor (LDLR) −/− mice; calcification occurs via Msx2 signaling that promotes the osteogenic differentiation of arterial myofibroblasts. We studied regulation of arterial osteogenesis by human parathyroid hormone (PTH) (1–34) (also called teriparatide) in LDLR −/− mice fed diabeticogenic diets for 4 weeks. LDLR −/− mice were treated with vehicle or 0.4 mg/kg of PTH(1–34) subcutaneously five times/week. Gene expression was determined from single aortas and hind limb RNA by fluorescence reverse transcription-PCR. Valve calcification was determined by histological staining of cardiac sections using image analysis to quantify valve leaflet mineralization. PTH(1–34) increased bone mineral content (by dual energy x-ray absorptiometry) in LDLR −/− mice, with induction of osseous osteopontin (OPN) expression and serum OPN levels (>150 nm); PTH(1–34) did not significantly change serum glucose, lipids, body weight, or fat mass. PTH(1–34) suppressed aortic OPN and Msx2 expression >50% and decreased cardiac valve calcification 80% (8.3 ± 1.5% versus 1.4 ± 0.5%; p < 0.001). Of the known circulating regulators of vascular calcification (OPN, osteoprotegerin, and leptin), PTH(1–34) regulated only serum OPN. We therefore studied actions at early stages of macrovascular disease progression, again exceeding blood pressure control, lipid control, and diabetes control in positive predictive value (12). Risk for stroke, particularly notable in post-menopausal women, is increased in the presence of aortic arch calcification (13). Multiple metabolic stimuli contribute to calcific vascular disease initiation and progression in patients with diabetes, hypercholesterolemia, poor glycemic control, and phosphate retention associated with renal failure contributing to vascular calcium accumulation (2). A fundamental understanding the molecular physiology of vascular calcification will provide insights useful for developing strategies to prevent and treat this macrovascular disease process.

Metabolic contributions to vascular calcification are well known and include hypercholesterolemia, diabetes, hyperphosphatemia, vitamin D toxicity, magnesium deficiency, and chronic renal insufficiency (2). The mechanisms whereby the metabolic insults of diabetes initiate and propagate vascular calcification are beginning to be examined in detail. Hyperglycemia, hyperlipidemia, and oxidative stress (3, 14) up-regulate the expression of vascular signaling molecules that promote mineral deposition in a process that resembles craniofacial bone formation (15). Hyperglycemia and hyperlipidemia have
been shown to up-regulate the expression of BMP2-Msx2 and leptin signaling cascades that promote vascular calcification (3, 14–17). Circulating inhibitors of mineralization, such as osteopontin (OPN) and osteoprotegerin (OPG), have been genetically demonstrated to play important roles in inhibiting mineralization (2); however, role and regulation in response to diabetes have not been systematically examined. Moreover, we know very little concerning the endocrine regulation of vascular calcium mobilization and the kinetics of vascular calcified tissue turnover.

Parathyroid hormone (PTH) and PTH-related polypeptide (PTHrP) play important roles in calcium homeostasis in bone, kidney, breast, and placenta, signaling via the PTH/PTHrP (PTH and PTHrP) and PTH2 (PTH) receptors (18). We wished to assess the regulation vascular calcification by human PTH(1–34). This osteoanabolic N-terminal polypeptide fragment of PTH promotes orthotopic bone formation via the PTH/PTHrP receptor; daily injections of human PTH(1–34), also known as teriparatide, represent the first robustly effective anabolic pharmacotherapy for osteoporotic fracture (19, 20). Thus, given its clinical relevance, we first examined the effect of human PTH(1–34), hence referred to as PTH(1–34), on vascular versus orthotopic osseous mineralization. We have emphasized study of the LDLR gene deletion mice fed high fat diets typical of westernized societies; in this model, obesity, diabetes, hypercholesterolemia, and vascular calcification arise in response to this clinically relevant stimulus. While promoting orthotopic mineral deposition, PTH(1–34) administration suppresses cardiovascular calcification and down-regulates aortic osteogenic programs driven by diabetes and dyslipidemia. Both PTH(1–34) and OPN, a circulating inhibitor of mineralization (21), inhibit Mxs2-dependent osteogenic mineralization in culture. Because osseous and circulating levels of the mineralization inhibitor OPN are markedly enhanced by PTH(1–34), this suggests that OPN may function as a component of an endocrine axis that controls vascular mineral homeostasis in concert with PTH(1–34).

MATERIALS AND METHODS

Reagents and Biochemical Assays—Molecular biology reagents were purchased from Promega (Madison, WI) and Fisher. RNA purification reagents were purchased from Qiagen. DNase I DNA removal reagent was purchased from Ambion (Diana-free kit; Austin, TX). Synthetic human PTH(1–34) was purchased from Calbiochem (San Diego, CA; catalog number 05-23-5501). Purified bovine milk OPN (catalog number 109-OP) was purchased from R & D Systems (Minneapolis, MN). Enzyme-linked immunosorbent assay methods were used to quantify serum OPN (Assay Designs, Ann Arbor, MI; catalog number 900-690), OPG (Quantikine M mouse OPG; R & D Systems, Minneapolis, MN; catalog number MOP00), and leptin (Crystal Chem, Inc., Chicago, IL; catalog number 90030), implementing the corresponding commercially available kits. Serum glucose, cholesterol, and triglycerides were measured as previously detailed (15).

Animals and Dual Energy X-ray Absorptiometry—LDLR–/– mice were maintained as detailed (15), following procedures approved by the Washington University Animal Studies Committee. At the initiation of the high fat diets (Harlan Teklad, Madison, WI; product TD 88137), male LDLR−/− mice (age 6 weeks) were injected subcutaneously daily, 5 days per week, with either vehicle (0.9% normal saline, 0.1 mM acetic acid, 0.01 mM mercaptoethanol) or 0.4 mg/kg PTH(1–34) in vehicle precisely following the protocol established by the Washington University Animal Studies Committee. At the termination of the experiment (Harlan Teklad, Madison, WI; product TD 88137), vehicle (0.1% acetylated bovine serum albumin in phosphate-buffered saline) or PTH(1–34) for 1 h prior to feeding with fresh mineralization medium containing 10% serum. The cDNA encoding our N-terminal Met-FLAG-tagged Msx2 (26) was subcloned into the NcoI and BamHI sites of SFG retroviral vector (27, 28). As a negative control, SFG-LacZ or SFG-Mxs2 transduced cells were maintained in mineralization medium (above growth medium supplemented with 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, and 100 ng/ml BMP2) for 3 weeks. For OPN, the cells were treated every Monday, Wednesday, and Friday morning with fresh mineralization medium containing either vehicle (0.1% acetylated bovine serum albumin in phosphate-buffered saline) or OPN in vehicle (0.1% final volume). OPN final concentrations were 50 ng/ml (15), 50 ng/ml (31), and 50 ng/ml (31). The cells were trypsinized every Monday, Wednesday, and Friday morning with either vehicle (0.1% acetylated bovine serum albumin in phosphate-buffered saline) or PTH(1–34) in vehicle for only 1 h prior to feeding with fresh mineralization medium and removal of PTH(1–34) for the subsequent 2 days. This treatment protocol was utilized as an in vitro approach that closely mimics the pulsatile exposure to PTH(1–34) also from daily subcutaneous dosing. PTH(1–34) final concentrations were 10, 75, and 200 ng/ml. At the end of the 3-week culture period, the cultures were stained for calcium phosphate mineral deposition using Alizarin red (calcium stain) as previously described (29), imaged with a Nikon CoolPix 990 3.5 megapixel digital camera using white light transmission. Relative mineralized culture calcium content was quantified by absorbometry (400 nm) of culture-associated Alizarin red dye eluted essentially as described (30). For valvular calcification studies, digital photomicrographs were captured with a Spot Enhanced camera mounted on a Zeiss Axiovert S1000 microscope. Mineral deposition was visualized with Alizarin red (calcium) (29) or von Kossa (phosphate) (15) stains, and the mineralized leaflet area was quantified using Kodak one-dimensional automated image analysis software version 3.5.3. The data are expressed as the percentage of mineralized area of total valve leaflet area (pixel counts) from digital photomicrograph images, averaging results over five adjacent 5-micron sections for each animal (n = 5–6 animals/treatment arm). Of note, a similar method has now been reported for quantifying vascular calcification in apolipoprotein E−/−OPN−/− mice (31). Immunohistochemical visualization of OPN was carried out on 5-micron paraffin sections as previously detailed (15, 25).

Statistics—Statistical analyses were performed using one-way analysis of variance and unpaired t test as previously detailed (25). Each experiment was performed at least twice, and the data are presented as the means ± S.E. of independent replicates (n ≥ 3, depending upon the assay; see above).

RESULTS

PTH(1–34) Up-regulates Skeletal Osteopontin Gene Expression but Suppresses Aortic Osteogenic Gene Expression in LDLR−/– Mice—Intermittent administration of PTH(1–34) exerts well described skeletal osteogenic actions in mice (22) and humans (19, 20). We wished to assess the effects of PTH(1–34) on heterotopic vascular osteogenesis. LDLR−/– mice fed high fat diets develop diabetes and vascular calcification (more pronounced in male animals; Ref. 15 and our unpublished observations). Therefore, we studied the effects of intermittent PTH(1–34) on osteogenic gene expression, using a dosing regu-
PTH(1–34) Promotes Skeletal Mineralization but Suppresses Vascular Mineral Accumulation in LDLR −/− Mice—Our gene expression data indicated that PTH(1–34) reciprocally regulates skeletal and vascular osteogenic differentiation. To extend and confirm these data, we assessed calcium accumulation in skeleton and the vasculature in response to PTH(1–34) treatment. As shown in Fig. 2A, PTH(1–34) promotes the accumulation of skeletal calcification as quantified by dual electron x-ray absorptometry, which is consistent with its well described anabolic actions in bone (19, 22). Both bone mineral content (Fig. 2A) and bone mineral density (Fig. 2B) were increased, but no significant change in weight or percentage of body fat was noted (Fig. 2B). A nonsignificant trend in the percentage of body fat was observed (p = 0.22). We next sought to characterize vascular mineralization in response to PTH(1–34). No standardized assay of vascular calcification has been established; however, the minute amounts of calcium phosphate deposited even at the early stages of disease can be visualized with histochemical stains (15, 31) (Alizarin for calcium, von Kossa for phosphate). Therefore, to study macrovascular calcium deposition, we quantified the extent of Alizarin-stained (Fig. 2C) and von Kossa-stained (not shown) valvular tissues in animals treated with vehicle versus PTH(1–34). PTH(1–34) suppressed the accumulation of vascular mineralization (Fig. 2C), quantified in the percentage areas of cardiac valve leaflets stained with Alizarin red (Fig. 2D). Similar results were obtained using von Kossa staining (data not shown). Thus, PTH(1–34) promotes skeletal mineral accumulation while suppressing valvular calcification in diabetic LDLR −/− mice.

PTH(1–34) Does Not Significantly Improve Glucose or Lipid Profiles in LDLR −/− Mice Fed High Fat Diets—Because PTH(1–34) suppresses aortic osteogenic gene expression and valvular calcification induced in response to diabetes and dyslipidemia, we assessed whether PTH(1–34) ameliorated fatty diet-induced dysmetabolic stimuli. As shown in Fig. 3, PTH(1–34) did not significantly reduce the fasting serum glucose (Fig. 3A), cholesterol (Fig. 3B), or free fatty acid (Fig. 3C) levels up-regulated by high fat diets. Although a nonsignificant reduction in glucose was observed, dyslipidemia worsened in this susceptible model (Fig. 3C). Thus, inhibition of diet-induced vascular osteogenic responses by PTH(1–34) does not arise from significant improvements in glucose, cholesterol, or free fatty acid levels.

Fig. 1. PTH(1–34) up-regulates skeletal OPN but suppresses vascular osteogenic programs in diabetic LDLR −/− mice. LDLR −/− mice (four to five animals/treatment) were fed high fat diabetogenic diets and treated with either vehicle or 0.4 mg/kg (0.4 mpk) PTH(1–34) for 4 weeks as described in the text. RNA was isolated from single aortas and hindlimbs, and the expression of OPN and Msx2 was quantified using fluorescence RT-PCR. A, note that PTH(1–34) markedly increases skeletal OPN expression but has little significant action on Msx2. B, note that, unlike regulation observed in bone, PTH(1–34) significantly suppresses aortic OPN and Msx2 expression. C, immunostaining for protein expression in cardiac valves and myocardium confirmed diminished OPN expression. Control, background that arises when primary anti-OPN antibody is omitted. Aby, antibody. See text for details.
Fig. 2. PTH(1–34) promotes skeletal bone mass but suppresses cardiovascular mineral deposition in diabetic LDLR −/− mice. LDLR −/− mice (five to six animals/treatment) were fed high fat diets.

**DISCUSSION**

The endocrine physiology of vascular calcification is incompletely studied and poorly understood (41). Cardiovascular toxicity in the clinical settings of chronic renal insufficiency and vitamin D toxicity has been emphasized (42). However, adult diabetes and obesity, promotes aortic vascular calcification in vitro (12). As shown in Fig. 4, high fat diabetogenic diets do not alter serum OPN levels (Fig. 4A) but increase circulating levels of OPG (Fig. 4B) and leptin (Fig. 4C). By contrast, PTH(1–34) markedly up-regulated circulating OPN levels (Fig. 4A) while concomitantly promoting bone formation and suppressing vascular mineral deposition (Fig. 2). Of note, PTH(1–34) did not further increase circulating OPG levels (Fig. 4B) or significantly reduce serum leptin levels (Fig. 4C) in male LDLR −/− mice. Thus, PTH(1–34) selectively increases circulating levels of the mineralization inhibitor OPN without altering serum OPG or leptin levels.

**PTH(1–34) and OPN Suppress in Vitro Osteogenic Mineralization Driven by BMP2-Msx2 in Multipotent Mesenchymal Vascular Progenitors**—High fat diabetogenic diets up-regulate aortic expression of Mx2 and BMP2 in LDLR −/− mice (15), similar to observations made in calcifying human arterial lesions (5, 16). Expression of Mx2 is observed in a subset of valvular and aortic adventitial myofibroblasts (15, 25). Transduction of aortic myofibroblasts (not shown; (25)) or C3H10T1/2 multipotent mesenchymal progenitors (39) (Fig. 5A) with an amphotrophic retrovirus (27) expressing Mx2 enhances culture calcification in synergy with BMP2 (Fig. 5A). We wished to assess whether PTH(1–34) and/or OPN directly regulate osteogenic mineralization driven by the aortic BMP2-Msx2 signals up-regulated in LDLR −/− mice. As shown in Fig. 5B, purified bovine milk OPN significantly suppresses Mx2-dependent mineralization in a dose-dependent fashion; spectrophotometric analysis of eluted Alizarin red matrix stain demonstrates that OPN treatment at the 50 nM concentration abrogates Mx2-dependent induction of calcification. Because PTHrP has been shown to inhibit vascular calcification in vitro (40), we tested the activity of PTH(1–34) in this assay. PTH(1–34) inhibits Mx2-dependent calcification of 10T1/2 cells by ~40% (Fig. 5A) but to a lesser extent than that of OPN (Fig. 5B). We next assessed whether PTH(1–34) could exert direct actions on OPN and Mx2 expression in primary aortic myofibroblasts (15, 25). As shown in Fig. 6B, PTH(1–34) significantly suppresses the accumulation of Mx2, but not OPN or BMP2, mRNA in cultures of primary aortic myofibroblasts. Thus, soluble OPN and PTH(1–34) can inhibit BMP2-Mx2-driven vascular osteogenesis processes recruited during arterial calcification (5, 15, 16) via direct and indirect mechanisms (Fig. 7).

**FIG. 2.** PTH(1–34) promotes skeletal bone mass but suppresses cardiovascular mineral deposition in diabetic LDLR −/− mice. LDLR −/− mice (five to six animals/treatment) were fed high fat diameters and treated with either vehicle (Veh) or 0.4 mg/kg (0.4 mpk) PTH(1–34) for 4 weeks as described in the text. Mineralization of the skeleton was assessed by dual energy x-ray absorptiometry (A and B), whereas mineralization of cardiac valves was assessed by histological staining and image analysis (B and C). A, note that PTH(1–34) enhances bone mass mineral accrual, consistent with its clinically useful and well described osteoanabolic actions. B, PTH(1–34) increases bone mineral density but does not alter total body weight or percent fat mass. C and D, by contrast, PTH(1–34) markedly suppresses cardiac valve calcification as revealed by histological staining and quantified by computer assisted digital image analysis. The arrows in panel C point to areas of calcium staining on cardiac valves. See text for details.
advanced age, diabetes, hypercholesterolemia, vascular inflammation, and hemodynamic excesses (hypertension, bicuspid aortic valve) all promote macrovascular calcium load (41). In the LDLR −/− mouse, macrovascular calcification occurs in part via heterotopic activation of an active ossification program (15). In this study, we have initiated a systematic evaluation of PTH/PTHrP receptor signaling on macrovascular calcification and osteogenic gene expression in LDLR −/− mice. We find that human PTH(1–34), a clinically useful fragment and ligand for the PTH/PTHrP receptor (20), suppresses heterotopic vascular osteogenesis and mineral accumulation while simultaneously promoting orthotopic skeletal bone formation. Because PTH(1–34), also known as teriparatide, has been very recently introduced into the clinic for treatment of osteoporosis (19, 20),

**FIG. 3.** PTH(1–34) does not significantly reduce fasting serum glucose, cholesterol, or free fatty acid levels. LDLR −/− mice (four to five per arm) were fed either mouse chow or high fat diets and were simultaneously treated with either vehicle (Veh) or PTH(1–34) at 0.4 mpk as indicated for 4 weeks. At the end of the treatment period, the animals were subjected to a 12-h fast, and sera were collected for biochemical analyses. Note that high fat diets up-regulated fasting serum glucose (A), serum cholesterol (B), and free fatty acids (C). Further note that administration of PTH(1–34) treatment did not significantly reduce fasting glucose or lipid levels. See text for details. *, p < 0.05 versus chow diet, vehicle treatment.

**FIG. 4.** PTH(1–34) selectively increases serum OPN, a circulating inhibitor of vascular mineralization. LDLR −/− mice (four to five per arm) were fed either mouse chow or high fat diets and were simultaneously treated with either vehicle (Veh) or PTH(1–34) at 0.4 mpk as indicated for 4 weeks. At the end of the treatment period, the animals were subjected to a 12-h fast, and sera were collected for measurement of OPN (34), leptin (37), and OPG (36), known circulating regulators of vascular calcification. A, note that PTH(1–34) increased serum OPN levels in mice fed either mouse chow or high fat diets. Further note that high fat diets did not up-regulate circulating OPN levels, even though vascular expression of OPN is enhanced under these conditions (15, 32). B, high fat diets up-regulate circulating leptin levels; however, PTH(1–34) did not significa ntly regulate serum leptin levels. C, as seen in humans, serum OPG levels are elevated in animals rendered diabetic from high fat diets; however, PTH(1–34) did not up-regulate serum OPG levels. *, p < 0.05 versus chow diet, vehicle treatment. #, p < 0.05 versus high fat diet, vehicle treatment.
it will be important to identify whether teriparatide regulates vascular calcification in humans as it does in this murine model of diabetes and dyslipidemia.

Our data demonstrating that PTH(1–34) can inhibit macrovascular osteogenesis in vivo is consistent with recent in vitro studies from two other labs. Using cultured bovine aortic smooth muscle cells, Morii and co-workers (40) first demonstrated that autocrine PTHrP signaling prevented vascular smooth muscle cell alkaline phosphatase induction and calcification. Huang et al. (43) very recently demonstrated that adrenomedullin, C-type natriuretic peptide, and PTHrP inhibit vascular smooth muscle cell calcification in vitro. To our knowledge, our data represent the first direct in vivo evaluation of PTH(1–34) signaling on vascular calcification and aortic osteogenesis. We have emphasized the study of the LDLR/H11002 mouse fed high fat diets typical of westernized societies, with obesity, diabetes, hypercholesterolemia, and vascular calcification arising in response to this clinically relevant stimulus. In this model, vascular calcification arises in part via active recruitment of a cardiovascular BMP2-Msx2 signaling cascade that drives osteogenic differentiation of vascular myofibroblasts (2). We show that intermittent administration of PTH(1–34) suppresses Msx2 expression and vascular mineral accumulation in vivo, and inhibits Msx2-induced osteogenic differentiation of 10T1/2 mesenchymal progenitors in vitro. We further demonstrate that PTH(1–34) markedly increases circulating levels of OPN, a matrix cytokine and inhibitor of vascular mineral accumulation. In vivo, PTH(1–34) increased circu-
lating OPN levels to 100 nM. Because PTH(1–34) is apparently less effective than OPN as an inhibitor of Msx2-driven calcium deposition, elevated circulating OPN may greatly contribute to net vascular calcification responses to PTH(1–34) in vivo. Because PTH(1–34) suppresses aortic osteogenic expression of OPN while up-regulating skeletal OPN and circulating OPN levels, we anticipate that the source of circulating OPN is the skeleton. Monocytes are not likely to be an OPN source in response to PTH; OPN+CD68+ cells are decreased with PTH(1–34) treatment.2

Vascular calcification is very severe in patients with end stage renal disease; phosphate retention and an elevated calcium-phosphate product no doubt contribute to pathophysiology (44). In this setting, however, both skeletal refactoriness to PTH and secondary hyperparathyroidism arise, the former in part because of PTH(7–84), a PTH fragment that antagonizes PTH(1–84) signaling (45). Of note, hyperparathyroidism arising from either congenital (46) or iatrogenic (47) causes appears to increase the risk for cardiovascular calcification. Thus, given our functional data, it is tempting to speculate that perturbed PTH/PTHrP receptor signaling directly contributes to the calcific vasculopathy in end stage renal disease and highlights one potential risk of excessive vitamin D levels (47).

The mechanisms whereby PTH(1–34) regulates tissue-specific (cardiovascular versus skeletal) OPN expression have yet to be elucidated. Our data suggest that in primary cultures of aortic myofibroblasts, PTH(1–34) does not directly suppress OPN expression, even though treatment down-regulates Msx2 mRNA accumulation. Given this observation, it is tempting to speculate that another cell type intimately associated with vascular and skeletal tissues, such as the endothelial cell (48, 49), may be providing signals that confer tissue-specific inductive responses to PTH(1–34). Alternatively, the post-receptor signaling complexes assembled in vitro may be tissue-specific and poorly recapitulated by cell culture models. Co-culture and organ culture models will be used to test these possibilities.

Like PTH(1–34), purified OPN inhibited Msx2-dependent mineralization in vitro. The mechanisms whereby OPN inhibits its mineralization are not completely clear. Matrix-associated OPN has been shown to promote influx and activation of cells derived from the monocyte lineage, including macrophages and osteoclasts (20). However, biologically active OPN exists in a minimum of three forms: a matrix-bound form, a circulating soluble form, and a secreted soluble form (51). In vitro soluble OPN inhibition of calcification is regulated by OPN phosphorylation state (34); however, the concentrations of OPN that inhibit mineralization are in the 5–50 nM range, concentrations that are far more reminiscent of hormonal signaling rather than calcium chelation. Our data demonstrate that although PTH(1–34) up-regulates circulating soluble OPN levels, it in fact suppresses OPN gene expression and matrix-associated protein accumulation in cardiovascular tissues. This suggests that soluble OPN, as originally suggested by Jono et al. (34), exerts direct actions on vascular mesenchymal cells that actively suppresses mineral deposition, in addition to the matricrine activities that promote monocyte/macrophage activation (21). OPN is a ligand for the αvβ3 integrin receptors that are also expressed on mesenchymal cells, such as vascular smooth muscle cells (52) and osteoblasts (53). In osteoblasts, activation of αvβ3 signaling suppresses mineral deposition in vitro (53).

Given our observations and the studies of Giachelli and co-workers (34, 52), this type of OPN-αvβ3 integrin signaling is very likely to occur in vascular myofibroblasts as well and is under investigation. Elegant genetic analyses suggest that circulating OPN may synergize matrix Gla protein, a vascular matricrine inhibitor of BMP2 signaling (54), to control vascular calcification in vivo (33).

As noted above, PTH plays an important immunomodulatory role as a Th1-type cytokine, again dependent upon OPN phosphorylation state (51). In addition, regional expression of osteopontin in vascular smooth muscle cells promotes medial thickening, neointima formation, and mesangio proliferative responses (55, 56). Moreover, even though the modest metabolic changes observed (trend for decrease in glucose, percentage of body fat, and leptin levels) were not of statistical significance, the experimental design was underpowered for these specific parameters and may contribute to net physiological responses. Thus, the cell culture models utilized cannot fully recapitulate the role and regulation of OPN in the diabetic vascular disease response, and analyses of mice with cell type-specific OPN deficiency will be required to clarify immunomodulatory versus paracrine versus endocrine contributions (31, 51).

There are several other limitations to our study. Our in vivo experiments were carried out in a prevention paradigm; the actions of PTH(1–34) on disease progression once vascular calcification has been initiated are unknown (treatment paradigm). In addition, we used only a single dose of PTH(1–34) for our in vivo studies; because many endocrine responses exhibit tissue-specific dose-response relationships, it remains to be determined whether the PTH(1–34) dose-response relationships for the induction of serum OPN and the reduction in valve calcification coincide. Moreover, the pharmacokinetic-pharmacodynamic relationships for the regulation of vascular calcification by PTH(1–34) have yet to be established. We have not measured the peak and trough serum levels of PTH(1–34) achieved by our dosing regimen. Of note, the in vitro treatment conditions used achieved short term (~1–2 h) daily exposure to PTH(1–34) during the culture period as a mechanism to mimic the pulsatile in vivo exposure that daily dosing provides. Moreover, it remains to be examined whether both PTH(1–34) and native, full-length PTH(1–84) will exhibit comparable activities in vivo. Finally, the sites and extent of OPN phosphorylation in the circulation are unknown. Giachelli and co-workers (21) have provided data demonstrating that phosphorylated OPN has in fact enhance the egress of calcium from mineralized valves in a porcine xenograft model. We are currently developing reagents to characterize the phosphorylation state of circulating OPN and its regulation by PTH(1–34). Future studies will examine the source of circulating OPN production and its contribution to the endocrinology of vascular calcium metabolism in vivo, including responses to PTH(1–34) in modes of prevention and treatment.

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