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Vitamin D Action and Regulation of Bone Remodeling: Suppression of Osteoclastogenesis by the Mature Osteoblast

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ABSTRACT: Vitamin D acts through the immature osteoblast to stimulate osteoclastogenesis. Transgenic elevation of VDR in mature osteoblasts was found to inhibit osteoclastogenesis associated with an altered OPG response. This inhibition was confined to cancellous bone. This study indicates that vitamin D–mediated osteoclastogenesis is regulated locally by OPG production in the mature osteoblast.

Introduction: Vitamin D stimulates osteoclastogenesis acting through its nuclear receptor (VDR) in immature osteoblast/stromal cells. This mobilization of calcium stores does not occur in a random manner, with bone preferentially removed from cancellous bone. The process whereby the systemic, humoral regulator is targeted to a particular region of the skeleton is unclear.
Materials and Methods: Bone resorption was assessed in mice with vitamin D receptor transgenically elevated in mature osteoblasts (OSVDR). Vitamin D–mediated osteoclastogenesis was examined in vitro using OSVDR osteoblasts and osteoblastic RANKL: osteoprotegerin (OPG) examined in vivo and in vitro after vitamin D treatment.
Results: Vitamin D–mediated osteoclastogenesis was reduced in OSVDR mice on chow and calcium-restricted diets, with effects confined to cancellous bone. OSVDR osteoblasts had a reduced capacity to support osteoclastogenesis in culture. The vitamin D–mediated reduction in OPG expression was reduced in OSVDR osteoblasts in vivo and in vitro, resulting in a reduced RANKL/OPG ratio in OSVDR compared with wildtype, after exposure to vitamin D.
Conclusions: Mature osteoblasts play an inhibitory role in bone resorption, with active vitamin D metabolites acting through the VDR to increase OPG. This inhibition is less active in cancellous bone, effectively targeting this region for resorption after the systemic release of activated vitamin D metabolites.
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Key words: vitamin D, osteoclastogenesis, mature osteoblast, osteoprotegerin

INTRODUCTION

Bone remodeling is the coordinated process that continuously renews small quanta of mineralized tissue throughout the skeleton to maintain an optimum bone structure corresponding to mechanical and metabolic demands. This apparently simple process makes up the basic multicellular unit (BMU) of bone remodeling, coordinated across multiple axes of regulation to allow the exquisite level of control required to respond to specific hormonal or metabolic requirements while maintaining structural integrity. Although the effectors of these changes in bone remodeling are often circulating factors, such as estrogen and $1,25(OH)_2D_3$, the effects can be mediated in a spatially restricted manner. For instance, in situations of calcium demand, remodeling is primarily initiated at cancellous sites releasing needed calcium but sparing more structurally critical cortical bone, thus preserving skeletal strength.[1,2] Similarly, bone loss related to estrogen deficiency, particularly in cancellous bone, can be confined to discrete skeletal sites[3] or even discrete trabeculae.[4] Thus, systemic modulators of bone remodeling are regulated at a local level within bone tissues.

Initiation of a BMU in site- and rate-specific manners[5] involves the formation and action of finite osteoclast teams.[6] For example, with dietary calcium deficiency,

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whereas the resorptive stimuli are systemically released hormones, the control of remodeling occurs at the local level. Elevation of PTH in response to calcium stress increases circulating $1,25(OH)_2D_3$, which acts on the immature osteoblastic cells to stimulate osteoclastogenesis through the RANKL/osteoprotegerin (OPG) regulatory system.[7] We previously reported envelope-specific bone remodeling activity in a transgenic mouse model overexpressing the vitamin D receptor (VDR) specifically in mature cells of the osteoblastic lineage (OSVDR). $1,25(OH)_2D_3$ can stimulate both bone formation and resorption, regulating bone turnover by acting on both osteoclastic and osteoblastic cell lineages.[7–12] Consistent with these pleiotropic effects of vitamin D on bone cells, both sides of the bone remodeling response were affected in OSDVR mice; however, the effects were location specific, with bone formation and mineral apposition increased solely on periosteal surfaces and resorption reduced, specifically in the cancellous compartment.[13] Those findings suggested that localized regulation of vitamin D action may occur through distinct responses in osteoblasts dependent on their stage of differentiation. The antiresorptive phenotype in these mice was consistent with a vitamin D regulated pathway enabling mature osteoblastic cells to inhibit bone resorption and, importantly, acting in this model in a location-specific manner. To study the nature of this osteoclastic repression by mature osteoblastic cells further, dietary calcium restriction was used to induce increased bone turnover in wildtype and OSVDR mice. The osteoclastogenic response of OSVDR osteoblasts was assessed in vivo and in vitro, and the role of the RANKL/OPG regulatory system was examined. This study reveals the in vivo function of a vitamin D–OPG interaction in mature osteoblastic cells acting to reduce the initiation of BMUs in a site-specific manner. Thus, OPG produced by mature cells of the osteoblast lineage may function to regulate the spatial control of bone remodeling, providing negative feedback to the osteoclast stimulatory effects of the immature osteoblast.

MATERIALS AND METHODS

Transgenic mice

The OSVDR transgenic line OSV3, carrying a single insertion of 5–10 copies of the transgene on the inbred FVB/N background as previously described,[13] was used throughout. Hemizygous OSV3 mice, bred by mating homozygous males to FVB/N wildtype females, were studied. All studies were carried out with the approval and monitoring of the Garvan Institute/St Vincent's Hospital Animal Experimentation Ethics Committee.

Dietary calcium restriction

Age-matched FVB/N wildtype and OSVDR female mice were housed together in groups of 10 from weaning. All mice were maintained on standard laboratory chow containing 1% calcium, 0.8% total phosphorous, and 1000 IU/kg vitamin D_3 (Glen Forrest Stockfeeders, Glen Forrest, Western Australia, Australia). All diets and water were supplied ad libitum throughout. At 3 months of age, 10 mice from each genetic group were anesthetized and killed by exsanguination as a baseline, with remaining mice allocated to chow or calcium-restricted diets (Fig. 1). The semisynthetic calcium-controlled diets were modifications of the AIN-93 diet[14] (Glen Forrest Stockfeeders) to contain 1% (high) or 0.1% (low) calcium, 0.4% total phosphorous, and 1000 IU/kg vitamin D. After 4 weeks on the semisynthetic diet (4 months of age), 10 mice from each genetic group were killed from the high and low calcium groups. After 12 weeks on diet (6 months of age), the remaining 10 mice from each genetic group on high and low calcium diets were killed. Mice were injected with calcein and demeclocycline (Sigma Chemical Co., St Louis, MO, USA), each at 25 mg/kg, 10 and 3 days before death. In a previous study, we assessed the relationship between serum calcium on different diets and transgene status and found there was no transgene effect on serum calcium in mice maintained on different diets, and thus, serum calcium was not measured in this study.[13]

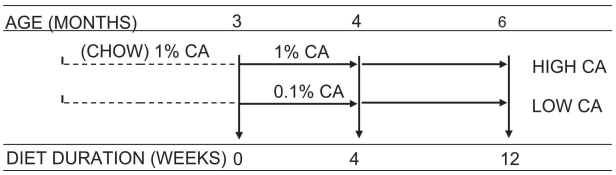

FIG. 1. Diet study protocol. All mice were maintained on standard laboratory chow containing 1% calcium until 3 months of age when baseline collection was made. Remaining animals were randomly assigned to a high (1%) or low calcium (0.1%) group, with tissue collection after 4 or 12 weeks on test diet, at 4 or 6 months of age, respectively.

Histomorphometry

Caudal vertebrae and femurs were collected, fixed in 4% paraformaldehyde, and prepared at 4°C for histomorphometry. Fourth caudal vertebrae and distal halves of the femur were embedded undecalcified in methyl methacrylate resin (Aldrich; Medim-Medizinische Diagnostik, Giessen, Germany) and 5-μm sagittal sections were analyzed by image analysis (Bioquant; R&M Biometrics, Nashville, TN, USA) connected to a tracing tablet (Calcomp Graphics). Vertebral measurements were made in a sample region encompassing all cancellous surfaces within the endosteal envelope, as previously described,[13] whereas femoral measurements were conducted in a sample region 2 mm distal from the midfemur, with measurements confined to the periosteal and endosteal cortical surfaces. Sections were stained for mineralized bone,[15] and cancellous bone volume (BV/TV), trabecular thickness (Tb.Th), and trabecular number (Tb.N) were quantitated.[16] For measurements of osteoclast surface (Oc.S), sections were stained for TRACP activity as described previously.[17] Mineralizing perimeter (double fluorochrome labeled surface + ½ single label) and mineral apposition rate (MAR) were assessed by fluorescence microscopy (Leica, Heerbrugg, Switzerland), and bone formation rate (BFR) was calculated (BFR = mineralizing fraction × MAR).

Long bone primary cell culture

Femora from 17-day-old mice were removed and cleaned of attached connective tissue and washed in PBS, and the epiphyses were removed. The remaining midshafts were minced and washed vigorously and repeatedly in PBS to remove marrow cells and transferred to digest mix (1 ml/6 femora) of 1 mg/ml collagenase (Boehringer-Mannheim, San Diego, CA, USA), 0.05% trypsin (Commonwealth Serum Laboratories, Canberra, Australia), and 0.02% EDTA (ICN, Costa Mesa, CA, USA) in PBS and stirred for 20 minutes at 37°C. Cells released from this first digest were discarded, and cells released from the second 20-minute digest were harvested and seeded at a density of 10^4 cells/cm^2 into 6-well plates in αMEM (Trace, Sydney, Australia) containing 10% FCS (Gibco, Grand Island, NY, USA), 100 U/ml penicillin, 100 mg/ml streptomycin, 40 mg/ml gentomycin, 20 mM HEPES, and 2 mM glutamine (all Gibco), supplemented with 10 mM β-glycerophosphate and 50 mg/ml ascorbic acid (Sigma) to promote matrix maturation and mineralization. Culture medium was changed after 3 days and every 2–3 days thereafter. Mineralized cultures (day 20) were treated with 10^{-8} M $1,25(OH)_2D_3$ or 0.1% isopropanol vehicle and collected 48 h later for gene expression studies, as previously described.[18]

Mouse osteoblast/human osteoclast co-cultures

Murine osteoblasts were prepared from femora by serial collagenase treatments as described above. Cells from the second digest were seeded in 75-cm^2 flasks at 10,000 cells/cm^2. Culture medium was as above but without β-glycerophosphate and ascorbic acid supplements to prevent mineralization. Confluence was judged to be at 14 days after seeding. Osteoblasts were harvested on day 20 (6 days after confluence) and co-cultured with osteoclast precursors, as described below. Osteoclast precursors were prepared from human umbilical cord blood mononuclear cells as previously described.[19]

Osteoclast in vitro assays

Mouse osteoblast (2.5×10^4 cells/culture) and osteoclast precursors (4×10^4 cells/culture) were combined and settled onto 4 × 4-mm sperm whale dentine slices in 96-well tissue culture plates and cultured in 200 μl MEM containing 10% FBS, nonessential amino acids, 50 IU/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 25 ng/ml macrophage-colony stimulating factor (M-CSF), and 10^{-7} M dexamethasone. After a 2-h settlement, 100 μl of culture supernatant was removed and replaced with 100 μl of medium containing $1,25(OH)_2D_3$. The cultures were refreshed twice weekly by replacing additives in one-half volume of medium. Cultures were fixed in 1% formalin and reacted for TRACP. The formation of osteoclasts and resorption activity were assessed as previously described.[19]

Tissue expression

For gene expression analysis, 4-month-old animals were treated with a single intraperitoneal injection of $1,25(OH)_2D_3$ (Calbiochem, La Jolla, CA, USA) at a dose of 2 μg per kg body weight. Femora were collected 6 h later. Marrow was excluded from the samples by removal of the ends of the long bones and flushing the cortical shafts using PBS to ensure removal of marrow cellular material. All samples were snap frozen and stored at –80°C before RNA preparation. RNA was isolated from whole bones or osteoblastic cell cultures using Trizol reagent (Gibco) with glycogen (5 μg/ml) as carrier according to manufacturer protocol. cDNA was generated by reverse transcriptase (Superscript II, Gibco) with random hexamer primers. PCR amplification was carried out with gene-specific primers (Sigma) using *Taq* polymerase (Amplitaq; Perkin Elmer, Boston, MA, USA). Reactions were carried out in foil-sealed 96-well plates (Thermofast 96; ABGene, Sydney, Australia) on a GeneAmp 9700 machine (Perkin Elmer). For PCR on cDNA from whole bones (OPG, RANKL, and GAPDH), conditions were optimized to produce linear amplification for the genes. Specific primers and conditions were as follows: OPG (annealing temperature = 65°C, 31 cycles, product 578 bp)—forward, 5′-TCCTGGCACCTACCTAAAACAGCA-3′ and reverse, 5′-CTACACTCTCGGCATTCACTTTGG-3′; RANKL (annealing temperature = 60°C, 37 cycles, product 790 bp)—forward, 5′-GGGAATTACAAAGTGCACCAG-3′ and reverse, 5′-GGTCGGGCAATTCTGAATT-3′; GAPDH (annealing temperature = 65°C, 27 cycles, product 983 bp)—forward, 5′-GGTCGGTGTGAACGGATTTGG-3′ and reverse, 5′-ATGTAGGCCATGAGGTCCACC-3′; human VDR (annealing temperature = 65°C, 29 cycles, product 472 bp)—forward, 5′-TCATTCTGACAGATGAGGAAGTGC-3′ and reverse, 5′-TCCTGGTATCATCTTAGCAAAGCC-3′. For osteopontin (OPN) expression in long bones, cDNA levels of OPN and GAPDH were quantitated by real-time RT-PCR on the Rotor-GeneTM300 Thermal Cycler (Corbett Research, Sydney, Australia) using SYBR Premix ExTaq (Takara Bio, Shiga, Japan) according to the manufacturer's protocol. Specific primers and conditions were as follows: GAPDH (annealing temperature = 60°C, 45 cycles, product 451 bp)—forward, 5′-ACCACAGTCCATGCCATCAC-3′ and reverse, 5′-TCCACCACCCTGTTGCTTA-3′; OPN (annealing temperature = 60°C, 45 cycles, product 80 bp)—forward, 5′-GATGCCACAGATGAGGACCTC-3′ and reverse, 5′-CTGGGCAACAGGGATGACAT-3′.

For PCR on cDNA from osteoblastic cultures, reactions for OPG and RANKL were duplexed with GAPDH after conditions were optimized to produce linear amplification for both genes. For both OPG-GAPDH and RANKL-GAPDH, annealing temperatures were 65°C, with the reactions run for 29 cycles. Specific primers were the same as for the bone tissue samples. PCR products were run on 1.5% agarose gels and stained with ethidium bromide. The UV image was captured using the GelDoc apparatus (Bio-Rad, Regents Park, Australia), and the bands were quantified using MolecularAnalyst software (BioRad). Data are the ratio to GAPDH PCR product.

Biochemistry

Serum intact PTH levels were determined by a mouse-specific ELISA (Immunotopics, San Clemente, CA, USA),

TABLE 1. EFFECT OF OSVDR TRANSGENE, DIETARY CALCIUM RESTRICTION AND DURATION OF TREATMENT ON CALCIOTROPIC HORMONE LEVELS IN SERUM

		Baseline	*4 weeks*		*12 weeks*	
		Chow	*High*	*Low*	*High*	*Low*
iPTH (pg/ml)	Wildtype	54 ± 44	41 ± 33	82 ± 27‡	50 ± 32	54 ± 44
	OSVDR	64 ± 30	48 ± 29	52 ± 32	44 ± 35	56 ± 39
$1,25(OH)_2D_3$ (pM)*	Wildtype	95 ± 34	85 ± 26	118 ± 38	115 ± 38.3	150 ± 51
	OSVDR	130 ± 42†	112 ± 57	114 ± 53	174 ± 53†	178 ± 45

Values are mean ± SD. n = 7–14.
* Significant difference between FVB/N and OSV3 across all nonbaseline groups.
† Significant difference between FVB/N and OSV3, within dietary group.
‡ Significant difference between high and low, within genetic group.

and 1,25-dihydroxyvitamin D levels were determined by radioimmunoassay after immunoextraction (Immunodiagnostic Systems, Boldon, UK).

Statistics

Statistical analyses assessing the effect of transgene status or diet were carried out by one-way ANOVA within diet or age groups separately. After ANOVA, linear contrasts selected a priori were used to compare results from the transgenic line with those from the FVB/N control line, with $p < 0.05$ considered significant (Statview 5.1 Software; Abacus Concepts, Berkeley, CA, USA). Analysis of co-culture data was also by ANOVA with posthoc linear contrasts (SuperANOVA Software; Abacus Concepts).

RESULTS

Biochemical response to dietary calcium restriction in OSVDR mice

Baseline PTH levels did not differ between wildtype and transgenic mice (Table 1); however, 4 weeks of dietary calcium restriction elevated serum PTH in wildtype mice to a level 60% higher than in OSVDR ($p < 0.05$). After 12 weeks of calcium restriction, wildtype PTH had returned to baseline (Table 1). In OSVDR mice, calcium restriction had no effect on PTH level after either 4 or 12 weeks. Serum $1,25(OH)_2D_3$ was 40% greater in OSVDR mice compared with wildtype mice at baseline and after 12 weeks on the high calcium diet (Table 1). Calcium restriction resulted in nonsignificant 30% increases in serum $1,25(OH)_2D_3$ after both 4 and 12 weeks in wildtype mice, resulting in levels similar to those seen in the OSVDR mice on the high calcium diet.

Effects of age and dietary calcium restriction on vertebral cancellous bone in OSVDR mice

In wildtype mice fed the high calcium diet, vertebral cancellous bone volume (Fig. 2A) declined with age between 3 and 6 months, consistent with an 80% rise in osteoclast surface (Fig. 2B) between 4 and 6 months ($p = 0.06$) and stable BFR (Fig. 2C). Dietary calcium restriction in wildtype mice resulted in significantly more resorption ($p < 0.02$) and a trend to decreased bone volume compared with the high calcium wildtype group after 4 weeks of treatment,

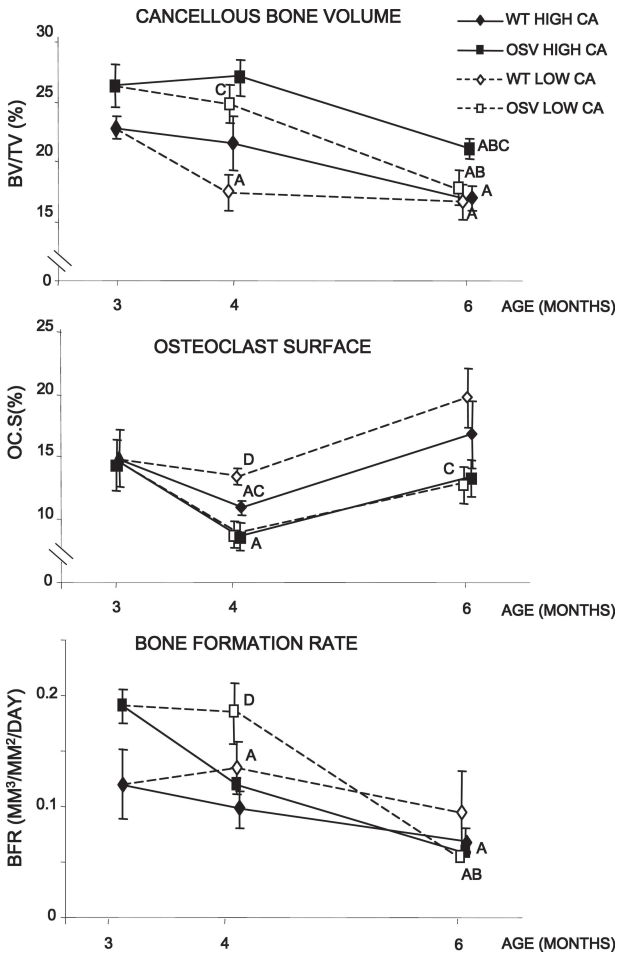

FIG. 2. Effect of elevated osteoblastic VDR on response of vertebral cancellous bone to dietary calcium deficiency. (A) Cancellous bone volume was elevated in OSVDR (OS) mice after 4 or 12 months on high calcium diet (black squares) and after 4 weeks on low calcium diet (white squares) compared with wildtype (wt) groups fed high and low calcium (black and white diamonds, respectively). (B) Osteoclast surface was significantly lower in OSVDR than wildtype mice after dietary calcium restriction. (C) Bone formation rate in this cancellous compartment was not significantly different between wildtype and OSVDR in any group. Statistical significance: [a]$p < 0.05$ vs. baseline (3 month) within diet group, [b]$p < 0.05$ vs. 4 months of age within diet group, [c]$p < 0.05$ vs. wildtype within diet/age group, [d]$p < 0.05$ vs. chow within age/genotype group. Values are mean ± SE, n = 7–12.

TABLE 2. EFFECT OF OSTEOBLASTIC VDR OVEREXPRESSION AND DIETARY CALCIUM RESTRICTION ON CORTICAL OSTEOCLAST SURFACE

		Baseline	*4 weeks*		*12 weeks*	
		Chow	*High*	*Low*	*High*	*Low*
Endo Oc.S	Wildtype	10.3 ± 3.2	12.2 ± 11.1	15.8 ± 7.9	25.5 ± 10.4*	24.9 ± 3.9*
(%BS)	OSVDR	13.9 ± 7.6	14.1 ± 5.4	15.4 ± 5.2	29.4 ± 12.6*	20.1 ± 10.1
Peri Oc.S	Wildtype	18.5 ± 5.8	7.2 ± 4.8†	3.3 ± 3.6†	5.3 ± 2.3	5.0 ± 6.7
(%BS)	OSVDR	17.2 ± 4.7	6.2 ± 5.7†	5.0 ± 5.0†	4.2 ± 4.0	4.7 ± 4.3

Values are mean ± SD.
* Significant difference between 4 and 12 weeks within dietary group.
† Significant difference between baseline and 4 weeks.

again with stable bone formation throughout the study period. The diet-induced bone loss after 4 weeks on the low calcium diet and the aging-associated loss between 4 and 6 months in the high calcium group resulted in equivalent bone volumes for both dietary groups of wildtype mice at 6 months reaching equivalent bone parameters (Fig. 2).

Consistent with previous observations,[13] cancellous bone volume was significantly elevated in OSVDR mice relative to wildtype at 4 and 6 months of age. Aging-related bone loss was also evident in the transgenics, with the decline in bone volume between 3 and 6 months associated with a 30% rise in resorption ($p = 0.06$) between 4 and 6 months and a 55% decline in bone formation ($p < 0.02$). The bone volume difference between wildtype and OSVDR mice fed high calcium persisted throughout the study period.

In contrast to wildtype mice, 4 weeks of dietary calcium restriction in OSVDR did not result in reduced cancellous bone volume at 4 months of age. Importantly, bone resorption was not different between high and low calcium OSVDR groups at this time-point, consistent with the transgene-associated resistance to calcium-induced bone loss previously observed.[13] This resistance did not prevent a decline in bone mass, however, because cancellous bone volume in the OSVDR low calcium group declined to wildtype levels after 12 weeks of diet treatment, in association with a nonsignificant trend to increased resorption between 4 and 6 months. This trend closely paralleled the change in bones of high calcium–fed OSVDR mice and was accompanied by a significant and marked decline in bone formation ($p < 0.0001$). This decline in formation in the OSVDR low calcium group resulted in a significant difference in bone volume between the OSVDR groups at 6 months.

On femoral cortical surfaces, periosteal osteoclast surface declined with age after baseline in both genotypes, independent of dietary calcium (Table 2). Conversely, endosteal osteoclast surface increased with age between 4 and 6 months of age in both genotypes, again independent of dietary calcium. Importantly, there were no transgene-associated changes in osteoclast surface on cortical bone surfaces.

Effects of transgenic osteoblast on osteoclastogenesis in monocyte co-cultures

To elucidate the underlying causes of the reduced resorption seen in transgenic vertebral cancellous bone, the osteoclastogenic potential of wildtype and OSVDR osteoblasts in co-cultures with human monocytes was analyzed (Fig. 3). In the presence of wildtype primary osteoblasts, there was a dose-dependent response to $1,25(OH)_2D_3$ in numbers of TRACP$^+$ multinucleated cells (Fig. 3A) and resorption pit area on bone slices (Fig. 3B). Both osteoclast formation and resorptive activity were increased with $1,25(OH)_2D_3$ treatment, with maximal effect at 10^{-9} and 10^{-10} M, respectively.

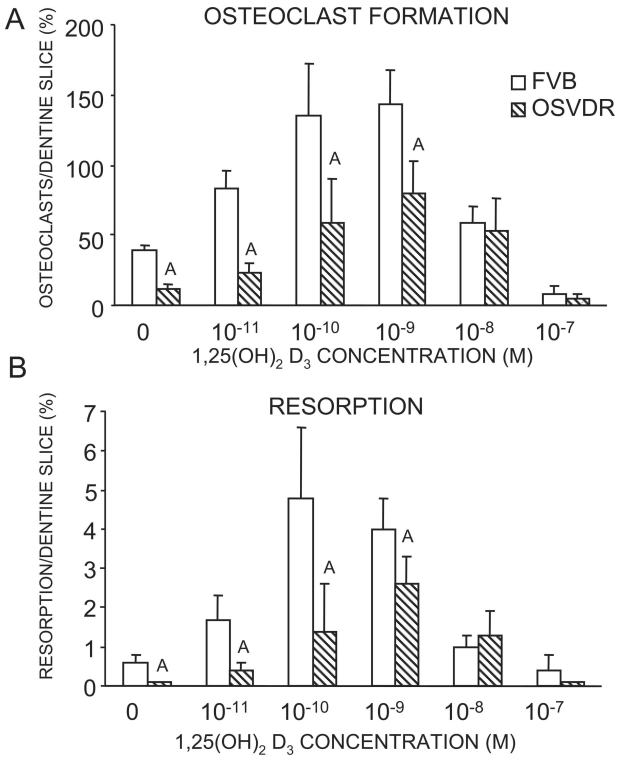

FIG. 3. Effect of elevated osteoblastic VDR levels on $1,25(OH)_2D_3$-stimulated bone resorption in co-cultures with human cord blood monocytes. (A) Production of TRACP$^+$ multinucleated cells in co-cultures with wildtype osteoblasts (wt) rose with increasing $1,25(OH)_2D_3$ concentration to 10^{-9} M, reducing thereafter at 10^{-8} and 10^{-7} M. TRACP$^+$ cell number in OSVDR cultures was lower than wildtype level in untreated cultures and remained ~50% lower than wildtype between 10^{-11} and 10^{-9} M $1,25(OH)_2D_3$, with no transgene-associated reduction evident at higher concentrations. (B) Pit area per bone slice results were similar to TRACP$^+$ cell number. [a]$p < 0.05$ vs. wildtype, within treatment concentration. Values are mean ± SE, $n = 4$.

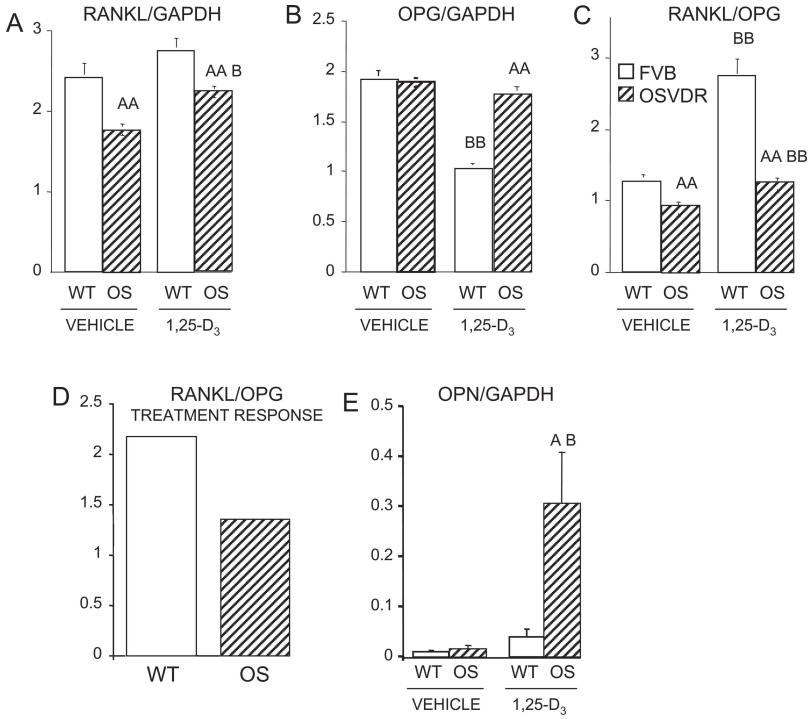

FIG. 4. Effect of elevated osteoblastic VDR levels on the expression of RANKL and OPG in the long bones of wildtype (wt) and OSVDR (OS) mice after $1,25(OH)_2D_3$ treatment. (A) RANKL expression was significantly decreased in vehicle and $1,25(OH)_2D_3$-treated OSVDR cells compared with wildtype. (B) OPG expression was similar in wildtype and OSVDR long bones. In vehicle-treated bones, it was significantly decreased in wildtype but not OSVDR after $1,25(OH)_2D_3$ treatment, with no change in OSVDR. (C) RANKL/OPG ratio was reduced in vehicle treated OSVDR mice compared with wildtype. $1,25(OH)_2D_3$ treatment increased RANKL/OPG in both groups. (D) RANKL/OPG response to treatment was markedly reduced in OSVDR bones. (E) Both basal levels and the $1,25(OH)_2D_3$ responsiveness of OPN were enhanced in the OSVDR bones. $^{a}p < 0.05$, $^{aa}p < 0.01$ vs. wildtype within treatment group; $^{b}p < 0.05$, $^{bb}p < 0.01$ vs. vehicle within genetic group. Values are mean ± SE, $n = 7–14$.

In monocytes co-cultured with OSVDR osteoblasts, both parameters were reduced in unstimulated and hormone-treated cultures, with levels of osteoclastogenesis and resorption ~50% lower than in wildtype co-cultures at $1,25(OH)_2D_3$ concentrations of 10^{-9} M or lower ($p < 0.05$; Fig. 3). Osteoclastogenesis with OSVDR osteoblasts decreased up to 10^{-9} M $1,25(OH)_2D_3$ as seen with wildtype osteoblasts, but at 10^{-8} M,$1,25(OH)_2D_3$ and above did not differ from the bone resorption parameters in wildtype co-cultures.

Transgene effects on RANKL and OPG expression

Levels of the principal cytokine mediators of osteoclastogenesis were assessed. RANKL, but not OPG, expression was reduced in long bones of untreated OSVDR mice compared with wildtype animals ($p < 0.005$; Fig. 4), resulting in a lower RANKL/OPG ratio in the OSVDR bones ($p < 0.05$). Treatment with $1,25(OH)_2D_3$ modestly increased RANKL expression in both wildtype (not significant) and OSVDR ($p < 0.05$) mice, but levels in treated OSVDR bones remained reduced compared with wildtype. In contrast, OPG expression after $1,25(OH)_2D_3$ treatment was reduced in wildtype mice by ~50% ($p < 0.0001$; Fig. 4B) but was not affected by the hormone in OSVDR mice. Thus, in wildtype bones, $1,25(OH)_2D_3$ treatment increased the RANKL/OPG ratio 2.3-fold, but in OSVDR mice treatment only increased the ratio 1.3-fold (Fig. 4D). To verify the enhanced vitamin D responsiveness of the OSVDR osteoblasts, the expression of osteopontin was also examined in the long bones (Fig. 4E). Basal levels of OPN mRNA were 60% higher in OSDVR than in FVB bones (not significant, $p = 0.15$). Treatment with $1,25(OH)_2D_3$ increased OPN expression 4-fold in FVB ($p = 0.08$). However, in the OSVDR bones, $1,25(OH)_2D_3$ treatment resulted in a 19-fold elevation in OPN expression ($p < 0.05$)

In mature mineralizing long bone primary osteoblastic cultures, in contrast to tissue levels, RANKL expression was higher in both vehicle and $1,25(OH)_2D_3$-treated OSVDR osteoblasts compared with wildtype ($p < 0.01$; Fig. 5A). Treatment with $1,25(OH)_2D_3$ increased RANKL expression in both wildtype and OSVDR cultures 1.2-fold, reaching statistical significance in the OSVDR cultures ($p < 0.05$; Fig. 5A). OPG levels were similar in vehicle-treated wildtype and OSVDR osteoblasts, and $1,25(OH)_2D_3$ treatment reduced OPG expression 1.3-fold in wildtype cultures ($p < 0.01$) but not in OSVDR cultures (Fig. 5B). Thus, the RANKL/OPG ratio was higher in vehicle-treated OSVDR cultures ($p < 0.05$) but comparable in $1,25(OH)_2D_3$-treated wildtype and transgenic cultures (Fig. 5C). Importantly, however, $1,25(OH)_2D_3$ increased the RANKL/OPG ratio 2.5-fold in wildtype but only 1.4-fold in OSVDR osteoblastic cultures (Fig. 5D).

DISCUSSION

The OSVDR mouse model has for the first time enabled in vivo analysis of the osteoblastic vitamin D response to focus specifically on the mature osteoblast. Enhanced vitamin D receptor expression specifically in the mature mouse osteoblast was associated with an inhibition of $1,25(OH)_2D_3$-mediated bone resorption, specifically on cancellous bone surfaces.[13] This OSVDR antiresorptive phenotype was mainly associated with a cell autonomous osteoblastic effect associated with an altered OPG response to $1,25(OH)_2D_3$. The findings of this study therefore provide evidence for a vitamin D responsive pathway originat-

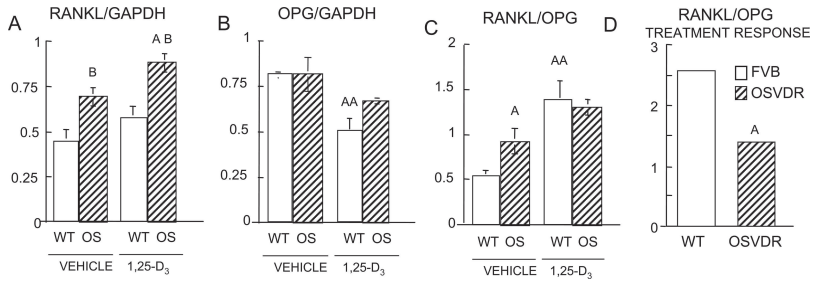

FIG. 5. Effect of elevated osteoblastic VDR levels on the expression of RANKL and OPG in primary osteoblastic cultures from long bones of wildtype (wt) and OSVDR (OS) mice after treatment with vehicle or 10^{-8} M $1,25(OH)_2D_3$. (A) RANKL expression was elevated to similar extents in OSVDR osteoblastic cells compared with wildtype in both vehicle and hormone-treated. (B) OPG expression was comparable in both vehicle-treated groups, but significantly decreased in wildtype cultures after treatment, with no significant change in OSVDR. (C) RANKL/OPG ratio was elevated in vehicle-treated OSVDR cells compared with wildtype but not in the $1,25(OH)_2D_3$-treated groups, with a significant rise in wildtype after treatment but no change in OSVDR cells after treatment. (D) RANKL/OPG response to treatment was substantially reduced in OSVDR cultures compared with wildtype, indicating a reduced osteoclastogenic capacity of the OSVDR osteoblasts. $^{a}p < 0.05$, $^{aa}p < 0.01$ vs. wildtype within treatment group; $^{b}p < 0.05$, $^{bb}p < 0.01$ vs. vehicle within genetic group. Values are mean ± SE, $n = 7$–14.

ing from mature osteoblasts that acts to repress bone resorption. The confinement of the OSVDR-mediated osteoclastic repression to cancellous bone in conditions of reduced dietary calcium is consistent with the preferential sparing of the cortices under conditions of calcium stress and would be compatible with a role for this pathway in normal cortical bone physiology.

The resistance to $1,25(OH)_2D_3$-mediated bone resorption in OSVDR mice was evident in all diet groups, but was manifested differently under the different dietary conditions. OSVDR mice maintained on a high calcium diet showed similar bone resorption to wildtype mice, despite significantly elevated serum $1,25(OH)_2D_3$ in the transgenic animals. In contrast, in the low calcium groups, bone resorption was lower in OSVDR than wildtype mice, despite comparable serum $1,25(OH)_2D_3$ levels in both groups. These results indicate that, in the presence of elevated VDR, the mature osteoblast is able to attenuate bone resorption regardless of circulating levels of active hormonal vitamin D. This resistance to the proresorptive effects of $1,25(OH)_2D_3$ was sufficient to maintain elevated cancellous bone volume in OSVDR mice compared with wildtype in the 4- and 12-week high calcium groups and after short-term calcium restriction.

It is well established that $1,25(OH)_2D_3$ action on BMU initiation is envelope specific, with calcium- and lactation-dependent resorption occurring primarily at cancellous and endocortical surfaces (i.e., areas of high bone turnover).[20,21] The finding that the OSVDR antiresorptive phenotype was not evident on endocortical or periosteal surfaces is consistent with effects of the transgene to locally reduce the bone proresorptive response to a systemic rise in active hormonal vitamin D levels. Mechanical load–responsive alterations in VDR expression have been reported, with microgravity suppressing[22] and hypergravity stimulating VDR activity.[23] These recent reports are consistent with our earlier observation of increased periosteal bone formation and enhanced mid-diaphyseal diameter and strength in OSVDR long bones, which suggested enhanced load responsiveness in OSVDR cortical bone.[13] The current data of resistance of OSVDR cancellous bone to reduction of dietary calcium support a vitamin D–regulated osteoclast inhibitory pathway within mature osteoblastic cells that can act to protect mechanically vulnerable cancellous surfaces from bone resorption.[13] The coordination of these envelope-specific modifications of remodeling induced by vitamin D action in the mature osteoblastic cells could thereby ensure the most efficient use of limited calcium available during calcium stress.

This study proposes the existence of a mechanism regulating osteoclasts present in the mature osteoblast. Because of the nature of this transgenic model, it was not possible to further study the extent of this regulatory system in other osteoblast cell populations. Inhibition of osteoclast activity by earlier osteoblasts would also seem logical, allowing these cells to lay down a matrix before resorption could occur, and conversely, perhaps in very mature osteoblasts (postosteocalcin expression), this inhibitory mechanism could be deactivated to allow appropriate remodeling of the newly laid bone to occur. Further studies will be required to elucidate whether other osteoblast populations do indeed regulate osteoclast activity in such a fashion.

The reduction in resorption in vivo was confirmed in co-culture experiments, where OSVDR osteoblasts reduced osteoclastogenesis and pit formation by human cord blood monocytes by ~50%. Such alterations in bone resorption suggested a possible modulation of the RANKL/OPG axis by the OSVDR transgene. Levels of RANKL and OPG expression in OSVDR bones were consistent with the reduction in osteoclastogenesis of OSVDR mice, with a significantly reduced RANKL/OPG response to $1,25(OH)_2D_3$ treatment in vivo. Whereas RANKL expression was moderately reduced in OSVDR, the change in RANKL/OPG ratio seemed to be primarily the result of a resistance to $1,25(OH)_2D_3$-induced inhibition[24,25] of OPG in OSVDR mice. Importantly, the reduced OPG response to $1,25(OH)_2D_3$ was also evident in OSVDR primary osteoblastic cultures. The biphasic response to $1,25(OH)_2D_3$ treatment seen in the osteoclast cultures is caused by $1,25(OH)_2D_3$ directly inhibiting osteoclastogenesis at higher concentrations ($>10^{-9}$ or 10^{-8} M).[26,27] At lower concentrations, the effect is to indirectly stimulate osteo-

clastogenesis through osteoblasts (through the RANKL/OPG system), and it is through this mechanism that we see an effect of the presence of the OSVDR transgene.

Thus, it seems likely that the mature osteoblast acts to restrict osteoclastogenesis by an OPG-related mechanism, which is supported by elevated OPG expression in mature osteoblasts.[18] Such a mechanism would contrast the strongly RANKL-based stimulation of osteoclastogenesis by the immature osteoblast.[28] Other studies have also suggested increased osteoblast maturation can reduce $1,25(OH)_2D_3$-regulated osteoclastogenesis in marrow/osteoblast co-culture, although a RANKL-associated mechanism was postulated rather than OPG.[29]

A recent study has suggested that *OPG* gene expression is rapidly and transiently reduced by $1,25(OH)_2D_3$ through c-*jun* activity at the activator protein-1 (AP-1) binding site.[30] This initial acute reduction in OPG by $1,25(OH)_2D_3$ played an important role in the stimulation of resorption, but chronic $1,25(OH)_2D_3$ exposure rendered the *OPG* gene insensitive to repression, thereby giving way to the anabolic phase of the vitamin D effect on bone. The constitutive high level of transgenic VDR expression in mature osteoblastic cells[13] in OSVDR mice may thus mimic chronic $1,25(OH)_2D_3$ treatment and block repression of OPG expression by c-*jun*, reducing the catabolic action of vitamin D in the transgenic model.

The findings of this study indicate the presence of a $1,25(OH)_2D_3$-regulated pathway in mature osteoblastic cells acting to stabilize OPG expression and thus reduce osteoclastogenesis in vivo. Acting in a site-dependent manner, stimulation of this pathway in OSVDR mice reduces resorption of cancellous bone under calcium replete and restricted conditions. Thus, differential regulation of OPG expression by $1,25(OH)_2D_3$ in immature versus mature osteoblasts may confer local protection of bone from the osteolytic effects of circulating $1,25(OH)_2D_3$. Signals of this nature may present a negative feedback system, acting through the mature osteoblastic cell to exert control over both the location and frequency of bone remodeling events initiated by immature osteoblastic cells in response to systemic signals such as active hormonal vitamin D. These data support the existence of distinct and in some ways diverse vitamin D regulatory pathways in immature and mature cells of the osteoblastic lineage. These different pathways may be central to the site-specific regulation of bone remodeling.

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