ORIGINAL ARTICLE

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Postnatal Skeletal Deletion of Dickkopf-1 Increases Bone Formation and Bone Volume in Male and Female Mice, Despite Increased Sclerostin Expression

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ABSTRACT

The Wnt antagonist Dickkopf-1 (Dkk1) is a negative regulator of osteoblast function and bone mass. However, because of the lack of appropriate models, many aspects of its role in the regulation of postnatal bone turnover and its cellular source have remained unknown. In this study, we deleted Dkk1 postnatally and in different cell types using various Cre-drivers (Rosa26-ERT2-Cre, Osx-cre, Dmp1-Cre) and assessed to which extent cells of the osteoblastic lineage contribute to the effects of Dkk1 on bone turnover and homeostasis. Female and male mice were examined at 12 weeks of age. Mice with a global or cell type–specific deletion of Dkk1 showed a two- to threefold higher bone volume compared with their Cre-negative littermates. The mineral apposition rate and the bone formation rate were increased two- to fourfold in all three mouse lines, despite a significant increase in systemic and skeletal levels of sclerostin. Dkk1 deletion further reduced the number of osteoclasts about twofold, which was accompanied by a strong decrease in the receptor activator of nuclear factor-κB ligand/osteoprotegerin mRNA ratio in femoral bone. Despite similar increases in bone mass, the deletion of Dkk1 in osterix-expressing cells reduced circulating Dkk1 significantly (males, −79%; females, −77%), whereas they were not changed in $Dkk1^{fl/fl}$;Dmp1-Cre mice. However, both lines showed significantly reduced Dkk1 mRNA levels in bone. In summary, we show that lack of Dkk1 in cells of the osteoblastic lineage leads to high bone mass with increased bone formation, despite increased levels of sclerostin. Moreover, the majority of systemic Dkk1 appears to originate from osteoprogenitors but not from mature osteoblasts or osteocytes. Nevertheless, the amount of Dkk1 produced locally by more mature osteogenic cells is sufficient to modulate bone mass. Thus, this study highlights the importance of local Wnt signaling on postnatal bone homeostasis. © 2018 American Society for Bone and Mineral Research.

KEY WORDS: DICKKOPF-1; WNT; SCLEROSTIN; BONE HOMEOSTASIS

Introduction

Bone homeostasis is tightly coordinated by the spatial and temporal interaction of bone-forming osteoblasts and bone-resorbing osteoclasts. Osteocytes in the bone matrix derive from osteoblasts and act as mechanosensing cells and coordinate the interaction and differentiation of osteoblasts and osteoclasts. Alterations in the activity or differentiation of these bone cells can be severe and can lead to various bone diseases such as osteoporosis, osteomalacia, hypophosphatemic rickets, or van Buchem's disease.[1]

One important pathway for the differentiation of osteoblasts is the highly conserved Wnt signaling pathway.[2] Wnt signaling has traditionally been categorized into canonical Wnt signaling, which requires β-catenin for its signal transduction, and noncanonical Wnt signaling, which is β-catenin-independent. However, these pathways are becoming more and more intertwined, as several cross-regulatory pathways have been discovered. Nevertheless, both pathways regulate bone homeostasis, and β-catenin, in particular, plays a critical role in osteoblast differentiation. Cells committed to the osteoblast lineage show an increased β-catenin, whereas removing β-catenin from osteoblast progenitors prevents their differentiation into mature osteoblasts.[3–5] Similarly, osteocyte-specific deletion of Wnt/β-catenin leads to progressive bone loss.[6]

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Wnt signaling is tightly regulated by various antagonists, including dickkopf-1 (Dkk1), which binds LRP5/6 and blocks its interactions with Wnts.[7–9] Overexpression of Dkk1 in mice results in osteopenia with reduced osteoblast numbers,[10] whereas deletion of a single allele of the Dkk1 gene increases bone mass, bone formation, and osteoblast numbers.[11] Dysregulation of Dkk1 expression is found in various bone-related disorders including rheumatoid arthritis,[12] various cancer types including multiple myeloma,[13] breast cancer,[14] and prostate cancer,[15] as well as in glucocorticoid-induced[16,17] and postmenopausal osteoporosis.[18] Thus, Dkk1 is a key negative regulator of osteoblast differentiation and is involved in the pathogenesis of various diseases affecting bone.

Despite these insights, several questions concerning the regulation of bone homeostasis via Dkk1 remain unanswered. For example, studying the role of Dkk1 in postnatal bone homeostasis was hampered due to the embryonic lethality of global homozygous Dkk1 knockout mice and the lack of inducible Dkk1 knockout mice. Moreover, the cellular source of Dkk1 in bone has not been conclusively addressed as Dkk1 is secreted by numerous cells in bone, including osteoprogenitors, osteoblasts, osteocytes, and adipocytes.[11,19,20] Thus, in this study, we aimed to address two questions: First, is Dkk1 important for postnatal bone homeostasis in males and females, and second, do osteoprogenitors, mature osteoblasts, and osteocytes produce similar amounts of Dkk1 and equally affect bone homeostasis? Using mice with a postnatal deletion of Dkk1 in various osteo-lineage cells, we show that lack of Dkk1 results in a high bone mass with decreased osteoclast number and increased bone formation, despite increased levels of sclerostin. Moreover, osteoprogenitors, mature osteoblasts, and osteocytes contribute to the regulation of bone homeostasis by producing Dkk1, despite differences in their contribution to systemic Dkk1 levels. Thus, this study highlights the importance of locally acting Wnt signaling for the control of bone homeostasis and shows that despite activating counter-regulatory feedback mechanisms, Dkk1 remains the key regulator of osteoblast function.

Materials and Methods

Animal models

Floxed Dkk1 mice ($Dkk1^{fl/fl}$)[20] in which exons 1 and 2 are flanked by *loxP* sites were crossed with Rosa26-CreERT2 mice (Jackson Laboratory, Bar Harbor, ME, USA) to generate tamoxifen-inducible global Dkk1 knockout mice ($Dkk1^{fl/fl}$;Rosa26-CreERT2). At the age of 7 weeks, male and female $Dkk1^{fl/fl}$;Rosa26-CreERT2-positive and -negative control mice were injected with 100 μL tamoxifen (10 g/L, Sigma, Munich, Germany) for 5 days to induce global deletion of Dkk1.

Moreover, $Dkk1^{fl/fl}$ mice were crossed with doxycycline-repressible Osx:Cre[5] and Dmp1:Cre[21] transgenic mouse lines. The mouse lines generated are referred to as $Dkk1^{fl/fl}$;Osx:Cre and $Dkk1^{fl/fl}$;Dmp1:Cre. $Dkk1^{fl/fl}$;Osx:Cre breeding pairs received doxycycline in their drinking water (10 mg/mL in a 3% sucrose solution) ad libitum to repress Cre activity during embryogenesis. $Dkk1^{fl/fl}$;Osx:Cre offspring received doxycycline drinking water until the age of 5 weeks. Respective Cre-negative littermates were used as controls. Sufficient recombination in the desired cell types/organs of all strains was validated via PCR analysis.

All experiments were performed with male and female mice at age 12 weeks. All mice were genotyped using standard PCR protocols. Mice were maintained in groups of up to 5 animals per cage and were kept in a dark cycle of 12/12 hours at room temperature in filter-top cages with cardboard houses as enrichment. All invasive procedures were approved by the local Institutional Animal Care Committee and the Landesdirektion Sachsen.

Assessment of bone length, bone mass, and microarchitecture

Bone length of the femur was assessed using a caliper. For μCT measurements, the femur and fourth vertebral body were analyzed ex vivo (vivaCT40, Scanco Medical, Bruttisellen, Switzerland) with an isotropic voxel size of 10.5 μm (70 kVp, 114 μA, 200 ms integration time). The metaphyseal scan region of the intact femur consisted of 100 slices beginning 1 mm proximal to and extending away from the growth plate. The diaphyseal region was located halfway between the femoral head and distal condyles and consisted of 400 slices. The fourth vertebral body (L_4) was scanned and 100 slices around the center were analyzed. Trabecular compartments of the L_4 vertebrae, femoral metaphysis, and midshaft were isolated by manual contouring and analyzed using established analysis protocols from Scanco Medical. The trabecular bone volume fraction (BV/TV) and bone mineral density (BMD) were assessed in the femoral metaphysis, and the cortical BV/TV, BMD, and periosteal diameter were calculated at the femoral mid-diaphysis. Micro-computed tomography (μCT) parameters were reported according to international guidelines.[22]

Bone histology and histomorphometry

Bone histomorphometry was performed as described previously.[23] In brief, calcein was injected intraperitoneally (20 mg/kg, Sigma) on days 5 and 2 before euthanizing the specimens. Bones from fourth lumbar vertebra and proximal tibia were fixated in 4% PBS-buffered paraformaldehyde for 48 hours and dehydrated in an ascending ethanol series. Afterwards, bones were embedded in methyl methacrylate (Technovit 9100, Hereaus Kulzer, Hanau, Germany) and cut into 7-μm sections to assess fluorescence labels. The mineral apposition rate (MAR) and the bone formation rate/bone surface (BFR/BS) were determined using Osteomeasure software (OsteoMetrics, Atlanta, GA, USA) following international standards.[23]

Tartrate-resistant acid phosphatase (TRAP) staining was performed on 2-μm paraffin sections of the fourth vertebral body and femora to assess the number of osteoclasts per bone surface (Oc.N/BS) as well as the number of osteoclasts per bone perimeter (N.Oc/B.Pm), osteoblast surface per bone surface (Ob.S/BS), and number of osteoblasts per bone perimeter (N.Ob/B.Pm).

Serum analysis

Blood was taken via heart punctuation, and serum was collected after 10 minutes centrifugation at 400*g*. Dkk1, sclerostin, C-terminal telopeptide (CTX), and type 1 procollagen amino-terminal-propeptide (P1NP) were measured using an immunoassay kit (Dkk1: R&D Systems, Minneapolis, MN, USA; sclerostin: Alpco, Salem, NH, USA; CTX and P1NP: Immundiagnostik, Bensheim, Germany) according to the manufacturer's protocols. For the sclerostin ELISA, serum samples were diluted 1:3.

RNA isolation, RT, and real-time PCR

RNA was extracted from the femur by crushing flushed femora of mice in liquid nitrogen and collecting the bone powder in Trifast

(Peqlab, Germany). RNA isolation was performed according to the manufacturer's protocol. Five hundred nanograms of RNA were reversed transcribed using Superscript II (Invitrogen, Darmstadt, Germany) and used for SYBR green-based real-time PCR using a standard protocol (Applied Biosystems, Carlsbad, CA, USA). The primer sequences were: β-actin s: ATCTGGCACCACACCTTCT, β-actin as: GGGGTGTTGAAGGTCT-CAAA; Dkk1 s: GAGGGGAAATTGAGGAAAGC, Dkk1 as: AGCC TTCTTGTCCTTTGGTG, RANKL s: CCGAGACTACGGCAAGTACC, RANKL as: GCGCTCGAAAGTACAGGAAC, OPG s: CCTTGCCCTG ACCACTCTTA, OPG as: ACACTGGGCTGCAATACACA, Sost s: CGTGCCTCATCTGCCTACTT, Sost as: TGACCTCTGTGGCATCATTC. PCR conditions were as follows: 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles with 95°C for 15 seconds and 60°C for 1 minute. The melting curve was assessed by the following program: 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 30 seconds. The results were calculated using the ΔΔCT method and are presented in x-fold increase relative to β-actin mRNA levels.

Statistical analysis

Results are presented as means SD. Statistical evaluations were performed using a Student's *t* test. Values of $p < 0.05$ were considered statistically significant.

Results

Global deletion of Dkk1 increases bone mass

As Dkk1 plays an important role during embryonic development, its global deletion is lethal.[24] Therefore, we generated Dkk1$^{fl/fl}$;Rosa26-CreERT2 conditional knockout mice in which Dkk1 is globally inactivated after tamoxifen administration. Tamoxifen was injected intraperitoneally for 5 days at the age of 7 weeks. All mice showed normal development before tamoxifen application, and no changes in weight or bone growth occurred after tamoxifen injections (Table 1).

At 12 weeks of age, male and female Cre-positive mice displayed a twofold higher bone volume in the distal femur (Fig. 1*A*) and the fourth lumbar vertebrae (Table 2), showing a more pronounced effect in the femur. Increased bone mass was independent of sex. At the structural level, the femur had a higher trabecular number and thickness, whereas trabecular separation was lower (Fig. 1*B–D*). Moreover, the conditional Dkk1 deletion increased cortical thickness (+6%) (Fig. 1*E*), whereas cortical bone mineral density was unchanged (Fig. 1*F*). The vertebral bone showed a similar pattern, and the data are summarized in Table 2. Representative 3D reconstructions of the femur are depicted in Fig. 1*G*.

Finally, the successful deletion of Dkk1 was confirmed by measurement of Dkk1 in the serum, which was strongly reduced in Cre-positive mice (male, –96%; female, –98%) (Fig. 1*H*), and in femoral bone, which likewise showed reduced Dkk1 mRNA levels (male, –90%; female, –93%) (Fig. 1*I*). As a counter-regulation of various Wnt inhibitors has previously been reported,[25,26] we also determined serum levels of sclerostin, which were significantly higher in Dkk1-deficient mice (Fig. 1*J*). Similarly, skeletal mRNA expression of sclerostin was higher in conditional Dkk1 knockout mice (male, +84%; female, +78%) (Fig. 1*K*). Thus, postnatal global deletion of Dkk1 in mice results in high bone mass, in spite of elevated sclerostin levels, further underlining its pivotal role in bone homeostasis.

Global conditional Dkk1 knock-out increases osteoblast activity

To determine whether the high bone mass is a result of increased bone formation or decreased bone resorption, we assessed bone turnover. The postnatal global deletion of Dkk1 in mice resulted in higher serum P1NP levels (Fig. 2*A*). At the microscopic level, the mineralizing surface was increased in the tibia of female Dkk1 knockout mice, whereas it did not differ in males (Fig. 2*B*). The mineral apposition rate and bone formation rate were significantly increased two- to fourfold in both sexes (Fig. 2*C*, *D*). The vertebrae of Cre-positive mice showed a similar increase in bone formation (Table 1). In both sexes, deletion of Dkk1 did not alter the osteoblast number (data not shown).

Serum levels of the bone resorption marker CTX-1 (Fig. 2*E*) were not altered in Dkk1-deficient mice, but the number of osteoclasts was reduced in the tibia (Fig. 2*F*) and vertebral body (Table 2).

Given that global deletion of Dkk1 resulted in a decreased number of osteoclasts, and Wnt signaling has been shown to modulate receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG), [27–29] we analyzed their expression in bone tissue. RANKL mRNA levels were significantly reduced by 85% to 88% (Fig. 2*G*), whereas OPG expression was increased

Table 1. Body Weight, Length and Periosteal Diameter of Femora of 12-Week-Old Dkk1;Rosa26-CreERT2, Dkk1;OsxCre, and Dkk1;Dmp1Cre Mice

	Male			Female		
	Cre− ($n = 9$)	Cre+ ($n = 12$)	*P* Value	Cre− ($n = 9$)	Cre+ ($n = 12$)	*p* Value
Dkk1$^{fl/fl}$;Rosa26-CreERT2						
Body weight (g)	24.9 ± 0.81	25.2 ± 0.49	0.582	22.3 ± 0.92	22.4 ± 0.86	0.787
Femur length (mm)	14.3 ± 0.10	14.4 ± 0.16	0.722	14.2 ± 0.16	14.2 ± 0.51	0.339
Periosteal diameter (mm)	0.97 ± 0.05	1.01 ± 0.06	0.184	0.84 ± 0.03	0.86 ± 0.04	0.115
Dkk1$^{fl/fl}$;Osx:Cre						
Body weight (g)	23.9 ± 0.41	24.6 ± 0.61	0.458	22.7 ± 0.67	21.8 ± 0.78	0.258
Femur length (mm)	13.7 ± 0.17	13.6 ± 0.23	0.560	13.7 ± 0.24	13.7 ± 0.33	0.705
Periosteal diameter (mm)	1.01 ± 0.05	1.02 ± 0.08	0.770	0.90 ± 0.05	0.93 ± 0.04	0.118
Dkk1$^{fl/fl}$;Dmp1:Cre						
Body weight (g)	24.3 ± 0.41	24.9 ± 0.70	0.784	22.9 ± 0.89	23.4 ± 0.77	0.457
Femur length (mm)	14.4 ± 0.12	14.4 ± 0.15	0.747	14.2 ± 0.10	14.3 ± 0.06	0.215
Periosteal diameter (mm)	1.00 ± 0.06	1.03 ± 0.04	0.129	0.91 ± 0.04	0.95 ± 0.05	0.121

Data represent the mean ± SD. Statistical analysis was performed by the Student's *t* test.

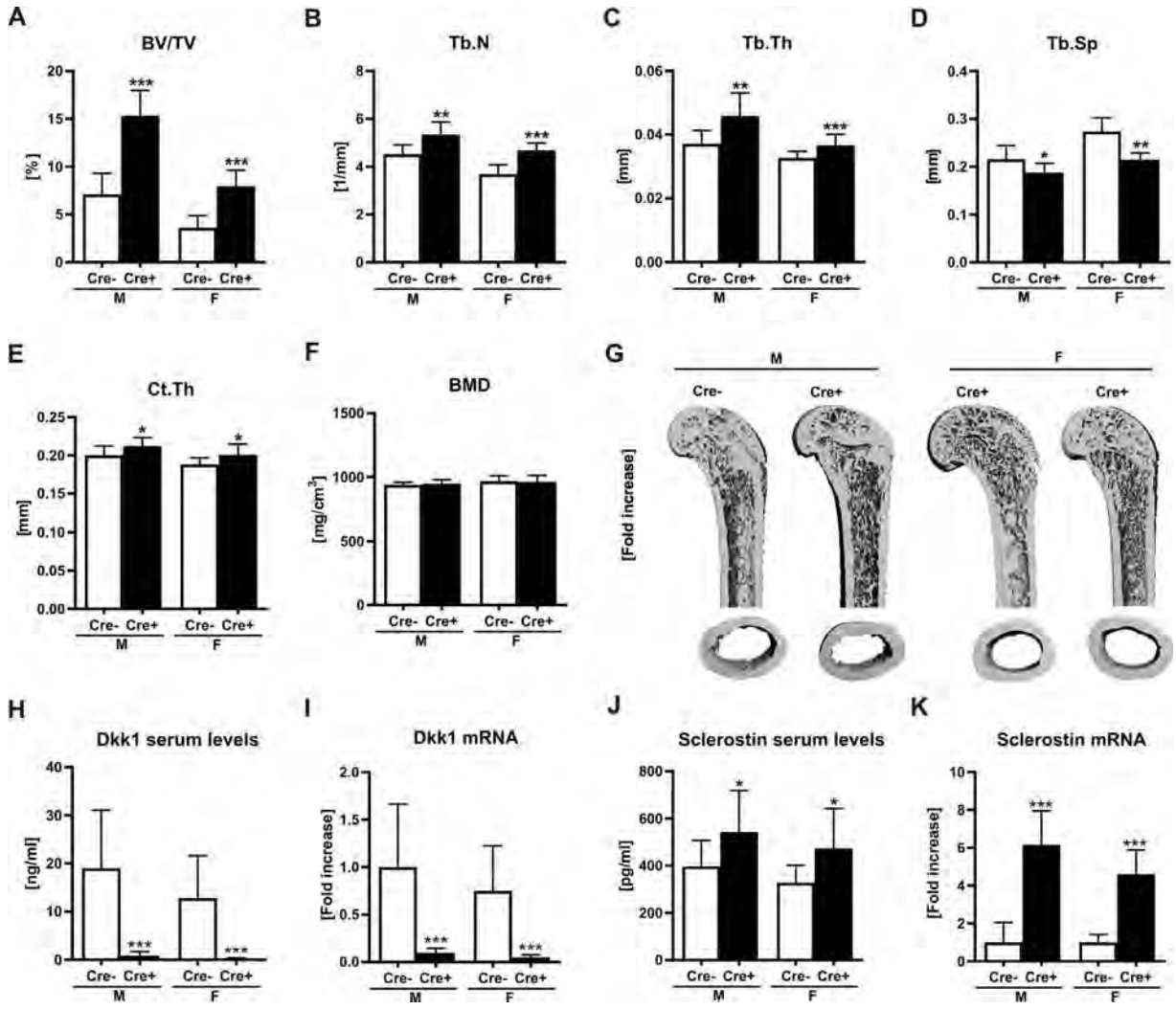

Fig. 1. Global deletion of Dkk1 results in high bone mass, despite increased sclerostin expression. The distal femora of 12-week-old male and female Dkk1$^{fl/fl}$;Rosa26-ERT2-Cre-positive and -negative mice were analyzed by μCT. (*A*) Trabecular bone volume per total volume (BV/TV), (*B*) trabecular number (Tb.N), (*C*) trabecular thickness (Tb.Th), and (*D*) trabecular separation (Tb.Sp) of the distal femur. (*E*) Cortical bone mineral density (BMD) and (*F*) cortical thickness (Ct.Th) of the femoral midshaft. (*G*) Representative 3D reconstruction of the whole femora. (*H*) Serum Dickkopf-1 (Dkk1) levels were assessed using a commercially available ELISA and (*I*) real-time PCR analysis was performed for Dkk1 in femoral bone tissue. (*J*) Sclerostin (SOST) serum levels were assessed using a commercially available ELISA and (*K*) sclerostin mRNA expression levels in the femora were assessed using real-time PCR analysis. Data represent the mean ± SD ($n = 9$–12/group). Statistical analysis was performed by the Student's *t* test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

five- to sevenfold in male and female mice (Fig. 2*H*). Taken together, postnatal global deletion of Dkk1 in mice enhances bone mass by increasing bone formation and reducing the number of osteoclasts, which may be mediated by a decreased RANKL/OPG ratio.

Conditional loss of Dkk1 at various stages of osteoblast maturation increases bone mass

Next, we investigated at which stage of osteoblast maturation Dkk1 plays a role. Therefore, we generated Dkk1$^{fl/fl}$;Osx-Cre and Dkk1$^{fl/fl}$;Dmp1-Cre mice, in which either cells are targeted early on (ie, at the osteoprogenitor stage and beyond using the Osx-cre) or later during osteoblast maturation (ie, late osteoblasts and osteocytes using the Dmp1-cre).[5,22,30]

Dkk1$^{fl/fl}$;Osx-Cre and Dkk1$^{fl/fl}$;Dmp1-Cre mice were born at a Mendelian ratio with no gross abnormalities, eg, size or body weight compared with control littermates (Table 1). The bone phenotype was analyzed in 12-week-old male and female mice. Both female Dkk1$^{fl/fl}$;Osx-Cre and Dkk1$^{fl/fl}$;Dmp1-Cre mice showed a similar increase in the bone volume fraction in the femur and vertebral body compared with littermate controls (Fig. 3*A*, Table 2). The bone microstructural data for male mice are shown in Table 3. In line with results from the global Dkk1 deletion, the appendicular skeleton was more severely affected by Dkk1 knockout (Table 2).

Although trabecular thickness was not changed in any of the Dkk1 cKO mice, trabecular number was higher and trabecular separation lower in both Dkk1 cKO strains (Fig. 2*B–D*; Table 3). Deletion of Dkk1 using the Osx-Cre did not alter the cortical

Table 2. Bone Microstructure and Histological Parameters of the Fourth Lumbar Vertebrae of 12-Week-Old Dkk1;Rosa26-CreERT2, Dkk1;OsxCre, and Dkk1;Dmp1Cre Mice

	Male			Female		
Lumbar spine	Cre− ($n=9$)	Cre+ ($n=12$)	p Value	Cre− ($n=9$)	Cre+ ($n=12$)	p Value
Dkk1$^{fl/fl}$;Rosa26-CreERT2						
μCT						
BV/TV (%)	12.9 ± 2.87	17.3 ± 4.97	<0.001	13.6 ± 2.72	17.9 ± 2.46	<0.001
Tb.N (1/mm)	4.02 ± 0.34	4.48 ± 0.48	<0.05	3.86 ± 0.36	4.45 ± 0.38	<0.01
Tb.Th (μm)	39.6 ± 5.66	48.0 ± 5.53	<0.01	40.2 ± 3.75	43.6 ± 2.76	<0.05
Tb.Sp (mm)	0.25 ± 0.03	0.23 ± 0.03	0.242	0.25 ± 0.03	0.25 ± 0.03	0.971
Histomorphometry						
MS/BS (%)	0.21 ± 0.15	0.38 ± 0.18	<0.05	0.19 ± 0.16	0.72 ± 0.49	<0.05
MAR (μm/d)	17.5 ± 2.24	18.1 ± 4.71	0.751	19.8 ± 3.81	24.6 ± 4.62	<0.05
BFR/BS ($\mu m^3/\mu m^2$/d)	1.12 ± 0.77	2.12 ± 1.07	<0.05	0.91 ± 0.79	2.78 ± 1.83	<0.05
N.Oc/B.Pm (#/mm)	5.53 ± 1.82	3.11 ± 1.98	<0.05	5.65 ± 2.86	2.81 ± 1,39	<0.05
Dkk1$^{fl/fl}$;Osx:Cre						
μCT						
BV/TV (%)	14.1 ± 3.30	17.7 ± 2.36	<0.05	9.31 ± 3.00	13.5 ± 2.28	<0.01
Tb.N (1/mm)	4.16 ± 0.71	4.88 ± 0.66	<0.05	3.35 ± 0.51	3.88 ± 0.39	<0.05
Tb.Th (μm)	40.3 ± 4.85	45.0 ± 3.12	<0.05	38.4 ± 3.39	42,1 ± 2.43	<0.05
Tb.Sp (mm)	0.22 ± 0.04	0.20 ± 0.02	0.064	0.29 ± 0.08	0.27 ± 0.03	0.271
Histomorphometry						
MS/BS (%)	17.1 ± 4.03	22.7 ± 10.4	0.135	26.8 ± 6.08	24.6 ± 4.67	0.374
MAR (μm/d)	1.18 ± 0.95	4.52 ± 2.02	<0.001	1.48 ± 0.64	5.20 ± 3.70	<0.01
BFR/BS ($\mu m^3/\mu m^2$/d)	0.20 ± 0.17	1.09 ± 0.65	<0.01	0.43 ± 0.22	1.31 ± 0.96	<0.05
N.Oc/B.Pm (#/mm)	5.38 ± 3.59	0.59 ± 0.92	<0.05	8.11 ± 2.81	4.24 ± 0.96	<0.05
Dkk1$^{fl/fl}$;Dmp1:Cre						
μCT						
BV/TV (%)	10.18 ± 2.95	14.6 ± 3.12	<0.01	9.2 ± 1.27	12.49 ± 1.83	<0.001
Tb.N (1/mm)	4.07 ± 0.33	4.75 ± 0.25	<0.001	3.52 ± 0.40	3.77 ± 0.38	0.186
Tb.Th (μm)	42.1 ± 7.28	42.0 ± 4.93	0.960	39.7 ± 3.06	41.5 ± 1.98	0.159
Tb.Sp (mm)	0.25 ± 0.02	0.21 ± 0.01	<0.001	0.29 ± 0.03	0.27 ± 0.03	0.200
Histomorphometry						
MS/BS (%)	19.4 ± 5.17	20.0 ± 6.93	0.825	22.8 ± 6.62	28.3 ± 3.76	0.113
MAR (μm/d)	0.98 ± 0.68	2.09 ± 0.93	<0.01	1.29 ± 0.57	2.89 ± 1.42	<0.05
BFR/BS ($\mu m^3/\mu m^2$/d)	0.21 ± 0.18	0.42 ± 0.19	<0.05	0.32 ± 0.20	0.80 ± 0.36	<0.05
N.Oc/B.Pm (#/mm)	6.43 ± 3.45	2.43 ± 2.29	<0.05	11.6 ± 5.14	7.44 ± 2.85	0.095

BV/TV = bone volume/total volume; Tb.N = trabecular number; Tb.Th = trabecular thickness; Tb.Sp = trabecular separation; MAR = mineral apposition rate; MS/BS = mineralizing surface/bone surface; BFR/BS = bone formation rate/bone surface; N.Oc/B.Pm = number of osteoclasts/bone perimeter.
Data represent the mean ± SD. Statistical analysis was performed by the Student's t test.

bone phenotype in male or female mice, but deletion using the Dmp1-Cre increased cortical thickness and cortical density in female mice but not in male mice (Fig. 3*E*, *F*; Table 3). Representative 3D reconstructions are shown in Fig. 3*G*.

Surprisingly, when we assessed the serum levels of Dkk1 to validate the efficient knockout, we detected reduced Dkk1 serum levels only in Dkk1$^{fl/fl}$;Osx-Cre mice (males, −79%; females, −77%) (Fig. 3*H*; Table 3). Serum levels of Dkk1 were neither changed in male nor in female Dkk1$^{fl/fl}$;Dmp1-Cre mice (Fig. 3*H*; Table 3). To test whether deletion of Dkk1 using the Dmp1-Cre results in a local downregulation of Dkk1, we analyzed Dkk1 mRNA levels in the bone tissue. Both mutant mouse strains showed a significant reduction of Dkk1 mRNA in the femur (Fig. 3*I*; Table 3). Similar to global Dkk1 deletion, also deficiency of Dkk1 in osteoprogenitors and mature osteoblasts/osteocytes resulted in higher serum levels and skeletal mRNA expression of sclerostin (Fig. 3*J*, *K*; Table 3).

These data show that most circulating Dkk1 originates from osteoprogenitors but not from mature osteoblasts or osteocytes. Nevertheless, the amount of Dkk1 in mature osteoblasts/osteocytes is sufficient to modulate bone mass, despite elevated sclerostin levels.

Dkk1 deletion in osteogenic cells increases bone formation and reduces numbers of osteoclasts

To investigate whether, similar to the global Dkk1 knockout mice, Dkk1$^{fl/fl}$;Osx-Cre and Dkk1$^{fl/fl}$; Dmp1-Cre mice display increased bone formation and a reduced number of osteoclasts, we performed further experiments including ELISA and histomorphometry. In line with increased P1NP serum levels, the mineral apposition (threefold) and bone formation rate (threefold) were significantly enhanced in the tibia of female mice of both strains, whereas the mineralizing surface did not differ (Fig. 4*A*–*D*). This increase in bone formation was independent of sex, as also males showed a similar trend (Table 3). Concordant with the more pronounced effects measured by μCT analysis of the femur, the bone formation parameters were only increased about twofold in the axial

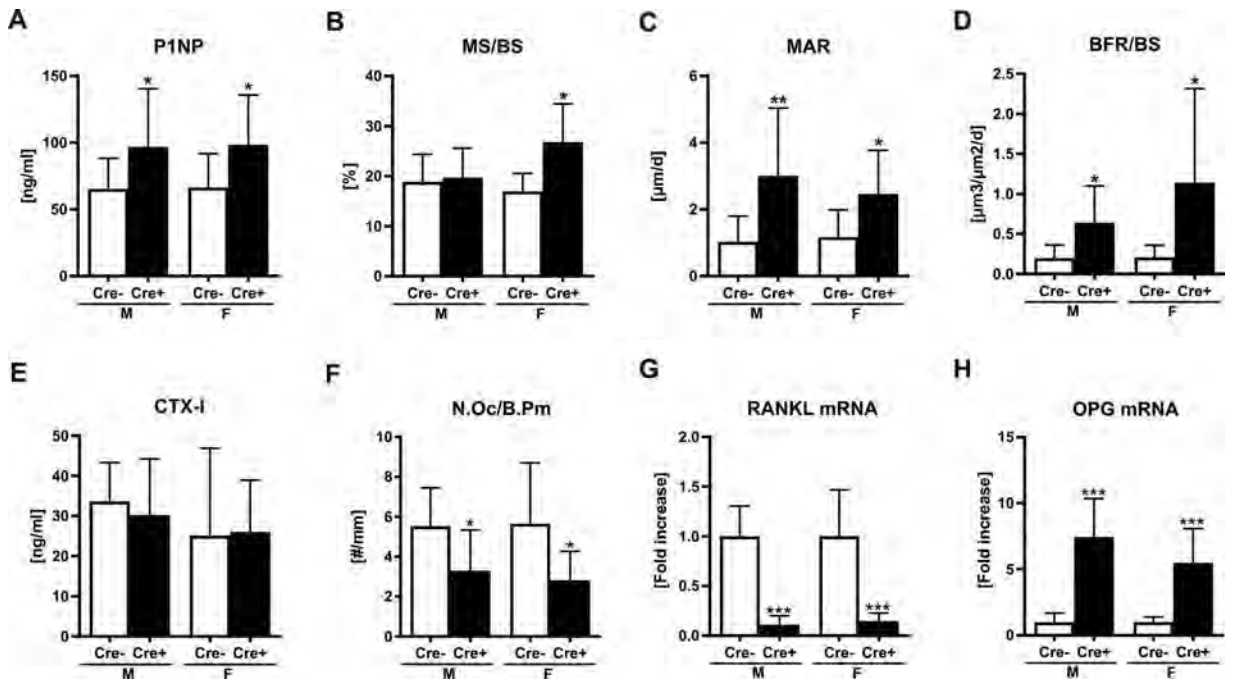

Fig. 2. Global Dkk1 deficiency increases bone formation and decreases osteoclast numbers. Histomorphometric and serum osteoblast and osteoclast parameters of 12-week-old Dkk1$^{fl/fl}$;Rosa26-ERT2-Cre mice and littermate controls. (*A*) Quantification of serum procollagen type 1 amino-terminal propeptide (P1NP) was performed by ELISA. (*B*, *C*) Histomorphometric analysis of calcein double labeling was performed to determine the (*B*) mineralizing surface/bone surface (MS/BS), (*C*) mineral apposition rate (MAR), and (*D*) bone formation rate/bone surface (BFR/BS). (*E*) Serum carboxy-terminal collagen cross-links (CTX-1), as a marker for bone resorption, was measured using ELISA. (*F*) Using tartrate-resistant acid phosphatase (TRAP) staining, the number of osteoclasts/bone parameter (N.Oc/B.Pm) was determined. (*G*) Receptor activator of nuclear factor-κB ligand (RANKL) mRNA and (*H*) osteoprotegerin (OPG) mRNA expression in femoral bone tissue was analyzed using real-time PCR analysis. Data represent the mean ± SD ($n = 9$–12/group). Statistical analysis was performed by the Student's *t* test. $^{*}p < 0.05$, $^{**}p < 0.01$.

skeleton of Dkk1$^{fl/fl}$;Osx-Cre and Dkk1$^{fl/fl}$;Dmp1-Cre mice (Table 2). In both cKO lines, the osteoblast number was not changed (data not shown). Serum CTX levels were unchanged (Fig. 2*E*, Table 3), whereas TRAP staining showed a decreased number of osteoclasts in the femur (Fig. 2*F*; Table 3) and in the spine (Table 2). Analysis of RANKL and OPG expression in the femur showed that deletion of Dkk1 using both Cre-lines resulted in a decreased RANKL (Fig. 2*G*; Table 3) and increased OPG expression (Fig. 2*H*; Table 3). Thus, even though osteoprogenitors produce more Dkk1, also late osteoblasts/osteocytes contribute to the negative regulation of bone mass by suppressing osteoblast activity and increasing osteoclast numbers.

Discussion

The Wnt signaling pathway regulates many aspects of bone homeostasis. It regulates osteoblast differentiation,[4,27] proliferation, and survival,[30] and thereby bone formation and also bone resorption.[29] Dkk1 is a potent Wnt antagonist functioning during embryonic development[24] and bone formation.[30] Overexpression of Dkk1 in osteoblasts decreases osteoblast numbers and results in osteopenia,[10] whereas loss of one Dkk1 allele increases bone formation and bone mass in mice.[11] Additionally, Dkk1 has been implicated in various diseases including arthritis,[12] postmenopausal osteoporosis,[18] as well as various cancers.[13–15] To elucidate the role of Dkk1 in the regulation of bone homeostasis after embryonic development, we investigated how postnatal global inactivation of Dkk1 in mice as well as the deletion of Dkk1 in various osteolineage populations affects bone remodeling.

As previous studies showed that homozygous global Dkk1 deletion is lethal and leads to severe developmental phenotypes including head defects and limb dysmorphogenesis,[24] we generated mice in which global deletion of Dkk1 can be induced with tamoxifen after birth. Using this approach, we found that Dkk1 is a critical negative regulator of bone homeostasis in mice. This finding was further supported by the postnatal inactivation of Dkk1 in osterix-expressing cells in mice. Importantly, even though the Osx-Cre is known to target hypertrophic chondrocytes, deletion of Dkk1 did not affect bone growth in either model.

Our results show that the high bone mass in all three mouse lines resulted from enhanced bone formation. This was mainly driven by increased osteoblast activity rather than enhanced recruitment of osteoblastic precursor cells as the mineral apposition rate was more significantly affected than the mineralizing surface. A previous study with Dkk1 overexpression showed decreased osteoblast numbers, although the mineralizing surface was not reduced.[10] Also, the bone formation rate was highly reduced, suggesting that this decrease was mainly caused by a decreased mineral apposition rate, although this parameter was not reported.[10] Another study using a single allele deletion of Dkk1 showed an increased mineralizing surface and mineral apposition rate in Dkk1-deficient mice.[11] Thus, in

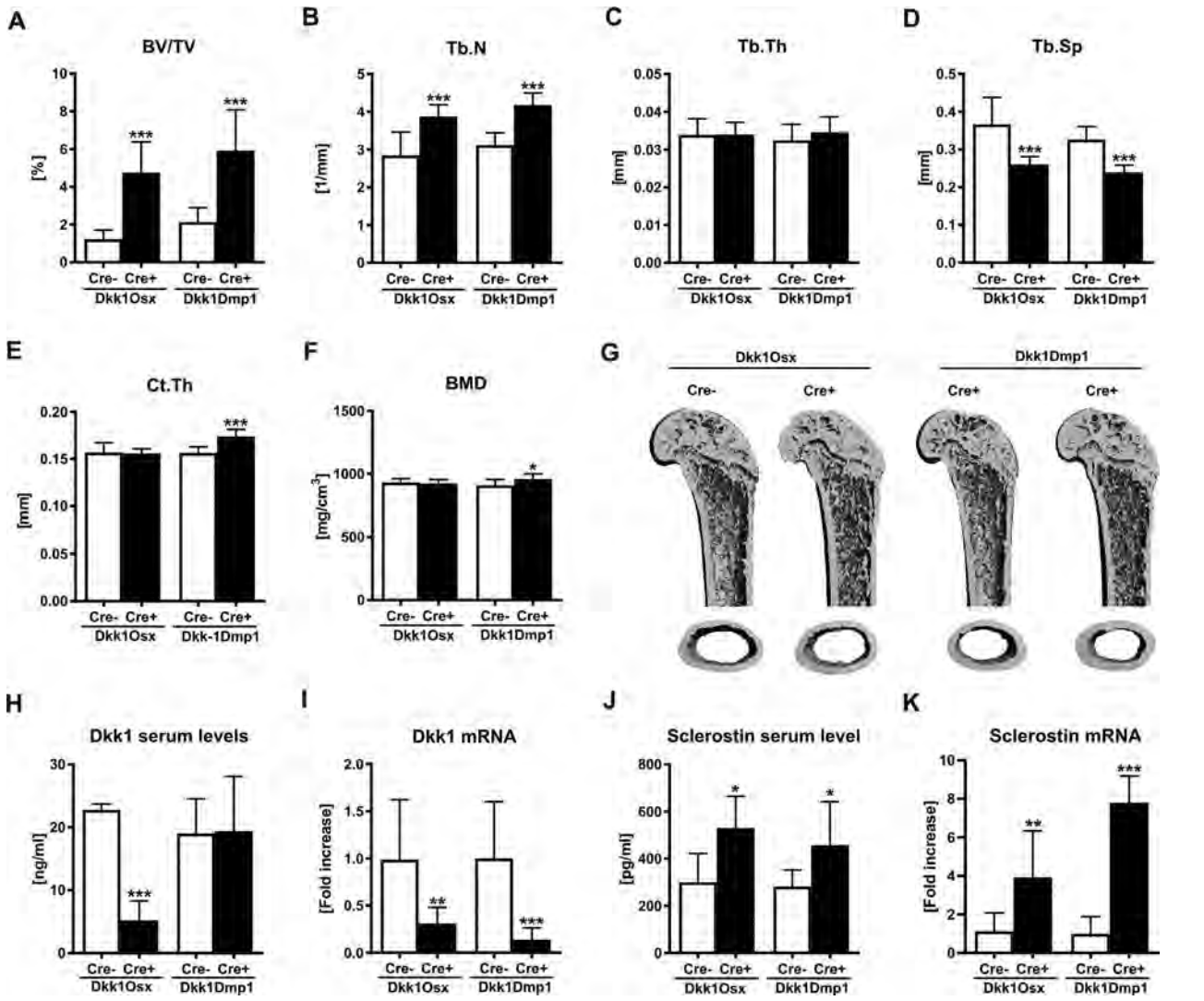

Fig. 3. Lack of Dkk1 in osteoprogenitors (Osx-Cre) and osteocytes (Dmp1-Cre) increases bone volume, despite increased sclerostin expression. The distal femora of 12-week-old female $Dkk1^{fl/fl}$;Osx-Cre and $Dkk1^{fl/fl}$;Dmp1-Cre (Cre-positive and Cre-negative) mice were analyzed by μCT. (*A*) Trabecular bone volume per total volume (BV/TV), (*B*) trabecular number (Tb.N), (*C*) trabecular thickness (Tb.Th), and (*D*) trabecular separation (Tb.Sp) of the distal femur. (*E*) Cortical bone mineral density (BMD) and (*F*) cortical thickness (Ct.Th) of the femoral midshaft. (*G*) Representative 3D reconstruction of the whole femora. (*H*) Serum Dickkopf-1 (Dkk1) levels were assessed using a commercially available ELISA and (*I*) real-time PCR analysis was performed for Dkk1 in femoral bone tissue. (*J*) Sclerostin (SOST) serum levels were assessed using a commercially available ELISA and (*K*) sclerostin mRNA expression levels in the femora were assessed using real-time PCR analysis. Data represent the mean ± SD ($n = 9$–12/group). Statistical analysis was performed by the Student's *t* test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

light of current literature and given that our data did not show consistent effects of Dkk1 on the abundance of osteoblasts on the bone surface, this suggests that Dkk1 has stronger effects on osteoblast function than on osteoblast proliferation or recruitment to bone surfaces. A possible explanation for this may be the increased serum sclerostin and mRNA levels found in all three knockout mouse strains, suggesting that elevated sclerostin restricts osteoblast recruitment or reactivation of lining cells when Dkk1 levels are low. In line with this finding, other studies have previously reported on counter-regulatory circuits between Wnt inhibitors. Florio and colleagues showed that sclerostin inhibition as well as Sost deficiency results in an upregulation of Dkk1 expression.[25] In addition, knockout of sFRP4 results in increased Sost expression.[26] Our study extends these compensatory circuits by showing that inhibition of Dkk1 also increases sclerostin expression. Interestingly, Dkk1 deficiency increased bone formation, even though sclerostin levels were increased. This suggests that sclerostin may only be able to inhibit bone formation in the presence of Dkk1 and that Dkk1 is the main determinant of bone formation. In any case, the regulation of osteoblast function by Wnt signaling, in particular its inhibitors, is a highly regulated and fine-tuned mechanism that requires further investigation to fully understand the regulatory circuits at play.

Furthermore, we show that lack of Dkk1 in our models affects bone resorption by decreasing the number of osteoclasts without affecting the serum level of the bone resorption marker CTX. The low number of osteoclasts may stem from the

Table 3. Bone Phenotype of Femora of 12-Week-Old Male Dkk1$^{fl/fl}$;Osx:Cre and Dkk1$^{fl/fl}$;Dmp1:Cre Mice

	Dkk1$^{fl/fl}$;Osx:Cre			Dkk1$^{fl/fl}$;Dmp1:Cre		
Males	Cre− ($n = 11$)	Cre+ ($n = 10$)	*P* Value	Cre− ($n = 10$)	Cre+ ($n = 12$)	*P* Value
μCT						
BV/TV (%)	6.72 ± 2.32	12.5 ± 3.55	<0.001	6.18 ± 3.00	10.6 ± 2.82	<0.01
Tb.N (1/mm)	4.33 ± 0.53	5.66 ± 0.40	<0.001	4.05 ± 0.53	5.38 ± 0.42	<0.001
Tb.Th (μm)	37.6 ± 3.54	40.5 ± 3.30	0.078	39.6 ± 7.40	38.9 ± 5.61	0.791
Tb.Sp (mm)	0.23 ± 0.03	0.18 ± 0.01	<0.001	0.25 ± 0.04	0.18 ± 0.02	<0.001
BMD (mg/μm^3)	932 ± 32.1	928 ± 47.6	0.828	918 ± 19.1	899 ± 45.5	0.295
Ct.Th (mm)	0.17 ± 0.01	0.17 ± 0.01	0.907	0.17 ± 0.01	0.17 ± 0.02	0.357
Histomorphometry						
MAR (μm/d)	0.97 ± 0.81	3.51 ± 2.05	<0.01	1.26 ± 0.69	2.30 ± 1.10	<0.05
MS/BS (%)	17.7 ± 3.53	26.3 ± 5.90	<0.001	20.7 ± 4.74	25.1 ± 4.88	<0.05
BFR/BS (μm^3/μm^2/d)	0.18 ± 0.17	0.92 ± 0.58	<0.001	0.27 ± 0.16	0.92 ± 1.29	0.087
N.Oc/B.Pm (#/mm)	4.72 ± 3.25	3.15 ± 3.06	0.358	7.13 ± 4.75	3.23 ± 2.27	0.079
Serum parameters						
Dkk1 (ng/mL)	25.5 ± 4.73	5.32 ± 1.80	<0.001	26.7 ± 7.09	22.9 ± 6.12	0.313
Sclerostin (pg/mL)	200 ± 70.9	437 ± 177	<0.05	290 ± 67.4	393 ± 78.8	<0.05
P1NP (ng/mL)	60.2 ± 13.9	79.3 ± 13.9	<0.05	59.9 ± 17.3	115 ± 63.7	<0.05
CTX (ng/mL)	33.8 ± 9.02	34.7 ± 6.80	0.522	29.3 ± 8.53	30.9 ± 8.05	0.737
mRNA level						
Dkk1 (fold increase)	1.00 ± 0.76	0.44 ± 0.08	<0.001	1.00 ± 0.60	0.14 ± 0.13	<0.001
Sclerostin (fold increase)	1.00 ± 0.72	4.49 ± 2.30	<0.001	1.00 ± 0.77	6.59 ± 2.03	<0.001
RANKL (fold increase)	1.00 ± 0.28	0.27 ± 0.17	<0.001	1.00 ± 0.39	0.24 ± 0.22	<0.01
OPG (fold increase)	1.00 ± 0.61	3.58 ± 0.63	<0.001	1.00 ± 0.65	4.01 ± 1.72	<0.01

BV/TV = bone volume per total volume; Tb.N = trabecular number; Tb.Th = trabecular thickness; Tb.Sp = trabecular separation; BMD = bone mineral density; Ct.Th = cortical thickness; MAR = mineral apposition rate; MS/BS = mineral surface/bone surface; BFR/BS = bone formation rate/bone surface; N.Oc/B.Pm = number of osteoclast/bone parameter.

Data represent the mean ± SD. Statistical analysis was performed by the Student's *t* test.

decreased skeletal RANKL/OPG ratio. This is consistent with previous studies in arthritic mice showing that blocking Dkk1 with antibodies decreases osteoclastogenesis by increasing OPG levels.[12] In addition, a previous study of experimental multiple myeloma using anti-Dkk1 antibodies showed increased bone formation, whereas osteoclast numbers were reduced in vivo.[31] Dkk1 antibody treatment in healthy mice, however, did not alter the eroded surface of trabecular bone.[25] Moreover, Dkk1 overexpression and the single allele deletion did not alter serum bone resorption markers or osteoclast numbers as assessed by histology.[11,12] Of note, the latter study depicted the osteoclast number relative to the total area. Considering that the heterozygous deletion of Dkk1 resulted in a twofold increase in the bone volume fraction and a 20% reduction in osteoclast numbers, it is likely that by relating numbers to the bone surface, osteoclast numbers may be reduced. Collectively, these data suggest that Dkk1 not only regulates osteoblast function but also affects osteoclast numbers. Why the decrease in osteoclast numbers in Dkk1 conditional knockout mice failed to decrease bone resorption as displayed by serum CTX levels remains to be further clarified. Likely, a transient effect on osteoclasts may be envisaged, similar to the suppression of osteoclast activity by sclerostin, which likewise remains to be conclusively addressed.[32]

Overall, the bone phenotype of Dkk1$^{fl/fl}$;Osx-Cre mice strongly resembles that of Dkk1$^{fl/fl}$;Dmp1-Cre mice. Importantly, one major difference between the mouse lines was the reduction of systemic Dkk1 levels. Although a significant reduction of serum Dkk1 was observed in the Dkk1$^{fl/fl}$;Osx-Cre mice, they were not altered in Dkk1$^{fl/fl}$;Dmp1-Cre mice. Nonetheless, both lines showed a marked reduction of Dkk1 in the bone tissue as well as a twofold increase in bone volume. Thus, osterix-expressing cells contribute to systemic Dkk1 levels, whereas Dmp1-expressing cells (ie, mainly late osteoblasts and osteocytes) only contribute to local Dkk1 levels. Nevertheless, local Dkk1 expression is sufficient to modulate bone mass. Thus, measuring Dkk1 in serum of patients or animals may not necessarily reflect local bone turnover. Of note, besides osteoprogenitors and their progeny, the Osx-Cre also targets a subset of hypertrophic chondrocytes, bone marrow adipocytes, and perivascular cells but not cells of the hematopoietic lineage, including osteoclasts.[33,34] Thus, it should be considered that in addition to osteoprogenitors, other bone marrow cells may contribute to systemic Dkk1 levels.

Finally, we observed sex-specific effects of Dkk1 in cortical bone. Although global Dkk1 deletion resulted in increased cortical thickness in male and female mice, lack of Dkk1 in osteocytes only affected the BMD and cortical thickness in females. The sex dimorphisms could be explained by the higher number of osteocytes in cortical bone of female compared with male mice,[35] resulting in a higher impact of Dkk1 deletion in females. In line with these findings, sex dimorphism has also been documented previously in various studies indicating that loss of function of Wnt/β-catenin signaling components affects the female skeleton more severely than the male skeleton.[6,36,37] At this point, the underlying mechanisms remain elusive, but interactions of the Wnt pathway with the sex steroid pathway may be envisaged or the effects may be related to overall sex-specific differences in bone mass, turnover, and biomechanics.[38–42]

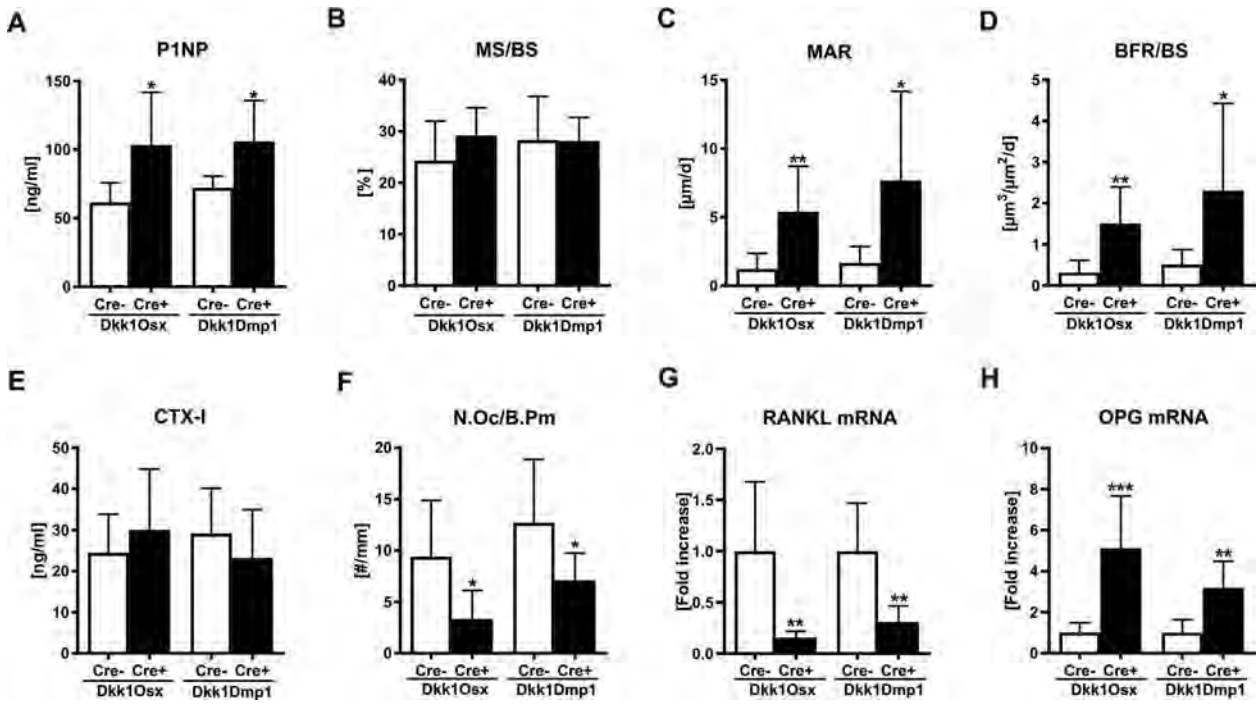

Fig. 4. Deficiency of Dkk1 in osteoprogenitors and osteocytes results in an increased bone formation and reduced osteoclast number. Histomorphometric and serum osteoblast and osteoclast parameters of 12-week-old Dkk1$^{fl/fl}$;Osx-Cre mice, Dkk1$^{fl/fl}$;Dmp1-Cre mice, and littermate controls were analyzed. (*A*) Quantification of serum procollagen type 1 amino-terminal propeptide (P1NP) was performed by ELISA. (*B*, *C*) Histomorphometric analysis of calcein double staining was performed to determine the (*B*) mineralizing surface/bone surface (MS/BS), (*C*) mineral apposition rate (MAR), and (*D*) bone formation rate/bone surface (BFR/BS). (*E*) Serum carboxy-terminal collagen cross-links (CTX-1) was measured using ELISA. (*F*) Tartrate-resistant acid phosphatase (TRAP) staining was used to determine the number of osteoclast/bone parameter (N.Oc/B.Pm). (*G*) Receptor activator of nuclear factor-κB ligand (RANKL) mRNA and (*H*) osteoprotegerin (OPG) mRNA expression in femoral bone tissue was analyzed using real-time PCR analysis. Data represent the mean ± SD ($n = 7$–10/group). Statistical analysis was performed by the Student's *t* test. $^{*}p < 0.05$, $^{**}p < 0.01$.

Taken together, our study shows that Dkk1 produced by osteolineage cells plays an essential role in bone remodeling by regulating osteoblast and, to a lesser extent, osteoclast function. Moreover, local levels of Dkk1 as produced by mature osteoblasts and osteocytes are sufficient to regulate systemic bone turnover, regardless of the compensatory increase of sclerostin.

Disclosures

All authors state that they have no conflicts of interest.

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Authors' roles: Study design: JC, ST, and MR. Data collection: JC, ST, and UB. Data analysis: JC, ST, UB, and MR. Data interpretation: JC, ST, UB, LFB, CN, LCH, and MR. Drafting manuscript: JC and MR. Revising and approving final version of manuscript: all authors. MR takes responsibility for the integrity of the data analysis.

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