A Rate-Limiting Role for Dickkopf-1 in Bone Formation and the Remediation of Bone Loss in Mouse and Primate Models of Postmenopausal Osteoporosis by an Experimental Therapeutic Antibody

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ABSTRACT

Genetic studies have linked both osteoporotic and high bone mass phenotypes to low-density lipoprotein receptor-related proteins (LRP4, LRP5, and LRP6). LRPs are receptors for inhibitory Dickkopf-1 (DKK1) protein, and treatment modalities that modulate LRP/DKK1 binding therefore may act as stimulators of bone mass accrual. Here, we report that RH2-18, a fully human monoclonal anti-DKK1 antibody elicits systemic pharmacologic bone efficacy and new bone formation at endosteal bone surfaces in vivo in a mouse model of estrogen-deficiency-induced osteopenia. This was paralleled by partial-to-complete resolution of osteopenia (bone mineral density) at all of the skeletal sites investigated in femur and lumbar-vertebral bodies and the restoration of trabecular bone microarchitecture. More importantly, testing of RH2-18 in adult, osteopenic rhesus macaques demonstrated a rate-limiting role of DKK1 at multiple skeletal sites and responsiveness to treatment. In conclusion, this study provides pharmacologic evidence for the modulation of DKK1 bioactivity in the adult osteopenic skeleton as a viable approach to resolve osteopenia in animal models. Thus, data described here suggest that targeting DKK1 through means such as a fully human anti-DKK1-antibody provides a potential bone-anabolic treatment for postmenopausal osteoporosis.

Introduction

Bone mass and bone turnover in adults is maintained locally by the balance between osteoclastic bone resorption and osteoblastic bone formation, and multiple coupling "factors" maintain this servo system at its physiological (homeostatic) steady state (Harada and Rodan, 2003). High bone turnover initiated during menopause and the succeeding progressive reduction in bone mass and microarchitecture result in an increase in the fragility of bone and its susceptibility to fractures, all characteristics of osteoporosis (Rodan and Martin, 2000).

The intermittent application of human parathyroid hormone fragment [hPTH 1–34] as the first bone-anabolic treatment has illustrated novel cues for effectively restoring lost bone mass, structure, and strength in the clinic.

ABBREVIATIONS: hPTH, human parathyroid hormone fragment; aBMD, areal bone mineral density; BMD, bone mineral density; BV/TV, bone volume per tissue volume; CT, computed tomography; DKK1, Dickkopf-1; DXA, dual-energy X-ray absorptiometry; HBM, high bone mass; HR-pQCT, high-resolution peripheral quantitative computed tomography; LRP, low-density lipoprotein receptor-related protein; MAR, mineral apposition rate; MS/BS, mineralizing surface/bone surface; Ovx, ovariectomized; PBST, phosphate-buffered saline with 0.05% Tween 20; QCT, quantitative computed tomography; Ctx, cross-linked C telopeptide of collagen type I; sCTX, serum CTx; TbN, trabecular number; TbSp, trabecular separation; TbTh, trabecular thickness; TbBMD, trabecular volumetric bone mineral density; NTx, N-telopeptides; uNTx, urinary NTx; vBMD, volumetric bone mineral density; rhDKK1, recombinant human DKK1; P1NP, procollagen type I NH2-terminal propeptide; TBS, Tris-buffered saline.
Recent genetic evidence has substantiated a central role for the Wnt-signaling components low-density lipoprotein receptor-related proteins (LRP4, LRP5, and LRP6) in bone mass accrual during development (Gong et al., 2001; Kokubu et al., 2004; Mani et al., 2007; Choi et al., 2009a). The keystone description of high-bone-mass (HBM) phenotypes in human triggered by single amino acid substitutions in LRP5 (Boyden et al., 2002; Little et al., 2002; Van Wesenbeek et al., 2003) has heightened interest in the Wnt/LRP pathway in bone homeostasis extending into adulthood (Baron and Rawadi, 2007). An increasing number of negative regulatory mechanisms intersecting at Wnt–LRP5/6 have been identified in bone, among those most relevant are endogenous inhibitors of the Wnt-signaling pathway including the DKK proteins (Krupnik et al., 1999), Kremen proteins (Mao et al., 2002), and Sclerostin (Seménov et al., 2005; Ellies et al., 2006). DKK1 binds with high affinity to LRP4/5/6 receptors and Kremen proteins (Bafico et al., 2001; Mao et al., 2001, 2002; Choi et al., 2009a; Fleury et al., 2010). It is interesting to note that LRP5 HBM is refractory to the inhibitory activities of DKK1 (Boyden et al., 2002; Ai et al., 2005), suggesting that this pathway promises enormous opportunities to identify novel treatment paradigms and to intervene pharmacologically with the course of low bone mass diseases (Khosla et al., 2008). Although DKK1 is expressed and secreted within the bone microenvironment (Robling et al., 2008), the protein is soluble and detectable in the peripheral circulation (Kaiser et al., 2008). These cues have led us to develop monoclonal antibodies as reagents that interfere functionally with the role of DKK1 in Wnt signaling and bone formation. In recent studies, DKK1 antibodies have been introduced into mice and have demonstrated efficacy in disease models of rheumatoid arthritis (Diaura et al., 2007) and multiple myeloma (Yaccoby et al., 2007; Pulciniti et al., 2009; Heath et al., 2009) where DKK1 levels are elevated. These findings corroborate a critical role for DKK1 in the bone microenvironment and point to the potential clinical applicability of DKK1-neutralizing agents (Choi et al., 2009b; Gavriatopoulou et al., 2009). However, proof of principle in crucial animal models with systemic osteopenia through estrogen-deficiency emulating postmenopausal osteoporosis in humans has not been demonstrated.

In this study, we describe a pharmacologic approach applying a novel fully human monoclonal anti-DKK1 antibody (Glantschnig et al., 2010). Our data support the hypothesis that systemic restoration of bone mass can be achieved via pharmacologic modulation of DKK1 bioavailability with concomitant resolution of osteopenia in murine animal models. More importantly, we provide evidence for the inhibitory activity and the rate-limiting role of DKK1 on bone mass accrual in the aged ovariectomized (OVX) primate. Thus, we provide the first description of an experimental therapeutic and putative clinically relevant treatment modality specifically targeting DKK1 for the treatment of low systemic bone mass, a characteristic of postmenopausal osteoporosis.

Materials and Methods

Rhesus DKK1 Protein. Rhesus DKK1 fusion protein was expressed and purified as described previously (Glantschnig et al., 2010) and used in all of the biological assays.

Generation of Anti-DKK1 Antibody RH2-18. Cambridge Antibody Technology (Cambridgeshire, UK) phage-displayed single-chain Fv antibody libraries were panned alternately against biotinylated rhesus or mouse DKK1 (R&D Systems, Minneapolis, MN) as described previously (Glantschnig et al., 2010). The single-chain Fv heavy chain variable regions were fused in-frame with the IgG2m4 constant region (An et al., 2009), and resulting anti-DKK1 antibody structures were published previously (Glantschnig et al., 2010). Anti-DKK1 antibody was expressed in Chinese hamster ovary cells using a mammalian expression vector, and the secreted antibodies were purified from the medium using standard protein A/G-based affinity chromatography following the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). Anti-DKK1 antibody RH2-18 binds to human DKK1 with high affinity, and its binding epitope is located within the C-terminal domain of DKK1 and does not show detectable binding to DKK2 or DKK4 proteins (Glantschnig et al., 2010). This experimental antibody was used in all of the procedures below.

Pharmacokinetic Studies. All of the animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals. These studies were carried out under the Institutional Animal Care and Use Committee of Merck Research Laboratories (West Point, PA). Female C57BL/6NTac mice (Taconic Farms, Germantown, NY) were OVX and housed for 7 weeks before use in pharmacokinetic studies and randomized by body weight (n = 3–4 per time point, 19 weeks of age, 28 g). Animals were provided with water and chow ad libitum and maintained on a 12-h light/dark cycle. Anti-DKK1 antibody RH2-18 dose solutions were prepared in a buffer consisting of 100 mM histidine, 100 mM arginine, and 6% sucrose, pH 6.0. Vehicle or RH2-18 at a dose of 2 or 15 mg/kg was administered subcutaneously in a dose volume of 0.1 ml. Individual mice were sacrificed at each time point (n = 3–4), and terminal blood samples were collected by cardiac puncture before dosing and on days 1, 4, 7, 11, 15, and 22 after dosing.

Male and female rhesus monkeys (n = 3 per group) from the University of Louisiana at Lafayette New Iberia Research Center (New Iberia, LA) weighing 4.7 to 12.0 kg were used. RH2-18 dose solutions were prepared in the same buffer as described above, and the antibody was administered at a dose of 0.5 or 2.5 mg/kg (0.16 ml/kg s.c.) or infused intravenously (0.5 mg/kg i.v.). Serial blood samples were collected from the saphenous or femoral vein predose, and subsequently blood samples were drawn on days 2, 3, 4, 8, 15, 22, 29, 36, 43, 50, 56, 64, 71, 78, and 85 after antibody administration. Serum was obtained after allowing blood samples to clot for 30 min in serum separator tubes and after centrifugation of coagulated blood (3200g, 10 min, 4°C). Serum samples were stored at −70°C, and anti-DKK1 antibody RH2-18 levels were determined as described in detail below.

Pharmacokinetic Analyses of Anti-DKK1 Antibody RH2-18 in Mouse and Rhesus Serum Samples. Recombinant rhDKK1 (235 ng/well) in phosphate-buffered saline was used to capture anti-DKK1 antibody in 96-well enzyme immunoassay plates (Thermo Fisher Scientific). Plates were washed once with wash buffer, phosphate-buffered saline with 0.05% Tween 20 (PBST), and 300 µl of blocking buffer (PBST containing 3% bovine serum albumin) was added to each well. Plates were then incubated at room temperature for 1.5 h. After being blocked, each well was washed three times with PBST, then dried, sealed, and stored at 4°C. All of the serum samples were diluted 1:20 with assay buffer (PBST containing 2% bovine serum albumin and NaCl) and subsequently diluted with 5% serum assay buffer (5% control serum in assay buffer) to concentrations of 1 to 100 ng/ml. Fifty-microliter aliquots of the previously diluted
samples were added to prepared rhDKK1–coated plates in duplicate, incubated at room temperature for 1 h, and then washed three times with PBST. Horseradish peroxidase-conjugated mouse anti-human IgG (1:3000; Southern Biotechnology Associates, Birmingham, AL) was used as the detection antibody. Turbo TMB (3,3′,5,5′-tetramethylbenzidine; Thermo Fisher Scientific) was used as the substrate with absorbance read at 450 nm after the addition of stop solution (1 M sulfuric acid). Data were analyzed, using a four-parameter curve fit for data reduction, as defined by SoftMax Pro (Molecular Devices, Sunnyvale, CA). The concentration of human IgG in the test samples was determined by interpolation from the constructed standard curve.

**Determination of Serum and Urinary Pharmacodynamic Markers in Mouse and Rhesus Monkey.** Biochemical markers of bone formation and resorption, including DKK1, were evaluated with commercially available kits following the manufacturer’s instructions: Metra-BAP, Metra-Osteocalcin, Metra-C1CP, Metra-Osteoprotegerin (Quidel Corporation, San Diego, CA), UniQ-P1NP (Orion Diagnostica, Espoo, Finland), urinary NTx (uNTx) Wampole Laboratories, Princeton, NJ), and total human DKK1 (Assay Designs, Ann Arbor, MI). Serum parameters in mouse were determined by serum CTx (cCTx) (Immunodiagnostic System Inc., Fountain Hills, AZ) and mouse P1NP (Immunodiagnostic System Inc., Fountain Hills, AZ) kits. Mouse serum DKK1 protein was determined in a competitive assay format, estimating free DKK1 in the presence of the treatment antibody RH2-18. Reacti-Bind White Opaque 96-well plates (Thermo Fisher Scientific) were coated with 200 ng of RH2-18 per well and blocked with starting block T20 blocking buffer in Tris-buffered saline (TBS) (Thermo Fisher Scientific). Fifty microliters of each mouse serum sample was added per well. Assay standards were prepared using mouse DKK1 (R&D Systems) from 62.5 to 4000 pg/ml. Two milligrams of N-terminal DKK1 detection antibody standards were prepared using mouse DKK1 (R&D Systems) from 62.5 to 4000 pg/ml. Two milligrams of N-terminal DKK1 detection antibody (RH1–28) was biotin-labeled (1.4 mol biotin/mol IgG) with the EZ-Link Sulfo-NHS-LC-Biotinylation Kit (Thermo Fisher Scientific) and used at 100 ng/well in the assays. Fifty microliters of the serum and biotinylated RH1–28 were added and incubated in assay plates for an additional 2 h. After four washes (TBS containing 0.02% Tween 20), europium-labeled streptavidin (PerkinElmer Life and Analytical Sciences–Wallac Oy, Turku, Finland) was added to achieve a final concentration of 100 ng/ml and then incubated for additional 2 h. After four washes (TBS containing 0.02% Tween 20), enhancement solution (PerkinElmer Life and Analytical Sciences) was added, and time-resolved fluorescence (400 μs, excitation/emission filter 340/615) was measured on a Victor3 plate reader (PerkinElmer Life and Analytical Sciences).

**Efficacy Study with Anti-DKK1 Antibody RH2-18 in the O VX Mouse.** All of the mice (C57BL/6Ntac) were received from Taconic Farms either O VX or sham-operated at age 12 weeks and then allowed to lose bone for an additional 12 weeks. Animals were provided with water and chow ad libitum and maintained on a 12-h light/dark cycle. C57BL/6Ntac mice were housed individually in the same temperature- and humidity-controlled room, with a 12-h light/dark cycle. Water and standard laboratory chow (high-protein monkey diet 5054; Purina, St. Louis, MO) were provided ad libitum. RH2-18 [50 mg/ml, 1% sucrose, 10 mM histidine, and 25 mM arginine (pH 6.0)] at a final dose level of 10 mg/kg (n = 14) or respective control buffer (n = 6) were administered as s.c. injections every 2 weeks (twice per month). All of the animals were fasted before the collection of serum and urine samples. Serum and urine samples were collected up to day 71 of the study. Imaging of bone for density and morphological measures was performed in-life at baseline and at 3, 6, and 9 months of dosing with the following modalities at the indicated anatomical sites: DXA (GE Lunar iDXA; GE Healthcare) for whole body, spine (L2–4), and hip; high-resolution peripheral quantitative CT (HR-pQCT) (Scanco XtremeCT; Scanco Medical, Zurich, Switzerland) at the distal radius and distal tibia; and quantitative CT (QCT) (GE-DST; GE Healthcare) for spine, femoral neck, and shaft. For QCT, animals were scanned in a head first supine position with a phantom with varying mineral densities (Mindways Software, Inc., Austin, TX) underneath the spine and hip of the animal for density calibration. Animals were scanned from L1 through midfemur using the following CT protocol: 80 kVp, 0.625-mm slice thickness, 300 mA s, pitch 1, bed height of 95, 250 mm reconstruction field of view corresponding to a pixel size of 0.244 μm, and bone convolution kernel. All of the image analyses were carried out using Mindways Three-Dimensional Volumetric QCT Bone Densitometry of the Spine and Hip (QCT Pro software; Mindways Software, Inc.). HR-pQCT was used to evaluate cortical and trabecular regions of bone below the growth plate in the ultra-distal radius and tibia by positioning the imaged region at a fixed distance below a landmark line of the radius and tibia, respectively.
The following scan parameters were used: 60 kVp, 900 mA, 1000 projections, 41-μm slice thickness; 3072 × 3072 pixels, integration time 200 ms, scan distance/dimension 9 mm, and number of slices 220. All rhesus macaques were returned after completion of the studies.

Statistical Analysis. All of the data are expressed as a mean ± S.E. Where appropriate, a one-way analysis of variance followed by Dunnett’s post hoc test to detect group differences was calculated using Prism 5.0 (GraphPad Software Inc., San Diego, CA).

Results

Anti-DKK1 Antibody (RH2-18) Neutralizes DKK1 Function In Vitro. Fully human anti-DKK1 antibody RH2-18 was identified from phage-displayed single-chain Fv antibody libraries and selected by consecutive panning on the basis of binding to both rhesus and mouse DKK1 proteins. Analyses of RH2-18 showed high affinity for human DKK1 (K_D = 249 pM) at a discrete C-terminal epitope on DKK1 (Glantschnig et al., 2010), a domain necessary and sufficient for LRPS/6 interaction (Brott and Sokol, 2002), and thus the antibody interferes with receptor-ligand interaction (Glantschnig et al., 2010). Interference of RH2-18 with DKK1 function was validated in a cell-based assay interrogating Wnt signaling through β-catenin/T cell factor in human embryonic kidney 293 cells (Supplemental Fig. 1A) and in mouse pluripotent mesenchymal C3H10T1/2 cells (Supplemental Fig. 1, B and C). Wnt3A induced expression of luciferase reporter, the selected markers alkaline phosphatase, insulin-like growth factor-binding protein 2 (Igfbp-2), Troy (TNRFSF-19), and Axin-2 were markedly diminished by rhDKK1 (50 nM). Addition of anti-DKK1 antibody RH2-18 re-stored Wnt-induced activities and expression of the selected markers to or above control levels in vitro.

DKK1 Neutralization Results in a Bone-Pharmacodynamic Response in OvX Mice. To evaluate pharmacokinetic properties in vivo, RH2-18 antibody (2 and 15 mg/kg) was administered to female OVX C57BL/6NTac mice by single-dose subcutaneous injection. RH2-18 concentration in serum revealed a close to linear dose-exposure relationship, with C_max 138.9 ± 11.4 and 913 ± 122 nM, respectively. In general, T_max was noted at 1 to 4 days after subcutaneous administration, and RH2-18 half-life (t(1/2)) was 12 to 19 days (Fig. 1A). In parallel, serum mouse DKK1 levels determined by a competitive assay format designed to detect DKK1 protein not bound to RH2-18 dropped from baseline levels as expected at all of the doses tested. The suppression of free DKK1 relative to the vehicle control group was nearly complete at 2.8% (2 mg/kg), or below the limit of quantification (15 mg/kg) on day 4 (Fig. 1B). Bioavailability of DKK1 increased in both groups up to 7.3% relative to that of vehicle-treated mice on day 23 (Fig. 1B). Thus, RH2-18 demonstrated binding to mouse DKK1 in serum and prolonged stability in vivo. A pharmacodynamic response of the mechanistically relevant serum collagen marker of bone formation (P1NP) was evident on day 5 (+97% above baseline in the 15 mg/kg group; p < 0.05 versus control) (Fig. 1C). No significant effects on serum levels of the collagen I degradation product sCTXs were detected (Fig. 1D). More importantly, ex vivo analyses of femurs and lumbar vertebrae (L3–4) on day 24 of the study showed significant increases (p < 0.05 versus vehicle control group) in femoral (Fig. 1E) and lumbar spine BMD (Fig. 1F) in both RH2-18 treatment groups. These results suggest that RH2-18 exposure mechanistically limited free DKK1 levels in parallel with increases in the bone formation marker P1NP and BMD in OVX mice.

Pharmacologic Suppression of DKK1 Function Increases Bone Formation and Resolves Low BMD in OVX Mice. To further evaluate the bone-mass-restoring potential of DKK1 inhibition in adult (age 23 weeks at study start) osteopenic mice, we studied RH2-18 effects in OVX mice with established estrogen-deficiency-induced osteopenia. Animals had OVX done at 12 weeks before study start to allow for bone loss (~6–7% lumbar spine-BMD versus sham control) and were treated for 8 weeks with 2 or 20 mg/kg RH2-18 per week. As a positive control, for a robust bone effect, hPTH (1–34) at a dose that is 140-fold over the equivalent weekly clinical exposure (6) resulted in expected changes in BMD and consistent effects on dynamic and static bone histomorphometry. Terminal RH2-18 serum concentrations after multiple dosing at week 8 were 113.8 ± 2.6 and 332.7 ± 38.9 nM, respectively. Bioavailable free DKK1 serum levels (baseline, 8.0 ± 3.4 ng/ml) were reduced on average by >90% in all of the RH2-18-treated groups.

Because bone formation biomarker and BMD responses in mouse are detectable after a single dose of RH2-18, we reasoned that bone efficacy might be detectable histologically after a relatively short treatment period. To test this hypothesis, we collected histological and BMD data from mouse femurs within a subset of treatment groups at day 24 (Table 1). Confirming this hypothesis, whole femur BMD was increased significantly in RH2-18 (2 mg/kg) group versus vehicle group by 4.5% (p < 0.05), with trends toward increased BMD in the central (4.0%) and distal (5.1%) femur. It is interesting to note that indices of new endosteal bone formation were increased significantly (p < 0.05) in the RH2-18 treatment group versus the vehicle group. From a mechanistic perspective, it is significant that endosteal bone formation rates were found to be increased in the RH2-18 treatment group on day 24, thus demonstrating the rapid onset of antibody-mediated bone-anabolic activity in adult osteopenic mice.

At 8 weeks of treatment, RH2-18 resolved low BMD in the femurs of OVX mice to sham OVX (intact) vehicle levels. Overall mean BMD in the whole femur was increased by 6.0% in the 20 mg/kg per week group (Fig. 2A). Likewise, BMD was higher in the distal femur (Fig. 2B) and central femur, representing cortical bone (Fig. 2C) with an increase of +7.2 and +6.6%, respectively, in the 20 mg/kg per week dose group. Consistent with the initial findings after 24 days, endosteal MS/BS in the central femur was increased significantly (p = 0.05) at 8 weeks of treatment (Table 2). The increased MS/BS was primarily responsible for a significant 2-fold rise in BFR/BS at this skeletal site. Periosteal MS/BS in the central femur rose modestly in response to treatment (Table 2), accounting for the modest increase in periosteal BFR/BS. Consequently, central femur mean BV/TV was 5.8% greater than that in the vehicle OVX control group. The increase in endosteal bone formation and the more modest effect on periosteal bone formation were paralleled by numerical increases in the cortical area as well as reductions in endosteal perimeter (Table 2).

More importantly, lumbar spine BMD (LV1–4) at 8 weeks was partially to completely restored in RH2-18 treatment groups to the levels seen in sham OVX (intact) vehicle-treated controls (Fig. 2D). This profound effect on lumbar spine aBMD was characterized further using ex vivo micro...
CT analysis of trabecular bone (LV3) in vehicle and RH2-18 treatment groups (Table 3). Volumetric analysis confirmed the significant effect on BMD by RH2-18 treatment and the overall increased bone volume (up to 27%) within the trabecular compartment. RH2-18 treatment (20 mg/kg) significantly augmented TbTh ($p < 0.001$ versus OVX vehicle; 13.6%) and TbN ($p = 0.068$; 15%) while reducing TbSp ($p < 0.01$; −25.7%). Thus, RH2-18 treatment produced pharmacological effects at multiple loci and improvement of bone microarchitectural parameters in the mature OVX mouse.

Fig. 1. Pharmacokinetics and pharmacodynamic responses of fully human anti-DKK1 antibody RH2-18 after single dose administration in OVX mouse in vivo. Vehicle (diamonds, dashed line) or RH2-18 (solid lines) at 2 mg/kg (triangles) or at 15 mg/kg (squares) was administered via subcutaneous injections, and serum samples were collected at the time points indicated up to day 23. A, serum levels and pharmacokinetic profile of RH2-18 over 23 days after administration. B, reduction in bioavailable DKK1 in serum of mice treated with increasing RH2-18 doses. C, P1NP serum levels show increases from baseline in RH2-18 treatment groups ($p = 0.02$; day 5; 15 mg/kg), and numerical increased P1NP levels were observed up to 16 days. D, serum CTx levels were determined at days 2 and 16. Whole femur aBMD (E) and lumbar spine aBMD (F) (L1–4) increase in RH2-18 treatment groups at day 24. Data are given as mean ± S.E. (n = 3–4 per group per time point). One-way analysis of variance, Dunnett’s test versus vehicle (*$p < 0.05$; **$p < 0.01$).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Osteoblast Activity</th>
<th>Osteoclast Activity</th>
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<tbody>
<tr>
<td>Whole Femur BMD (mg/cm²)</td>
<td>Vehicle</td>
<td>RH2-18</td>
</tr>
<tr>
<td>Distal Femur BMD (mg/cm²)</td>
<td>48.28 ± 0.358</td>
<td>50.45 ± 0.759</td>
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<tr>
<td>Central Femur BMD (mg/cm²)</td>
<td>44.81 ± 0.434</td>
<td>54.35 ± 1.199</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>45.28 ± 0.637</td>
<td>45.87 ± 0.612</td>
</tr>
<tr>
<td>MAR (µm/day)</td>
<td>1.21 ± 0.073</td>
<td>1.36 ± 0.052</td>
</tr>
<tr>
<td>MS/BS (%)</td>
<td>16.63 ± 3.140</td>
<td>27.76 ± 2.520</td>
</tr>
<tr>
<td>BFR/BS (µm²/µm² per year)</td>
<td>73.17 ± 14.770</td>
<td>136.10 ± 12.180</td>
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Data are given as mean ± S.E. One-way analysis of variance, Dunnett’s test versus vehicle (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).
Physiological and Biological Properties of the Anti-DKK1 Antibody (RH2-18) Enable the Testing of DKK1’s Role in Nonhuman Primates (Rhesus Macaque). To address pharmacokinetic properties in nonhuman primates, we first administered single doses of RH2-18 (0.5 or 2.5 mg/kg) via subcutaneous or intravenous routes to rhesus macaque and then drew blood samples at the indicated time points for analysis (Fig. 3). A comparison of exposure after administration of 0.5 mg/kg s.c. and i.v. doses suggests essentially complete absorption of RH2-18, an apparent \( t_{1/2} \) of approximately 2 weeks, and a very low serum clearance of 2.7 \( \mu \)l/\( \text{min} \cdot \text{kg}^{-1} \) and a small volume of distribution of 56 ml/kg revealing an exceptionally favorable pharmacokinetic profile of the antibody for further testing of the role of DKK1 in primates.

**DKK1 Functions as a Rate-Limiting Suppressor of BMD in Aged and OVX Nonhuman Primates (Rhesus Macaque).** We next introduced RH2-18 (10 mg/kg, twice per month) into long-term OVX rhesus macaques (14–20 years of age, >8 years after OVX). Serum RH2-18 levels at days 7 and 14 (the last time point tested) were measured in the mid shaft of the central femur, in lumbar vertebral bodies (L1–4). Data are given as mean ± S.E. \((n = 10–14 \text{ per group})\). One-way analysis of variance, Dunnett’s test versus vehicle OHX control group (*, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \)).

**Table 2**

<table>
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<tr>
<th>Treatment</th>
<th>Intact</th>
<th>Vehicle</th>
<th>RH2-18</th>
<th>RH2-18</th>
<th>hPTH (1–34)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>2 mg/kg</td>
<td>20 mg/kg</td>
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<tr>
<td>Central femur BV/TV (%)</td>
<td>49.67 ± 0.597***</td>
<td>42.86 ± 0.602</td>
<td>45.79 ± 0.532**</td>
<td>45.49 ± 0.725*</td>
<td>48.37 ± 0.435***</td>
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<tr>
<td>CtAr (mm²)</td>
<td>0.81 ± 0.013*</td>
<td>0.75 ± 0.021</td>
<td>0.78 ± 0.014</td>
<td>0.80 ± 0.018</td>
<td>0.82 ± 0.015*</td>
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<tr>
<td>Endosteal EcPm (mm)</td>
<td>3.40 ± 0.038***</td>
<td>3.74 ± 0.050</td>
<td>3.60 ± 0.025</td>
<td>3.67 ± 0.070</td>
<td>3.50 ± 0.026*</td>
</tr>
<tr>
<td>MAR (µm/day)</td>
<td>1.33 ± 0.046</td>
<td>1.13 ± 0.497</td>
<td>1.22 ± 0.038</td>
<td>1.28 ± 0.065</td>
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<td>MS/BS (%)</td>
<td>16.17 ± 2.766</td>
<td>13.34 ± 2.719</td>
<td>17.84 ± 2.288</td>
<td>22.85 ± 2.879*</td>
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<td>BFR/BS (µm²/µm² per year)</td>
<td>79.99 ± 14.620</td>
<td>56.19 ± 13.200</td>
<td>70.10 ± 9.828</td>
<td>109.00 ± 15.150*</td>
<td>77.42 ± 12.688</td>
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<tr>
<td>Periosteal PsPm (mm)</td>
<td>4.67 ± 0.029**</td>
<td>4.85 ± 0.065</td>
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<td>MAR (µm/day)</td>
<td>0.96 ± 0.055</td>
<td>1.09 ± 0.069</td>
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<tr>
<td>MS/BS (%)</td>
<td>4.94 ± 1.415</td>
<td>1.23 ± 0.353</td>
<td>5.99 ± 1.791*</td>
<td>2.69 ± 0.758</td>
<td>5.44 ± 1.466</td>
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<tr>
<td>BFR/BS (µm²/µm² per year)</td>
<td>7.79 ± 3.094</td>
<td>2.38 ± 0.937</td>
<td>9.62 ± 4.051</td>
<td>7.32 ± 2.682</td>
<td>18.11 ± 5.413**</td>
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</tbody>
</table>

CtAr, cortical area; EcPm, endocortical perimeter; PsPm, periosteal perimeter.

Data are given as means ± S.E. \((n = 9–14 \text{ per group})\). One-way analysis of variance, Dunnett’s test versus OVX vehicle (*, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \)).
TABLE 3
Histomorphometric indices at the lumbar vertebrae (L3) acquired by ex vivo micro computed tomography (micro CT) in sham-OVX and OVX mice after 56 days of treatment with vehicle or anti-DKK1 antibody RH2-18

<table>
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<th>OVX</th>
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<tr>
<td></td>
<td>Vehicle</td>
<td>RH2-18</td>
</tr>
<tr>
<td>Lumbar vertebra</td>
<td>vBMD (mg/cm²)</td>
<td>248.20 ± 0.70804***</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>0.161 ± 0.0088*</td>
<td>0.134 ± 0.0064</td>
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<tr>
<td>TbTh (mm)</td>
<td>0.023 ± 0.0006***</td>
<td>0.23 ± 0.0034***</td>
</tr>
<tr>
<td>TbN (no./mm)</td>
<td>7.044 ± 0.3874</td>
<td>7.037 ± 0.3782</td>
</tr>
<tr>
<td>TbSp (mm)</td>
<td>0.124 ± 0.0080</td>
<td>0.127 ± 0.0070</td>
</tr>
</tbody>
</table>

TbN, trabecular number; TbSp, trabecular spacing.

Data are given as mean ± S.E. One-way analysis of variance, Dunnett’s test versus OVX vehicle (*, p < 0.05; ***, p < 0.001).

![Representative micro CT slice images by vBMD from respective control and RH2-18 treatment groups.](image)

Fig. 3. Serum levels and pharmacokinetic profile of fully human anti-DKK1 antibody RH2-18 after administration of a single 0.5 mg/kg i.v. (closed triangles) or s.c. (open triangles) dose or a single 2.5 mg/kg s.c. (open squares) dose in rhesus macaque. Data are given as mean ± S.E. (n = 3 per group).

line 0.15 ± 0.005 to 29.9 ± 7.7 nM (day 8) and remained essentially unchanged to the last time point tested (27.3 ± 6.6 nM; day 71). Within this time-frame, P1NP, a serum marker of bone formation, was consistently elevated over levels in vehicle-treated controls (Fig. 4A), whereas C1CP showed a significant albeit transient increase (Fig. 4B) in the RH2-18 treatment group. Other bone formation indices, bone-specific alkaline phosphatase and osteocalcin (Fig. 4, C and D), both consistently trended upward in the RH2-18 treatment group at later time points, without achieving statistical significance. There was no significant change in levels of the collagen I degradation markers sCTx and uNTx or of osteoprotegerin (Fig. 4, E–G) within the observation period.

aBMD was analyzed longitudinally by DXA in the vehicle and RH2-18 treatment groups at baseline and at 3-month intervals thereafter. The baseline lumbar spine aBMD of the OVX animal cohort was 0.695 ± 0.075 g/cm² compared with historic in-house data of aged non-OVX animals (0.814 ± 0.119 g/cm²). Several measures of bone density and morphology investigated by imaging showed statistically significant improvement from baseline and compared with the vehicle group. Whole-body and spine aBMD increased longitudinally from baseline by 4.6% (p < 0.05 versus control group) and by 5.0% (p < 0.05 versus control group), respectively, in RH2-18-treated animals at 9 months of treatment, whereas no significant changes were observed in the vehicle-treated group (Fig. 5, A and B). These findings are supplemented by vBMD at the lumbar spine (L1–4) by QCT (Fig. 5C). Trabecular-vBMD (TbvBMD) of the spine showed an early treatment effect at 3 months, with a significant increase from baseline of 9.5 ± 1.8% (mean ± S.E.; p = 0.037), which is consistent with the concept that treatment with anti-DKK1 antibody RH2-18 primarily affected trabecular bone BMD. TbvBMD increased further at 6 months in most animals by 2.3 ± 0.6 to 11.5 ± 1.6% (p < 0.05) above baseline and reached a plateau at 9 months (11.5%; p < 0.001). TbBMD changes from baseline (4.6 ± 1.1%) in the vehicle group at 6 months were nonsignificant. Individual response size in lumbar spine TbBMD at 6 months within the RH2-18 treatment group correlated well with early individual changes in levels of the bone formation marker P1NP from baseline at day 12 of the study (Supplemental Fig. 2). In the aggregate, data presented here demonstrate significant increases in bone mass affecting the whole body and regional lumbar spine BMD in adult primates. Furthermore, a trend to increased femoral neck total aBMD by DXA was observed in the RH2-18 treatment group (p = 0.047 versus vehicle) at 9 months.

To further investigate fracture relevant sites, we extended the analyses to distal tibia and distal radius, examining vBMD and trabecular microarchitecture by HR-pQCT. Integral vBMD (combined cortical/trabecular, represented by D100 in the Scanco analysis software) of the distal tibia was found to be increased by 3.7% (p < 0.001) by treatment at 9 months (Fig. 5D), with detectable increases in TbTh of 3.1% (p < 0.01), 4.6% (p < 0.005), and 5.6% (p < 0.0001) at 3, 6, and 9 months, respectively (Supplemental Fig. 3). Similar efficacies were observed in the distal radius of the treatment group at 6 and 9 months treatment, because integral vBMD showed increases of 3.6% (p < 0.005) and 5.4% (p < 0.005), respectively (Fig. 5E), and TbTh showed increases of 3.0% (p < 0.01) and 3.3% (p < 0.005), respectively (Fig. 5F). Thus,
anti-DKK1 antibody RH2-18 treatment positively affected bone parameters at both axial and appendicular skeletal regions in aged and OVX nonhuman primates.

**Discussion**

Bone-anabolic treatments targeting the Wnt-signaling pathway offer novel therapeutic approaches for systemic diseases such as postmenopausal or iatrogenic osteoporosis (Khosla et al., 2008; Deal, 2009; McCarthy and Marshall, 2010) and for localized bone loss secondary to metastatic bone disease (Yaccoby et al., 2007; Fulciniti et al., 2009; Gavriatopoulou et al., 2009; Heath et al., 2009) and rheumatoid arthritis (Diarra et al., 2007) or in fracture repair (Bajada et al., 2009). Though genetic models have provided overwhelming evidence for the role of DKK1 as a crucial regulator during bone growth and development (Li et al., 2006; Morvan et al., 2006), documentation of a role for DKK1 in the maintenance of the adult skeleton and the therapeutic potential for functional modulation of DKK1 is critical. In this study, we provide evidence for the inhibitory function of DKK1 in systemic low-bone-mass conditions (osteopenia) in adult bone using an experimental therapeutic antibody. Furthermore, we show that targeting the DKK1 mechanism results in bone efficacy at multiple skeletal sites in both osteopenic murine and primate models.

The anti-DKK1 antibody described in this study displays several characteristics to address the above paucity of data. First, the antibody identification strategy permitted cross-reactivity for testing in murine as well as primate species. Second, the antibody exhibits no measurable interaction with the closely related DKK2/DKK4 proteins, thus assuring data interpretation that is tightly focused on DKK1 function (Krupnik et al., 1999; Glantschnig et al., 2010). HBM mutations of LRP5 diminish binding of DKK1 and confer resistance to the inhibitory actions of DKK1 on LRP5/6 and Wnt signaling (Boyden et al., 2002; Ai et al., 2005; Fleury et al., 2010), thus providing a rationale for the mechanism of action of RH2-18 on bone and its efficacy via the modulation of DKK1 function (Glantschnig et al., 2010). In addi-

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**Fig. 4.** Serum bone formation and bone resorption marker profiles in aged OVX rhesus macaques by anti-DKK1 antibody RH2-18 treatment (10 mg/kg, 2 weeks). All of the serum and urinary analytes were determined at baseline and at time points indicated up to day 71 in vehicle and RH2-18 treatment groups. Data are given as the percentage change from baseline corrected for respective values in the vehicle control group (Δ) at the individual time points. A, collagen I synthesis by-product P1NP; B, collagen I synthesis by-product C1CP; C, bone-specific alkaline phosphatase; D, osteocalcin; E, urinary collagen I degradation product uNTx; F, serum collagen I degradation product sCTx; G, osteoprotegerin. Data are given as mean ± S.E. One-way analysis of variance, Dunnett’s test versus baseline (*, p < 0.05; **, p < 0.01).
tion, DKK1 interaction with LRP4 (Choi et al., 2009a) and Kremen receptors (Mao et al., 2002) may be affected by the pharmacological action of anti-DKK1 antibody; however, this was not tested experimentally.

We demonstrate here the profound systemic effects of anti-DKK1 antibody in adult osteopenic mice at multiple skeletal sites, including endosteal surfaces composed of trabecular and cortical compartments, leading to partial to complete resolution of the osteopenic phenotype.

The most notable finding in this study is that when introduced de novo into estrogen-deficient osteopenic adult mice RH2-18 treatment elevated BMD to a level comparable with that of sham-operated controls within 8 weeks of treatment. Wnt/β-catenin signaling has been reported previously to influence both bone formation and resorption (Baron and Rawadi, 2007; Pinzone et al., 2009). The anti-DKK1 antibody-induced osteoanabolic activity was demonstrated by increased circulating P1NP in treated mice and primates and confirmed by the observation of augmented bone formation at all of the endosteal surfaces examined in mouse. The approximate 2-fold increase in endosteal bone formation and detectable but nonsignificant effects on periosteal bone formation were paralleled by a progression toward an increase in the cortical area. The cellular process underlying this effect likely involves Wnt/β-catenin and thus a DKK1 inhibitable mechanism in the differentiation of mesenchymal progenitor cells to preosteoblast progenitors as well as in preosteoblast to osteoblast transitions (Pinzone et al., 2009). This interpretation is consistent with the genetic DKK1 loss-of-function phenotype in mice that includes both an increase in osteoblast number and elevated bone formation paralleled by improved biomechanical strength (Morvan et al., 2006). Thus, an augmenting effect on bone strength by a pharmacologic DKK1 inhibitor is inferred; however, this awaits confirmation by future studies. Further validation of on-target efficacy by the experimental antibody RH2-18 could be gained using genetic models where DKK1 protein expression is strongly reduced or preferably completely absent in the adult mouse, and thus treatment effects by anti-DKK1 antibody are expected to diminish.

Evidence suggesting a role for local Wnt-pathway modulation through DKK1 in murine bone tissue has been provided recently by models of inflammatory joint disease (Diarra et al., 2007) and multiple myeloma in immune compromised mice (Yaccoby et al., 2007; Fulciniti et al., 2009; Heath et al., 2009) in which DKK1 levels are elevated mechanistically. Anti-DKK1 antibodies have been shown to suppress bone resorption activity in these murine disease models (Diarra et al., 2007; Yaccoby et al., 2007), whereas in other studies the lack of an effect on bone resorption indices has been reported (Fulciniti et al., 2009; Heath et al., 2009). In general, RH2-18 treatments elicited no long-term effects on markers of bone resorption but a transient suppressive effect on sCTx and uNTx levels in primates, leaving open the possibility of an associated though modest bone resorptive component in the current study. Studies in primates were noninvasive and
Therapeutic Effect of Anti-DKK1 Antibody in Osteopenia

Thus, modulation of DKK1 function through an experimental fully human anti-DKK1 antibody exhibits translational potential as a bone-anabolic agent for the treatment of human low bone mass disease, a characteristic of postmenopausal osteoporosis.

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We thank Diane Posavec and Richa Jayakar (Imaging Department, Merck Research Laboratories) for support in conducting HR-pQCT studies, Mona Purcell and Steve Krause (Imaging Department, Merck Research Laboratories) for support in conducting QCT studies, and Brenda Pennypacker and Pat Masarachia for support in conducting studies in mice. This article is dedicated to the memory of Shun-ichi Harada.

Authorship Contributions

Participated in research design: Glantschnig, Scott, McCracken, Fisher, Sandhu, Cook, Williams, Streth, Flores, Kimmel, Wang, and An.

Conducted experiments: Glantschnig, Scott, Wei, McCracken, Nantermet, Cook, Williams, and Kimmel.

Contributed new reagents or analytic tools: Hampton, Wei, McCracken, Zhao, Vitelli, Huang, Wang, and An.

Performed data analysis: Glantschnig, Scott, Wei, McCracken, Nantermet, Sandhu, Kimmel, and An.

Wrote or contributed to the writing of the manuscript: Glantschnig, Scott, Hampton, Fisher, Sandhu, Williams, Kimmel, and An.

References


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Supplemental Information

A rate-limiting role for DKK1 in bone formation and the remediation of bone loss in mouse and primate models of postmenopausal osteoporosis by an experimental therapeutic antibody

Helmut Glantschnig, Kevin Scott, Richard Hampton, Nan Wei, Paul McCracken, Pascale Nantermet, Jing Z. Zhao, Salvatore Vitelli, Lingyi Huang, Peter Haytko, Ping Lu, John E. Fisher, Punam Sandhu, Jacquelynn Cook, Donald Williams, William Strohl, Osvaldo Flores, Donald Kimmel, Fubao Wang, and Zhiqiang An

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Materials and Methods

Preparation of Wnt3A conditioned media (CM). CM from cells expressing Wnt3A was harvested from L-cells stably expressing mouse-Wnt3A (CRL-2647; American Type Culture Collection Manassas, VA) or from the parent L-cell clone (CRL-2648; ATCC) after 4 days and an additional harvest following 3 days in culture. CM were combined, sterile filtered (0.2 μM PES filter; Corning Lifesciences, Lowell, MA) and stored at 4°C.

Wnt-signaling assay (Topflash). In vitro functional activity of anti-DKK1 antibody on the Wnt-signaling pathway was studied in stable HEK293\textsuperscript{bLnp5} cells seeded in 10% FBS, Dulbecco's modified Eagles medium (Invitrogen, Carlsbad, CA) at 25,000 cells/well in a 96-well plate (Becton Dickinson & Co, Franklin Lakes, NJ). Cells were cultured overnight at 37°C in a humidified chamber with 5% CO\textsubscript{2}. Cells were transfected overnight with 3.75 ng of Topflash (Lef-1/β-catenin reporter; Promega, Madison, WI), 0.08 ng of pTK-renilla (Promega, Madison, WI) and 50 ng of plasmid encoding Lef-1/well using Fugene-6 (Roche, Branchburg, NJ). Antibody was then added into the cell system followed by addition of rhDKK1 (25 nM). The tissue culture plates were incubated for 15 minutes to allow DKK1 binding to RH2-18 or cell surface receptors. Thereafter, 100 μl of Wnt3A-CM (100-200 ng/ml) was added to a final concentration of 50-100 ng/ml. An equal volume of parent cell CM was added to control wells. After an
additional 24 hours, cells were lysed and reporter gene expression evaluated using Dual-Reporter-Assay system kits (Promega, Madison, WI).

**Mesenchymal progenitor cell assay.** The effect of anti-DKK1 antibodies on osteoblastic differentiation were characterized in C3H10T1/2 cells (CCL-226, clone 8: American Type Culture Collection, Manassas, VA). Cells were passaged in DMEM with phenol red supplemented with 10% heat-inactivated FBS (Invitrogen, Carlsbad, CA). For experimentation, 20,000 cells were seeded per well in MEM-alpha (Invitrogen, Carlsbad, CA) supplemented with 10% FBS into 96-well plates. After 2 days media was replenished and antibody was added to a final concentration in the range of 3 to 100 nM followed by addition of rhDKK1 protein (50 nM). After 15 minutes of equilibration 25 μl of Wnt3A-CM or respective control-CM was added and incubation continued over 2-3 more days. Alkaline phosphatase (AP) activity was determined after washing cells twice in buffer (20 mM Hepes, pH 7.5; 150 mM NaCl) and cell lysis in buffer (10 mM Tris HCl pH 9.0, 150 mM NaCl, 0.025% Tween-20). AP enzymatic activity was determined with Attophos substrate (Promega, Madison, WI) using standards prepared from calf intestinal phosphatase (Roche, Branchburg, NJ).

**RNA preparation and quantitative RT-PCR.** Total RNA was extracted using the 6100 Nucleic prep station (Applied Biosystems, Foster City, CA) from C3H10T1/2 cells. Quantitative RT-PCR was carried out using Brilliant one step RT-PCR kit and the MX3000 sequence detection system according to the manufacturer’s protocols (Stratagene, La Jolla, CA). In brief, 75 ng aliquots of RNA were reverse transcribed and amplified using the following primers and Taqman probes. Tumor necrosis factor receptor superfamily, member 19 (TNFRSF19; Troy) Forward 5'-CGCCACTGTGCCCATAGAG-3', Reverse 5'-GTGAACAGGCCCATACATTGG-3', and Probe 5'-6Fam-ATGCTGTCAGTATCACCGGGACTCAGC-Tamra-3'; Insulin-like Growth Factor Binding Protein-2 (IGFBP-2) Forward 5'-CGCGGGTACCTGTGAAAAGA-3', Reverse 5'-TCGTCATCACTACCACGACTCAGC-Tamra-3'; and Probe 5'-6Fam-TCGTCACTGCTGCAACCT-3'; Axin-2 Forward 5'-GGTTCCGGCTATGTCTTTGC-3', Reverse 5'-CAGTGCGTCGCTGGATAACTC-3' and Probe 5'-6Fam-AGCCACCAGCGCCAACGACAG-Tamara-3'. Data were normalized to Cyclophilin.
Forward 5′-CAAATGCTGGACCAAACACAA-3′, Reverse 5′-GCCATCCAGCCACTCAGTCT-3′ and Probe 5′-Vic-TGGTTCCCAGTTTTATCTGCACTGCCT. Average cycle threshold (Ct) values from duplicate PCR reactions were normalized to average Ct values for Cyclophilin from the same RNA preparations. The ratio of expression of each gene in experimental vs. control samples was calculated as $2^{-(\text{mean}\Delta\Delta\text{Ct})}$. 
SI Figure 1

**A**

Wnt3A+DKK1

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**C**

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**Supplemental Fig. 1.** Anti-DKK1 antibody RH2-18 neutralized DKK1 function and increased Wnt-signaling and cell differentiation *in vitro*. (A) Wnt-signaling was monitored by Topflash assay in transiently transfected HEK293 cells treated with control-CM (control), or Wnt3A-CM (Wnt3A), or rhDKK1 in absence or presence of RH2-18 at final concentration of 10 or 30 nM over 20 hours. (B) C3H10T1/2 cell differentiation as determined by Wnt3A-induced endogenous AP activities is suppressed by DKK1 and can be rescued by anti-DKK1 antibody RH2-18. Cells were treated over 3-days with control-CM or Wnt3A-CM, and rhDKK1 in absence or presence of 3, 10, 30 or 100 nM RH2-18. (C) Anti-DKK1 antibody RH2-18 neutralizes inhibitory DKK1 function on gene expression (C3H10T1/2 cells). Cells were concomitantly treated as indicated with Wnt3A-CM, rhDKK1 and RH2-18 (100 nM). After 16 hrs cells were lysed and transcripts for Igfbp-2 (top) Axin-2 (middle) and Troy (bottom) analyzed by RT-qPCR (Taqman) with relative expression levels given versus untreated cells set to 1. Data given as Means ± SE. One-way ANOVA, Dunnetts’ test vs. 0 nM control (** p<0.01, *** p< 0.001).
Supplemental Fig. 2. Correlation analyses of early changes in bone formation biomarker P1NP levels (percent change from baseline on day 12) and change from baseline in spine trabecular-vBMD (percent change from baseline at 6-month) in individual rhesus macaque in response to treatment with anti-DKK1 antibody RH2-18 (Pearson r²=0.348; p=0.26). Control treatment (open diamonds, n=6), RH2-18 treatment (closed squares, n=14).
Supplemental Fig. 3. Bone-efficacy of fully human anti-DKK1 antibody RH2-18 reveals a rate limiting role for DKK1 in aged and ovariectomized rhesus macaque. Changes in trabecular thickness of the distal tibia in rhesus macaque evaluated by hr-pQCT. Vehicle (open diamonds) or anti-DKK1 antibody RH2-18 (closed squares) was administered (s.c., 10mg/kg/2-weeks) up to 9-month and multiple bone endpoints were determined. X-axis indicates time points of data acquisition at baseline and at 3-month intervals. Y-axis indicates percent change from baseline. All data given as percent change from baseline (mean ± SE). (n=6 in vehicle treatment group; n=14 in anti-DKK1 antibody treatment group). One-way ANOVA, Dunnett’s test vs. vehicle control group (** p<0.01, *** p<0.001).