Generation and Selection of Novel Fully Human Monoclonal Antibodies That Neutralize Dickkopf-1 (DKK1) Inhibitory Function \textit{in Vitro} and Increase Bone Mass \textit{in Vivo}\textsuperscript{5}

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Wnt/LRP5 signaling is a central regulatory component of bone formative and resorptive activities, and the pathway inhibitory DKK1 is a suppressor of bone formation and bone mass accrual in mice. In addition, augmented DKK1 levels are associated with high bone turnover in diverse low bone mass states in rodent models and disease etiologies in human. However, examination of the precise role of DKK1 in the normal skeleton and in higher species requires the development of refined DKK1-specific pharmacological tools. Here, we report the strategy resulting in isolation of a panel of fully human anti-DKK1 antibodies applicable to studies interrogating the roles of mouse, rhesus, and human DKK1. Selected anti-DKK1 antibodies bind primate and human DKK-1 with picomolar affinities yet do not appreciably bind to DKK2 or DKK4. Epitopes mapped within the DKK1 C-terminal domain necessary for interaction with LRP5/6 and consequently effectively neutralized DKK1 function \textit{in vitro}. When introduced into naïve normal growing female mice, IgGs significantly improved trabecular bone volume and structure and increased both trabecular and cortical bone mineral densities in a dose-related fashion. Furthermore, fully human DKK1-IgG displayed favorable pharmacokinetic parameters in non-human primates. In summary, we demonstrate here a rate-limiting function of physiologic DKK1 levels in the regulation of bone mass in intact female mice, amenable to specific pharmacologic neutralization by newly identified DKK1-IgGs. Importantly the fully human IgGs display a profile of attributes that recommend their testing in higher species and their use in evaluating DKK1 function in relevant disease models.

The Wnt signaling pathway plays a key role in embryonic differentiation, adult tissue maintenance, stem cell biology, oncogenesis, and the etiology of degenerative diseases and has been directly linked to regulation of bone metabolism (1–5). Loss of function mutations in the Wnt coreceptor LRP5 result in osteoporosis-pseudoglioma syndrome in humans and mice characterized by low bone mass with reduced bone formation (6). Consistently, allelic LRP5 variants have been associated with altered bone mineral densities (BMD)\textsuperscript{3} and fracture risk in human populations (7). Importantly, discrete LRP5 gain-of-function mutations lead to a high bone mass (HBM) phenotype in humans and mice (8–11). Interestingly, HBM LRP5 mutations have been shown to be refractory to the inhibitory effects of Dickkopf-1 (DKK1) on canonical Wnt-signaling (9, 12), thus, strongly suggesting that the impairment of DKK1 signaling plays a role in the etiology of the HBM phenotype (9, 12). Recently this mechanism has been expanded to include other inhibitors of Wnt signaling such as sclerostin (13). Analyses of genetically DKK1 insufficient mice provides compelling evidence for an inhibitory role of DKK1 on new bone formation and a titratable effect on bone mass accrual resulting in a HBM phenotype that is maintained throughout adulthood (14, 15). DKK1 is a high affinity ligand for LRP5 and LRP6 cell surface receptors \textit{in vitro} and is a negative regulator of the Wnt/\textbeta-catenin pathway \textit{in vivo} (16–20). Although the protein is expressed and secreted within the bone microenvironment, it is highly soluble and readily detectable in the peripheral circulation (21–23). The second cysteine-rich domain (CRD-2) of DKK1 is necessary and sufficient for receptor binding and antagonism via interaction with LRP5/6 domains, and the tertiary structure of the CRD-2 has been reported to resemble a distinct globular folding pattern (24–26). DKK1 forms a trimeric complex with Kremen proteins (Krm) to mediate inhibitory effects on Wnt signaling (27). Consistently, loss of Krm leads to a high bone density phenotype in mice (28).

Skeletal mass is maintained through a complex and tightly regulated dynamic balance between osteoclastic bone resorption and osteoblastic bone formation (4, 29). Dysregulation of DKK1 has been implicated as a causal or disease-modifying factor in rodent models and has, therefore, been proposed as a

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\textsuperscript{3} The abbreviations used are: BMD, bone mineral density; HBM, high bone mass; DKK1, Dickkopf-1; rhDKK1, rhesus DKK1; CRD, cysteine-rich domain; CM, conditioned media; CT, micro-computed tomography; HC, heavy chain; ANOVA, analysis of variance; CM, conditioned media; hPTH, human parathyroid hormone.
Fully Human Antibodies That Neutralize DKK1 Function

therapeutic target for the treatment of diseases associated with low bone mass (30–32), multiple myeloma (33–35), and rheumatoid inflammatory disease (36, 37), the promotion of fracture repair (38, 39), and in disorders primarily affecting extra-osseous tissues (40, 41). Despite corroboration of a role for DKK1 in human disease, animal studies are currently limited to murine disease models in which DKK1 levels are experimentally elevated. Thus, there is a clear need for specific pharmacological agents that allow testing of disease-modifying activities uniquely applicable to higher species, preferably primates, while demonstrating attributes compatible with continuing preclinical development (42). Here we report the discovery and characterization of fully human anti-DKK1 monoclonal antibodies that not only potently neutralize DKK1 physiological activities and significantly augment bone mass and structure in normal mice but also have suitable pharmacokinetic profiles in non-human primates. Together these data offer pharmacological evidence for a role of DKK1 in bone metabolism and provide tools for continuing translational studies of DKK1 in murine and primate disease models.

**EXPERIMENTAL PROCEDURES**

**DKK1 Proteins**—Rhesus DKK1 cDNA was cloned by RT-PCR from rhesus macaque (*Macaca mulatta*) bone tissue and cloned into the pCDNA3.1-Myc-His expression vector (Invitrogen). A subclone in pFastbac1 (Invitrogen) was used to express recombinant protein in the baculovirus/His- Insect cell expression system. Cell lysate obtained through 0.5-μm filtration was loaded on a SP-sepharose fast flow column (GE Healthcare, Piscataway, NJ) and step-eluted with 20 mM Tris-HCl, pH 7.5, 300 mM NaCl. After a sterile filtration step, the eluate was loaded onto a XK16/10-cm nickel-nitrilotriacetic acid column (4 ml/min). After washing and equilibration with buffer containing 200 mM imidazole and then stored at −70 °C. The rhesus DKK1 (rhDKK1) protein was used in all biological assays. For panning of the phage-displayed human scFv libraries, rhesus DKK1 and mouse DKK1 protein (R&D Systems, Minneapolis, MN) were labeled with biotin (Bt-DKK1) using the EZ-link Sulfo-NHS-LC biotinylation kit (Pierce) following the manufacturer’s instructions.

**Phage-displayed scFv Library Construction, Panning, Screening, and Sequencing**—The Cambridge Antibody Technology (Cambridgeshire, UK) phage-displayed scFv libraries BMV, CS, and DP47 (43) were panned with a 10-fold excess coverage of library diversity (1–8 × 10^10) using 100 nm rhesus or mouse Bt-DKK1 proteins. Phage bound to Bt-DKK1 was separated by streptavidin-coated magnetic beads (Invitrogen) and proteolytically cleaved from the Bt/DKK/streptavidin bead complex via the addition of bovine pancreas trypsin (10 μg/ml; Sigma) in 0.1 M sodium phosphate buffer, pH 7.0, for 30 min at 37 °C. The phage-containing supernatant was used to infect *Escherichia coli* TG1 (A_{600 nm} = 0.5–0.8) for 1 h at 37 °C in an orbital shaking incubator (150 rpm). Titers of the phage were determined by serial dilutions on 2YTAG plates with ampicillin (100 μg/ml) and glucose (2%) (Teknova, Hollister, CA). Rescued phage was re-amplified in log-phase *E. coli* TG1 super-infected with KO7 helper phage (10 multiplicity of infection) at 30 °C in 2YT containing ampicillin (100 μg/ml) and 50 μg/ml kanamycin (2YTAK) overnight. Aliquots of supernatants containing rescued phage clones were collected for use in subsequent rounds of panning. Output clones from the second and third panning iterations were analyzed for their ability to recognize both rhesus and mouse DKK1 in an ELISA as outlined below. A total of 176 and 88 individual phage clones enriched in each panning experiment from the second and third round, respectively, were transferred to 96-well plates containing 100 μl of 2YTAG, incubated overnight (30 °C, 500 rpm) in a HiGro cell culture system (Genomic Solutions, Ann Arbor, MI), and archived at −80 °C in 17% glycerol. Clones were sequenced using primers for sequencing through the gene III leader region (PUC/M13 reverse primer 5'-CAGGAACAGCTATGAC-3') and into the scFv region (forward sequence 5'-GTCGTCCTTCCAGCATGATGT-3') (GeneWiz, North Brunswick, NJ). Affinity maturation of scFv clone RH2-18 was achieved by constructing and panning of randomized light chain CDR3 libraries using degenerate primers described previously (44) and the pCANTAB8s scFv phage display vector (43). Libraries were randomized in five amino acid blocks of the light chain CDR3 with resulting size and diversity in all greater than 10^8 and panned in four consecutive rounds using rate-limiting amounts of Bt-rhDKK1 and resulted in isolation of RH2-18LC01.

For scFv phage screening by ELISA, Bt-DKK1 was coated on clear Reacti-bind streptavidin-coated plates (Pierce) for 16–18 h at 4 °C. Eighty microliters of clarified scFv supernatant from overnight culture plates were added to the assay plates and incubated for 1 h at room temperature. Plates were stringently washed 6 times with Dulbecco’s phosphate-buffered saline containing 0.1% Tween 20 (Sigma). Eighty μl of horseradish-peroxidase (HRP) anti-M13gpVIII-conjugated secondary antibody (1:5000 in Dulbecco’s PBS, 3% non-fat dry milk) was added to each well and incubated for an additional hour at room temperature. After a repeat of the wash steps as above, bound phage was detected by the addition of 100 μl/well TMB substrate system for ELISA (Sigma) for 5 min at room temperature, and reactions were stopped by the addition of 0.5 μl of H_2SO_4 (50 μl/well). Absorbance was read at 450 nm using a SpectraMax M5 plate reader ( Molecular Devices, Sunnyvale, CA).

**Large Scale scFv Purification**—A single *E. coli* TG1 colony was inoculated in 2–5 ml of 2YTAG (2% glucose), and the overnight culture was expanded to 500 ml of 2YTAG (0.1% glucose) and then incubated at 37 °C until an A_{600 nm} reading of 0.6 – 0.8 was achieved. Isopropyl 1-thio-β-D-galactopyranoside (0.5 mM) was then added to induce scFv expression, and cultures were continued overnight (200 rpm, room temperature). Cells were harvested by pelleting (3000 g, 10 min) and frozen for 1 h at −20 °C. His-tagged scFv was purified from lysed cell pellets using a Qiagen Ni-NTA Superflow BioRobot (Qiagen, Valencia, CA) following the vendor’s instructions and then concentrated in a Centricon Plus 20 PL-10K tube (Millipore, Billerica, MA). Concentrated samples were transferred to a Slide-A-Lyzer Dialysis Cassette (Pierce).
and dialed against Dulbecco’s PBS in the absence of calcium and magnesium. Dialed scFv samples were quantified for total protein by the BCA method (Pierce).

IgG Conversion—The heavy chain variable regions were fused in-frame with the IgG2m4 (45) constant region and the light chain variable regions were fused with either \( \lambda \) or \( \kappa \) constant region in alignment with the corresponding variable regions of the scFv clones. Clone RH2-18L to was also converted using the IgG1 constant region. The heavy and light chain variable regions were amplified by PCR from scFv vectors and cloned into IgG expression vectors using the In-Fusion method (Clontech, Mountain View, CA). The forward and reverse PCR primers contain 21 nucleotides overlaps with the IgG expression vector sequences and 15 nucleotides overlaps with the variable regions. The constructs were verified by DNA sequencing (GeneWiz, North Brunswick, NJ). The antibodies were expressed transiently in HEK 293 cells (46) and purified using a standard protein A/G affinity chromatography protocol (Pierce).

Measurement of Binding Affinities—The binding affinity between DKK1 and the antibodies was measured using a Biacore 3000 instrument (GE Healthcare). Briefly, goat anti-human IgG Fc-specific antibody (Calbiochem) was diluted to 50 \( \mu \text{g/ml} \) in 10 \( \text{mM} \) sodium acetate, pH 5.0, and immobilized covalently at \( \sim 6000 \) resonance units on the surface of a flow cell on a CM4 chip using standard amine coupling chemistry. The anti-DKK1 IgG antibodies were diluted to 5 \( \text{nM} \) HBS-EP buffer (10 \( \text{mM} \) HEPES, pH 7.4, 150 \( \text{mM} \) NaCl, 3.4 \( \text{mM} \) EDTA, 0.005\% Surfactant P20), injected over the flow cells at a rate of 10 \( \mu \text{l/min} \) for 1 min, and captured by the anti-goat anti-human IgG Fc-specific antibodies. Various concentrations of DKK1 or buffer controls were injected over the flow cells at a rate of 60 \( \mu \text{l/min} \) for 2.5 min followed by a 30-min dissociation phase. The binding data were processed and fit to a 1:1 interaction model using the BioEvaluation software (GE Healthcare) to calculate the \( K_D \).

Selection of scFv and IgG Antibodies in in Vitro Cell-based DKK1 Binding Assays—Rhesus DKK1 protein was labeled by Lys-directed conjugation of Eu\(^{3+}\) chelate following the manufacturer’s instruction (PerkinElmer Life Sciences) and purified with Sephadex G-25 PD-10 columns (GE Healthcare). Human Lrp5 cDNA (47) was generously provided by Fred Hess (Merck Research Laboratories, West Point, PA). HEK293 Flp-In cells (Invitrogen) stably transfected with human Lrp5 (HEK293\(^{\text{hLrp5}}\)) were seeded in poly-D-lysine-coated 96-well plates at 100,000 cells/well (Biocoat, BD Biosciences) and incubated overnight. Eu\(^{3+}\)-labeled DKK1 protein diluted in DELFIA assay buffer without detergent (PerkinElmer Life Sciences) was added at a final assay concentration of 100 \( \text{pM} \), and the binding reaction was continued at 37 °C for 15 min. Nonspecific binding was determined by competition binding using a 250-fold molar excess of non-labeled rhDKK1. Unbound rhDKK1 was removed by three consecutive washes with wash buffer (50 \( \text{mM} \) Tris, 0.138 \( \text{mM} \) NaCl, 2.7 \( \text{mM} \) KCl, pH 8.0, 0.02% Tween 20). Cells were treated with 100 \( \mu \text{l} \) of DELFIA Enhancement Solution (PerkinElmer Life Sciences) per well for 20 min, and time-resolved fluorescence was determined using a Victor3 plate reader at excitation/emission 340/615 nm (PerkinElmer Life Sciences). scFv antibodies were allowed to preincubate with DKK1 protein for 60 min before the addition to cells, whereas IgG antibodies were tested by adding IgGs and DKK1 to cells consecutively. Polyclonal goat anti-DKK1 IgG (R&D Systems) was used as a control.

Wnt Signaling Assay—Wnt3A was harvested from conditioned media (CM) of L-cells stably expressing and secreting mWnt3A (CRL-2647; American Type Culture Collection, Manassas, VA) after 4 and 8 days of culture and combining the harvested CM (Wnt3A-CM). The identical procedure was followed with the parent L-cell clone (CRL-2648) to obtain control CM (L-CM). HEK293\(^{\text{hLrp5}}\) cells were seeded in 10% FBS, high glucose DMEM (Invitrogen) at 25,000 cells/well in a 96-well plate (BD Biosciences) and cultured overnight at 37 °C in a humidified chamber with 5% \( \text{CO}_2 \). Cells were transfected overnight with 3.75 ng of Topflash (Lef-1/\beta\text{-catenin reporter}; Promega, Madison, WI), 0.08 ng of pTK-renilla (Promega), and 50 ng of plasmid encoding Lef-1/well using FuGENE 6 (Roche Applied Science). In a 96-well polystyrene plate, 1 \( \mu \text{g} \) of anti-DKK1 scFv antibody was combined with 1 \( \mu \text{g} \) of rhDKK1 (50 nm) for 15 min, and the DKK1/scFv mix was then added to 96-well plates seeded with HEK293\(^{\text{hLrp5}}\) cells. The tissue culture plates were incubated for 15 min to allow DKK1 binding to cell surface LRP-5 and LRP-6. Thereafter, 100 \( \mu \text{l} \) of Wnt3A-CM was added at an estimated final concentration of 50–100 ng/ml. An equal volume of L-CM was added to control wells. The experimental plates were incubated for 16 h followed by cell lysis, and determination of luciferase activities by relative light units was normalized to a pTK-renilla control reporter (Promega).

Specificity of Anti-DKK1 IgGs—Cynomolgus (Macaca fascicularis) DKK4–Myc-His protein (cyDKK4), and a chimera of rhesus DKK1 N terminus (residues 1–158) with rhesus DKK2 CRD-2 (residues 143–259)-Myc-His protein (rhDKK2) were expressed in baculovirus/Hi5-Insect cells expression system and purified as described above for rhesus DKK1 protein. CXCL16-His (R&D Systems) was used as a non-relevant loading control. A range of 1–100 ng of the respective recombinant proteins was added to nitrocellulose membranes (Millipore), and anti-DKK1 IgGs or mouse anti-His-tag antibody (Invitrogen, 1 \( \mu \text{g/ml} \) ) was allowed to bind overnight. After washing with buffer, bound antibodies were detected using anti-human Fc-AP conjugate (Sigma) or goat anti-mouse IgG-AP conjugate (Santa Cruz Biotechnology, Santa Cruz, CA).

Domain Mapping by Alanine Scanning—Rhesus DKK1 (M1-H266) was subcloned in pEGFP-N2 (Clontech) to fuse with GFP to the C terminus of DKK1. \( \Delta N\text{-DKK1} \) (Met-1—Val-60, Ser-157—His-266) and \( \Delta C\text{-DKK1} \) (Met-1—Pha-158) were generated as described (25). Site-directed mutagenesis of \( \Delta N\text{-DKK1} \) was done using QuikChange (Stratagene, Carlsbad, CA) by substituting single or paired amino acid residues with alanine (Ala). All proteins were expressed by transient transfection of HEK293 cells for 72 h, and CM containing the secreted recombinant proteins were collected. Protein expression was verified by immunoblotting onto nitrocellulose membrane using anti-GFP antibody ab6556 (Abcam Inc., Cambridge, MA) or anti-DKK1-IgG (1 \( \mu \text{g/ml} \)).
Epitope Excision Mapping of Anti-DKK1 IgGs—CNBr-activated Sepharose beads (Amersham Biosciences) in 1 mM HCl at a concentration of 50 mg/ml were packed into a 0.8-ml micro-columns (Mobitec, Göttingen, Germany) and equilibrated with 0.1 M NaHCO₃, 0.5 M NaCl (pH 8.3). A total of 500 μg of antibody was immobilized on beads at 37 °C for 2 h and then blocked with 0.1 M Tris-HCl, 0.5 M NaCl, pH 8.0, followed by washes and equilibration with PBS (pH 7.4). The rhDKK1/IgG immune complex was prepared by incubating with the antigen (0.25 mg/ml) in 300 μl of PBS, for 2 h at 37 °C. Unbound DKK1 was then removed by washing the column with PBS. An aliquot was sampled to a MALDI target plate and subjected to direct matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analysis to verify that rhDKK1 was bound to the mAb-immobilized beads. All digestions for epitope excision were performed in PBS, pH 7.4, at 37 °C in presence of 0.01–1 mg/ml endoproteases. Multiple additions of endoproteases were required to fully digest the antigen. Trypsin (Promega) was used as the first enzyme, after which the trypsin-cleaved immune complex was split into three aliquots and independently subjected to further digestion by chymotrypsin (Sigma), GluC, AspN (Roche Applied Science), or aminopeptidase (Sigma). Following each proteolysis step, the compact reaction column containing the antigenic peptide-antibody complex was drained and washed three times with PBS buffer to remove unbound peptides. Aliquots of the eluates were analyzed by MALDI-TOF MS before and after DTT reduction. DTT reduction was performed by adding 10 mM DTT in 0.1M NH₄HCO₃ at 37 °C for 30 min, after which a 1-μl matrix was added to the beads for MS analysis of the reduced samples. Bead samples were mixed on to the target plate with 1 μl of a saturated solution of sinapinic acid (Fluka, St. Louis, MO) in acetonitrile/water/1% TFA and air-dried. Analyses were performed using a Voyager DE-STR (Applied Biosystems, Carlsbad, CA) operating in linear mode (500 scans were averaged). Samples were ionized with a nitrogen laser (λ = 337 nm), and spectra were internally calibrated using Sequazyme™ Peptide Mass Standards kits (Applied Biosystems).

Mesenchymal Progenitor Cell Assay—C3H10T1/2 cells (#CCL-226, clone 8; American Type Culture Collection) were passaged in high glucose DMEM with phenol red (Invitrogen) supplemented with 10% heat-inactivated FBS (Invitrogen). For experiments, cells were seeded into 96-well plates (20,000 cells/well) in MEM-α (Invitrogen) supplemented with 10% FBS. Media were replenished after 2 days, and treatment begun by the addition of IgG (2.7–80 nm) followed by rhDKK1 protein (50 nm) and then by control CM or Wnt3A-CM to a final concentration of 10 ng/ml. Wnt3A stimulated expression of alkaline phosphatase activity in this cell system over 2–3 days. After washing cells twice in buffer (20 mM Hepes, pH 7.5, 150 mM NaCl) and cell lysis (10 mM Tris HCl, pH 9.0, 150 mM NaCl, 0.025% Tween 20), alkaline phosphatase enzymatic activity was determined with the Attophos-kit (Promega) and calculated with standards prepared from calf intestinal phosphatase (Roche Applied Science).

In Vivo Pharmacodynamic and Efficacy Study in Female Mice—All procedures performed on animals were in accordance with established guidelines and were reviewed and approved by the Merck West Point Institutional Animal Care and Use Committee. Five-week-old C57BL/6NTac female mice were obtained from Taconic Inc. (Hudson, NY). After acclimating to the animal facility for 1 week, mice were randomized by body weight (~30 grams) to eight treatment groups (n = 10–12). Anti-DKK1 antibodies were administered subcutaneously in 0.1 ml of phosphate buffer per mouse. Animals were treated twice weekly for 4 weeks with vehicle, 0.15, 0.5, 1.5, 5, or 15 mg/kg (subcutaneously) of antibody RH2-59 IgG2m4 or 1.5 mg/kg of antibody RH2-18 IgG2m4. A control group was administered human PTH-(1–34) (Bachem Inc., Torrance, CA) at 80 μg/kg (3×/week). At necropsy, body weights were recorded, and femora were dissected free of soft tissues and fixed in 70% ethanol. Whole femora were scanned by Piximus (GE/Lunar; Schenectady, NY). During additional analyses the femur was subdivided into two regions of interest; that is, a distal region comprising the distal most 3 mm and a central region beginning 5 mm from the distal end and extending proximally 5 mm. The central region of interest is composed of 100% cortical bone, whereas the distal region of interest consisted of ~80% cortical bone and ~20% trabecular bone. Piximus software was used to calculate BMD (mg/cm²) for whole femur, distal femur, and central femur as defined. A quality control phantom (hydroxyapatite) was run each day before sample analyses. Cortical area was assessed in a transverse section of mid-femur. In preparation for histological evaluation, the central femur was dehydrated in graded concentrations of ethanol and then embedded undecalcified in 90% methyl methacrylate, 10% dibutylphthalate solution (Polysciences, Warrington, PA). At the mid-point of the femur, transverse histological sections were cut at a 100-μm thickness using a Leica SP1600 Saw Microtome (Leica Instruments GmbH; Nussloch, Germany). The sections were mounted on glass slides and coverslipped with Eukitt’s mounting media. All evaluations were done on coded slides with the investigator blinded to treatment group. The microscope used in the study was an Eclipse 80i (Nikon, Japan) equipped with an Optronics DEI-750 CE (Tuttlingen, Germany) video camera interfaced to a computer system running Bioquant Nova Software (Bioquant Image Analysis Corp., Nashville, TN).

Micro-computed Tomography (CT)—Micro-CT was performed using a volumetric scanner μCT40 (Scanco Medical, Zurich, CH) with data collected by MicroView (GE Healthcare) and additional cutaway views using SCIRun (Scientific Computing and Imaging Institute, University of Utah, Salt Lake City, UT). Instrument calibration was performed with a hydroxyapatite standard before sample analyses. Bone density and volumetric measurements were obtained from the distal end of each right femur using a cylindrical tube (2 × 0.8 mm) centered ~0.7 mm distal to the growth plate. Individual thresholding values were averaged per group.

Pharmacological Studies in Rhesus Monkey—Male and female rhesus macaque (n = 3/group; 6–10 years of age) from the University of Louisiana-Lafayette, New Iberia Research...
Center (New Iberia, LA) weighing 4.7–12.0 kg were used for single dose administration studies. Anti-DKK1 IgG dose solutions were prepared in a buffer (100 mM histidine, 100 mM arginine, and 6% sucrose, pH 6.0) and administered at a dose of 10 mg/kg subcutaneously (0.16 ml/kg). Serial blood serum samples were collected from the saphenous or femoral vein into serum separator tubes pre-dose and after dose administration on day 4, 8, 15, 22, 29, 36, 43, 50, and 57. Serum was prepared by allowing blood samples to clot for 5–10 min and centrifuging the coagulated blood (3200 × g, 10 min, 4 °C). Serum samples were stored at −70 °C immediately after collection. Serum anti-DKK1 antibody levels were determined using recombinant rhDKK1 (235 ng/well) in PBS coated as the antigen to capture anti-DKK1 antibodies in 96-well EIA plate (Fisher). Briefly, plates were rinsed once with wash buffer PBST (PBS, 0.05% Tween 20) and then blocked with 300 μl of blocking buffer (3% BSA, PBST) at room temperature for 1.5 h. After three additional washes with PBST, plates were dried, sealed, and stored at 4 °C. For assays, all samples were diluted 1:20 with assay buffer (2% BSA, PBST containing NaN3) and subsequently diluted with assay buffer containing 5% serum (5% control serum in assay buffer) to concentrations in the range of 1–100 ng/ml. A 50-μl aliquot of each previously diluted sample was added to the prepared plate in duplicate and incubated for 1 h. After three washes with PBST, HRP-conjugated mouse anti-human IgG (1:3000; SouthernBiotech, Birmingham, AL) was added as a detection antibody. Turbo TMB (3,3’5,5’ tetramethylbenzidine; Pierce) was used as a substrate, and absorbance read at 450 nm after the addition of stop solution (1 m sulfuric acid). A four-parameter curve fit was used for data reduction, as defined by SoftMax® Pro (Molecular Devices). The concentration of human IgG in test samples was determined by interpolation from the constructed standard curve.

**Statistical Analysis—** All data are expressed as the means ± S.E. Where appropriate, a one-way ANOVA followed by Dunnett’s post-hoc test to detect group differences was calculated using Graphpad Prism 5.0 (GraphPad Software, La Jolla, CA).

**RESULTS**

**Isolation of Anti-DKK1 scFvs—** Identification and characterization of efficacious anti-DKK1 antibodies are outlined in supplemental Fig. 1. Each of the three scFv phage display libraries was subjected to three rounds of solution-based panning against biotinylated rhesus and mouse DKK1 proteins. Overall, 110–530-fold enrichment was achieved during the first to second round of panning using rhesus DKK1 and relative minor enrichment for the third round of panning using mouse-DKK1 (Table 1). A total of 176 and 88 hits from the second and third round of panning, respectively, was isolated from each of the three libraries. These individual clones from the second and third round pannings were assayed for ELISA-based binding to rhesus and mouse DKK1 to allow for selection of antibody sequences conferring pan-species applicability. Among the hits selected from all libraries, 56.6% dual ELISA positive rhesus/mouse cross reactive hits were observed from the round 2 pannings, whereas 88% dual positive hits were observed from the round 3 pannings with the largest number of cross-reactive antibodies obtained from the CS sublibrary (Table 1).

All 232 dual-positive scFv clones from the third round pannings were sequenced, and 24 unique sequences were identified. Some of the scFv clones from within sublibraries were identified repeatedly (e.g. sequences repeated up to 17 times among the third round panning output clones), suggesting that dominant scFv sequences out-competed others for binding to DKK1 and, therefore, that particular epitope and binding affinity might be of significance. Interestingly, several clones (RH2-18, RH2-31, RH2-59, and RH2-80) had similar heavy chain (HC) sequences with an uncharacteristically short CDR3 region comprising three amino acid residues. All clones displayed EDY heavy chain CDR3 sequences, and all were derived from within the CS sublibrary. The variable region amino acid sequences of these four originally isolated clones are also given and differ from the third round panning output clones, whereas RH2-18LC01 was converted into full-length IgG1 isotype. The IgG1 isotype was used to investigate a possible impact of the effector function on in vivo efficacy and safety. The IgG clones were expressed and purified from HEK293 cells and subjected to a series of in vitro and in vivo functional analyses. Purified RH2-18LC01 IgG1 and RH2-18lgG2 m4 proteins are shown on reduced and native gels (Fig. 1B).

**Inhibition of DKK1 Binding to hLRP5 Expressed on HEK293 Cells and Wnt3A Signaling by scFv and IgG Antibody Clones—** To identify anti-DKK1 scFv, which can inhibit DKK1 binding to hLRP5, a HEK293bHLR5 cell line that stably expresses hLRP5 was used for monitoring the scFv inhibition of europium-labeled DKK1 binding to the cell surface. In the

| Sublibrary | Round 1 | Round 2 | Round 3 |
|------------|---------|---------|---------|
| BMV        |         |         |         |
| Input      | 1.0E+12 | 7.0E+09 | 5.6E+10 |
| Output     | 5.0E+05 | 4.7E+05 | 1.4E+09 |
| % Recovery | 5.0E-05 | 6.7E-03 | 2.6E-03 |
| Enrichment | 1.3E+02 | 3.8E+01 |         |
| Dual ELISA (+) | 81/176 (46%) | 79/88 (90%) |
| CS         |         |         |         |
| Input      | 1.0E+12 | 7.2E+09 | 7.0E+09 |
| Output     | 5.5E+05 | 4.3E+05 | 2.0E+07 |
| % Recovery | 5.5E-05 | 6.0E-03 | 2.9E-03 |
| Enrichment | 1.1E+02 | 4.8E+02 |         |
| Dual ELISA (+) | 114/176 (65%) | 88/88 (100%) |
| DP47       |         |         |         |
| Input      | 1.0E+12 | 1.0E+10 | 6.2E+09 |
| Output     | 1.2E+05 | 6.1E+05 | 2.3E+07 |
| % Recovery | 1.1E-05 | 6.1E-03 | 3.7E-03 |
| Enrichment | 5.3E+02 | 6.0E+01 |         |
| Dual ELISA (+) | 104/176 (59%) | 65/88 (74%) |
absence of neutralizing scFv, europium-labeled DKK1 is capable of binding to cell surface receptors (LRP5 and LRP6). Functionally interfering scFv prevented DKK1 binding to cell surface receptors and consequently reduced the fluorescent signal. Inhibition is expressed as the percentage of specific DKK1 binding in absence of an inhibitor. As an example, significant inhibitory activity of RH2-31 scFv on DKK1 binding was detectable when used at 0.3 \( \mu \)g/well, and specific DKK1 binding was completely inhibited by the scFv at 1–3 \( \mu \)g/well. This effect was comparable with the effect of a reference polyclonal goat anti-DKK1 IgG (Fig. 2A). In contrast, a non-relevant control scFv (8B4) did not affect DKK1 binding to the surface of HEK293hLrp5 cells (Fig. 2A).

Functional interference of scFv in a Wnt3A signaling assay was measured by a T-cell factor/\( \beta \)-catenin activity assay (Fig. 2B). In this assay Wnt3A induced activity was completely inhibited by 100 nm rhDKK1. RH2-31 scFv (1 \( \mu \)g/well) neutralized DKK1 activity by 52\%, whereas a non-relevant control scFv (8B4) had no effect (2\%), demonstrating specific functional interference of selected anti-DKK1 scFv clones (Fig. 3A).

Guided by the experimental data using anti-DKK1 scFv protein, 24 scFv clones with unique amino acid sequences were converted to full IgG2m4 format as described under “Experimental Procedures.” Functional validation of purified IgGs was performed by dose titration in a HEK293hLRP5-based DKK1 binding assay (Fig. 2C), and RH2-18, RH2-31, RH2-59, and RH2-80 IgGs were examples of potent inhibitors of cell-based DKK1 binding. Selected IgG isotypes RH2-18 (IgG2m4) and RH2-18LC01 (IgG1) were further tested in full titration curves from 0.2 to 690 nM with calculated EC50 values of 4.0 to 8.0 nM (Fig. 2D). Equally important, both base line and plateau were reached at the two ends of the titration range. These results indicate that selected IgG clones not only bind
to DKK1, but they also functionally inhibit DKK1 interaction with cell surface receptors (LRP5/6) and moreover can promote Wnt-\(\beta\)-catenin pathway activation in HEK293hLRP5 cells. RH2-18 IgG2m4 antibody and DKK1 binding kinetics were examined using surface plasmon resonance. Several independent affinity studies were performed using either human or rhesus DKK1 proteins. Table 2 summarizes the mean rate constants and \(K_D\) values of repeat experiments and were calculated based on published methods (48). The overall affinity to human DKK1 ranged from 202 to 269 pM with a mean \(K_D\) value of 249 pM. Values determined for rhesus DKK1 ranged from 771 to 934 pM, with a mean \(K_D\) value of 855 pM.

Epitope Mapping—It is noteworthy that, despite their high affinities, neutralizing anti-DKK1 antibodies RH2-18, RH2-31, RH2-59, RH2-80, and RH2-18LC01 described here fail to bind denatured DKK1 protein in immunoblots and fail to bind to discrete peptides (20-mers) that span the human DKK1 sequence (data not shown), suggesting the antibodies recognize conformational epitopes. Specificity of antibody binding to rhDKK1 versus other DKK-protein family members was, therefore, determined by dot-blot of non-denatured His-tagged native proteins (Fig. 3A). In this assay format the detection limit for native rhDKK1 was 1–3 ng, and no specific binding of RH2-18 IgG2m4 to rhDKK2 or cyDKK4 was observed when compared with an irrelevant His-tagged protein. Anti-His loading controls confirmed the presence of all recombinant proteins.

To map the DKK1 domains necessary for functional interaction with anti-DKK1 IgGs, N-terminal (\(\Delta\text{C}-\text{DKK1}\)) and C-terminal CRD (\(\Delta\text{N}-\text{DKK1}\)) of DKK1 were constructed.

FIGURE 2. Anti-DKK1 scFv and IgG clones inhibit DKK1 functional activities in HEK-293hLrp5 cells. A, anti-DKK1 scFv (RH2-31) and negative control scFv (8B4) were tested at indicated increasing doses in Eu-rhDKK1 binding assay (\(\mu\)g/well). Polyclonal anti-DKK1 IgG (1 \(\mu\)g/well) served as the positive control (IgG). B, RH2-31 and 8B4 scFv tested at 1 \(\mu\)g/well in Wnt3A-T-cell factor/\(\beta\)-catenin reporter assay over 24 h. Data are given as relative light units normalized to control reporter. Polyclonal goat anti-DKK1 IgG (1 \(\mu\)g/well) served as positive control (DKK1-IgG). C, primary validation of selected crude anti-DKK1 RH2-18 (\(\Delta\)), RH2-31 (\(\Box\)), RH2-59 (\(\bigtriangleup\)), and RH2-80 (\(\bigcirc\)) antibodies inhibited Eu-rhDKK1 HEK-293hLRP5 cell binding at final concentrations of 0.2, 0.6, 2.6, and 20 nm. Antibody 8B4 (\(\blacksquare\)) was used as a negative control (20 nm). D, purified anti-DKK1 RH2-18 (\(\Delta\)) and RH2-18LC01 (\(\bigcirc\)) antibodies were re-titrated using an extended dose range in the same assay format for determination of an effective concentration 50 (EC50). Data are given as the means ± S.E; one-way ANOVA, Dunnett’s test versus PBS-control treatment (\(*\), \(p < 0.05\); **, \(p < 0.01\); *** , \(p < 0.001\)).
blotting analysis showed that the neutralizing antibody RH2-18 bound within the CRD-2 (159–266) domain of \( \text{H9004} \) N-DKK1 (Fig. 3B). Specific binding to CRD-2 was also found for RH2-80, RH2-31, RH2-59, and RH2-18LC01. Epitope mapping for RH2-18 by alanine (Ala) substitutions within the CRD-2 identified Ser-187 and Val-188 together with Arg-203, His-204, Phe-205, and Lys-208 as residues necessary for interaction, whereas Ala substitution at the predicted DKK1 N-glycosylation site (Asn-256) had no effect on binding (data not shown). Consistent with the notion of a conformational epitope, the distant residues Glu-241 and Gly-243 and the disruption of a Cys-bond (C220A) in CRD2 also resulted in loss of RH2-18 binding. An epitope-excision approach (49) comprising stepwise limited proteolysis of the immune-complex with a series of endo- and exo-peptidases identified protected peptides entirely confined to sequences within CRD-2. In particular, two continuous protected fragments Val-188—Lys-217 and Cys-239—Cys-245 are in strong agreement with the Ala-scanning data (Fig. 3C).

We next examined the effect of DKK1 and leading neutralizing anti-DKK1 IgGs on \( \text{in vitro} \) cell differentiation. The mouse mesenchymal pluripotent cell line, C3H10T1/2, differentiates toward an osteoblastic cell lineage by treatment with osteogenic factors (50, 51). Wnt3A treatment over 72 h induced the activity of the early differentiation marker alkaline phosphatase, whereas concomitant treatment with rhDKK1 (50 nM) inhibited alkaline phosphatase activities, indicating that DKK1 functions as a negative regulator of C3H10T1/2 cell differentiation \( \text{in vitro} \) (Fig. 4). The presence of anti-DKK1 IgGs RH2-18LC01, RH2-18, and RH2-59 neutralized the inhibitory function of rhDKK1 on cell differentiation. Consistent neutralizing effects were evident at antibody concentrations of 8 nM and further diminished the inhibitory function of rhDKK1 on the cell differentiation marker in a dose-related manner (Fig. 4). In addition, physical stability of RH2-18LC01, RH2-18, and RH2-59 was sufficient to produce and maintain neutralizing effects over a 3-day assay period. As expected, the non-relevant control IgG (8B4) at the same dose range did not result in significant effects.

Based on \( \text{in vitro} \) analyses of DKK1 binding and Wnt activation as well as C3H10T1/2 cell differentiation assays, three antibodies (RH2-18, RH2-59, and RH2-18LC01) were selected for further study. All three lead antibodies displayed a similar HC sequence with a short CDR3 (three amino acids). How-

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**FIGURE 3.** Specificity of binding to DKK1 and epitope mapping of RH2-18 IgG. A, immunoblot, recombinant native DKK proteins were probed at increasing mass as indicated (ng), and bound RH2-18 was detected by human Fc Ig-AP conjugates. Protein loading control used an anti-His tag antibody (Anti-tag). B, immunoblot, native rhDKK1 full-length protein, N-terminal deletion of CRD1 (Met-1—Val-60, Ser-157—His-266; \( \text{H9004} \) N-DKK1), and C-terminal deletion of CRD2 (Met-1—Phe-158) were probed with RH2-18 and tag antibody (anti-EGFP). C, shown is a summary of epitope mapping results by Ala substitutions within the CRD2 domain and through epitope excision/MS analyses of full-length rhDKK1. CRD-1 (dashed box) and CRD-2 (solid box) are highlighted. Arrows (↓) point to residues where Ala substitutions resulted in complete loss of RH2-18 binding, whereas asterisks (*) indicate Ala-substituted residues without effect. ND, not determined. Three CRD-2 peptides bound to RH2-18 after proteolysis are indicated by solid lines, and specific residues found to be protected within the epitope-excision experiments are underlined (e.g. R203).

**TABLE 2**

Anti-DKK1-IgG RH2-18 binding affinities to rhesus and human DKK1 proteins

| Antibody | Antigen       | Repeats | \( K_D \) (\( \text{pM} \)) | \( k_a \) (\( \text{s}^{-1} \)) | \( k_d \) (\( \text{s}^{-1} \)) |
|----------|---------------|---------|-----------------|-----------------|-----------------|
| RH2-18   | Human DKK1    | 4       | 249 ± 32        | 5.4 ± 1.4 × 105 | 1.3 ± 0.4 × 10^{-4} |
|          | Rhesus DKK1   | 3       | 855 ± 82        | 1.7 ± 0.7 × 105 | 1.5 ± 0.5 × 10^{-4} |
Fully Human Antibodies That Neutralize DKK1 Function

ever, all three antibodies have to have significant different light chain sequences, suggesting that the short HC CDR3 plays a critical role in inhibiting DKK1 binding to receptors and function.

Anti-DKK1 Antibodies Show Efficacy on Cortical and Trabecular BMD and Improve Bone Structural Parameters in Growing Female Mice—Lead anti-DKK1 clones RH2-59 and RH2-18 were analyzed in 6-week-old intact female mice to determine whether the in vitro DKK1 neutralization and effects on osteoblast differentiation could be validated in vivo. 10–12 female mice per group were subcutaneously administered RH2-59 in a dose range of 0.15–15 mg/kg or RH2-18 at 1.5 mg/kg (2×/week). A control group was treated with hPTH-(1–34) at 80 μg/kg (3×/week, subcutaneously), a regimen expected to produce an osteoanabolic stimulus. At the conclusion of the 4-week dosing period, mice were subjected to necropsy, and BMD was assessed in the whole femur, the central femur, and distal femur (Fig. 5). BMD analysis of the whole femur, accounting for bone activities observed in both cortical and cancellous compartments, showed a dose-related increase in response to RH2-59, achieving significance at 5 mg/kg (p < 0.05) and 15 mg/kg (p < 0.01) dose levels with a relative mean BMD increase of 4.9 and 6.1% versus vehicle, respectively (Fig. 5A). Similarly, the RH2-18 IgG group showed a 4.7% increase in areal BMD (p < 0.05). The hPTH-(1–34) control group displayed a 7.2% (p < 0.001) increase in whole femur BMD. In the distal femur, a region comprised of both cancellous and cortical bone, BMD was increased in a dose-related manner by RH2-59 treatment, achieving a 7.2% increase at 15 mg/kg RH2-59 groups (p < 0.01) and rising by 4.5% (p < 0.05) with RH2-18 treatment (Fig. 5B). The hPTH-(1–34) controls showed a mean 6.3% increase in distal femur BMD. Measurements of central femur BMD (Fig. 5C) and cortical bone area in histological sections (Fig. 5D) were undertaken to evaluate the effect of anti-DKK1 IgGs on compact bone. At week 4, central femur BMD was found to have risen dose-dependently in response to RH2-59, reaching significance (p < 0.01) at 15 mg/kg, whereas RH2-18 dosing produced a numerical increase in mean areal BMD by 2.9% (not significant) (Fig. 5C). This was paralleled by non-significant numerical increases in cortical area in anti-DKK1 IgG treatment groups (Fig. 5D), whereas the hPTH-(1–34) treatment group showed significant increases in mean BMD (8.0%) and cortical area at the femoral mid-shaft (p < 0.01). There were no treatment-related body weight changes during the course of study (Fig. 5E). Evaluation of distal femora from RH2-18-treated mice was extended into volumetric micro-CT analyses of trabecular bone (Fig. 6). In agreement with areal BMD analyses, a substantial increase in trabecular volumetric BMD (11.3% over control-group, p < 0.001) was detected. Moreover, close to a 2-fold increase in bone volume/tissue volume indicated a substantial treatment effect on trabecular bone volume in the distal femur (p < 0.05) (Table 3). Consistent with this result, RH2-18 treatment was associated with significantly increased trabecular number and trabecular thickness (p < 0.001), improved connectivity density, and commensurately decreased trabecular spacing (p < 0.001), all indices of a robust treatment effect on trabecular micro-architecture in normal female mice. Highly significant treatment effects (p < 0.001) were noted by micro-CT analyses of trabecular bone of distal tibia in the hPTH-(1–34) treatment group (Table 3).

Pharmacological Studies in Rhesus Macaque—To evaluate the pharmacokinetic characteristics of the anti-DKK1 antibodies, a single dose of RH2-18LC01 (10 mg/kg) was administered subcutaneously to male and female rhesus macaques. Serum RH2-18LC01 antibody concentration peaked at 94.4 ± 8.3 μg/ml at post-dosing day 4 and declined thereafter to day 21 (30.7 ± 4.2 μg/ml) and, in a second phase to day 57 (3.9 ± 2.0 μg/ml), the last day tested, with an apparent half-life of 13.3 ± 1.3 days (Fig. 7). RH2-18LC01 IgG was detectable in all samples at all time points tested (detection limit, <20 ng/ml) and an RH2-18LC01 exposure of 287.9 ± 12.4 μM·h was achieved over the observation period, from days 4 to 57.

DISCUSSION

Human and mouse genetics have linked Wnt-signaling components including cell surface receptors and soluble ligands with roles in bone formation, bone growth and remodeling as well as bone regeneration and repair (52, 53). Thus, the potent pathway inhibitor DKK1 presents an attractive therapeutic target for the treatment of iatrogenic or pathologic low bone mass diseases. We report here fully human anti-DKK1 monoclonal antibodies isolated from phage-displayed scFv libraries specifically targeting full-length rhesus and mouse DKK1 and selected for their neutralizing effects on DKK1 function. Interestingly, the novel antibodies described here have a short heavy chain CDR3 consisting of only three amino acids, which may suggest a unique interaction with its corresponding topological DKK1 epitope. The recently described novel IgG2m4 isotype (45) was chosen for its
reduced effector function with an overall reduction in complement and Fcγ receptor binding while maintaining the normal in vivo serum half-life. Substitutions within LRP5 leading to HBM confer resistance to the inhibitory activity of DKK1 (9, 12). Although the precise molecular mechanism underlying the HBM phenotype has not been fully elucidated, the diminished interaction and in vitro binding of DKK1 to LRP5-HBM variants is well established (9, 12), and this is consistent with the pharmacologic effects of efficacious anti-DKK1 IgGs described here.

DKK1 protein topology is composed of two CRD located in the N- and C-terminal domain, respectively. The CRD-2 globular domain (24) is necessary and sufficient for DKK1 binding to the receptor LRP6 (25). Consistent with this, we have noted that all neutralizing IgGs derived from the three phage-displayed scFv sublibraries recognized only non-denatured native DKK1, and IgG binding was dependent on CRD-2, but not CRD-1, indicating that the functional epitope of anti-DKK1 IgGs is defined by CRD-2 sequence and its tertiary structure. Alanine substitutions within residues Ser-157—His-266 and epitope-excision mapping of full-length rhDKK1 for RH2-18 further strengthen this observation.

Although the anti-DKK1 IgGs were selected in a hLrp5-overexpressing cell background, the isolated antibodies effec-

**FIGURE 5. Increase in areal-BMD by 4-week treatment with fully human anti-DKK1 IgGs in normal female mice.** Intact female mice (n = 10–12) were dosed (2×/week) with RH2-59 (0.15–15 mg/kg), RH2-18 (1.5 mg/kg), or PTH1–34 (80 µg/kg, 3×/week). A, shown is femoral areal BMD acquired in the whole femur by ex vivo analyses. B, shown is areal BMD of the cancellous/cortical subregion of the distal femur. C, shown is areal BMD in the cortical bone region of the central femur. D, histological analysis of the cortical bone area on cross-sections in the femoral midshaft is shown. E, body weight gains in treatment groups throughout study are shown. Data are given as the means ± S.E. (n = 10–12); one-way ANOVA, Dunnett’s test versus control group (*, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, non-significant).
Fully Human Antibodies That Neutralize DKK1 Function

A single dose of RH2-18LC01 (10 mg/kg, subcutaneously) was administered to rhesus macaque (n = 3). Serum samples were drawn at the indicated time points up to day 57 post-dosing, and serum levels of human IgG1 determined are as described under “Experimental Procedures.”

Results of micro-CT analyses of distal femur trabecular bone in control, RH2-18, and hPTH(1–34) treatment groups

|BV/TV (cm²/cm³) | 740.9 ± 9.1 | 715.2 ± 5.1 | 782.7 ± 5.6 |
|----------------|-------------|-------------|-------------|
|Conn. Den. (1/mm³) | 54.629 ± 2.172 | 50.345 ± 2.132 | 67.942 ± 2.623 |
|SMI | 3.188 ± 0.043 | 3.105 ± 0.023 | 3.188 ± 0.043 |
|BMD (mg/cm³) | 665.8 ± 7.6 | 715.2 ± 5.1 | 740.9 ± 9.1 |
|Tb.Th. (mm) | 0.028 ± 0.001 | 0.033 ± 0.001 | 0.033 ± 0.001 |
|Tb.Sp. (mm) | 0.291 ± 0.004 | 0.245 ± 0.006 | 0.004 ± 0.001 |
|Tb.N. (1/mm³) | 3.449 ± 0.055 | 3.105 ± 0.023 | 3.105 ± 0.023 |
|BV/TV | 0.036 ± 0.003 | 0.031 ± 0.003 | 0.031 ± 0.003 |

*p < 0.001; one-way ANOVA, Dunnett’s test vs. control group.

*p < 0.05; one-way ANOVA, Dunnett’s test vs. control group.

Compelling evidence from analyses of life-long DKK1-insufficient mice (14) indicates an inhibitory role for DKK1 on bone formation and bone mass accrual that is maintained throughout adulthood. HBM phenotypes are apparent in Lrp5 G171V transgenics (11) and in DKK1-insufficient mice (12, 14) before longitudinal growth and skeletal development are complete. Therefore, efficacy testing of novel DKK1-neutralizing agents invites validation in the developing mouse before introduction into relevant disease models or higher species. We hypothesized that introducing pharmacologic inhibitors of DKK1 anew in 6-week-old mice may recapitulate the genetic loss of function phenotype in the adolescent skeleton. Indeed, temporary pharmacological interference with DKK1 function in vivo improved bone architecture and increased bone mineral densities. Moreover, and similar to genetic DKK1 deficiencies (15), the bone phenotype was titratable and penetrated into both cortical and trabecular bone compartments. In particular, the effects of DKK1 inhibition were comparable with those of a potent bone anabolic hPTH(1–34) on trabecular bone mineral density, bone volume, and architectural parameters of the distal femur. Although these findings underscore the therapeutic potential of the fully human IgGs, this awaits further confirmation in adult mice and higher species to assess the effect on remodeling mechanisms in skeletally mature bone.

Wnt signaling plays a major role in mesenchymal stem-cell fate by actively suppressing adipogenic and chondrogenic cell fates while promoting cellular programs leading to osteoblastogenic differentiation (58). As expected, rhDKK1 blocked the Wnt3A-mediated phenotype in pluripotent mesenchymal precursor C3H10T1/2-cells, whereas all efficacious IgGs demonstrated neutralizing activity on DKK1 function in vitro.

Akin to the effects of genetic DKK1 insufficiency in mice (14), the cellular mechanism in vivo likely involves increased osteoblast activities and new bone formation. Although exploration of the underlying cellular mechanism was not within the scope of the current report, follow-up studies in adult mice revealed 2-fold increases in bone formation rates, supporting the engagement of a bone forming mechanism by anti-DKK1 antibodies.

4 H. Glantschnig, K. Scott, R. Hampton, N. Wei, P. McCracken, P. Nantermet, J. Z. Zhao, S. Vitelli, L. Huang, P. Haytko, P. Lu, J. Fisher, P. Sandhu, J. Cook, D. Williams, W. Strohl, D. Kimmel, O. Flores, F. Wang, and Z. An, submitted for publication.
Fully Human Antibodies That Neutralize DKK1 Function

Rat and goat anti-DKK1 antibodies have been tested recently in murine models of rheumatoid arthritis (36) and in immune-compromised mouse multiple myeloma models (33, 34, 59). Results from these studies convincingly illustrate disease-modifying activities under conditions where DKK1 expression is augmented locally via inflammatory insult or secreted by myeloma cells. The data presented here further expand the rate-limiting role for DKK1 in bone expressed at physiological levels in intact mice and the potential for achieving marked improvements in volume, mass, and microarchitecture of normal bone.

To begin addressing the translational value of these findings, we undertook pharmacokinetic analyses in rhesus monkeys. The fully human anti-DKK1 IgGs showed favorable pharmacokinetics with an extended half-life in higher species.

In conclusion, we describe here the discovery of novel high affinity DKK1-specific IgGs that neutralize DKK1 receptor binding and function. When introduced into intact mice, these IgGs stimulate increases in bone mass and positively impact bone structural parameters, demonstrating a rate-limiting role for physiologic DKK1 in normal mouse bone. We reveal pharmacological actions of these IgGs as well as pharmacokinetic and pharmacodynamic properties that recommend their applicability to efficacy testing in adult rodent and non-human primate disease models. A wealth of information, although mostly from murine studies, corroborates a key role for DKK1 in low-bone mass disorders and the potential for therapeutic intervention (30, 33, 34, 36, 38, and 59). Thus, the fully human monoclonal anti-DKK1 IgGs described here will enable the further elucidation of these roles as well as testing translational outcomes in higher species.

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