ARTICLE

Journal of Cellular Biochemistry 108:1066–1075 (2009)

Journal of Cellular Biochemistry

A Cell-Based Dkk1 Binding Assay Reveals Roles for Extracellular Domains of LRP5 in Dkk1 Interaction and Highlights Differences Between Wild-Type and the High Bone Mass Mutant LRP5(G171V)

Richard J. Murrills,[1*] Jeanne J. Matteo,[1] Bheem M. Bhat,[1] Valerie E. Coleburn,[1] Kristina M. Allen,[2] Wei Chen,[2] Veronique Damagnez,[2] Ramesh A. Bhat,[1] Frederick J. Bex,[1] and Peter V.N. Bodine[1]

[1] *Department of Osteoporosis and Frailty, Women's Health and Musculoskeletal Biology, Wyeth Research, Collegeville, Pennsylvania 19426*

[2] *Human Genetics, Oscient Pharmaceuticals, Waltham, Massachusetts 02451*

ABSTRACT

Dkk1 is a secreted antagonist of the LRP5-mediated Wnt signaling pathway that plays a pivotal role in bone biology. Because there are no well-documented LRP5-based assays of Dkk1 binding, we developed a cell-based assay of Dkk1/LRP5 binding using radioactive ^{125}I-Dkk1. In contrast to LRP6, transfection of LRP5 alone into 293A cells resulted in a low level of specific binding that was unsuitable for routine assay. However, co-transfection of LRP5 with the chaperone protein MesD (which itself does not bind Dkk1) or Kremen-2 (a known Dkk1 receptor), or both, resulted in a marked enhancement of specific binding that was sufficient for evaluation of Dkk1 antagonists. LRP5 fragments comprising the third and fourth β-propellers plus the ligand binding domain, or the first β-propeller, each inhibited Dkk1 binding, with mean IC_{50}s of 10 and 196 nM, respectively. The extracellular domain of Kremen-2 ("soluble Kremen") was a weaker antagonist (mean IC_{50} 806 nM). We also found that cells transfected with a high bone mass mutation LRP5(G171V) had a subtly reduced level of Dkk1 binding, compared to wild type LRP5-transfected cells, and no enhancement of binding by MesD. We conclude that (1) LRP5-transfected cells do not offer a suitable cell-based Dkk1 binding assay, unless co-transfected with either MesD, Kremen-2, or both; (2) soluble fragments of LRP5 containing either the third and fourth β-propellers plus the ligand binding domain, or the first β-propeller, antagonize Dkk1 binding; and (3) a high bone mass mutant LRP5(G171V), has subtly reduced Dkk1 binding, and, in contrast to LRP5, no enhancement of binding with MesD. J. Cell. Biochem. 108: 1066–1075, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: LRP5; LRP6; MesD; KREMEN-2; HIGH BONE MASS; BINDING ASSAY

Dkk1 is a secreted 256 amino acid protein antagonist of the Wnt signaling pathway that is currently being investigated as a potential therapeutic target for cancer, neurodegenerative diseases and osteoporosis. Dkk1 is associated with the metastasis and pathophysiology of certain bone-associated cancers such as myeloma [Politou et al., 2006; Kaiser et al., 2008; Heider et al., 2009], osteosarcoma [Lee et al., 2007], prostate cancer [Hall and Keller, 2006] and breast cancer [Voorzanger-Rousselot et al., 2007] and has been proposed to be the principal mediator of neurodegeneration associated with cerebral ischemia and Alzheimer's disease [Zhang et al., 2008; Mastroiacovo et al., 2009]. Dkk1's importance in the skeleton is reflected in the observations that the Dkk1+/− mouse has increased bone mass [Morvan et al., 2006], a Dkk1 over-expressing transgenic mouse has osteopenia [Li et al., 2006], and an anti-Dkk1 antibody has been shown to increase bone mass in a preclinical model [Glantschnig et al., 2008]. Dkk1 antagonizes Wnt signaling by binding to receptors from two different families, namely LRP6 and LRP5, on the one hand [Bafico et al., 2001], and Kremen 1 and Kremen 2 on the other [Mao et al., 2002].

LRP5 and LRP6 are transmembrane proteins of the LDL receptor family that contain four extracellular β-propeller domains and a classical LDL ligand binding domain as well as a transmembrane domain and a short intracellular signaling domain [Brown et al.,

*Correspondence to: Dr. Richard J. Murrills, PhD, Women's Health & Musculoskeletal Biology, Wyeth Research, 500 Arcola Road, Collegeville, PA 19426. E-mail: murrills@aol.com

Received 31 July 2009; Accepted 3 August 2009 • DOI 10.1002/jcb.22335 • © 2009 Wiley-Liss, Inc.

Published online 10 September 2009 in Wiley InterScience (www.interscience.wiley.com).

1998; Hey et al., 1998; Springer, 1998; Jeon et al., 2001; Bhat et al., 2007]. They act as co-receptors for Wnts, with receptors of the Frizzled family, such that Frizzled proteins cannot activate the canonical Wnt pathway in the absence of LRP5 or LRP6 [Tamai et al., 2000; Wehrli et al., 2000]. The extracellular regions of LRP5 and LRP6 interact with the Wnt antagonists Dkk1 [Mao et al., 2001a] and sclerostin (SOST) [Ellies et al., 2006; Balemans et al., 2008], while the intracellular regions interact with the scaffolding protein Axin [Mao et al., 2001b] and the canonical Wnt pathway antagonist glycogen synthase kinase-β [Piao et al., 2008]. Wnt binding to a Frizzled receptor results in the formation of a complex that includes Wnt, Frizzled, and LRP5 or LRP6, and that leads to activation of the Wnt pathway; but the details of the intermolecular binding of this complex are not well understood [Cadigan and Liu, 2006].

Kremens (Kremen-1 and Kremen-2) are also transmembrane receptors for Dkk1 but do not associate with Frizzled receptors. Instead they antagonize Wnt signaling by binding to the opposite side [Wang et al., 2008] of Dkk1 that binds LRP5/6, thus forming a ternary complex with Dkk1 acting as a scaffold protein. In one model of Wnt antagonism, the ternary complex of Kremen/Dkk/LRP5/6 dissociates from its Frizzled co-receptor and is internalized, thereby terminating Wnt signaling [Mao et al., 2002]. In another model, that does not require Kremen binding, Dkk1 disrupts the Wnt-induced Frizzled/LRP5/6 complex [Semenov et al., 2001], and Dkk1 does not induce internalization of LRP6 [Semenov et al., 2008]. Furthermore, there appears to be additional complexity, as there is recent evidence that, in the absence of Dkk1, Kremens can act as LRP6 chaperones, promoting Wnt signaling through cell surface expression of the receptor [Hassler et al., 2007], rather than inhibiting it.

MesD is a 195 amino acid protein (in the mouse) that acts as a chaperone for LRP5 and LRP6, playing an important role in the conversion of low molecular weight, immature forms of LRP5/6 into higher molecular weight, mature protein [Hsieh et al., 2003], and also preventing LRP5/6 intermolecular disulfide-bonded aggregates [Hsieh et al., 2003]. Cell surface expression of LRP5/6 is essential for Dkk1 binding, and MesD has been shown to enhance cell surface expression of LRP5 [Hsieh et al., 2003]. The C-terminal region (residues 150–195) of MesD is necessary and sufficient for both LRP6 folding and LRP6 binding [Li et al., 2005]. A recently published solution structure suggests that a highly charged, polarized core region in MesD (residues 104–177) may be involved in protein-protein interaction [Kohler et al., 2006]. It should also be noted that MesD has been reported to potentially have dual, opposing, roles in LRP5/6 biology. In addition to functioning as a chaperone intracellularly, MesD can, if applied extracellularly, compete with Dkk1 for binding to LRP6 [Li et al., 2005].

Our group is interested in exploiting the central role that Wnt signaling plays in bone physiology [Westendorf et al., 2004; Levasseur et al., 2005; Krishnan et al., 2006; Yavropoulou and Yovos, 2007] for the development of an osteoporosis treatment. Wnt signaling is instrumental in osteoblast differentiation and survival, and is activated as part of the osteogenic response to parathyroid hormone [Murrills, 2006; Kousteni and Bilezikian, 2008], mechanical loading [Hens et al., 2005; Robinson et al., 2006] and BMP [Chen et al., 2007]. Additionally, Wnt/β-catenin signaling is implicated in the control of osteoclast differentiation through modulation of osteoclastogenic regulators OPG and RANKL [Glass et al., 2005; Holmen et al., 2005; Jackson et al., 2005]. In addition to the effects of Dkk1 knockout on bone mass, transgenic and knockout mice of other Wnt signaling proteins such as Axin2, LRP5, β-catenin, SFRP-1 and sclerostin have significant skeletal phenotypes [Babij et al., 2003; Bodine et al., 2004; Yu et al., 2005; Glass and Karsenty, 2006; Li et al., 2008], and mutations in LRP5 and sclerostin are each associated with significant high or low bone mass conditions in humans [Gong et al., 2001; Boyden et al., 2002; Little et al., 2002; Van Wesenbeeck et al., 2003]. Of particular interest to us, one specific mutation, the LRP5(G171V), located in the first β-propeller of the extracellular region of LRP5, was identified as the cause of high bone mass in a group of related patients in the United States [Little et al., 2002], and additional mutations have subsequently been identified in the first β-propeller of LRP5 that also result in high bone mass conditions [Van Wesenbeeck et al., 2003; Balemans et al., 2007]. The cellular mechanism whereby LRP5(G171V) induces high bone mass has been widely investigated. Transfection of the high bone mass mutant LRP5 alone does not activate the canonical Wnt signaling pathway [Boyden et al., 2002]; however, transfection of LRP5(G171V) and other high bone mass propeller 1 mutants can blunt the inhibitory effects of Dkk1 on canonical Wnt signaling [Boyden et al., 2002; Balemans et al., 2007; Bhat et al., 2007]. The most straightforward mechanism to explain the data would be for Dkk1 to bind LRP5(G171V) with lower affinity than native LRP5, thus resulting in reduced inhibition. However, deletion studies with LRP5 implicate β-propellers 3 and 4 as requirements for the binding of the closely related LRP6 to Dkk1 [Mao et al., 2001a], whereas the mutation for the high bone mass LRP5(G171V) is in the first β-propeller [Little et al., 2002]. An alternate mechanism for the reduction in Dkk1 sensitivity has been proposed. This involves a decreased interaction of LRP5(G171V) with the chaperone protein MesD that is responsible for trafficking LRP5 to the cell surface, resulting in less LRP5(G171V) reaching the cell surface and being available for Dkk1 inhibition [Zhang et al., 2004]. Supporting this idea, MesD has been reported to have a reduced interaction with both the high bone mass mutation LRP5 (G171V) [Zhang et al., 2004; Ai et al., 2005], which is located in the first β-propeller of LRP5, and also the LRP6 hypomorphic mutation R886W (Ringelschwanz) [Kubota et al., 2008], which is located in third YWTD β-propeller domain of LRP6. However, not all high bone mass mutants with mutations in the first β-propeller fail to bind MesD, reducing the likelihood that reduced MesD interaction is responsible for the high bone mass phenotype, and suggesting an effect of first β-propeller mutations on other aspects of LRP5 biology [Ai et al., 2005]. Following the discovery that sclerostin (SOST), which is related to the classic BMP antagonists Dan and Cerberus, is also a Dkk1 antagonist that interacts with the first β-propeller, it was shown that high bone mass mutants all exhibited reduced inhibition by SOST in functional assays, in addition to reduced inhibition by Dkk1 [Ellies et al., 2006; Balemans et al., 2008].

In order to enhance our efforts to discover a canonical Wnt pathway activator that would mimic the anabolic effects of the high bone mass LRP5 mutations, we wanted to develop a reliable Dkk1 cell-based binding assay based on LRP5, and in addition, wished to

learn more about the effects on Dkk1 binding of the high bone mass mutation LRP5(G171V). There have been several reports of labeled Dkk1 binding to cells expressing LRP6 [Bafico et al., 2001; Mao et al., 2001a; Brott and Sokol, 2002; Li et al., 2005; Binnerts et al., 2007; Wang et al., 2008], but only two reports [Bafico et al., 2001; Glantschnig et al., 2004], one of them preliminary [Glantschnig et al., 2004], that mention binding of a labeled Dkk1 to cells expressing LRP5, and none that describe antagonists of Dkk1 binding to LRP5. Given the observed differences between Dkk1 binding to LRP5 and LRP6 noted in the published literature [Bafico et al., 2001], we also wished to explore ways of enhancing the binding of Dkk1 to LRP5 in a cell-based assay format.

MATERIALS AND METHODS

CELLS, PLASMIDS, AND REAGENTS

293A cells. 293A cells were obtained from ATCC and maintained in growth medium DMEM (Invitrogen) containing 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 1% sodium pyruvate and 1% Glutamax.

DNA plasmids. The full-length cDNAs encoding human LRP5, the LRP5–G171V (HBM) mutant, LRP6, MesD and Kremen-2 were engineered into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA) with a consensus Kozak sequence immediately upstream of the initiating ATG.

Dkk1. C-terminally c-myc- and 6xHis-tagged human Dkk1 (NP_036374.1) was cloned into pDNA3.1 vector and transfected into HEK293T using Lipofectamine 2000/Opti-MEM (Invitrogen) and, following removal of the Lipofectamine after 4–6 h, cultured in Opti-MEM for 48 h. Conditioned media was collected and Dkk1 purified using a Ni column followed by graded imidazole elutions. Dkk1-containing fractions were identified on dot blots using HRP anti-myc antibody (Invitrogen cat # R951-25) and the ECL Plus Western Blot Detection System (Amersham cat # RPN2132). Fractions containing Dkk1 were then pooled and EDTA and Tween20 added to final concentrations of 0.5 mM and 0.1%. The pooled fractions were then concentrated by centrifugation at 3,000 rpm using a Centricon YM-30 cellulose membrane (Millipore), protein concentration and purity determined using the Bradford assay and SDS–PAGE and aliquots frozen with liquid nitrogen and stored at −80°C. Activity of the purified Dkk1 was confirmed by its ability to suppress a Wnt 3a-stimulated TCF-luciferase signal in U2OS cells. The purified hDkk1 ran at ~35 kDa on a Western, and its activity in the TCF assay was sensitive to tunicamycin treatment, consistent with it being glycosylated. The procedure is also described in detail in US Patent US20080038775A1. Additional hDkk1 (same construct) was obtained from Roche (Nutley, NJ), and purified from conditioned media of hDkk1-transfected HEK293 EBNA cells using a single step Ni IMAC chromatography. Aliquots were ^{125}I-labeled at GE Healthcare (Amersham) using the Chloramine-T method, freeze-dried in 50 μCi aliquots and stored at −20°C.

LRP5 prop 3,4-LBD. C-terminally V5- and 6xHis-tagged human LRP5 prop 3,4-LBD (Val642–Pro1376) was cloned into pDNA3.1 vector and co- transfected into HEK293T cells along with RAP in pCMVSport6 vector, using Lipofectamine/Opti-MEM (Invitrogen) and, following removal of the Lipofectamine after 4–6 h, cultured in Opti-MEM for 48 h. Conditioned media was collected and stored frozen. Thawed conditioned medium was adjusted to 0.5 M NaCl, 5 mM imidazole containing 1 mM PMSF and protease inhibitor cocktail (Roche Cat # 1873580). LRP5 prop 3,4-LBD was then purified using a Ni column and graded imidazole (up to 1,000 mM) elution and identified in the ensuing fractions on dot-blots using HRP anti-V5 antibody (Invitrogen cat # R961-25) and the ECL Plus Western Blot Detection System (Amersham cat # RPN2132). Fractions containing LRP5 prop 3,4-LBD were then pooled and concentrated by centrifugation at 3,000 rpm using a Centricon YM-30 cellulose membrane (Millipore), protein concentration and purity determined using the Bradford assay and SDS–PAGE and aliquots frozen with liquid nitrogen and stored at −80°C.

LRP5 prop 1. C-terminally 6xHis-tagged LRP5 prop 1 (Ser32-Glu341) was transiently transfected into 293 cells. Conditioned media was collected and purified by Ni column, using a loading buffer of 25 mM Tris pH 7.5 containing 0.5 M NaCl, 5% glycerol and 5 mM imidazole, followed by size exclusion chromatography (Superdex 200) at room temperature using 25 mM Tris pH 7.5 buffer containing 0.5 M NaCl and 5% glycerol.

Soluble Kremen. Monomeric recombinant human Kremen-2 extracellular domain ("soluble Kremen") was obtained from R&D Systems, Inc. (catalog #1946-KR). This is a C-terminally 10xHis-tagged construct of hKremen-2 19-364 with an N-terminal 16 amino-acid CD33 signal peptide that has been shown to bind to Dkk1 in an immobilized ELISA assay. The calculated molecular weight of 38K was used to determine molarity; however glycosylation may bring the actual molecular weight up to 59–63K (R&D Systems Product sheet).

Dkk1 binding assay. Dkk1 binding was assayed using a protocol modified from Bafico et al. [2001]. 293A cells were plated at a density of 40–70 K (48-well plates) or 150 K (24-well plates) in growth medium (DMEM containing 10% FBS, 1% sodium pyruvate, and 1% Glutamax) and incubated 24 h without antibiotics until 70–80% confluent, before being transfected with appropriate DNA plasmids using Lipofectamine 2000 (Invitrogen) or FuGENE 6 (Roche Applied Science), according to the manufacturers' instructions. In addition to the construct(s) of interest, cells were also co-transfected, as appropriate, with pcDNA to ensure equal amounts of transfected DNA in each treatment, including control wells.

Following transfection, cells were incubated 24 h to permit protein expression, after which they were washed 2× with serum-free growth medium containing 25 mM HEPES pH 7.5, 1 μg/ml heparin and 1% BSA (assay buffer). Cells were then exposed to 0.4 nM ^{125}I-Dkk1 in assay buffer for 3 h at room temperature, after which they were washed 2× with assay buffer and lysed by adding 0.5% SDS in PBS. The lysate was then transferred to 12 mm tubes and radioactivity assayed using a γ-counter. Non-specific binding was estimated by blocking binding of the radiolabeled Dkk1 with an excess (40–400 nM) of cold Dkk1. Specific binding was calculated by subtracting non-specific binding from the total counts.

STATISTICS

Specific counts were analyzed for statistical significance using ANOVA, with or without log transformation as appro-

priate, followed by tests of least significant difference (SAS Excel).

RESULTS

BINDING OF ^{125}I-DKK1 TO LRP5- AND LRP6-TRANSFECTED CELLS

In contrast to LRP6, which resulted in robust binding of ^{125}I-Dkk1, LRP5 transfection of 293A cells resulted in only modest increases in specific ^{125}I-Dkk1 binding to 293A cells (Fig. 1). The amount of ligand bound specifically to LRP5-transfected cells was generally well below the 70% preferred for a binding assay (Fig. 1, Table I).

ENHANCEMENT OF DKK1 BINDING TO LRP5 BY MESD AND KREMEN-2

We were able to increase the level of ^{125}I-Dkk1 binding to LRP5-transfected cells by co-transfecting either Kremen-2 or MesD, or both (Fig. 2). MesD, when transfected alone into 293A cells, resulted in no increase in Dkk1 binding, while transfection of Kremen-2 alone (750–1,500 ng/well) increased specific ^{125}I-Dkk1 binding to 293A cells consistent with its known ability to bind Dkk1 (Fig. 2B, Table I). Cells co-transfected with LRP5 together with MesD, or Kremen-2, bound ^{125}I-Dkk1 robustly (Fig. 2A,B, Table I). A combination of LRP5, Kremen-2 and MesD resulted in even greater binding of ^{125}I-Dkk1 (Fig. 2B). The level of ligand bound specifically was >70% only when LRP5 was co-transfected with Kremen-2, MesD or both (Table I).

BLOCKING OF DKK1 BINDING

As expected, cold Dkk1 was able to reproducibly displace ^{125}I-Dkk1 from cells transfected with LRP5, with mean IC_{50}s of 0.124 nM (LRP5 + Kremen transfection) (Fig. 3A) and 0.437 nM (LRP5 + MesD transfection), confirming the specific nature of ^{125}I-Dkk1 binding. A fragment of LRP5 containing propellers 3 and 4 and the " ligand binding domain," "LRP5 prop 3,4-LBD," was a potent antagonist of ^{125}I-Dkk1 binding to LRP5 under various co-transfection conditions (mean $IC_{50} = 0.010\ \mu M$; Fig. 3B). An LRP5 fragment containing just propeller 1 was an approximately 20-fold weaker inhibitor of Dkk1 binding (mean $IC_{50} = 0.196\ \mu M$; Fig. 3C). Finally, the weakest of the antagonists tested was a soluble Kremen-2 construct, which had a mean an IC_{50} of 0.806 μM (Fig. 3D).

THE LRP5(G171V) HBM MUTATION

Transfection of the LRP5(G171V) "high bone mass" mutant resulted in low levels of ^{125}I-Dkk1 binding (Fig. 4), that were generally lower than that observed with wild type LRP5 (median ratio of HBM:LRP5 specific counts was 0.589:1 at 375 ng/well dose; 0.574:1 at 750 ng/well dose, with 8 of 20 direct comparisons being significantly lower than LRP5). Co-transfection of LRP5(G171V) with Kremen-2 resulted in enhanced binding of ^{125}I-Dkk1, similar to LRP5. However, analysis of all direct comparisons, including those without a pcDNA comparator, revealed that the combination of

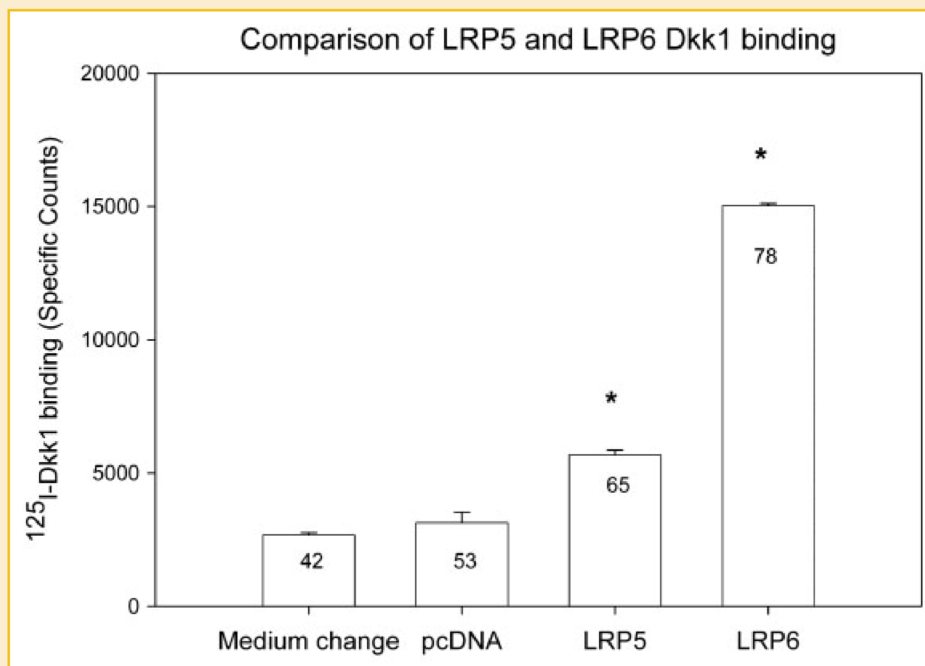

Fig. 1. Comparison of ^{125}I-Dkk1 binding to 293A cells transfected with LRP5 or LRP6. 293A cells were plated at 150 K cells/well in 24-well plates and transfected with 1.2 μg DNA/well using Lipofectamine 2000. Labels inside bars indicate the % of radioactive label that was specifically bound, that is (specific counts/total counts) × 100. Significantly different from pcDNA transfection $^{*}P < 0.0001$. Values are means ± SEM, three replicates per treatment.

TABLE I. Effect of Various Transfectants on the Amount of Specific ^{125}I-Dkk1 Binding to 293A Cells

Transfectant	Dose (ng/well)	Fold increase over pcDNA (specific counts)					% counts specifically bound				
		Mean	Min	Max	Median	Sig/n	Mean	Min	Max	Median	n
LRP5	375	2.01	1.09	2.83	2.09	6/12	60	38	86	61	12
”	750	2.43	1.32	3.56	2.81	3/5	57	35	76	57	5
”	1,125	1.96	1.57	2.54	1.77	2/3	52	48	59	50	3
LRP6	375	3.35	1.50	5.21	3.33	3/3	80	78	83	80	3
MesD	750	0.80	0.53	0.98	0.83	0/5	31	16	57	32	5
LRP5 + MesD	375 + 750	4.17	2.61	6.30	3.97	8/8	73	64	84	72	8
Kremen-2	750	3.20	2.87	3.58	3.16	4/4	64	61	67	64	4
LRP5 + Kremen-2	375 + 750	9.69	5.88	15.03	8.93	4/4	81	74	87	81	4
LRP5 + MesD + Kremen-2	375 + 375 + 375	10.45	9.39	11.51	10.45	2/2	84	81	87	84	2
LRP5(G171V)	375	1.49	0.98	2.02	1.48	1/4	44	35	57	43	4
”	750	2.54	1.63	3.69	2.42	3/4	38	23	48	40	4
LRP5(G171V) + MesD	375 + 750	1.71	0.96	2.67	1.50	1/3	45	38	56	41	3
LRP5(G171V) + Kremen-2	375 + 750	10.89	6.13	15.65	10.89	2/2	84	82	85	84	2

Data are expressed as fold increase over pcDNA transfection alone, and also as the % of the total radioactive counts that are bound specifically, that is, cannot be displaced by excess cold Dkk1. Statistical significance, relative to pcDNA alone, was determined using ANOVA and the method of least significant difference. Experiments in which the pcDNA alone level was below 200 were omitted, as these introduced much higher apparent fold increases.

LRP5(G171V) with Kremen-2 also trended lower than that of LRP5 with Kremen-2 (median ratio of HBM + Kremen:LRP5 + Kremen specific counts was 0.872:1 at 750 ng/well dose n = 6, and 0.778:1 at 1,500 ng/well dose n = 2, with five of eight comparisons being significantly lower). In sharp contrast to LRP5, however, LRP5(G171V) showed no enhancement of Dkk1 binding when co-transfected with MesD (Fig. 4).

DISCUSSION

In this study, LRP5 transfection into 293A cells generally resulted in low levels of Dkk1 binding. This observation is consistent with several other workers' experiences with LRP5-transfected cells. Bafico et al. [2001] noted that LRP5-transfected NIH3T3 cells had lower levels of Dkk1 binding than LRP6-transfected cells; Mao et al. [2001a] did not detect any binding of Dkk1-AP to LRP5; and Glantschnig et al. [2004] could not demonstrate binding of fluorescently labeled Dkk1 to LRP5 in transiently transfected cells, only a stable cell line [Glantschnig et al., 2004]. The presence of immature or aggregated protein may be responsible for the low binding of LRP5-transfected cells, as there is evidence in the literature for LRP5 transfected cells showing two bands of LRP5, one mature and one immature [Li et al., 2005].

Accordingly, we co-transfected LRP5 with the chaperone protein MesD and found that this greatly enhanced binding of Dkk1 to LRP5-transfected cells, presumably through improved protein maturation and/or transport to the cell surface. Transfection of Kremen-2 alone, itself a known Dkk1 binder, resulted in a detectable increase in ^{125}I-Dkk1 binding and when Kremen-2 was co-transfected with LRP5, this also enhanced binding over LRP5 alone to an extent that seemed to be at least additive, and frequently more than additive. It should be noted that the ternary model of Dkk1 binding to LRP5 and Kremen-2, where one molecule of Dkk1 binds to one molecule each of Kremen-2 and LRP5, would predict that the introduction of Kremen-2 to cells already expressing LRP5 would result in formation of ternary complexes containing Kremen-2, Dkk1 and LRP5, which would in turn result in no net increase in the amount of Dkk1 bound. Our observations are therefore not explained by this model, and suggest that Kremen is either not forming a ternary complex with Dkk1 and LRP5 in our system or is also playing other roles such as acting as a chaperone for LRP5, or somehow enhancing the binding affinity of LRP5 for Dkk1. It is interesting that a recent publication [Hassler et al., 2007], in addition to a preliminary report [Glantschnig et al., 2004], have described the formation of an intracellular complex of Kremen and LRP5/6 that is independent of Dkk1, which may represent a chaperone-like function of Kremen [Hassler et al., 2007].

Our studies with potential antagonists of Dkk1 binding both support and extend the published information on Dkk1 binding to LRP5/6. Although there are no published antagonist studies in the literature, our observation that a soluble LRP5 construct comprising the third and fourth β-propellers (and the LBD) was a potent inhibitor of Dkk1 binding is consistent with the deletion studies conducted on LRP6 by Mao et al. [2001a], which highlighted these regions as important for binding of Dkk1, and with the finding that an internally truncated LRP5 mutant found in hyperparathyroid tumors and breast cancers that lacks propeller 3 was not inhibited by Dkk1 [Bjorklund et al., 2007, 2009]. However, it is interesting that we also detected Dkk1 antagonist activity, albeit somewhat weaker, of an LRP5 construct containing the first β-propeller only. This suggests that the first β-propeller of LRP5 may also bind to Dkk1, which may provide a rational explanation for the reduced functional Dkk1 inhibition observed in high bone mass mutants bearing mutations in the first β-propeller [Bhat et al., 2007]. A soluble Kremen receptor was a very weak antagonist of Dkk1 binding. While Kremen has been shown to bind Dkk1 with high, sub-nanomolar affinity [Mao et al., 2002], current evidence suggests that Kremen and LRP5 bind to opposite sides of the Dkk1 molecule [Wang et al., 2008]. According to this model, a Dkk1 molecule that is bound by soluble Kremen would still be available for binding to LRP5, and hence soluble Kremen would induce little inhibition of Dkk1/LRP5 binding.

In contrast to wild-type LRP5, Dkk1 binding to the HBM mutant LRP5(G171V) was not enhanced by co-transfection with MesD, although it was with Kremen-2. The LRP5(G171V) mutant has

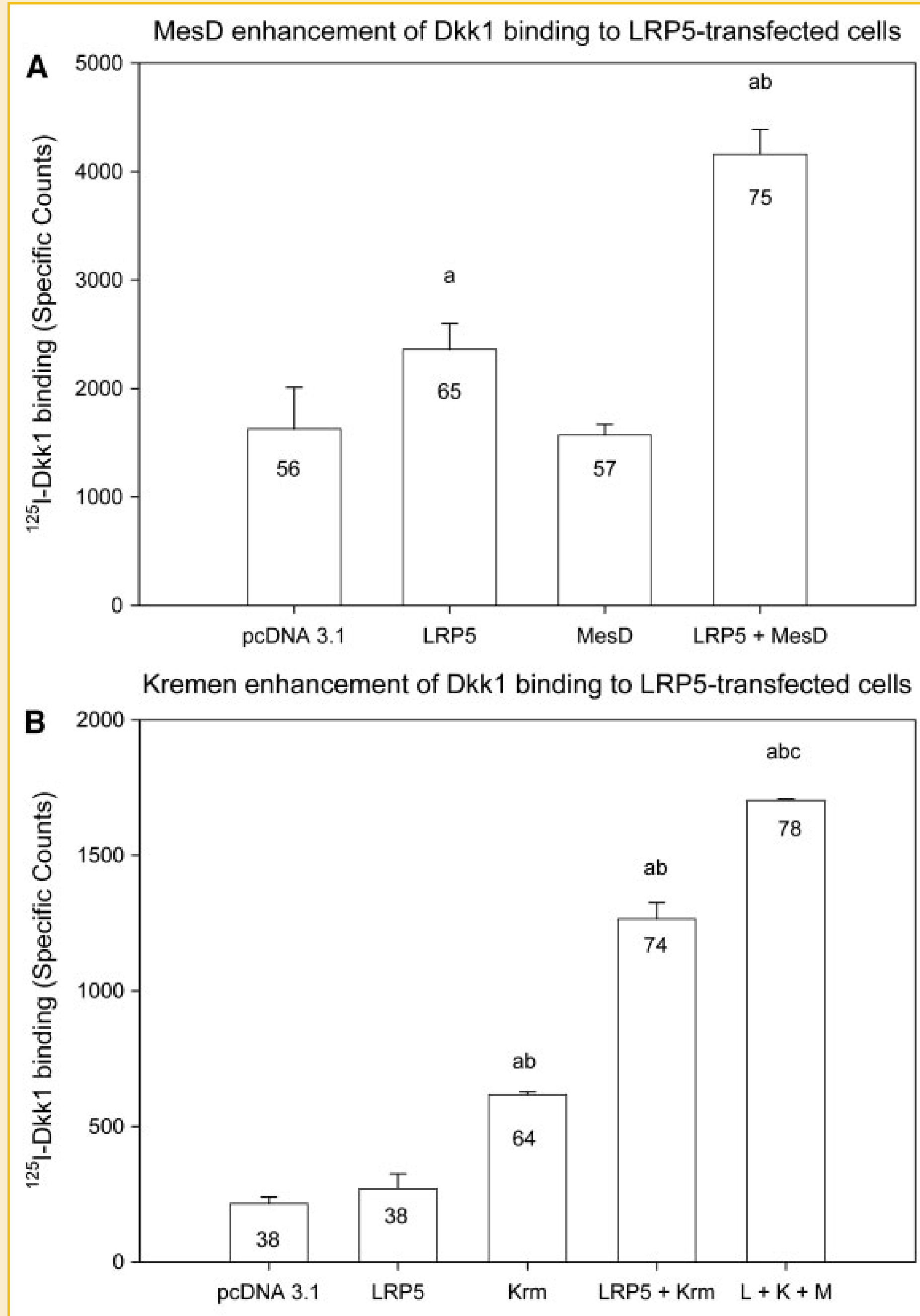

Fig. 2. Enhancement of Dkk1 binding to LRP5-transfected cells by co-transfection of MesD or Kremen-2. A: Enhancement by MesD: 293A cells were plated at 70 K per well in a 48-well plate and transfected 24 h later with a total of 1.125 μg of DNA/well for 4 h using Lipofectamine 2000, and assayed for ^{125}I-Dkk1 binding the next day. Actual doses of DNA per well for each treatment: pcDNA 3.1 = 1.125 μg; LRP5 = 375 ng LRP5 + 750 ng pcDNA; MesD = 750 ng MesD + 350 ng pcDNA; LRP5 + MesD = 375 ng LRP5 + 750 ng MesD. Significantly greater than, at $P < 0.05$, a = pcDNA control, b = LRP5. Values are means ± SEM, three replicates per treatment. (B). Enhancement by Kremen-2: 293A cells were plated at 40 K per well in a 48-well plate and transfected 24 h later with 1.125 μg DNA/well with FuGene 6, and assayed for ^{125}I-Dkk1 binding the next day. Actual doses of DNA per well for each treatment: pcDNA 3.1 = 1.125 μg; LRP5 = 375 ng LRP5 + 750 ng pcDNA; Kremen-2 = 750 ng Kremen-2 + 350 ng pcDNA; LRP5 + Kremen-2 = 375 ng LRP5 + 750 ng Kremen-2; LRP5 + Kremen-2 + MesD = 375 ng LRP5 + 375 ng Kremen-2 + 375 ng MesD. Significantly greater than, at $P < 0.05$: a = pcDNA; b = LRP5; c = LRP5 + Kremen-2. Labels inside bars indicate the % of radioactive label that was specifically bound, that is (specific counts/total counts) × 100. Values are means ± SEM, two replicates per treatment.

previously been noted to have a lower interaction with MesD in immunoprecipitation studies conducted by both Zhang et al. [2004] and by Ai et al. [2005], and our studies are consistent with this data. The significance of this aspect of HBM mutant biology is however controversial. Zhang's group suggested that reduced interaction of LRP5(G171V) with the chaperone MesD may limit the expression of LRP5(G171V) on the cell surface and hence render the cell less "inhibitable" by Dkk1, resulting in enhanced Wnt signaling.

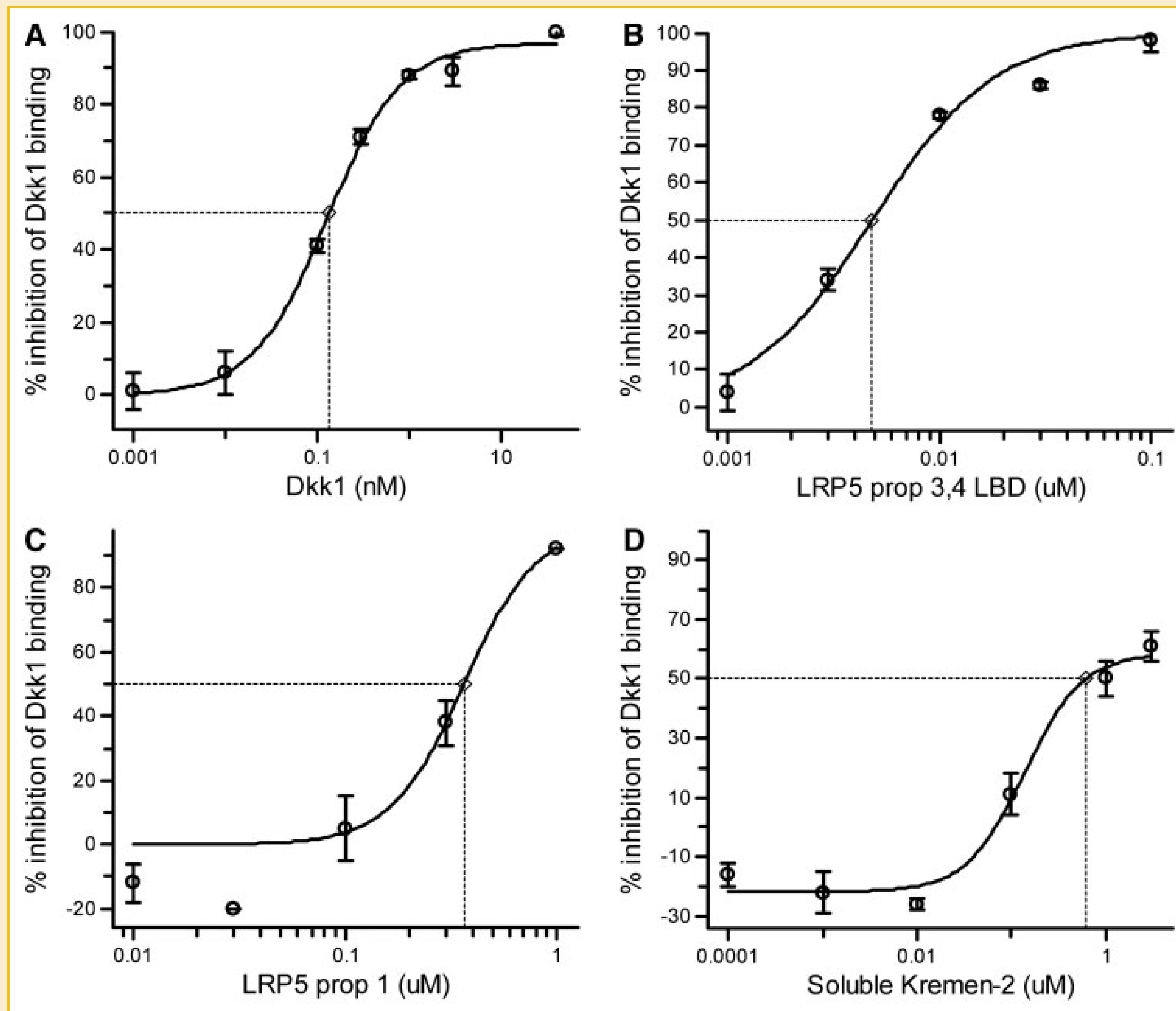

Fig. 3. Dose–response curves of Dkk1 antagonists in the ^{125}I-Dkk1 radioactive whole cell binding assay. A: Cold Dkk1 on LRP5/Kremen-2 transfected cells (375 ng/well LRP5, 750 ng/well Kremen-2). The IC_{50} in this experiment was 0.134 nM (confidence limits 0.096–0.186 nM) B: LRP5 prop 3,4-LBD on LRP5/Kremen transfected cells. The IC_{50} in this experiment was 4.8 nM; LRP5 prop 3,4-LBD was also tested using other transfectant conditions (LRP5/MesD, LRP5/MesD/Kremen) with similar results. The median IC_{50} from all the LRP5 prop 3,4-LBD experiments was 5.9 nM ($n = 6$). C: LRP5 prop 1 on LRP5/MesD/Kremen-2 transfected cells. The IC_{50} in this experiment was 322 nM; LRP5 prop 1 was also tested using other transfectant conditions (LRP5/Kremen, LRP5/MesD) with similar results. The median IC_{50} from all LRP5 prop 1 determinations was 166 nM ($n = 4$). D: Soluble Kremen-2 on LRP5/MesD transfected cells. The IC_{50} in this experiment was 845 nM, which was also the median IC_{50} of three determinations. Values are means ± SEM, two replicates per dose.

However, Ai's group argued that, because there are other prop 1 HBM mutants that do not appear to have a reduced interaction with MesD but nonetheless were functionally less "inhibitable" by Dkk1, that interaction with MesD was irrelevant to Dkk1 inhibition. Recent data suggests that the HBM mutants of LRP5 have enhanced Wnt signaling not only through reduced Dkk1 inhibition but also through reduced sclerostin inhibition [Ellies et al., 2006; Balemans et al., 2008]. Hence, the significance of the reduced interaction of the HBM mutant LRP5(G171V) with MesD, although reproducible, requires further research.

In conclusion, we have shown that transfection of LRP5 into 293A cells, in contrast to the robust effects of LRP6, results in only a modest increase in Dkk1 binding that requires enhancement by co-transfection with either MesD, Kremen-2 or both MesD and Kremen-2 to achieve a level of specific binding suitable for assay. Testing of soluble fragments of Dkk1 receptors in this assay reveals that prop 3,4 LRP5 (LBD) is a potent inhibitor of Dkk1 binding, whereas prop 1 LRP5, and soluble Kremen-2 are weaker antagonists, highlighting the primary role that prop 3,4 has in the binding of Dkk1 to LRP5. The high bone mass mutation LRP5(G171V) trended toward a lower level of Dkk1 binding compared with LRP5, but its most dramatic difference from LRP5 was its failure to be enhanced by co-transfection with MesD. This cell-based radioactive Dkk1 binding assay, incorporating co-transfection of MesD and/or Kremen-2, is useful for the evaluation of Dkk1 antagonists and highlights both the role of

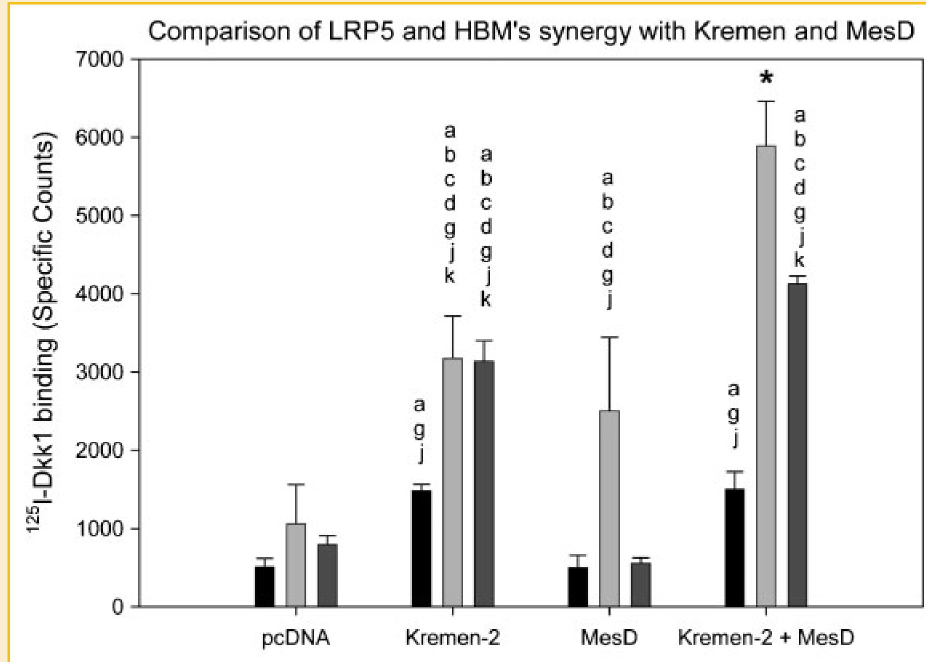

Fig. 4. Comparison of the effect of MesD or Kremen-2 co-transfection on ^{125}I-Dkk1 binding to cells transfected with LRP5 or the HBM mutant LRP5(G171V) (HBM). Cells were plated at 40 K per well in 48-well plates and transfected using FuGene 6 with a total of 1.125 μg DNA as follows: LRP5 or HBM—375 ng/well; MesD or Kremen-2—750 ng/well; MesD in combination with Kremen-2—375 ng/well each; pcDNA added as appropriate to make up to total DNA of 1.125 ng/well. Significantly greater than, at $P < 0.05$ level, a: pcDNA, b: LRP5 alone, c: HBM alone, d: Kremen-2 alone, e: Kremen-2 + LRP5, f: Kremen-2 + HBM, g: MesD alone, h: MesD + LRP5, j: MesD + HBM, k: Kremen-2 + MesD, *from all treatments except Kremen-2 + LRP5, Kremen-2 + HBM and MesD + Kremen-2 + HBM. Values are means ± SEM, two replicates per treatment. Key: black bars: transfectant(s) alone; light gray bars: transfectant(s) + LRP5; dark gray bars: transfectant(s) + HBM.

prop 3,4 LRP5 in Dkk1 binding and some differences in the biology of a high bone mass mutation of LRP5.

ACKNOWLEDGMENTS

We are grateful to Dr. Tony Anisowicz (Oscient Pharmaceuticals) for his key role in the cloning, expression, and purification of Dkk1 and LRP5 (prop 3,4) LBD.

REFERENCES

Ai M, Holmen SL, Van Hul W, Williams BO, Warman ML. 2005. Reduced affinity to and inhibition by DKK1 form a common mechanism by which high bone mass-associated missense mutations in LRP5 affect canonical Wnt signaling. Mol Cell Biol 25(12):4946–4955.

Babij P, Zhao W, Small C, Kharode Y, Yaworsky PJ, Bouxsein ML, Reddy PS, Bodine PV, Robinson JA, Bhat B, Marzolf J, Moran RA, Bex F. 2003. High bone mass in mice expressing a mutant LRP5 gene. J Bone Miner Res 18(6):960–974.

Bafico A, Liu G, Yaniv A, Gazit A, Aaronson SA. 2001. Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. Nat Cell Biol 3(7):683–686.

Balemans W, Devogelaer JP, Cleiren E, Piters E, Caussin E, Van Hul W. 2007. Novel LRP5 missense mutation in a patient with a high bone mass phenotype results in decreased DKK1-mediated inhibition of Wnt signaling. J Bone Miner Res 22(5):708–716.

Balemans W, Piters E, Cleiren E, Ai M, Van Wesenbeeck L, Warman ML, Van Hul W. 2008. The binding between sclerostin and LRP5 is altered by DKK1 and by high-bone mass LRP5 mutations. Calcif Tissue Int 82(6):445–453.

Bhat BM, Allen KM, Liu W, Graham J, Morales A, Anisowicz A, Lam HS, McCauley C, Coleburn V, Cain M, Fortier E, Bhat RA, Bex FJ, Yaworsky PJ. 2007. Structure-based mutation analysis shows the importance of LRP5 beta-propeller 1 in modulating Dkk1-mediated inhibition of Wnt signaling. Gene 391(1–2):103–112.

Binnerts ME, Kim KA, Bright JM, Patel SM, Tran K, Zhou M, Leung JM, Liu Y, Lomas WE 3rd, Dixon M, Hazell SA, Wagle M, Nie WS, Tomasevic N, Williams J, Zhan X, Levy MD, Funk WD, Abo A. 2007. R-Spondin1 regulates Wnt signaling by inhibiting internalization of LRP6. Proc Natl Acad Sci USA 104(37):14700–14705.

Bjorklund P, Akerstrom G, Westin G. 2007. An LRP5 receptor with internal deletion in hyperparathyroid tumors with implications for deregulated WNT/beta-catenin signaling. PLoS Med 4(11):e328.

Bjorklund P, Svedlund J, Olsson AK, Akerstrom G, Westin G. 2009. The internally truncated LRP5 receptor presents a therapeutic target in breast cancer. PLoS ONE 4(1):e4243.

Bodine PV, Zhao W, Kharode YP, Bex FJ, Lambert AJ, Goad MB, Gaur T, Stein GS, Lian JB, Komm BS. 2004. The Wnt antagonist secreted frizzled-related protein-1 is a negative regulator of trabecular bone formation in adult mice. Mol Endocrinol 18(5):1222–1237.

Boyden LM, Mao J, Belsky J, Mitzner L, Farhi A, Mitnick MA, Wu D, Insogna K, Lifton RP. 2002. High bone density due to a mutation in LDL-receptor-related protein 5. N Engl J Med 346(20):1513–1521.

Brott BK, Sokol SY. 2002. Regulation of Wnt/LRP signaling by distinct domains of Dickkopf proteins. Mol Cell Biol 22(17):6100–6110.

Brown SD, Twells RC, Hey PJ, Cox RD, Levy ER, Soderman AR, Metzker ML, Caskey CT, Todd JA, Hess JF. 1998. Isolation and characterization of LRP6, a

novel member of the low density lipoprotein receptor gene family. Biochem Biophys Res Commun 248(3):879–888.

Cadigan KM, Liu YI. 2006. Wnt signaling: Complexity at the surface. J Cell Sci 119(Pt 3):395–402.

Chen Y, Whetstone HC, Youn A, Nadesan P, Chow EC, Lin AC, Alman BA. 2007. Beta-catenin signaling pathway is crucial for bone morphogenetic protein 2 to induce new bone formation. J Biol Chem 282(1):526–533.

Ellies DL, Viviano B, McCarthy J, Rey JP, Itasaki N, Saunders S, Krumlauf R. 2006. Bone density ligand, Sclerostin, directly interacts with LRP5 but not LRP5G171V to modulate Wnt activity. J Bone Miner Res 21(11):1738–1749.

Glantschnig H, Zuck PD, Kasparcova V, Zhang H, Hess FJ, Freedman LP, Harada S. 2004. Characterization of protein-protein interactions of LRP5/6 with Dkk-, Sclerostin- and Kremen-proteins. J Bone Miner Res 19(Suppl 1): S80.

Glantschnig H, Hampton R, Wei N, Scott K, Nantermet P, Zhao J, Chen F, Fisher J, Su Q, Pennypacker B, Cusick T, Sandhu P, Reszka A, Strohl W, Flores O, Wang F, Kimmel D, An Z. 2008. Fully human anti-Dkk1 antibodies increase bone formation and resolve osteopenia in mouse models of estrogen-deficiency induced bone loss. J Bone Miner Res 23(Suppl 1):S60–S61.

Glass DA 2nd, Karsenty G. 2006. Canonical Wnt signaling in osteoblasts is required for osteoclast differentiation. Ann NY Acad Sci 1068:117–130.

Glass DA II, Bialek P, Ahn J, Starbuck M, Patel M, Clevers H, Take M, Long F, McMahon A, Lang R. 2005. Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. Dev Cell 8(5):751–764.

Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, Wang H, Cundy T, Glorieux FH, Lev D, Zacharin M, Oexle K, Marcelino J, Suwairi W, Heeger S, Sabatakos G, Apte S, Adkins WN, Allgrove J, Arslan-Kirchner M, Batch JA, Beighton P, Black GC, Boles RG, Boon LM, Borrone C, Brunner HG, Carle GF, Dallapiccola B, De Paepe A, Floege B, Halfhide ML, Hall B, Hennekam RC, Hirose T, Jans A, Juppner H, Kim CA, Keppler-Noreuil K, Kohlschuetter A, LaCombe D, Lambert M, Lemyre E, Letteboer T, Peltonen L, Ramesar RS, Romanengo M, Somer H, Steichen-Gersdorf E, Steinmann B, Sullivan B, Superti-Furga A, Swoboda W, van den Boogaard MJ, Van Hul W, Vikkula M, Votruba M, Zabel B, Garcia T, Baron R, Olsen BR, Warman ML. 2001. LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. Cell 107(4):513–523.

Hall CL, Keller ET. 2006. The role of Wnts in bone metastases. Cancer Metastasis Rev 25(4):551–558.

Hassler C, Cruciat CM, Huang YL, Kuriyama S, Mayor R, Niehrs C. 2007. Kremen is required for neural crest induction in Xenopus and promotes LRP6-mediated Wnt signaling. Development 134(23):4255–4263.

Heider U, Kaiser M, Mieth M, Lamottke B, Rademacher J, Jakob C, Braendle E, Stover D, Sezer O. 2009. Serum concentrations of DKK-1 decrease in patients with multiple myeloma responding to anti-myeloma treatment. Eur J Haematol 82(1):31–38.

Hens JR, Wilson KM, Dann P, Chen X, Horowitz MC, Wysolmerski JJ. 2005. TOPGAL mice show that the canonical Wnt signaling pathway is active during bone development and growth and is activated by mechanical loading in vitro. J Bone Miner Res 20(7):1103–1113.

Hey PJ, Twells RC, Phillips MS, Yusuke N, Brown SD, Kawaguchi Y, Cox R, Guochun X, Dugan V, Hammond H, Metzker ML, Todd JA, Hess JF. 1998. Cloning of a novel member of the low-density lipoprotein receptor family. Gene 216(1):103–111.

Holmen SL, Zylstra CR, Mukherjee A, Sigler RE, Faugere MC, Bouxsein ML, Deng L, Clemens TL, Williams BO. 2005. Essential role of beta-catenin in postnatal bone acquisition. J Biol Chem 280(22):21162–21168.

Hsieh JC, Lee L, Zhang L, Wefer S, Brown K, DeRossi C, Wines ME, Rosenquist T, Holdener BC. 2003. Mesd encodes an LRP5/6 chaperone essential for specification of mouse embryonic polarity. Cell 112(3):355–367.

Jackson A, Vayssiere B, Garcia T, Newell W, Baron R, Roman-Roman S, Rawadi G. 2005. Gene array analysis of Wnt-regulated genes in C3H10T1/2 cells. Bone 36(4):585–598.

Jeon H, Meng W, Takagi J, Eck MJ, Springer TA, Blacklow SC. 2001. Implications for familial hypercholesterolemia from the structure of the LDL receptor YWTD-EGF domain pair. Nat Struct Biol 8(6):499–504.

Kaiser M, Mieth M, Liebisch P, Oberlander R, Rademacher J, Jakob C, Kleeberg L, Fleissner C, Braendle E, Peters M, Stover D, Sezer O, Heider U. 2008. Serum concentrations of DKK-1 correlate with the extent of bone disease in patients with multiple myeloma. Eur J Haematol 80(6):490–494.

Kohler C, Andersen OM, Diehl A, Krause G, Schmieder P, Oschkinat H. 2006. The solution structure of the core of mesoderm development (MESD), a chaperone for members of the LDLR-family. J Struct Funct Genomics 7(3–4):131–138.

Kousteni S, Bilezikian JP. 2008. The cell biology of parathyroid hormone in osteoblasts. Curr Osteoporos Rep 6(2):72–76.

Krishnan V, Bryant HU, Macdougald OA. 2006. Regulation of bone mass by Wnt signaling. J Clin Invest 116(5):1202–1209.

Kubota T, Michigami T, Sakaguchi N, Kokubu C, Suzuki A, Namba N, Sakai N, Nakajima S, Imai K, Ozono K. 2008. Lrp6 hypomorphic mutation affects bone mass through bone resorption in mice and impairs interaction with Mesd. J Bone Miner Res 23(10):1661–1671.

Lee N, Smolarz AJ, Olson S, David O, Reiser J, Kutner R, Daw NC, Prockop DJ, Horwitz EM, Gregory CA. 2007. A potential role for Dkk-1 in the pathogenesis of osteosarcoma predicts novel diagnostic and treatment strategies. Br J Cancer 97(11):1552–1559.

Levasseur R, Lacombe D, de Vernejoul MC. 2005. LRP5 mutations in osteoporosis-pseudoglioma syndrome and high-bone-mass disorders. Joint Bone Spine 72(3):207–214.

Li Y, Chen J, Lu W, McCormick LM, Wang J, Bu G. 2005. Mesd binds to mature LDL-receptor-related protein-6 and antagonizes ligand binding. J Cell Sci 118(Pt 22):5305–5314.

Li J, Sarosi I, Cattley RC, Pretorius J, Asuncion F, Grisanti M, Morony S, Adamu S, Geng Z, Qiu W, Kostenuik P, Lacey DL, Simonet WS, Bolon B, Qian X, Shalhoub V, Ominsky MS, Zhu Ke H, Li X, Richards WG. 2006. Dkk1-mediated inhibition of Wnt signaling in bone results in osteopenia. Bone 39(4):754–766.

Li X, Ominsky MS, Niu QT, Sun N, Daugherty B, D'Agostin D, Kurahara C, Gao Y, Cao J, Gong J, Asuncion F, Barrero M, Warmington K, Dwyer D, Stolina M, Morony S, Sarosi I, Kostenuik PJ, Lacey DL, Simonet WS, Ke HZ, Paszty C. 2008. Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone strength. J Bone Miner Res 23(6):860–869.

Little RD, Carulli JP, Del Mastro RG, Dupuis J, Osborne M, Folz C, Manning SP, Swain PM, Zhao SC, Eustace B, Lappe MM, Spitzer L, Zweier S, Braunschweiger K, Benchekroun Y, Hu X, Adair R, Chee L, FitzGerald MG, Tulig C, Caruso A, Tzellas N, Bawa A, Franklin B, McGuire S, Nogues X, Gong G, Allen KM, Anisowicz A, Morales AJ, Lomedico PT, Recker SM, Van Eerdewegh P, Recker RR, Johnson ML. 2002. A mutation in the LDL receptor-related protein 5 gene results in the autosomal dominant high-bone-mass trait. Am J Hum Genet 70(1):11–19.

Mao B, Wu W, Li Y, Hoppe D, Stannek P, Glinka A, Niehrs C. 2001a. LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. Nature 411(6835):321–325.

Mao J, Wang J, Liu B, Pan W, Farr GH 3rd, Flynn C, Yuan H, Takada S, Kimelman D, Li L, Wu D. 2001b. Low-density lipoprotein receptor-related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway. Mol Cell 7(4):801–809.

Mao B, Wu W, Davidson G, Marhold J, Li M, Mechler BM, Delius H, Hoppe D, Stannek P, Walter C, Glinka A, Niehrs C. 2002. Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling. Nature 417(6889):664–667.

Mastroiacovo F, Busceti CL, Biagioni F, Moyanova SG, Meisler MH, Battaglia G, Caricasole A, Bruno V, Nicoletti F. 2009. Induction of the Wnt antagonist,

Dickkopf-1, contributes to the development of neuronal death in models of brain focal ischemia. J Cereb Blood Flow Metab 29(2):264–276.

Morvan F, Boulukos K, Clement-Lacroix P, Roman Roman S, Suc-Royer I, Vayssiere B, Ammann P, Martin P, Pinho S, Pognonec P, Mollat P, Niehrs C, Baron R, Rawadi G. 2006. Deletion of a single allele of the Dkk1 gene leads to an increase in bone formation and bone mass. J Bone Miner Res 21(6):934–945.

Murrills RJ. 2006. Parathyroid hormone and bone cells. Clin Rev Bone Miner Metab 4(4):233–258.

Piao S, Lee SH, Kim H, Yum S, Stamos JL, Xu Y, Lee SJ, Lee J, Oh S, Han JK, Park BJ, Weis WI, Ha NC. 2008. Direct inhibition of GSK3beta by the phosphorylated cytoplasmic domain of LRP6 in Wnt/beta-catenin signaling. PLoS ONE 3(12):e4046.

Politou MC, Heath DJ, Rahemtulla A, Szydlo R, Anagnostopoulos A, Dimopoulos MA, Croucher PI, Terpos E. 2006. Serum concentrations of Dickkopf-1 protein are increased in patients with multiple myeloma and reduced after autologous stem cell transplantation. Int J Cancer 119(7):1728–1731.

Robinson JA, Chatterjee-Kishore M, Yaworsky PJ, Cullen DM, Zhao W, Li C, Kharode Y, Sauter L, Babij P, Brown EL, Hill AA, Akhter MP, Johnson ML, Recker RR, Komm BS, Bex FJ. 2006. Wnt/beta-catenin signaling is a normal physiological response to mechanical loading in bone. J Biol Chem 281(42):31720–31728.

Semenov MV, Tamai K, Brott BK, Kuhl M, Sokol S, He X. 2001. Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. Curr Biol 11(12):951–961.

Semenov MV, Zhang X, He X. 2008. DKK1 antagonizes Wnt signaling without promotion of LRP6 internalization and degradation. J Biol Chem 283(31):21427–21432.

Springer TA. 1998. An extracellular beta-propeller module predicted in lipoprotein and scavenger receptors, tyrosine kinases, epidermal growth factor precursor, and extracellular matrix components. J Mol Biol 283(4):837–862.

Tamai K, Semenov M, Kato Y, Spokony R, Liu C, Katsuyama Y, Hess F, Saint-Jeannet JP, He X. 2000. LDL-receptor-related proteins in Wnt signal transduction. Nature 407(6803):530–535.

Van Wesenbeeck L, Cleiren E, Gram J, Beals RK, Benichou O, Scopelliti D, Key L, Renton T, Bartels C, Gong Y, Warman ML, De Vernejoul MC, Bollerslev J, Van Hul W. 2003. Six novel missense mutations in the LDL receptor-related protein 5 (LRP5) gene in different conditions with an increased bone density. Am J Hum Genet 72(3):763–771.

Voorzanger-Rousselot N, Goehrig D, Journe F, Doriath V, Body JJ, Clezardin P, Garnero P. 2007. Increased Dickkopf-1 expression in breast cancer bone metastases. Br J Cancer 97(7):964–970.

Wang K, Zhang Y, Li X, Chen L, Wang H, Wu J, Zheng J, Wu D. 2008. Characterization of the Kremen-binding site on Dkk1 and elucidation of the role of Kremen in Dkk-mediated Wnt antagonism. J Biol Chem 283(34):23371–23375.

Wehrli M, Dougan ST, Caldwell K, O'Keefe L, Schwartz S, Vaizel-Ohayon D, Schejter E, Tomlinson A, DiNardo S. 2000. arrow encodes an LDL-receptor-related protein essential for Wingless signalling. Nature 407(6803):527–530.

Westendorf JJ, Kahler RA, Schroeder TM. 2004. Wnt signaling in osteoblasts and bone diseases. Gene 341:19–39.

Yavropoulou MP, Yovos JG. 2007. The role of the Wnt signaling pathway in osteoblast commitment and differentiation. Hormones (Athens) 6(4):279–294.

Yu HM, Jerchow B, Sheu TJ, Liu B, Costantini F, Puzas JE, Birchmeier W, Hsu W. 2005. The role of Axin2 in calvarial morphogenesis and craniosynostosis. Development 132(8):1995–2005.

Zhang Y, Wang Y, Li X, Zhang J, Mao J, Li Z, Zheng J, Li L, Harris S, Wu D. 2004. The LRP5 high-bone-mass G171V mutation disrupts LRP5 interaction with Mesd. Mol Cell Biol 24(11):4677–4684.

Zhang QG, Wang R, Khan M, Mahesh V, Brann DW. 2008. Role of Dickkopf-1, an antagonist of the Wnt/beta-catenin signaling pathway, in estrogen-induced neuroprotection and attenuation of tau phosphorylation. J Neurosci 28(34):8430–8441.