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Mesd Is a Universal Inhibitor of Wnt Co-receptor LRP5/6 and Blocks Wnt/β-catenin Signaling in Cancer Cells[†]

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Abstract

Mesd is a specialized chaperone for the low-density lipoprotein receptor-related protein-5 (LRP5) and LRP6. In our previous studies, we found that Mesd binds to mature LRP6 on the cell surface and blocks the binding of Wnt antagonist Dickkopf-1(Dkk1) to LRP6. Herein, we demonstrated that Mesd also binds to LRP5 with a high affinity, and is a universal inhibitor of LRP5/6 ligands. Mesd not only blocks Wnt antagonists Dkk1 and Sclerostin binding to LRP5/6, but also inhibits Wnt3A and Rspondin1-induced Wnt/β-catenin signaling in LRP5/6 expressing cells. We also found that Mesd, Dkk1 and Sclerostin compete with one another for binding to LRP5 and LRP6 at the cell surface. More importantly, we demonstrated that Mesd is able to suppress LRP6 phosphorylation and Wnt/β-catenin signaling in prostate cancer PC-3 cells, and inhibits PC-3 cell proliferation. Our results indicate that recombinant Mesd protein is a useful tool for studying Wnt/β-catenin signaling on the cell surface, and has a potential therapeutic role in Wnt-dependent cancers.

The Wnt/β-catenin signaling pathway is involved in various differentiation events during embryonic development and can lead to tumor formation when aberrantly activated. The low density lipoprotein receptor-related protein-5 (LRP5)[1] and LRP6 are two members of the expanding low density lipoprotein receptor (LDLR) family (1). Wnt binds to a receptor complex composed of members of the Frizzled (Fz) family of seven transmembrane, serpentine receptors and LRP5/6 to activate the Wnt/β-catenin signaling pathway. The cytoplasmic tails of LRP5/6, upon receptor activation by Wnt proteins, are phosphorylated, and recruit the cytosolic scaffold protein Axin to the membrane. As a result, β-catenin protein is stabilized, and then enters the nucleus to form a complex with transcription factors of the T-cell factor/lymphoid enhancing factor (TCF/LEF) family to activate transcription of Wnt target genes (1).

By binding to the extracellular domain of LRP5/6, several secreted proteins can regulate Wnt/β-catenin signaling on the cell surface (1). The R-spondin (Rspo) proteins constitute a novel class of ligands that are implicated in the amplification of Wnt/β-catenin signaling (2). There are four human Rspo proteins; Rspo1 has a specific proliferative effect on intestinal crypt cells

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[1]The abbreviations used are: ALP, Alkaline phosphatase; CHO, Chinese hamster ovary; CM, conditioned medium; Dkk1, Dickkopf-1; EGF, epidermal growth factor; ER, endoplasmic reticulum; FBS, fetal bovine serum; Fz, Frizzled; HSPG, heparan sulfate proteoglycan; LDLR, low density lipoprotein receptor; LRP5, low-density lipoprotein receptor-related protein-5; OPG, osteoprotegerin; RAP, receptor-associated protein; Rspo, R-spondin; TCF/LEF, T-cell factor/lymphoid enhancing factor.

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(3). The Dickkopf (Dkk) family and the Wise/Sclerostin family are two distinct classes of Wnt inhibitors. Both Dkks and Sclerostin are LRP5/6 ligands/antagonists. By binding to LRP6, Dkk1 and Sclerostin disrupt Wnt-induced Fz-LRP6 complex *in vitro* (4,5). In the adult, Dkks are implicated in bone formation and bone disease, cancer and Alzheimer's disease (1). Sclerostin is predominantly expressed in skeletal tissues, and mutations in its gene cause Sclerosteosis, which is characterized by massive bone overgrowth (1)

Mesd is a specialized molecular chaperone for members of the LDLR family (6–11), particularly the Wnt co-receptors LRP5 and LRP6. Mesd was discovered due to its requirement for the folding of LRP5/6 (6,7). In mice, the consequences of *Mesd* deficiency resemble what is seen in *wnt3*-deficient mutants (7). Similar to other ER chaperones, Mesd also carries an endoplasmic reticulum (ER) retention signal (KDEL in *Drosophila*, REDL in mammals) at its carboxyl terminus and localizes to the ER by immunohistochemistry (6). All members of the LDLR family have at least one six-bladed β-propeller domain, which is immediately followed by an epidermal growth factor (EGF) repeat. Mesd is specifically required for the maturation of these β-propeller/EGF modules through the secretory pathway (8). In the absence of Mesd, LRP5/6 form aggregates in the ER and fail to reach the cell surface (6–11).

The specialized chaperone function of Mesd resembles that of a 39 kDa receptor-associated protein (RAP), a well-characterized molecular chaperone for members of the LDLR family with dual functions in both receptor folding and in regulation of ligand-receptor interactions (12). The most dramatic effect observed when RAP is bound to LRP1 is the inhibition of binding and/or uptake of all known LRP1 ligands. In addition to LRP1, RAP also binds to other members of the LDLR family and inhibits their ligand interactions (12). In our previous studies, we found that Mesd binds to mature LRP6 on the cell surface and blocks Dkk1 binding to LRP6 (9). In the present studies, we tested whether Mesd also binds to LRP5 with a high affinity and is a universal inhibitor of LRP5/6 ligands. We also studied the role of Mesd in Wnt/β-catenin signaling in cancer cells.

MATERIALS AND METHODS

Materials

Plasmid pcDNA3.1C-Myc-hLRP5 containing the full-length human LRP5 cDNA and plasmid pCS-Myc-hLRP6 containing the full-length human LRP6 cDNA were from Dr. Cindy Bartels (Case Western Reserve University, Cleveland) and Dr. Christof Niehrs (Deutsches Krebsforschungszentrum, Heidelberg, Germany), respectively. Plasmid pGST-E-cadherin was provided by Dr. Gail Johnson (University of Alabama at Birmingham, Alabama). Preparation of recombinant mouse Mesd protein has been described before (9). Recombinant human Rspo1 protein was kindly provided by Dr. Kyung-Ah Kim (Nuvelo Inc., California). Recombinant Dkk1 protein was kindly provided by Dr. Helmut Glantschnig (Merck Research Laboratories, Pennsylvania). Recombinant Wnt3A and Sclerostin proteins were purchased from R&D Systems. The TOPFlash luciferase construct was from Upstate Biotechnology. A β-galactosidase-expressing vector was from Promega. Anti-Dkk1 antibody and anti-osteoprotegerin (OPG) antibody were obtained from R&D Systems. Monoclonal anti-phosphorylated-LRP6 was purchased from Cell Signaling Technology. Monoclonal anti-β-catenin was from BD Biosciences. Monoclonal anti-actin was from Sigma. Peroxidase labeled anti-mouse antibody and ECL system were purchased from Amersham Life Science. The dual luciferase and β-galactosidase assay systems were from Promega. Alkaline phosphatase (ALP) assay kit, IODO-GEN, sulfo-NHS-biotin and streptavidin-HRP were from Pierce. Tissue culture media, fetal bovine serum (FBS), and plastic-ware were obtained from Life Technologies, Inc. Proteinase inhibitor cocktail Complete™ was obtained from Boehringer Mannheim. Proteins were iodinated by using the IODO-GEN method as described previously (9).

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Cell Culture and Conditioned Media

LDLR- deficient Chinese hamster ovary (CHO) cell line ldlA7 was kindly provided by Dr. Monty Krieger (Massachusetts Institute of Technology, Cambridge). ldl-7 cells were stably transfected with human LRP5 or control pcDNA3.1 vector with the standard method, and cultured in Ham's F-12 medium containing 10% of FBS and 350 μg/ml of G418. LRP6-transduced HT1080 cells and the control cells have been described before (13). HEK293 cells, C2C12 cells, Wnt3A-secreting L cells, and control L cells were obtained from American Type Culture Collection. LRP6 HT1080 cells, LRP5 HEK293 cells, Wnt3A-secreting L cells were cultured in DMEM medium containing 10% of FBS and 350 μg/ml of G418. HEK293 and C2C12 cells were cultured in the same above medium without G418. Wnt3A-conditioned medium (CM) and L cell control CM were prepared according to manufacturer's specifications. For our Wnt3A CM preparation, we found that 20% (v/v) Wnt3A CM was equivalent to 200 ng/ml Wnt3A recombinant protein (R&D Systems) in term of activation of Wnt/β-catenin signaling in C2C12 cells (data not shown).

Ligand Binding Assay

Ligand binding assay was carried out exactly as previously described (9). Cells (2×10^5) were seeded into 12-well dishes 1 day prior to assay. Ligand-binding buffer (minimal Eagle's medium containing 0.6% BSA with a different concentration of radioligand, 0.6 ml/well) was added to cell monolayers, in the absence or the presence of 500 nM unlabeled Mesd, followed by incubation for 4 h at 4°C. Thereafter, overlying buffer containing unbound ligand was removed, and cell monolayers were washed and lysed in low-SDS lysis buffer (62.5 mM Tris-HCl pH 6.8, 0.2% SDS, 10% v/glycerol) and counted. The protein concentration of each cell lysate was measured in parallel dishes that did not contain the ligands.

Luciferase reporter assay

HEK293 cells were plated into 24-well plates. After overnight culture, the cells were transiently transfected with 0.06 μg of the TOPFlash luciferase construct (Upstate Biotechnology), 0.06 μg of β-galactosidase-expressing vector (Promega, Madison, WI), and 0.06 μg of pcDNA3.1C-Myc-hLRP5, pCS-Myc-hLRP6 or control vector. After 24 h incubation, cells were treated with Mesd, Rspo1 and Wnt3A CM. Cells were then lysed 24 h later and both luciferase and β-galactosidase activities were determined. The luciferase activity was normalized to the β-galactosidase activity.

Western Blotting

Cells in 6-well plates were lysed in 0.5 ml of lysis buffer (phosphate-buffered saline containing 1% Triton X-100 and 1 mM PMSF) at 4°C for 10 min. Equal quantities of protein were subjected to SDS-PAGE under reducing conditions. Following transfer to immobilon-P transfer membrane, successive incubations with anti-β-catenin, anti-OPG, anti-phosphorylated-LRP6, or anti-actin, and horseradish peroxidase-conjugated secondary antibody were carried out for 60–120 min at room temperature. The immunoreactive proteins were then detected using the ECL system. Films showing immunoreactive bands were scanned by Kodak Digital Science DC120 Zoom Digital Camera.

Cytosolic Free β-catenin Analysis with GST-E-cadherin Binding Assay

The GST-E-cadherin binding assay was carried out exactly as previously described (14). Uncomplexed cytosolic free β-catenin present in 100 μg of total cell lysate was subjected to SDS-PAGE and detected using the monoclonal antibody to β-catenin.

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Measurement of ALP Activity

C2C12 cells in 24-well plates were treated for indicated amounts of various reagents indicated in each figure legend. Cells were harvested 48 h later for assay of ALP activity by determining the amount of p-nitrophenol synthesized from p-nitrophenylphosphate after 30 min of incubation at room temperature as previously described (14). Cell lysates were analyzed for protein concentrations using a Bio-Rad protein assay kit, and ALP activity was normalized for total protein content in each well.

Biotinylation of Cell Surface Proteins and Immunoprecipitation

C2C12 cells in 6-well plates were treated with Mesd and Wnt3A CM, and then the cells were incubated with 0.5 mg/ml sulfo-NHS-biotin at 4°C for 30 minutes. After quenching of excess biotin with 50 mM NH4Cl, the cells were lysed in 0.5 ml of lysis buffer. The lysates with same amounts of proteins were immunoprecipitated with anti-LRP6 antibody, and the immunoprecipitates were analyzed by immunoblotting with streptavidin-HRP (cell-surface LRP6).

Cell proliferation assay

Cells were seeded into 6-well plates at a density of 10000 cells/well. RPMI-1640 medium containing 2% FBS was used as assay media. After 16 h incubation, the cells were treated with Mesd for 10 days. Media were changed every other day. At the end of the experiment, cells were harvested and counted using the trypan blue exclusion assay.

Colony formation assay

PC-3 cells were seeded in low serum growth medium (0.5% FBS) at 500 cells/well into 6-well plates. Sixteen hours after plating, Mesd was added at concentration of 2 μM, and medium was replenished every other day. After 14 days incubation, colonies were fixed with 4% formaldehyde and stained by 0.5 mg/ml crystal violet and imaged on FluorChem HD2 Imager System (Alpha Innotech).

Statistics

Statistical analyses were performed using Student's unpaired t-test. Data were presented as mean ± SD.

RESULTS

Mesd Binds to LRP5 at the Cell Surface with a High Affinity

In our previous study, we showed that Mesd binds to mature LRP6 at the cell surface with a Kd of 3.3 nM (9). However, the binding affinity between Mesd and LRP5 was unclear. Therefore, we stably transfected human LRP5 cDNA into LDLR-deficient ldl-7 cells, and performed ^{125}I-Mesd binding assay. As expected, LRP5-expressing ldl-7 cells exhibited a significantly higher level of ^{125}I-Mesd binding than the control ldl-7 cells, which express pcDNA3.1 vector. Furthermore, inclusion of unlabeled Mesd (500 nM) greatly eliminated ^{125}I-Mesd binding to LRP5 (Figure 1A). Saturation binding and scatchard analysis of the binding data revealed that Mesd binds LRP5 with a Kd of 3.3 nM (Figure 1B & 1C), indicating that Mesd binds to LRP5 and LRP6 with a similar high affinity.

Mesd Antagonizes Dkk1 Binding to LRP5 and LRP6 at the Cell Surface

Dkk1 is a specific ligand and antagonist of LRP5 and LRP6 (15). Dkk1 binds to LRP5/6 and prevents the formation of the Fz-Wnt-LRP5/6 complex in response to Wnt. In our previous study, we found that Mesd is able to block Dkk1 binding to LRP6 (9). To test whether Mesd

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is also able to block Dkk1 binding to LRP5, we performed ^{125}I-Dkk1 binding analysis with LRP5-expressing ldl-7 cells and LRP6-expressing HT1080 cells. Similar to the LRP6-expressing HT1080 cells (Figure 2B), LRP5-expressing ldl-7 cells also exhibited a significantly higher level of ^{125}I-Dkk1 binding than the control ldl-7 cells. The increased Dkk1 binding was greatly inhibited by Mesd (Figure 2A).

Mesd Antagonizes Sclerostin Binding to LRP5 and LRP6 at the Cell Surface

Sclerostin is another specific ligand and antagonist of LRP5 and LRP6 (5,16). Similar to Dkk1, Sclerostin binds to LRP5/6 and disrupts Wnt induced Fz-Wnt-LRP5/6 complex formation. By performing ^{125}I- Sclerostin binding analysis, we found that both LRP5-expressing ldl-7 cells and LRP6-expressing HT1080 cells exhibited significantly higher levels of ^{125}I-Sclerostin binding than the corresponding control cells, and that the increased Sclerostin binding was abolished by Mesd (Figure 2C & 2D).

Mesd, Dkk1and Sclerostin Compete with One another for Binding to LRP5 and LRP6 at the Cell Surface

Having established that Mesd blocks the binding of Dkk1 and Sclerostin to LRP5 and LRP6, we then tested whether Mesd, Dkk1and Sclerostin compete with one another for binding to the receptors on the cell surface. Interestingly, we found that Dkk1 and Sclerostin were able to block Mesd binding to LRP6 (Figure 3) and LRP5 (Figure S1, Supporting Information), and that Dkk1and Sclerostin competed with each other for binding to LRP6 (Figure 3) and LRP5 (Figure S1, Supporting Information).

Mesd Blocks Wnt/β-catenin Signaling Induced by Wnt3A and Rspo1 in LRP5 or LRP6 Expressing HEK293 Cells

Wnt3A is one of about 19 vertebrate members of the Wnt family. There are four members in the Rspo family. LRP6 has been shown to act as a receptor for Wnts (17,18) and Rspos (19–22). Initially, we performed ^{125}I-Wnt3A and ^{125}I-Rspo1 binding assays to test whether Mesd blocks Wnt3A and Rspo1 binding to LRP5/6. Due to low binding affinities between LRP5/6 and Wnt3A/Rspo1 (19–22) and high binding levels to cell surface heparan sulfate proteoglycans (HSPGs) (19,20,23), LRP5-expressing ldl-7 cells and LRP6-expressing HT1080 cells did not display a consistently higher level of ^{125}I-Wnt3A or ^{125}I-Rspo1 binding than the corresponding control cells (data not shown). Therefore, we performed Wnt/β-catenin signaling reporter assay to test whether Mesd blocks Wnt/β-catenin signaling activation induced by Wnt3A and Rspo1. HEK293 cells were transiently transfected with LRP5 or LRP6 along with Wnt/β-catenin signaling reporter TOPFLash, and treated with Wnt3A CM or Rspo1 protein in the presence or absence of Mesd. As expected, LRP5 or LRP6 expression resulted in an increase of TOPFlash activity in HEK293 cells (Figure 4A & 4B), which was further enhanced by Wnt3A or Rspo1 treatments (Figure 4C–4F). Importantly, the increased TOPFlash activity induced by LRP5, LRP6, LRP5 plus Wnt3A, LRP6 plus Wnt3A, LRP5 plus Rspo1, or LRP6 plus Rspo1 was blocked by Mesd in a dose dependent manner (Figure 4C–4F).

Mesd Blocks LRP6 Phosphorylation, ALP production and OPG expression Induced by Wnt3A and Rspo1 in C2C12 Cells

Wnt3A can induce osteoblast differentiation through a mechanism involving the activation of Wnt/β-catenin signaling (14,24,25). C2C12 cells are uncommitted mesenchymal progenitor cells that can be differentiated into osteoblasts upon the activation of Wnt/β-catenin signaling (14,24,25). We employed C2C12 cells to further examine the effects of Mesd on Wnt3A induced Wnt/β-catenin signaling. LRP6 phosphorylation is critical for Wnt/β-catenin signaling induced by Wnt proteins (1), and ALP is a specific marker of osteoblast differentiation (14, 24,25), and OPG is a direct target gene of Wnt/β-catenin signaling in osteoblasts (26,27). As

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the Wnt3A treatment lasted only for 4h, we pretreated C2C12 cells with Mesd for 2h to effectively inhibit Wnt3A effects. We found that treatment with Mesd greatly reduced Wnt3A-induced endogenous LRP6 phosphorylation without affecting the cell surface level of LRP6 (Figure S2A & S2B, Supporting Information). Furthermore, treatment with Mesd blocked Wnt3A-induced ALP activity and OPG expression (Figure S2C & S2D, Supporting Information)

In our previous study, we demonstrated that Rspo1 synergizes with Wnt3A in inducing osteoblast differentiation and OPG expression in C2C12 cells, although Rspo1 itself has minor effects (14). Thus, we examined the effects of Mesd on Rspo1 plus Wnt3A-induced ALP activation and OPG expression in C2C12 cells. We found that Mesd was able to block LRP6 phosphorylation and attenuate cytosolic free β-catenin accumulation induced by either Rspo1 (10 ng/ml), Wnt3A CM (1%), or Rspo1 plus Wnt3A CM (Figure 5A). Furthermore, Mesd treatment significantly reduced ALP production (Figure 5B) and completely abolished OPG expression induced by Rspo1 plus Wnt3A (Figure 5C).

Mesd Attenuates Wnt/β-catenin Signaling in Prostate Cancer PC-3 Cells

Accumulating evidence indicates that activation of Wnt/β-catenin signaling is due to up-regulation of Wnt proteins and their receptors and/or down-regulation of secreted antagonists of the Wnt/β-catenin signaling pathway in several types of cancers such as prostate, breast and lung cancer (28–31). In our previous study, we demonstrated that the androgen-independent PC-3 cell line exhibits a higher level of Wnt/β-catenin signaling than other prostate cancer cell lines and non-cancerous prostate cells (32). Thus, we selected PC-3 cells to test whether Mesd attenuates Wnt/β-catenin signaling in Wnt-dependent cancer cells. We found that Mesd treatment greatly reduced the levels of endogenous LRP6 phosphorylation (Figure 6A). When PC-3 cells were transiently transfected with the Wnt/β-catenin signaling reporter TOPFlash, the TOPFLash luciferase activity in PC3 cells was significantly decreased after Mesd treatment (Figure 6B).

Axin2 and cyclin D1 are transcriptional targets of Wnt/β-catenin signaling. To confirm the effects of Mesd on Wnt/β-catenin signaling in PC-3 cells, we examined the expression of Axin2 and cyclin D1 by Western blotting. As expected, Mesd treatment greatly reduced the expression of Axin2 and cyclin D1 in PC-3 cells (Figure 6A).

Mesd Inhibits Prostate Cancer PC-3 Cell Proliferation

Having established that Mesd blocks Wnt/β-catenin signaling in PC-3 cells, we then examined the effects of Mesd on PC-3 cell proliferation. As seen in Figure 6C & 6D, Mesd displayed inhibitory effect on PC-3 cell proliferation in a dose- and time-dependent manner. Mesd treatment for 10 days significantly inhibited PC-3 cell proliferation (13% decrease with 0.125 μM Mesd, $P<0.05$; 28% with 0.5 μM Mesd, $P<0.01$; 65% with 2 μM Mesd, $P<0.01$). The long-term effect of Mesd treatment was further estimated by clonogenic assay. PC-3 cells were cultured in the presence of vehicle or 2 μM Mesd for 14 days. As shown in Figure 6E, Mesd treatment resulted in almost complete inhibition of clone formation of PC-3 cells. These results clearly demonstrate that Wnt/β-catenin signaling is crucial for the proliferation of prostate cancer PC-3 cells, which can be antagonized by Mesd.

DISCUSSION

Mesd and RAP are two specialized molecular chaperones for members of the LDLR family. RAP is particularly important for the giant receptors LRP1, LRP1B and LRP2 (12), while Mesd is crucial for Wnt co-receptors LRP5 and LRP6 (6–11). It is well established that RAP is a unique receptor antagonist for members of the LDLR family (12). Wnt, Rspo, Dkk and

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Sclerostin are four major types of LRP5/6 ligands. In the present study, we demonstrated that Mesd not only blocks Dkk1 and Sclerostin binding to LRP5 and LRP6, but also inhibits Wnt3A and/or Rspo1-induced Wnt/β-catenin signaling in LRP5 or LRP6 expressing cells. Our results indicate that Mesd is a unique receptor antagonist for LRP5 and LRP6 and should be a useful research tool to study the function of LRP5/6 in various pathophysiological conditions such as bone metabolism, stem cells and cancer.

LRP5 and LRP6 are essential co-receptors for the Wnt/β-catenin signaling pathway and are subjected to modulation by several secreted proteins (1). With the ^{125}I-ligand binding assays, we herein demonstrated that Dkk1 and Sclerostin directly bind to LRP5 and LRP6 on the cell surface, which is consistent with reported results (5,15,16). We also found that LRP5-expressing ldl-7 cells and LRP6-expressing HT1080 cells did not exhibit a higher level of cell surface ^{125}I-Wnt3A or ^{125}I-Rspo1 binding compared to the corresponding control cells, yet Mesd is able to block Wnt3A and/or Rspo1 induced Wnt/β-catenin signaling in LRP5 or LRP6 expressing cells. Wnt proteins are known to bind to LRP6 with a low affinity (1,33), however, the interaction between LRP6 and Rspos remains controversial (19–22). While attempting to establish binding between Rspo2 with LRP6 was unsuccessful (22), it was reported that Rspo3 physically interacts with extracellular domain of LRP6 as demonstrated by co-immunoprecipitation experiments (19). Rspo1 binds to LRP6 *in vitro* with a high affinity (Kd = 1.2 nM) as measured by a solid phase enzyme-linked binding assay (20); however, binding between Rspo1 and LRP6 on the surface of LRP6-expressing HEK293 cells was difficult to detect (21). Both Wnts and Rspos are high affinity heparin-binding proteins (19,20,23). Heparan sulfate proteoglycans (HSPGs) are involved in Wnt/β-catenin signaling (34–36). In the present study, we found both LRP5/6 expressing cells and the corresponding control cells displayed high levels of cell surface ^{125}I-Wnt3A or ^{125}I-Rspo1 binding, likely because of their binding to cell surface HSPGs. It was speculated that the high affinity Rspo1 binding to HSPGs masks the Rspo1 binding to LRP6 on the cell surface; however, Binnerts *et al.* failed to detect an interaction between Rspo1 and LRP6 when the binding assays were carried out in the presence of heparin (21). Future studies will be necessary to dissect the exact mechanism underlying Rspo-mediated LRP6/β-catenin signaling.

LRP5 and LRP6 are closely related cell surface receptors that belong to the expanding low-density lipoprotein receptor (LDLR) family (37–39). The organization of structural modules, i.e. ligand-binding repeats, EGF repeats, and the YWTD motifs, in LRP5/6 is unique among the LDLR family members. For example, LRP5/6 each contains only three ligand-binding repeats, which are located close to the transmembrane domain (37–39). It is interesting to note that all the identified LRP5/6 extracellular ligands including Mesd bind to the β-propeller/EGF repeat modules (1,8,33,40), whereas ligands including RAP for other LDLR family members bind to the clusters of ligand-binding repeats (12). Therefore, as specialized molecular chaperones for the LDLR family, it is likely that Mesd functions primarily in promoting the folding of the β-propeller/EGF modules, whereas RAP plays a major role in promoting the folding of cysteine-rich ligand-binding repeats. Indeed, Culi *et al* reported that Boca, the Mesd ortholog in *Drosophila*, interacts preferentially with the immature β-propeller/EGF modules, and is specifically required for the maturation of these β-propeller/EGF modules through the secretory pathway (8). LRP5/6 has four β-propeller/EGF modules. We previously demonstrated that Mesd binds to cell surface LRP6 with high affinity (9), and that both secreted mature β-propeller/EGF modules 1–2 and 3–4 of LRP6 bind to Wnt3A, Dkk1 and Mesd (33). In the present study, we found that Mesd also binds to cell surface LRP5 with high affinity. Therefore, the fact that Mesd binds to both unfolded and folded LRP5/6 indicates that Mesd resembles RAP as a folding chaperone and an escort protein (12). In the present study, we further demonstrated that Mesd, Dkk1and Sclerostin compete with one another for binding to LRP5 and LRP6 at the cell surface. Our data suggest that at least part of one ligand-binding site on LRP5/6 is likely similar or common to the binding sites utilized by the other two LRP5/6

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ligands. However, we cannot rule out the possibility that binding of one ligand to LRP5/6 might lead to conformational changes that reduce or prevent LRP5/6 interaction with other ligands.

While genetic mutations of certain intracellular components of the Wnt/β-catenin pathway, such as *APC* and *CTNNB1*, are significant contributing factors for colorectal cancers, they are typically not the predominating mechanism associated with other cancer types such as breast, prostate and lung cancer. Instead, it appears that dysregulation of cell surface Wnt/β-catenin signaling components leads to aberrant activation of this pathway in these types of cancer (28–31). Secreted Frizzled-related protein1, a member of the secreted Wnt antagonist family which binds to Wnt proteins and prevents the latter from binding Fz receptors, is down-regulated in breast and lung cancer (41–43). On the other hand, it has been reported that Wnt1 is up-regulated in prostate, breast and lung cancer (44–47), and that LRP6 is up-regulated in breast cancer (48). Furthermore, treatment of prostate cancer LNCaP cells with Wnt3A CM and purified recombinant Wnt3A protein significantly enhances cell growth (49). Consistent with these findings, our results showed that Mesd inhibits Wnt/β-catenin signaling and proliferation in prostate cancer PC-3 cells, in which Wnt/β-catenin signaling is highly activated (32). Our results support the notion that the Wnt/β-catenin signaling pathway is a promising therapeutic target in cancer treatment (50).

Recent studies reveal that, in addition to LRP5/6, several other members of the LDLR family are able to modulate Wnt/β-catenin signaling (51–55). For example, LRP4 was recently demonstrated as a novel receptor for Dkk1 and Sclerostin, and is expressed by osteoblasts to regulate bone growth and turnover *in vivo* (55). Although our present study in general reflects that Mesd can inhibit Wnt/β-catenin signaling through LRP5/6, we cannot rule out the possibility that the effects of Mesd on PC-3 cell proliferation and C2C12 cell differentiation can be partially attributed to the interaction between Mesd and other members of the LDLR family. Further studies are required to address this possibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev Cell 2009;17:9–26.
2. Kim KA, Wagle M, Tran K, Zhan X, Dixon MA, Liu S, Gros D, Korver W, Yonkovich S, Tomasevic N, Binnerts M, Abo A. R-Spondin family members regulate the Wnt pathway by a common mechanism. Mol Biol Cell 2008;19:2588–2596. [PubMed: 18400942]
3. Kim KA, Kakitani M, Zhao J, Oshima T, Tang T, Binnerts M, Liu Y, Boyle B, Park E, Emtage P, Funk WD, Tomizuka K. Mitogenic influence of human R-spondin1 on the intestinal epithelium. Science 2005;309:1256–1259. [PubMed: 16109882]
4. Semënov MV, Tamai K, Brott BK, Kühl M, Sokol S, He X. Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. Curr Biol 2001;11:951–961.

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5. Semënov M, Tamai K, He X. SOST is a ligand for LRP5/LRP6 and a Wnt signaling inhibitor. J Biol Chem 2005;280:26770–26775. [PubMed: 15908424]
6. Culi J, Mann RS. Boca, an endoplasmic reticulum protein required for wingless signaling and trafficking of LDL receptor family members in Drosophila. Cell 2003;112:343–354.
7. Hsieh JC, Lee L, Zhang L, Wefer S, Brown K, DeRossi C, Wines ME, Rosenquist T, Holdener BC. Mesd encodes an LRP5/6 chaperone essential for specification of mouse embryonic polarity. Cell 2003;112:355–367.
8. Culi J, Springer TA, Mann RS. Boca-dependent maturation of beta-propeller/EGF modules in low-density lipoprotein receptor proteins. EMBO J 2004;23:1372–1380.
9. Li Y, Chen J, Lu W, McCormick LM, Wang J, Bu G. Mesd binds to mature LDL-receptor-related protein-6 and antagonizes ligand binding. J Cell Sci 2005;118:5305–5314. [PubMed: 16263759]
10. Koduri V, Blacklow SC. Requirement for natively unstructured regions of mesoderm development candidate 2 in promoting low-density lipoprotein receptor-related protein 6 maturation. Biochemistry 2007;46:6570–6577. [PubMed: 17488095]
11. Li Y, Lu W, He X, Bu G. Modulation of LRP6-mediated Wnt signaling by molecular chaperone Mesd. FEBS Lett 2006;580:5423–5428.
12. Bu G. The roles of receptor-associated protein (RAP) as a molecular chaperone for members of the LDL receptor family. Int Rev Cytol 2001;209:79–116. [PubMed: 11580203]
13. Li Y, Lu W, He X, Schwartz AL, Bu G. LRP6 expression promotes cancer cell proliferation and tumorigenesis by altering beta-catenin subcellular distribution. Oncogene 2004;23:9129–9135.
14. Lu W, Kim KA, Liu J, Abo A, Feng X, Cao X, Li Y. R-spondin1 synergizes with Wnt3A in inducing osteoblast differentiation and osteoprotegerin expression. FEBS Lett 2008;582:643–650.
15. Niehrs C. Function and biological roles of the Dickkopf family of Wnt modulators. Oncogene 2006;25:7469–7481. [PubMed: 17143291]
16. Li X, Zhang Y, Kang H, Liu W, Liu P, Zhang J, Harris SE, Wu D. Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling. J Biol Chem 2005;280:19883–19887. [PubMed: 15778503]
17. Yamamoto H, Komekado H, Kikuchi A. Caveolin is necessary for Wnt-3a-dependent internalization of LRP6 and accumulation of beta-catenin. Dev Cell 2006;11:213–223.
18. Tamai K, Semenov M, Kato Y, Spokony R, Liu C, Katsuyama Y, Hess F, Saint-Jeannet JP, He X. LDL-receptor-related proteins in Wnt signal transduction. Nature 2000;407:530–535.
19. Nam JS, Turcotte TJ, Smith PF, Choi S, Yoon JK. Mouse cristin/R-spondin family proteins are novel ligands for the Frizzled 8 and LRP6 receptors and activate β-catenin-dependent gene expression. J Biol Chem 2006;281:13247–13257.
20. Wei Q, Yokota C, Semenov MV, Doble B, Woodgett J, He X. R-spondin1 is a high affinity ligand for LRP6 and induces LRP6 phosphorylation and beta-catenin signaling. J Biol Chem 2007;282:15903–15911.
21. 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. R-Spondin1 regulates Wnt signaling by inhibiting internalization of LRP6. Proc Natl Acad, Sci USA 104:14700–14705.
22. Kazanskaya O, Glinka A, del Barco Barrantes I, Stannek P, Niehrs C, Wu W. R-Spondin2 is a secreted activator of Wnt/beta-catenin signaling and is required for Xenopus myogenesis. Dev Cell 2004;7:525–534.
23. Cumberledge S, Reichsman F. Glycosaminoglycans and WNTs: just a spoonful of sugar helps the signal go down. Trends Genet 1997;13:421–423. [PubMed: 9385834]
24. Winkler DG, Sutherland MS, Ojala E, Turcott E, Geoghegan JC, Shpektor D, Skonier JE, Yu C, Latham JA. Sclerostin inhibition of Wnt-3a-induced C3H10T1/2 cell differentiation is indirect and mediated by bone morphogenetic proteins. J Biol Chem 2005;280:2498–2502.
25. Tian E, Zhan F, Walker R, Rasmussen E, Ma Y, Barlogie B, Shaughnessy JD Jr. The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. N Engl J Med 2003;349:2483–2494. [PubMed: 14695408]
26. Glass DA, Bialek P, Ahn JD, Starbuck M, Patel MS, Clevers H, Taketo MM, Long F, McMahon AP, Lang RA, Karsenty G. Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. Dev Cell 2005;8:751–764.

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27. Jackson A, Vayssiere B, Garcia T, Newell W, Baron R, Roman-Roman S, Rawadi G. Gene array analysis of Wnt-regulated genes in C3H10T1/2 cells. Bone 2005;36:585–598.
28. Turashvili G, Bouchal J, Burkadze G, Kolar Z. Wnt signaling pathway in mammary gland development and carcinogenesis. Pathobiology 2006;73:213–223.
29. Mazieres J, He B, You L, Xu Z, Jablons DM. Wnt signaling in lung cancer. Cancer Lett 2005;222:1–10. [PubMed: 15837535]
30. Verras M, Sun Z. Roles and regulation of Wnt signaling and beta-catenin in prostate cancer. Cancer Lett 2006;237:22–32. [PubMed: 16023783]
31. Rubin JS, Barshishat-Kupper M, Feroze-Merzoug F, Xi ZF. Secreted WNT antagonists as tumor suppressors: pro and con. Front Biosci 11:2093–2105.
32. Lu W, Tinsley HN, Keeton A, Qu Z, Piazza GA, Li Y. Suppression of Wnt/β-catenin signaling inhibits prostate cancer cell proliferation. Eur J Pharmacol 2009;602:8–14. [PubMed: 19026633]
33. Liu CC, Pearson C, Bu G. Cooperative folding and ligand-binding properties of LRP6 beta-propeller domains. J Biol Chem 2009;284:15299–15307.
34. Dhoot GK, Gustafsson MK, Ai X, Sun W, Standiford DM, Emerson CP. Regulation of Wnt signaling and embryo patterning by an extracellular sulfatase. Science 2001;293:1663–1666. [PubMed: 11533491]
35. Ai X, Do AT, Lozynska O, Kusche-Gullberg M, Lindahl U, Emerson CP Jr. QSulf1 remodels the 6-O sulfation states of cell surface heparan sulfate proteoglycans to promote Wnt signaling. J Cell Biol 2003;162:341–351.
36. Tsuda M, Kamimura K, Nakato H, Archer M, Staatz W, Fox B, Humphrey M, Olson S, Futch T, Kaluza V, Siegfried E, Stam L, Selleck SB. The cell-surface proteoglycan Dally regulates Wingless signalling in Drosophila. Nature 1999;400:276–280.
37. Kim DH, Inagaki Y, Suzuki T, Ioka RX, Yoshioka SZ, Magoori K, Kang MJ, Cho Y, Nakano AZ, Liu Q, Fujino T, Suzuki H, Sasano H, Yamamoto TT. A new low density lipoprotein receptor related protein, LRP5, is expressed in hepatocytes and adrenal cortex, and recognizes apolipoprotein E. J Biochem 1988;124:1072–1076.
38. Hey PJ, Twells RC, Phillips MS, Nakagawa Y, Brown SD, Kawaguchi Y, Cox R, Xie G, Dugan V, Hammond H, Metzker ML, Todd JA, Hess JF. Cloning of a novel member of the low-density lipoprotein receptor family. Gene 1988;216:103–111.
39. Brown SD, Twells RC, Hey PJ, Cox RD, Levy ER, Soderman AR, Metzker ML, Caskey CT, Todd JA, Hess JF. Isolation and characterization of LRP6, a novel member of the low density lipoprotein receptor gene family. Biochem Biophys Res Commun 1988;248:879–888.
40. Kikuchi A, Yamamoto H, Kishida S. Multiplicity of the interactions of Wnt proteins and their receptors. Cell Signal 2007;19:659–671.
41. Ugolini F, Charafe-Jauffret E, Bardou VJ, Geneix J, Adelaide J, Labat-Moleur F, Penault-Llorca F, Longy M, Jacquemier J, Birnbaum D, Pebusque MJ. WNT pathway and mammary carcinogenesis: loss of expression of candidate tumor suppressor gene SFRP1 in most invasive carcinomas except of the medullary type. Oncogene 2001;20:5810–5817.
42. Klopocki E, Kristiansen G, Wild PJ, Klaman I, Castanos-Velez E, Singer G, Stohr R, Simon R, Sauter G, Leibiger H, Essers L, Weber B, Hermann K, Rosenthal A, Hartmann A, Dahl E. Loss of SFRP1 is associated with breast cancer progression and poor prognosis in early stage tumors. Int J Oncol 2004;25:641–649.
43. Fukui T, Kondo M, Ito G, Maeda O, Sato N, Yoshioka H, Yokoi K, Ueda Y, Shimokata K, Sekido Y. Transcriptional silencing of secreted frizzled related protein 1 (SFRP 1) by promoter hypermethylation in non-small-cell lung cancer. Oncogene 2005;24:6323–6327.
44. Chen G, Shukeir N, Potti A, Sircar K, Aprikian A, Goltzman D, Rabbani SA. Up-regulation of Wnt-1 and β-catenin production in patients with advanced metastatic prostate carcinoma: potential pathogenetic and prognostic implications. Cancer 2004;101:1345–1356.
45. Huang CL, Liu D, Ishikawa S, Nakashima T, Nakashima N, Yokomise H, Kadota K, Ueno M. Wnt1 overexpression promotes tumour progression in non-small cell lung cancer. Eur J Cancer 2008;44:2680–2688. [PubMed: 18790633]

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46. Huguet EL, McMahon JA, McMahon AP, Bicknell R, Harris AL. Differential expression of human Wnt genes 2, 3, 4, and 7B in human breast cell lines and normal and disease states of human breast tissue. Cancer Res 1994;54:2615–2621.
47. Milovanovic T, Planutis K, Nguyen A, Marsh JL, Lin F, Hope C, Holcombe RF. Expression of Wnt genes and frizzled 1 and 2 receptors in normal breast epithelium and infiltrating breast carcinoma. Int J Oncol 2004;25:1337–1342.
48. Lindvall C, Zylstra CR, Evans N, West RA, Dykema K, Furge KA, Williams BO. The Wnt co-receptor Lrp6 is required for normal mouse mammary gland development. PLoS One 2009;4:e5813.
49. Verras M, Brown J, Li X, Nusse R, Sun Z. Wnt3a growth factor induces androgen receptor-mediated transcription and enhances cell growth in human prostate cancer cells. Cancer Res 2004;64:8860–8866.
50. Barker N, Clevers H. Mining the Wnt pathway for cancer therapeutics. Nat Rev Drug Discov 2006;5:997–1014.
51. Zilberberg A, Yaniv A, Gazit A. The low density lipoprotein receptor-1, LRP1, interacts with the human frizzled-1 (HFz1) and down-regulates the canonical Wnt signaling pathway. J Biol Chem 2004;279:17535–17542.
52. Lindner I, Hemdan NY, Buchold M, Huse K, Bigl M, Oerlecke I, Ricken A, Gaunitz F, Sack U, Naumann A, Hollborn M, Thal D, Gebhardt R, Birkenmeier G. Alpha2-macroglobulin inhibits the malignant properties of astrocytoma cells by impeding beta-catenin signaling. Cancer Res 2010;70:277–287. [PubMed: 20048078]
53. Chen Y, Hu Y, Lu K, Flannery JG, Ma JX. Very low density lipoprotein receptor, a negative regulator of the wnt signaling pathway and choroidal neovascularization. J Biol Chem 2007;282:34420–34428. [PubMed: 17890782]
54. Li Y, Pawlik B, Elcioglu N, Aglan M, Kayserili H, Yigit G, Percin F, Goodman F, Nürnberg G, Cenani A, Urquhart J, Chung BD, Ismail S, Amr K, Aslanger AD, Becker C, Netzer C, Scambler P, Eyaid W, Hamamy H, Clayton-Smith J, Hennekam R, Nürnberg P, Herz J, Temtamy SA, Wollnik B. LRP4 Mutations Alter Wnt/beta-Catenin Signaling and Cause Limb and Kidney Malformations in Cenani-Lenz Syndrome. Am J Hum Genet. 2010 [Epub ahead of print].
55. Choi HY, Dieckmann M, Herz J, Niemeier A. Lrp4, a novel receptor for Dickkopf 1 and sclerostin, is expressed by osteoblasts and regulates bone growth and turnover in vivo. PLoS One 2009;4:e7930.

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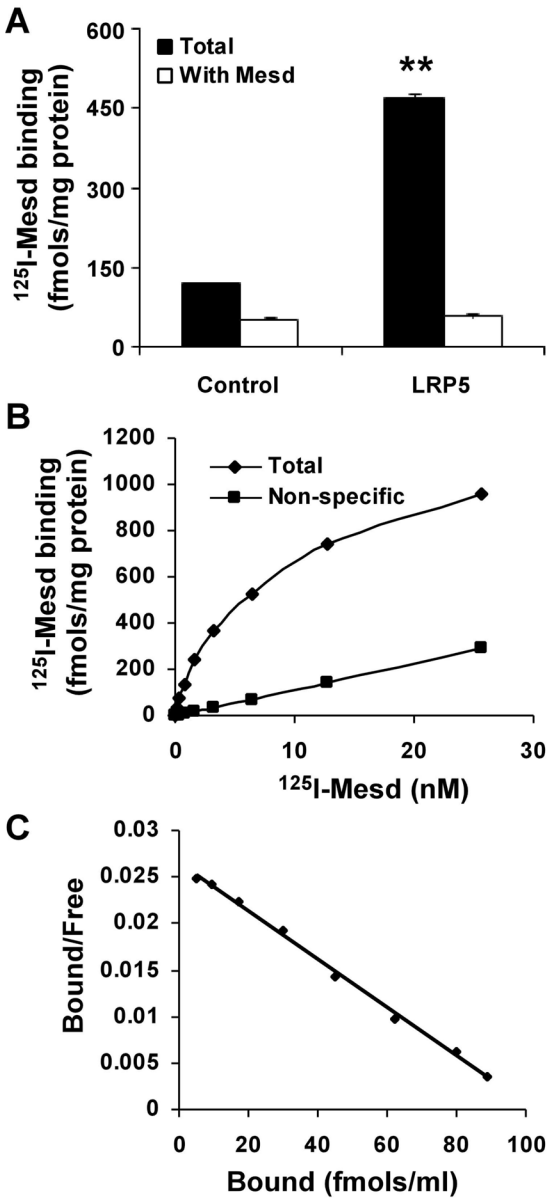

Figure 1.

Mesd binds to LRP5 with high affinity at the cell surface. (A) ^{125}I-Mesd (5 nM) binding to LRP5-transfected ldl-7 cells and the control cells was carried out for 4 h at 4°C in the absence or presence of 500 nM Mesd. Values are the average of triple determinations with the s.d. indicated by error bars. (B) Saturation binding of ^{125}I-Mesd to LRP5-transfected ldl-7 cells. Assay was carried out at indicated concentrations for 3 h at 4°C in the absence (total) or presence (non-specific) of 500 nM Mesd. (C). Scatchard plots of Fig. 1B data. Values are the average of triple determinations with the s.d. indicated by error bars.

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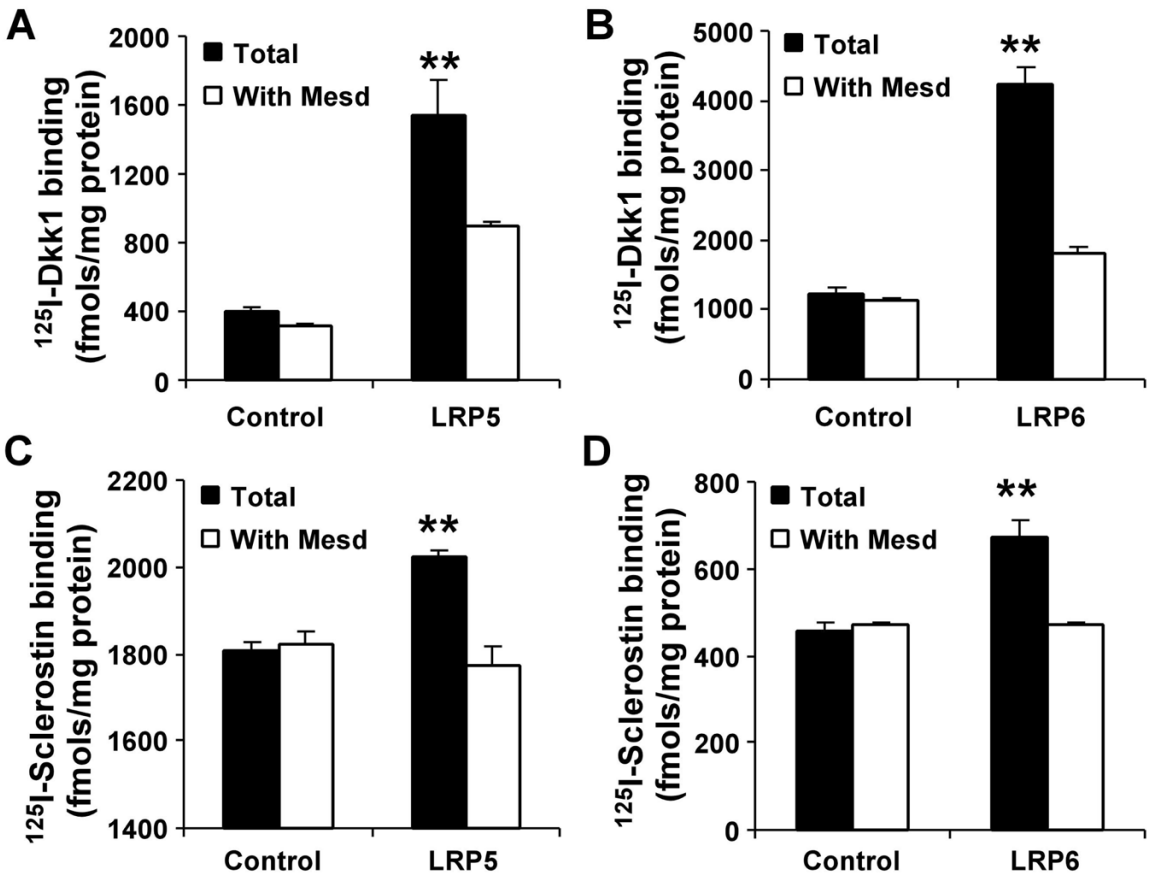

Figure 2.

Mesd blocks Dkk1 and Sclerostin binding to LRP5 and LRP6 at the cell surface. (A, B) ^{125}I-Dkk1 (5 nM) binding to LRP5-transfected *ldl*-7 cells, LRP6-transduced HT1080 cells and the corresponding control cells was carried out for 4 h at 4°C in the absence or presence of 500 nM Mesd. (C, D) ^{125}I-Sclerostin (1.5 nM) binding to LRP5-transfected *ldl*-7 cells, LRP6-transduced HT1080 cells and the corresponding control cells was carried out for 4 h at 4°C in the absence or presence of 500 nM Mesd. Values are the average of triple determinations with the s.d. indicated by error bars. $^{**}P < 0.01$ compared to the cells treated with 500 nM Mesd or the corresponding control cells.

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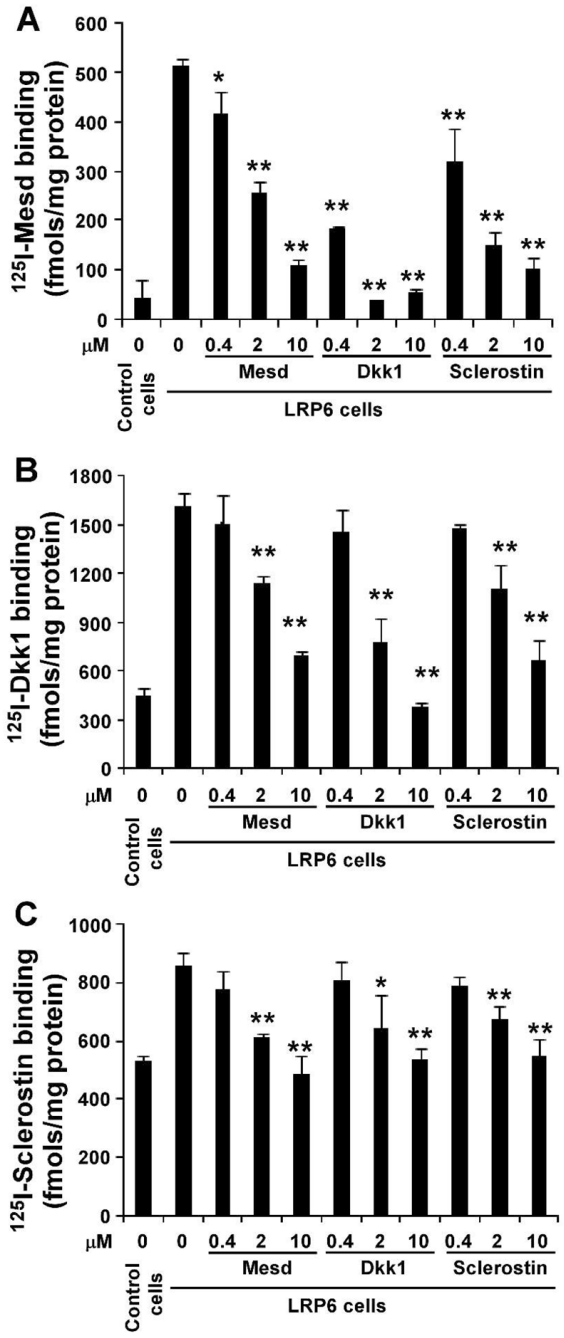

Figure 3.

LRP6 ligand competition binding assays. (A) ^{125}I-Mesd (2 nM), (B) ^{125}I-Dkk1 (2 nM) and (C) ^{125}I- Sclerostin (2 nM) binding to LRP6-transduced HT1080 cells and the corresponding control cells was carried out for 4 h at 4°C in the absence or presence of indicated concentrations of unlabelled Mesd, Dkk1 or Sclerostin. Values are the average of triple determinations with the s.d. indicated by error bars. $^{**}P < 0.01$ compared to the LRP5/6 cells in the absence of unlabelled Mesd, Dkk1 or Sclerostin.

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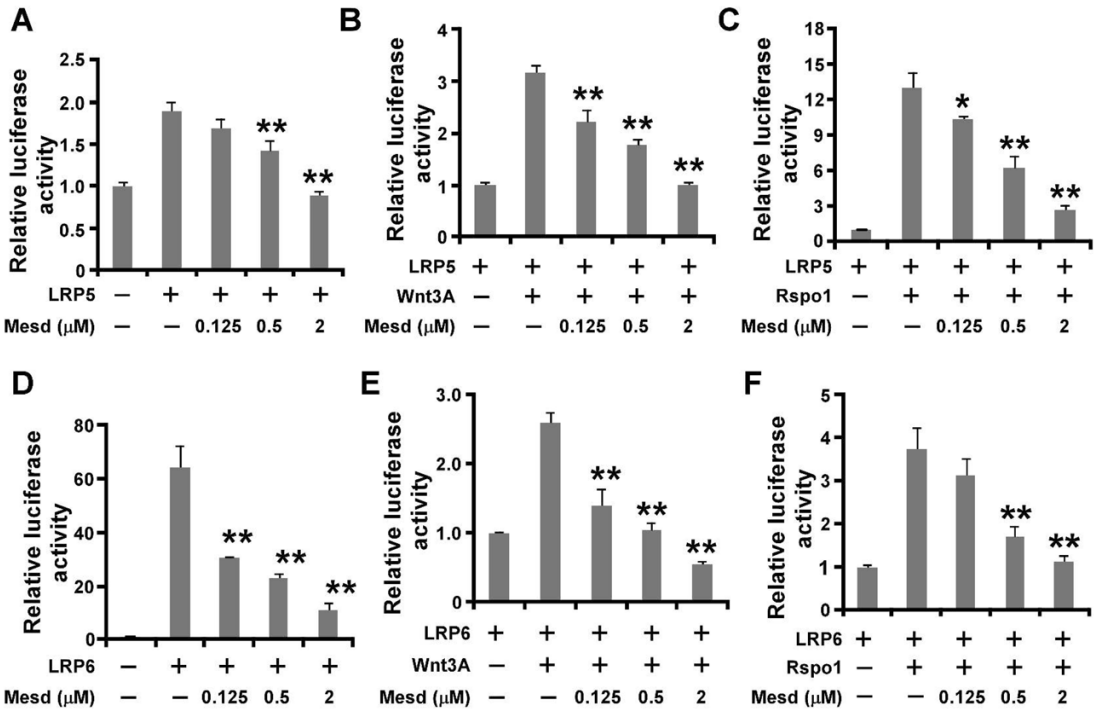

Figure 4.

Mesd blocks Wnt/β-catenin signaling induced by LRP5, LRP6, Wnt3A and Rspo1 in HEK293 cells. HEK293 cells in 24-well plates were transiently transfected with the LRP5 plasmid (A-C), the LRP6 plasmid (D–F) or the corresponding control vector, along with the TOPFlash luciferase construct and the β-galactosidase-expressing vector in each well. After 24 h incubation, cells were treated with Mesd (A, D), Wnt3A CM (5%) plus Mesd (B, E), or Rspo1 (40 ng/ml) plus Mesd (C, F) at the indicated concentrations. The luciferase activity was then measured 24 h later with normalization to the activity of the β-galactosidase. Values are the average of triple determinations with the s.d. indicated by error bars. **P* < 0.05, ***P* < 0.01 compared to the control cells without Mesd treatment.

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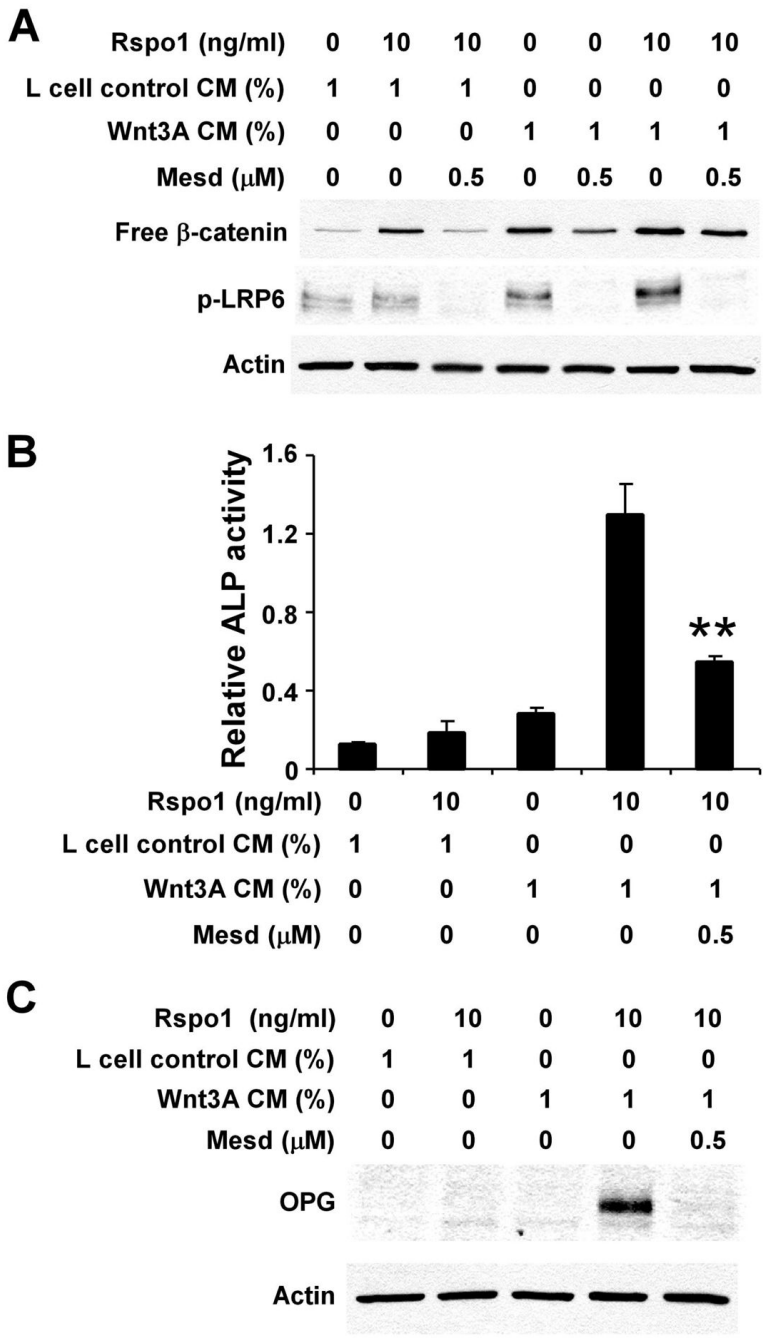

Figure 5.

Mesd blocks LRP6 phosphorylation, cytosolic free β-catenin accumulation, osteoblastic differentiation and OPG expression induced by Rspo1 and Wnt3A. (A) C2C12 cells in 6-well plates were pretreated with or without Mesd (500 nM) for 2 h, and then incubated with Mesd plus Rspo1 CM (10 ng/ml) and/or Wnt3A CM (1%) for 4 h. The levels of phosphorylated LRP6 and cytosolic free β-catenin were analyzed. Samples were also probed with the anti-actin antibody to verify equal loading. (B) C2C12 cells in 12 well plates were incubated with Rspo1 (10 ng/ml) and/or Wnt3A CM (1%) in the presence or absence of Mesd (500 nM). Cells were harvested 48 h later to assay for ALP activity. Values are the average of triple determinations with the s.d. indicated by error bars. $^{**}p < 0.01$ verse cells treated with Rspo1 plus Wnt3A.

(C) C2C12 cells in 6-well plates were incubated with Rspo1 (10 ng/ml) and/or Wnt3A CM (1%) in the presence or absence of Mesd (500 nM) for 48 h. The levels of total cellular OPG were analyzed by Western blotting. Samples were also probed with the anti-actin antibody to verify equal loading.

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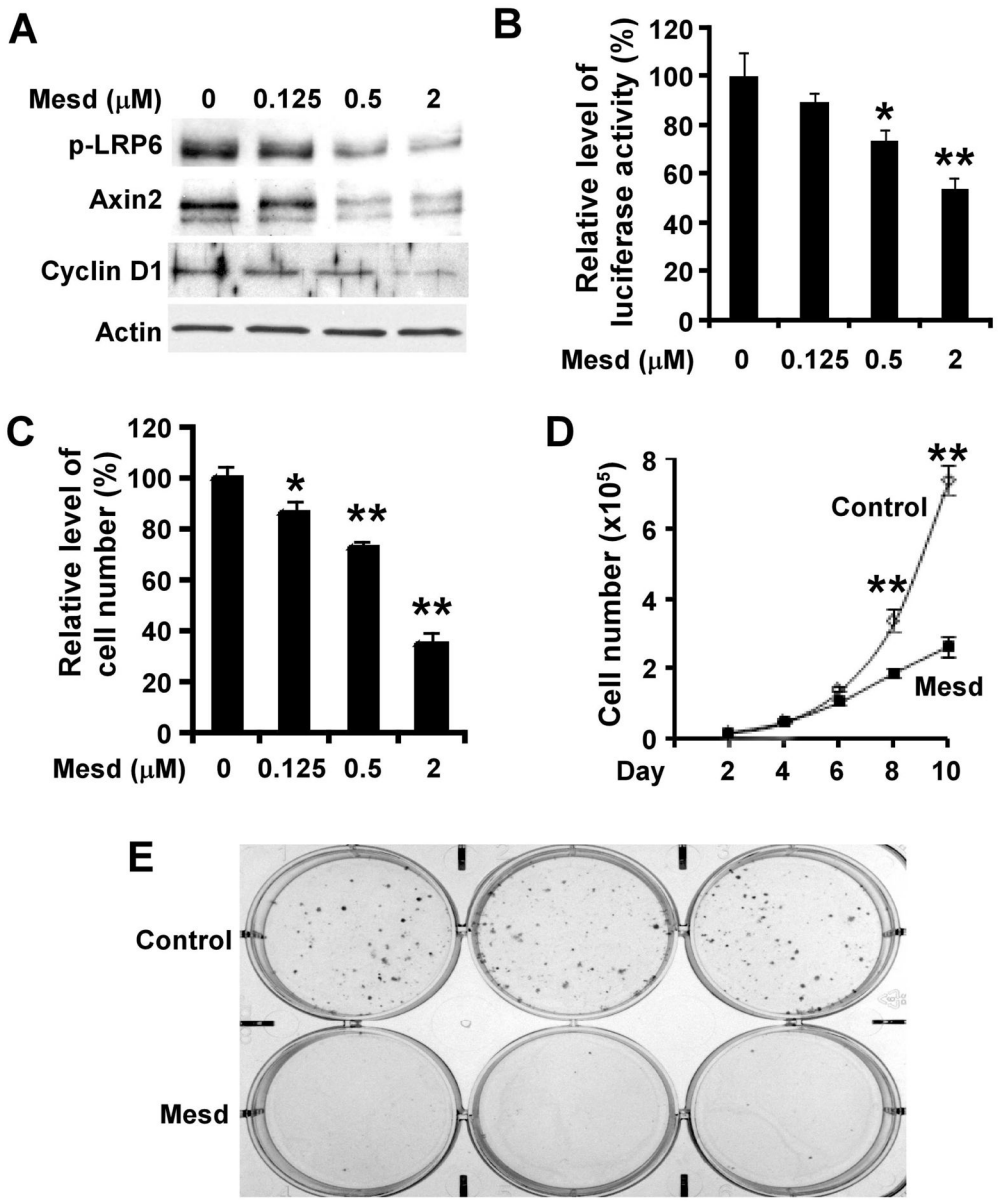

Figure 6.

Mesd blocks Wnt/β-catenin signaling in prostate cancer PC-3 cells and inhibits PC-3 cell proliferation. (A) PC-3 cells in 6-well plates were treated with Mesd at indicated concentrations for 24 h. The levels of Axin2, cyclin D1 and phosphorylated LRP6 were then analyzed by Western blotting. Samples were also probed with the anti-actin antibody to verify equal loading. (B) PC-3 cells in 24-well plates were transiently transfected with the TOPFlash luciferase construct and β-galactosidase-expressing vector in each well. After 24 h incubation, cells were treated with Mesd at the indicated concentrations. The luciferase activity was then measured 24 h later with normalization to the activity of the β-galactosidase. Values are the average of triple determinations with the s.d. indicated by error bars. *$P < 0.05$, **$P < 0.01$ compared to the control cells without Mesd treatment. (C) PC-3 cells in 6-well plates were treated with Mesd at indicated concentrations in RPMI-1640 medium containing 2% FBS for 10 days. Media were changed every other day, and cells were harvested and counted using the trypan blue exclusion assay. Values are the average of triple determinations with the s.d.

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indicated by error bars. *$P < 0.05$, **$P < 0.01$ compared to the control cells without Mesd treatment. (D) PC-3 cells in 6-well plates were treated with 2 μM Mesd in RPMI-1640 medium containing 2% FBS. Media were changed every other day, and cells were harvested and counted using the trypan blue exclusion assay at days 2, 4, 6, 8 and 10. Values are the average of triple determinations with the s.d. indicated by error bars. **$P < 0.01$ compared to the corresponding control. (E) PC-3 cells in 6-well plates were treated with 2 μM Mesd in RPMI-1640 medium containing 0.5% FBS for 14 days. Media were changed every other day. Colonies were fixed with formaldehyde and stained by crystal violet.