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Mesd Is a Universal Inhibitor of Wnt Coreceptors LRP5 and LRP6 and Blocks Wnt/β-Catenin Signaling in Cancer Cells†

Wenyan Lu,‡ Chia-Chen Liu,§ Jaideep V. Thottassery,‡ Guojun Bu,§ and Yonghe Li*,‡

‡*Department of Biochemistry and Molecular Biology, Drug Discovery Division, Southern Research Institute, 2000 Ninth Avenue South, Birmingham, Alabama 35255, and* §*Department of Pediatrics and Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110*

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ABSTRACT: Mesd is a specialized chaperone for 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 demonstrate that Mesd also binds to LRP5 with a high affinity and is a universal inhibitor of LRP5 and LRP6 ligands. Mesd not only blocks binding of Wnt antagonists Dkk1 and Sclerostin to LRP5 and LRP6 but also inhibits Wnt3A and Rspondin 1-induced Wnt/β-catenin signaling in LRP5- and LRP6-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. 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 or LRP6 to activate the Wnt/β-catenin signaling pathway. The cytoplasmic tails of LRP5 and LRP6, 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 and LRP6, 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 (*3*). The Dickkopf (Dkk) family and the Wise/Sclerostin family are two distinct classes of Wnt inhibitors. Both Dkks and Sclerostin are LRP5 and LRP6 ligands and/or antagonists. By binding to LRP6, Dkk1 and Sclerostin disrupt the 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 coreceptors LRP5 and LRP6. Mesd was discovered because of its requirement for the folding of LRP5 and LRP6 (*6*, *7*). In mice, the consequences of *Mesd* deficiency resemble what is seen in *wnt3*-deficient mutants (*7*). Similar to other endoplasmic reticulum (ER) chaperones, Mesd also carries an ER retention signal (KDEL in *Drosophila* and 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-blade 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 and LRP6 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 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

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*To whom correspondence should be addressed: Department of Biochemistry and Molecular Biology, Drug Discovery Division, Southern Research Institute, 2000 Ninth Avenue South, Birmingham, AL 35255-5305. Telephone: (205) 581-2750. Fax: (205) 581-2093. E-mail: y.li@sri.org.

[1]Abbreviations: 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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binding of Dkk1 to LRP6 (*9*). In this study, we tested whether Mesd also binds to LRP5 with a high affinity and is a universal inhibitor of LRP5 and LRP6 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 C. Bartels (Case Western Reserve University, Cleveland, OH) and C. Niehrs (Deutsches Krebsforschungszentrum, Heidelberg, Germany), respectively. Plasmid pGST-E-cadherin was provided by G. Johnson (University of Alabama, Birmingham, AL). Preparation of recombinant mouse Mesd protein has been described previously (*9*). Recombinant human Rspo1 protein was kindly provided by K.-A. Kim (Nuvelo Inc.). Recombinant Dkk1 protein was kindly provided by H. Glantschnig (Merck Research Laboratories). 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. The anti-Dkk1 antibody and anti-osteoprotegerin (OPG) antibody were obtained from R&D Systems. Monoclonal antiphosphorylated LRP6 was purchased from Cell Signaling Technology. Monoclonal anti-β-catenin was from BD Biosciences. Monoclonal anti-actin was from Sigma. The 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. The alkaline phosphatase (ALP) assay kit, IODO-GEN, sulfo-NHS-biotin, and streptavidin-HRP were from Pierce. Tissue culture media, fetal bovine serum (FBS), and plasticware 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*).

Cell Culture and Conditioned Media. LDLR-deficient Chinese hamster ovary (CHO) cell line ldlA7 was kindly provided by M. Krieger (Massachusetts Institute of Technology, Cambridge, MA). 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% FBS and 350 μg/mL G418. LRP6-transduced HT1080 cells and the control cells have been described previously (*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, and Wnt3A-secreting L cells were cultured in DMEM containing 10% FBS and 350 μg/mL G418. HEK293 and C2C12 cells were cultured in the same medium described above without G418. Wnt3A conditioned medium (CM) and L cell control CM were prepared according to the 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 terms of activation of Wnt/β-catenin signaling in C2C12 cells (data not shown).

Ligand Binding Assay. The ligand binding assay was conducted exactly as previously described (*9*). Cells (2×10^5) were seeded into 12-well dishes 1 day prior to the 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 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, and 10% (v/v) glycerol] and counted. The protein concentration of each cell lysate was measured in parallel dishes that did not contain the ligands.

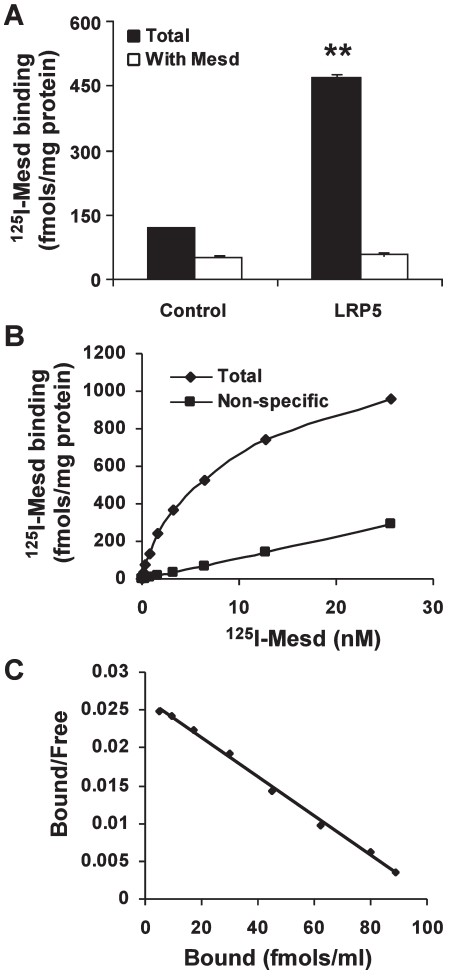

FIGURE 1: Mesd binds to LRP5 with high affinity at the cell surface. (A) Binding of [^{125}I]Mesd (5 nM) to LRP5-transfected ldl-7 cells and control cells for 4 h at 4 °C in the absence or presence of 500 nM Mesd. Values are the averages of three determinations with the standard deviations indicated by error bars. (B) Saturation binding of [^{125}I]Mesd to LRP5-transfected ldl-7 cells. The assay was conducted at the indicated concentrations for 3 h at 4 °C in the absence (total) or presence (nonspecific) of 500 nM Mesd. (C) Scatchard plots of the data from panel B. Values are averages of three determinations with the standard deviations indicated by error bars.

Luciferase Reporter Assay. HEK293 cells were plated into 24-well plates. After being cultured overnight, 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 being incubated for 24 h, 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 six-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.

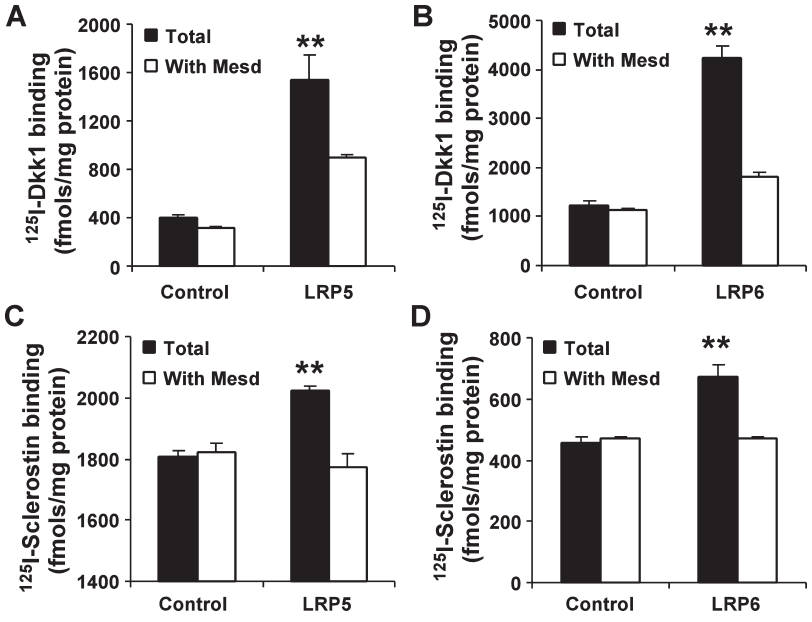

FIGURE 2: Mesd blocks binding of Dkk1 and Sclerostin to LRP5 and LRP6 at the cell surface. (A and B) Binding of [^{125}I]Dkk1 (5 nM) to LRP5-transfected ldl-7 cells, LRP6-transduced HT1080 cells, and the corresponding control cells was conducted for 4 h at 4 °C in the absence or presence of 500 nM Mesd. (C and D) Binding of [^{125}I]Sclerostin (1.5 nM) to LRP5-transfected ldl-7 cells, LRP6-transduced HT1080 cells, and the corresponding control cells was conducted for 4 h at 4 °C in the absence or presence of 500 nM Mesd. Values are the averages of three determinations with the standard deviations indicated by error bars. Asterisks indicate $P < 0.01$ compared to the cells treated with 500 nM Mesd or the corresponding control cells.

Following the 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 conducted for 60−120 min at room temperature. The immunoreactive proteins were then detected using the ECL system. Films showing immunoreactive bands were scanned with a Kodak Digital Science DC120 Zoom Digital Camera.

Cytosolic Free β-Catenin Analysis with the GST−E-Cadherin Binding Assay. The GST−E-cadherin binding assay was conducted 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.

Measurement of ALP Activity. C2C12 cells in 24-well plates were treated for the indicated amounts of various reagents indicated in each figure legend. Cells were harvested 48 h later for the assay of ALP activity by determining the amount of *p*-nitrophenol synthesized from *p*-nitrophenylphosphate after incubation for 30 min 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 six-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 min. After the excess biotin had been quenched with 50 mM NH4Cl, the cells were lysed in 0.5 mL of lysis buffer. The lysates with the 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 six-well plates at a density of 10000 cells/well. RPMI-1640 containing 2% FBS was used as assay medium. After being incubated for 16 h, 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 a density of 500 cells/well into six-well plates. Sixteen hours after the plates had been set up, Mesd was added at a concentration of 2 μM, and medium was replenished every other day. After being incubated for 14 days, colonies were fixed with 4% formaldehyde, stained with 0.5 mg/mL crystal violet, and imaged on a FluorChem HD2 Imager System (Alpha Innotech).

Statistics. Statistical analyses were performed using a Student's unpaired *t* test. Data are presented as means ± the standard deviation.

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 K_d of 3.3 nM (*9*). However, the affinity between Mesd and LRP5 was unclear. Therefore, we stably transfected human LRP5 cDNA into LDLR-deficient ldl-7 cells and performed the [^{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 the pcDNA3.1 vector. Furthermore, inclusion of unlabeled Mesd (500 nM) greatly eliminated binding of [^{125}I]Mesd to LRP5 (Figure 1A). Saturation binding and Scatchard analysis of the binding data revealed that Mesd binds LRP5 with a K_d of 3.3 nM (Figure 1B,C), indicating that Mesd binds to LRP5 and LRP6 with a similar high affinity.

Mesd Antagonizes Binding of Dkk1 to LRP5 and LRP6 at the Cell Surface. Dkk1 is a specific ligand and antagonist of LRP5 and LRP6 (*15*). Dkk1 binds to LRP5 and LRP6 and prevents the formation of the Fz−Wnt−LRP5 or −LRP6 complex in response to Wnt. In our previous study, we found that Mesd is

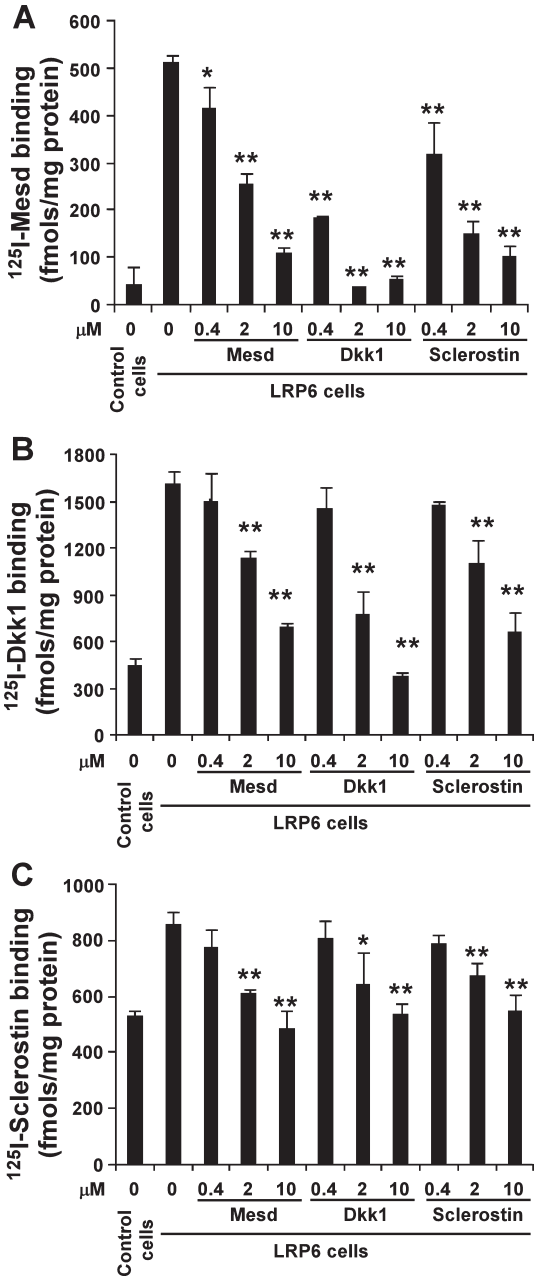

FIGURE 3: LRP6 ligand competition binding assays. Binding of (A) [^{125}I]Mesd (2 nM), (B) [^{125}I]Dkk1 (2 nM), and (C) [^{125}I] Sclerostin (2 nM) to LRP6-transduced HT1080 cells and the corresponding control cells was conducted for 4 h at 4 °C in the absence or presence of the indicated concentrations of unlabeled Mesd, Dkk1, or Sclerostin. Values are averages of three determinations with the standard deviations indicated by error bars. One asterisk indicates $P < 0.05$ and two asterisks indicate $P < 0.01$ compared to the LRP5 and LRP6 cells in the absence of unlabeled Mesd, Dkk1, or Sclerostin.

able to block binding of Dkk1 to LRP6 (*9*). To test whether Mesd is also able to block binding of Dkk1 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 strengthened Dkk1 binding was greatly inhibited by Mesd (Figure 2A).

Mesd Antagonizes Binding of Sclerostin 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 and LRP6 and disrupts Wnt-induced formation of the Fz−Wnt−LRP5 or −LRP6 complex. 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 level of Sclerostin binding was abolished by Mesd (Figure 2C,D).

Mesd, Dkk1, and 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, Dkk1, and 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 the binding of Mesd to LRP6 (Figure 3) and LRP5 (Figure S1 of the Supporting Information) and that Dkk1 and Sclerostin competed with each other for binding to LRP6 (Figure 3) and LRP5 (Figure S1 of the Supporting Information).

Mesd Blocks Wnt/β-Catenin Signaling Induced by Wnt3A and Rspo1 in LRP5- or LRP6-Expressing HEK293 Cells. Wnt3A is one of ~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 binding of Wnt3A and Rspo1 to LRP5 and LRP6. Because of the low binding affinities between LRP5 or LRP6 and Wnt3A/Rspo1 (*19*−*22*) and high levels of binding 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 a 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 in TOPFlash activity in HEK293 cells (Figure 4A,B), which was further enhanced by Wnt3A or Rspo1 treatments (Figure 4C−F). Importantly, the increased TOPFlash activity induced by LRP5, LRP6, LRP5 with Wnt3A, LRP6 with Wnt3A, LRP5 with Rspo1, or LRP6 with Rspo1 was blocked by Mesd in a dose-dependent manner (Figure 4C−F).

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*); 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 the Wnt3A treatment lasted for only 4 h, we pretreated C2C12 cells with Mesd for 2 h to effectively inhibit Wnt3A effects. We found that treatment with Mesd greatly reduced the level of Wnt3A-induced endogenous LRP6 phosphorylation without affecting the cell surface level of LRP6 (Figure S2A,B of the Supporting Information).

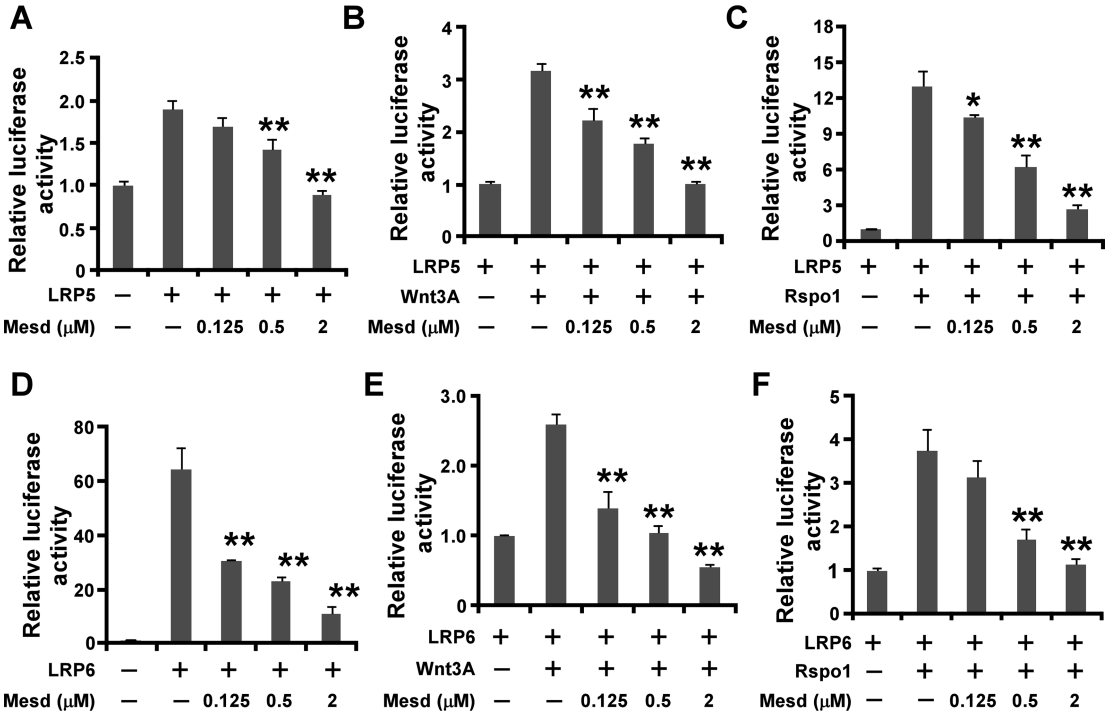

FIGURE 4: Mesd blocks Wnt/β-catenin signaling induced by LRP5, LRP6, Wnt3A, and Rspo1 in HEK293 cells. HEK293 cells in 24-well plates were transiently transfected with the LRP5 plasmid (A−C), the LRP6 plasmid (D−F), or the corresponding control vector, along with the TOPFlash luciferase construct and the β-galactosidase-expressing vector in each well. After being incubated for 24 h, cells were treated with Mesd (A and D), Wnt3A CM (5%) with Mesd (B and E), or Rspo1 (40 ng/mL) with Mesd (C and 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 averages of three determinations with the standard deviations indicated by error bars. One asterisk indicates $P < 0.05$ and two asterisks indicate $P < 0.01$ compared to the control cells without Mesd treatment.

Furthermore, treatment with Mesd blocked Wnt3A-induced ALP activity and OPG expression (Figure S2C,D of the 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 and 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 with Wnt3A CM (Figure 5A). Furthermore, Mesd treatment significantly slowed ALP production (Figure 5B) and completely abolished OPG expression induced by Rspo1 and 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 upregulation of Wnt proteins and their receptors and/or downregulation 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 noncancerous 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 level of 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 panels C and D of Figure 6, Mesd exhibited an 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 a 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 giant receptors LRP1, LRP1B, and LRP2 (*12*), while Mesd is crucial for Wnt coreceptors 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 Sclerostin are four major types of LRP5 and LRP6 ligands. In this study, we demonstrate that Mesd not only blocks binding of Dkk1 and

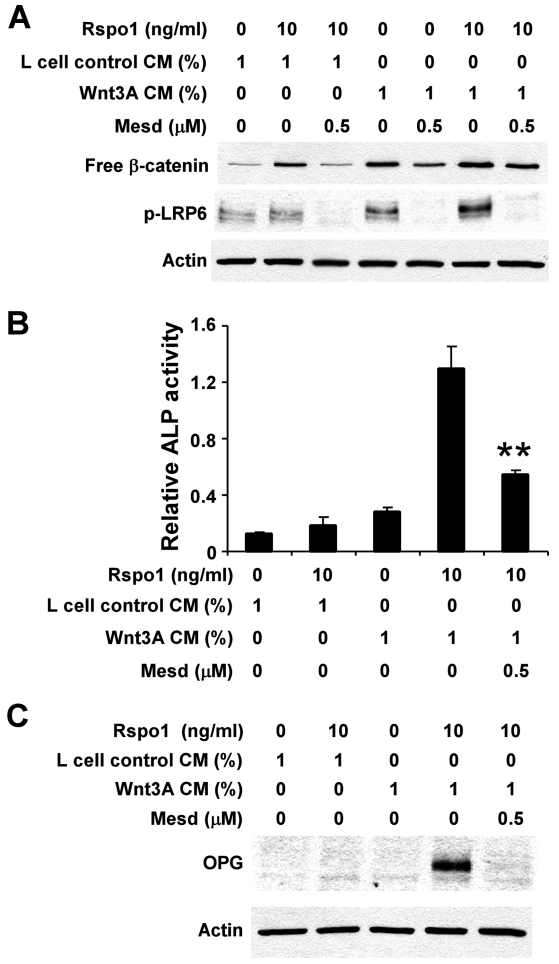

FIGURE 5: Mesd blocks LRP6 phosphorylation, cytosolic free β-catenin accumulation, osteoblastic differentiation, and OPG expression induced by Rspo1 and Wnt3A. (A) C2C12 cells in six-well plates were pretreated with or without Mesd (500 nM) for 2 h and then incubated with Mesd and Rspo1 CM (10 ng/mL) 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 averages of three determinations with the standard deviations indicated by error bars. Asterisks indicate $P < 0.01$ vs cells treated with Rspo1 and Wnt3A. (C) C2C12 cells in six-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.

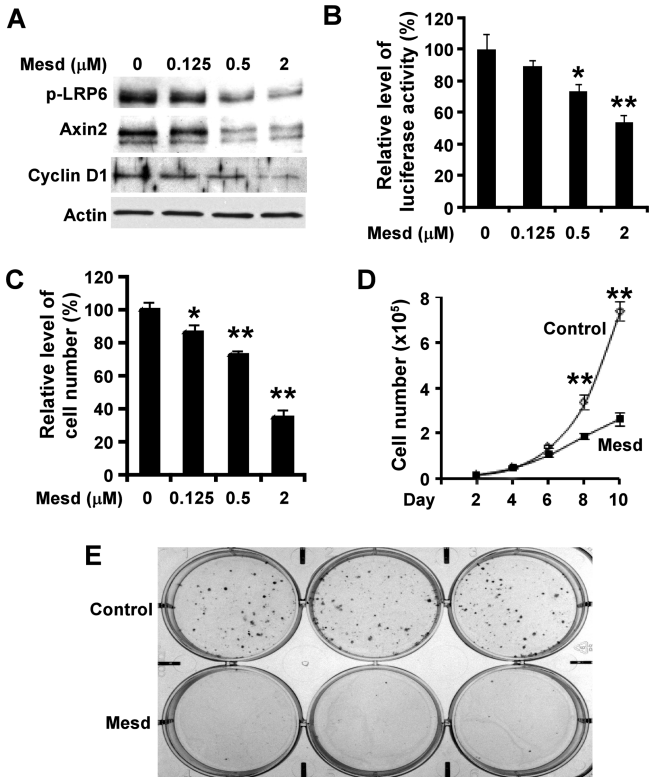

FIGURE 6: Mesd blocks Wnt/β-catenin signaling in prostate cancer PC-3 cells and inhibits PC-3 cell proliferation. (A) PC-3 cells in six-well plates were treated with Mesd at the indicated concentrations for 24 h. The levels of Axin2, cyclin D1, and phosphorylated LRP6 were then analyzed by Western blotting. 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 being incubated for 24 h, 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 averages of three determinations with the standard deviations indicated by error bars. One asterisk indicates $P < 0.05$ and two asterisks indicate $P < 0.01$ compared to the control cells without Mesd treatment. (C) PC-3 cells in six-well plates were treated with Mesd at the indicated concentrations in RPMI-1640 containing 2% FBS for 10 days. The medium were changed every other day, and cells were harvested and counted using the trypan blue exclusion assay. Values are averages of three determinations with the standard deviations indicated by error bars. One asterisk indicates $P < 0.05$ and two asterisks indicate $P < 0.01$ compared to the control cells without Mesd treatment. (D) PC-3 cells in six-well plates were treated with 2 μM Mesd in RPMI-1640 containing 2% FBS. The medium were changed every other day, and cells were harvested and counted using the trypan blue exclusion assay on days 2, 4, 6, 8, and 10. Values are averages of three determinations with the standard deviations indicated by error bars. Asterisks indicate $P < 0.01$ compared to the corresponding control cells. (E) PC-3 cells in six-well plates were treated with 2 μM Mesd in RPMI-1640 containing 0.5% FBS for 14 days. The medium were changed every other day. Colonies were fixed with formaldehyde and stained with crystal violet.

Sclerostin 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 for studying the function of LRP5 and LRP6 in various pathophysiological conditions such as bone metabolism, stem cells, and cancer.

LRP5 and LRP6 are essential coreceptors for the Wnt/β-catenin signaling pathway and are subjected to modulation by several secreted proteins (*1*). With the ^{125}I-labeled ligand binding assays, we demonstrate 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 the attempt to establish binding between Rspo2 with LRP6 was unsuccessful (*22*), it was reported that Rspo3 physically interacts with the extracellular domain of LRP6 as demonstrated

by co-immunoprecipitation experiments (*19*). Rspo1 binds to LRP6 in vitro with a high affinity (K_d = 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 this study, we found both LRP5- and LRP6-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. There was speculation that the high-affinity binding of Rspo1 to HSPGs masks the binding of Rspo1 to LRP6 on the cell surface; however, Binnerts et al. (*21*) failed to detect an interaction between Rspo1 and LRP6 when the binding assays were conducted in the presence of heparin. 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 YWTD motifs, in LRP5 and LRP6 is unique among the LDLR family members. For example, LRP5 and LRP6 each contain 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 and LRP6 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. (*8*) 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. LRP5 and LRP6 have 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 this 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 and LRP6 indicates that Mesd resembles RAP as a folding chaperone and an escort protein (*12*). In the study presented here, we further demonstrate that Mesd, Dkk1, and 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 and LRP6 is likely similar or common to the binding sites utilized by the other two LRP5 and LRP6 ligands. However, we cannot rule out the possibility that binding of one ligand to LRP5 and LRP6 might lead to conformational changes that weaken or prevent the interaction of LRP5 and LRP6 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 protein 1, a member of the secreted Wnt antagonist family that binds to Wnt proteins and prevents the latter from binding Fz receptors, is downregulated in breast and lung cancer (*41*−*43*). On the other hand, it has been reported that Wnt1 is upregulated in prostate, breast, and lung cancer (*44*−*47*), and that LRP6 is upregulated 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 and LRP6, several other members of the LDLR family are able to modulate Wnt/β-catenin signaling (*51*−*55*). For example, LRP4 was recently shown to be as a novel receptor for Dkk1 and Sclerostin and is expressed by osteoblasts to regulate bone growth and turnover in vivo (*55*). Although the study presented here in general reflects the fact that Mesd can inhibit Wnt/β-catenin signaling through LRP5 and LRP6, 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.

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SUPPORTING INFORMATION AVAILABLE

LRP5 ligand competition binding assays (Figure S1) and data illustrating how Mesd blocks LRP6 phosphorylation, osteoblastic differentiation, and OPG expression induced by Wnt3A in C2C12 cells (Figure S2)

. This material is available free of charge via the Internet at http://pubs.acs.org.

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