Supporting Information

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SI Materials and Methods

Western Blotting and Immunohistochemistry. Cells were lysed in PBS containing 0.5% Triton, protease inhibitor mixture (Roche), and 1 mM PMSF at 4°C for 30 min. Equal quantities of protein were subjected to SDS/PAGE under reducing conditions. Following transfer to Immobilon-P membranes, successive incubations with primary and HRP-conjugated secondary antibodies (Amersham Life Science) were performed according to the manufacturer's specifications. Immunoreactive proteins were detected using the ECL system. For immunohistochemical staining, tumors were fixed in 10% buffered formalin for 48 h, transferred to 70% ethanol/PBS, and embedded in paraffin. Sections were cleared in a graded xylene/ethanol series and treated with 3% hydrogen peroxide/H_2O for 20 min. Antigens were retrieved by steaming in citrate buffer (pH 6.0) for 20 min, and sections were blocked and incubated with the primary antibody at 4°C overnight. After successive incubations with the corresponding biotin-conjugated secondary antibody and ABC Elite reagent (Vector Laboratories), antigens were detected using diaminobenzidine (DAB) (Vector Laboratories) as the chromogenic substrate. Slides were counterstained with hematoxylin, dehydrated, and mounted. H&E staining was performed on tumor xenograft tissue, skin, and intestine harvested at the end of Mesd treatment.

Construct, Nucleofection, and Transfection. LRP6 was subcloned into the mLRP4T100 backbone the construction of which has been described previously (1). For rescue experiments, a codon-modified LRP6-Res construct was generated using the QuikChange Site-Directed Mutagenesis kit (Stratagene). The shRNA-targeted sequence was replaced with CTGAGGTGTAAC, which does not change the amino acid composition of LRP6 but renders the construct insensitive to LRP6-specific shRNA. The modified LRP6 cDNA was confirmed by sequencing. Nucleofactor (Amaxa Biosystems) was used for transfection of DNA into MDA-MB-231 or T-47D cells according to the manufacturer's instructions. Fugene 6 (Roche) was used for transfection of HCC1187 cells according to the manufacturer's instructions.

Quantitative Real-Time PCR. Total RNAs isolated from breast cancer and control cells using TRIzol (Invitrogen) and RNeasy Mini Kit (Qiagen) were reverse-transcribed with SuperScript First-Strand Synthesis System (Invitrogen). The reaction mix was subjected to quantitative real-time PCR to detect expression levels of LRP6 and other Wnt-related genes using human quantitative PCR primers specific for corresponding genes and RT^2 Profiler PCR Arrays (SABiosciences). All primers for real-time PCR were ordered from SABiosciences. Triplicate reactions were prepared using a 25-μl mixture containing Platinum SYBR Green qPCR Super Mix UDG (Invitrogen). Real-time quantification was performed on a Bio-Rad iCycle iQ system. Serial 10-fold dilutions of cDNA were used as references for the standard curve in SuperArray. All data were normalized to the endogenous actin expression.

Lentiviral Production. 293T cells were cotransfected with pLKO.1 or pLKO.1-LRP6 shRNA and packaging plasmids pHR'CVM8.2ΔR and pCMV-VSV-G using FuGENE 6 (Roche Diagnostics). The virus-containing supernatant was collected for infection of breast cancer cells. For stable cell line generation, cells were selected in growth medium containing 2 μg/mL puromycin.

Cell Growth and Cell Proliferation Assays. Cell growth was measured by MTT assay (Promega). Breast cancer cells expressing control or LRP6 shRNA were seeded into 48-well plates. MTT-labeling reagent was added to each well 24 h later, and plates were incubated at 37°C for 4 h. After the incubation period, the formazan crystals were dissolved in an MTT solubilization reagent and the resulting color was quantified spectrophotometrically. Cell proliferation was measured by BrdU incorporation using the BrdU ELISA kit (Roche Molecular Systems) according to the manufacturer's instructions. Briefly, cancer cells expressing control or LRP6 shRNA were plated on 96-well plate. Cells were incubated with BrdU for 24 h, and the newly synthesized BrdU-DNA was then determined by colorimetry using an ELISA reader. All experiments were performed three times in triplicate.

Soft Agar Tumorigenicity Assays. A 1-mL bottom layer consisting of 1% agar medium was added to six-well plates. MDA-MB-231 cells expressing control or LRP6 shRNA were trypsinized, centrifuged, resuspended in 0.5% agar medium (equal volumes of 1% agar and 2× culture medium), and plated at 1,000 cells/well as a top layer. Cells were incubated for 3 weeks at 37°C until colony formation and colonies were stained with 0.5% Crystal Violet for counting.

Apoptosis Analysis. Apoptosis of cancer cells expressing control shRNA or LRP6 shRNA was detected using a TUNEL kit (Upstate) according to the manufacturer's protocol. Cells were counterstained with DAPI and examined by fluorescent microscopy.

GST–E-Cadherin Pull-Down Assay. The GST–E-cadherin pull-down assay was performed as previously described (2). Cells expressing control or LRP6 shRNA were treated with L cells or Wnt3a-conditioned media (CM) for 4 h. Free β-catenin was determined using a GST–E-cadherin pull-down assay. Cells were lysed for 30 min at 4°C. Protein concentrations in lysates were quantified, and equal quantities of total proteins from different samples were incubated with GST–E-cadherin Sepharose beads for 4 h at 4°C. After incubation, the beads were washed three times, and the bound proteins were eluted and separated via SDS/PAGE. Western blotting was performed using antibody to β-catenin.

Luciferase Reporter Assay. To examine reporter activities in response to Wnt3a stimulation and/or β-catenin expression, cells expressing control or LRP6 shRNA were transfected with TOPFlash/FOPFlash plasmids (Upstate) and treated with conditioned media from parental L cell or Wnt3a-expressing cultures (3) for 24 h. A β-gal reporter cDNA was cotransfected to normalize data for transfection efficiency. The luciferase and β-gal activities were measured by the Luciferase and β-gal Assay Systems, respectively, following the instructions of the manufacturer (Promega).

1. Bu G, Rennke S (1996) Receptor-associated protein is a folding chaperone for low density lipoprotein receptor-related protein. *J Biol Chem* 271:22218–22224.
2. Bafico A, Gazit A, Wu-Morgan SS, Yaniv A, Aaronson SA (1998) Characterization of Wnt-1 and Wnt-2 induced growth alterations and signaling pathways in NIH3T3 fibroblasts. *Oncogene* 16:2819–2825.
3. Shibamoto S, et al. (1998) Cytoskeletal reorganization by soluble Wnt-3a protein signalling. *Genes Cells* 3:659–670.

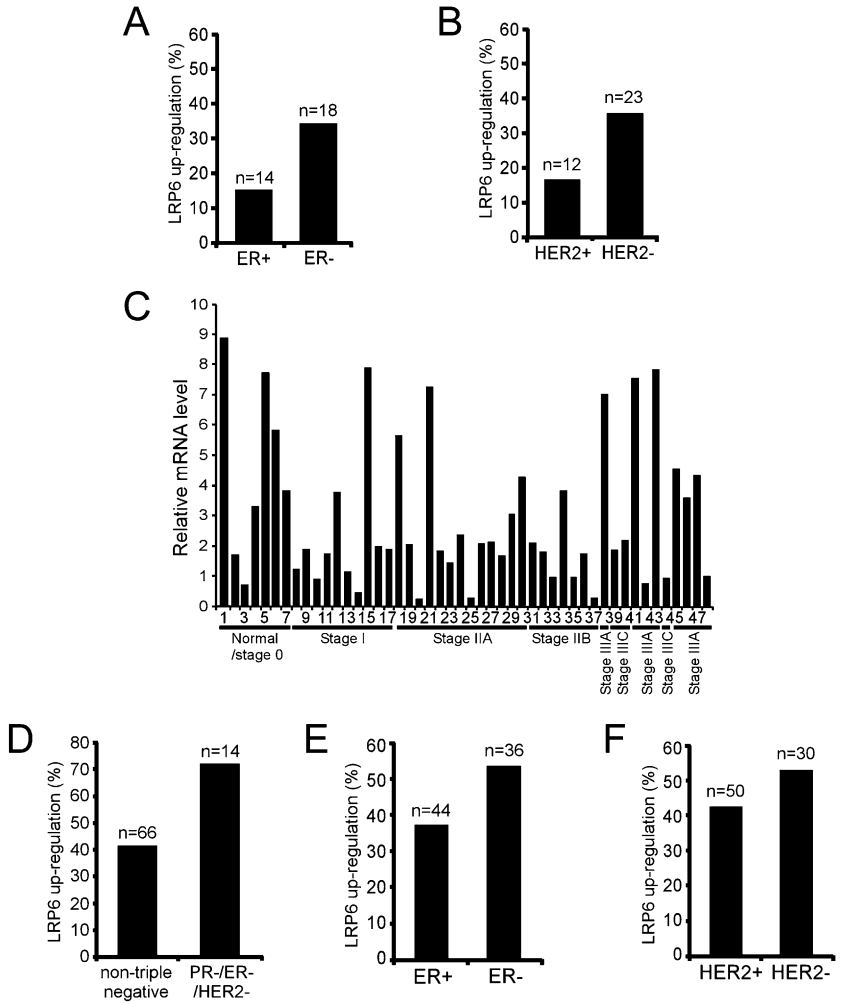

Fig. S1. LRP6 expression is up-regulated more frequently in ER-, HER2-, or triple-negative human breast tumors. (*A* and *B*) TissueScan Real-Time Breast Cancer Disease Panels (OriGene) were used for real-time PCR to quantitate LRP6 transcripts. Breast tumors with up-regulated LRP6 expression were analyzed against ER and HER2 status. Note that LRP6 is up-regulated more frequently in ER- or HER2-negative breast tumors. (*C*) Breast cancer TissueScan array was analyzed for LRP5 expression by real-time PCR. There are no significant changes of LRP5 expression between normal mammary tissues and breast cancer tissues. (*D–F*) Breast cancer tissue microarray (Biomax) was used for immunohistochemistry staining of LRP6 and the quantification of staining was evaluated as described in Fig. 1*C*. Breast tumors with up-regulated LRP6 expression were analyzed against ER and HER2 status. Note that LRP6 expression is up-regulated more frequently in ER-, HER2-negative, or ER/PR/HER2–triple-negative breast tumors.

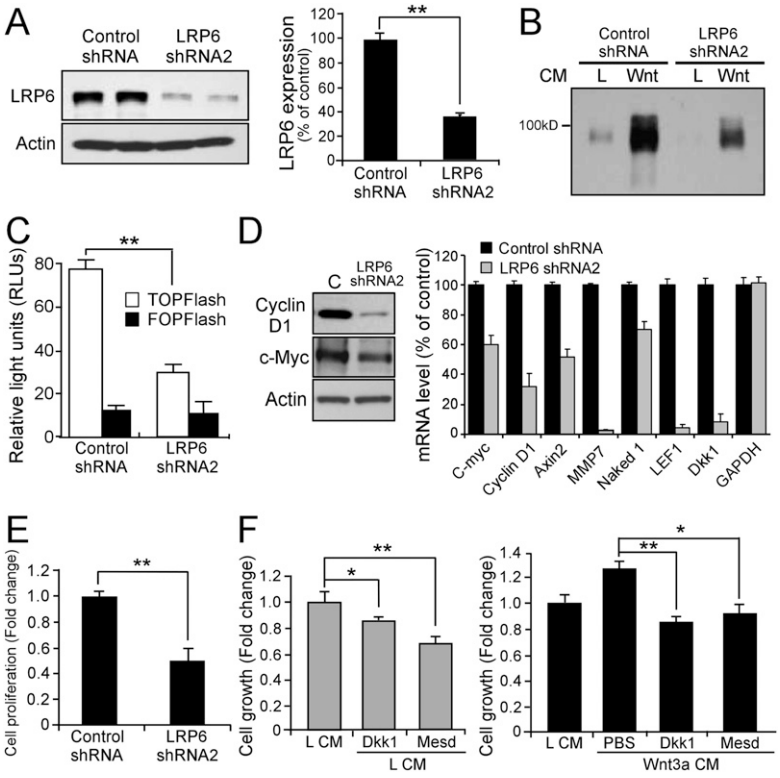

Fig. S2. Knockdown of LRP6 in HCC1187 breast cancer cells decreases Wnt signaling and cell proliferation. (*A*) Western blot analysis of LRP6 knockdown by lentiviral shRNA. (*Right*) Densitometric analysis of Western blots from triplicate samples. (*B*) Knockdown of LRP6 decreased Wnt signaling shown by GST–E-cadherin pull-down assay. (*C*) Cells expressing control or LRP6 shRNAs were transfected with TOPFlash or FOPFlash firefly luciferase reporter plasmids. Data are expressed as relative light units (RLUs). (*D*) Western blot analysis (*Left*) and quantitative real-time PCR (*Right*) of Wnt target gene expression after cells treated with control or LRP6 shRNAs. Data were normalized to actin expression and GAPDH was used as a control. (*E*) Proliferation of LRP6 knockdown cells was decreased by 50% as measured by BrdU assay. (*F*) (*Left*) HCC1187 cells were treated with L cell CM together with PBS, Dkk1 (50 nM) or Mesd (5 μM). (*Right*) HCC1187 cells were treated with L CM or Wnt3a CM together with PBS, Dkk1 (10 nM) or Mesd (1 μM). Viable cells were measured by the MTT assay. $*P < 0.05$; $**P < 0.01$.

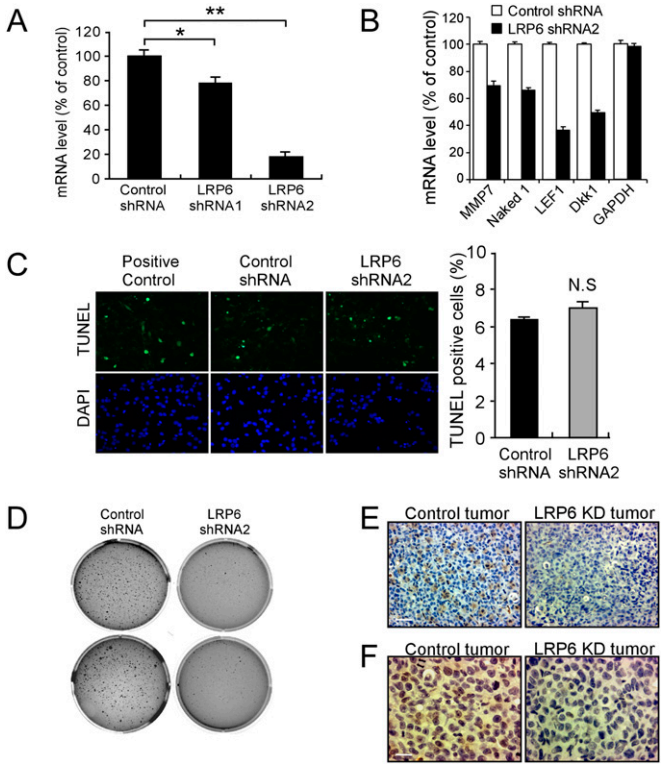

Fig. S3. LRP6 knockdown in MDA-MB-231 breast cancer cells and xenograft tumors. (*A–D*) Effects of LRP6 knockdown on apoptosis and soft agar colony formation in MDA-MB-231 breast cancer cells. (*A*) Quantitative real-time PCR showing reduction of LRP6 transcript after LRP6 shRNA lentiviral infection. (*B*) Quantitative real-time PCR of Wnt target gene expression after cells were treated with control or LRP6 shRNAs. GAPDH was used as a control. (*C*) Effect of LRP6 silencing on cell apoptosis evaluated by TUNEL staining. (*Right*) Quantification of TUNEL-positive cells from three randomly chosen fields. Note that LRP6 knockdown does not affect apoptosis. Data are mean ± SD from three independent experiments. $*P < 0.05$; $**P < 0.01$. (*D*) Representative images showing that LRP6 knockdown leads to a decrease in colony formation in soft agar assay. (*E* and *F*) Decreased LRP6 level and nuclear β-catenin staining in LRP6 knockdown xenograft tumors. (*E*) Lower-power images of LRP6 IHC staining in control and LRP6 KD xenograft tumors. (Scale bars, 100 μm.) (*F*) Immunohistochemical staining for β-catenin in control and LRP6 KD xenograft tumors. (Scale bars, 50 μm.)

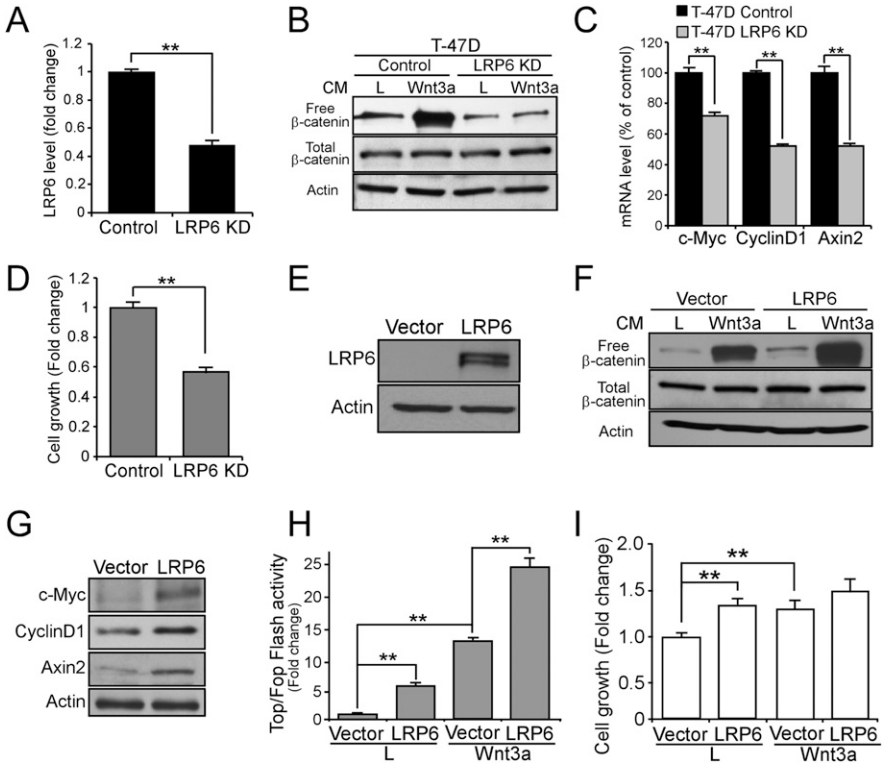

Fig. S4. Modulation of LRP6 expression in T-47D breast cancer cells alters Wnt signaling and cell growth. (*A–D*) T-47D cells were transduced with control or LRP6 lentiviral shRNAs. (*A*) Quantitative real-time PCR showing reduction of LRP6 transcript after knocking down of LRP6 (LRP6 KD). (*B*) Knockdown of LRP6 decreased Wnt signaling shown by GST–E-cadherin pull-down assay. (*C*) Quantitative real-time PCR shows that Wnt target gene expression (*c-Myc, Cyclin D1* and *Axin2*) was down-regulated in cancer cells expressing LRP6 shRNA. (*D*) Knockdown of LRP6 in T-47D cells decreased cell viability assessed by MTT assay. (*E–I*) T-47D cells were transfected with vector control or human LRP6 cDNA. (*E*) Western blot analysis showing the overexpression of LRP6. (*F*) Overexpression of LRP6 increased Wnt signaling shown by GST–E-cadherin pull-down assay. (*G*) Western blot analysis shows that Wnt target gene expression (*c-Myc, Cyclin D1,* and *Axin2*) was up-regulated in cancer cells overexpressing LRP6. (*H*) Cells experssing vector or LRP6 were treated with L or Wnt3a CM. Overexpression of LRP6 in T-47D cells enhanced Wnt signaling detected by TOPFlash reporter assay. (*I*) Overexpression of LRP6 in T-47D cells increased cell growth assessed by MTT assay.

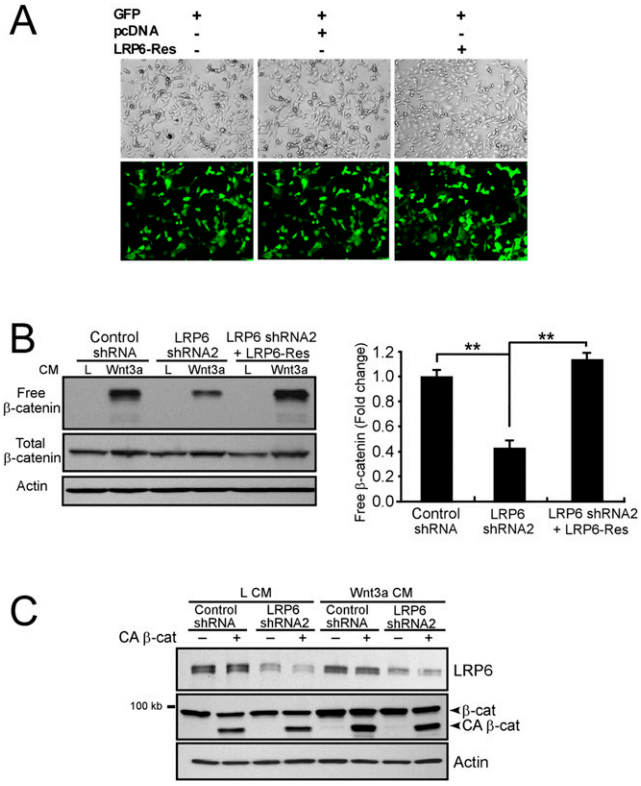

Fig. S5. shRNA-resistant LRP6 and CA β-catenin rescue Wnt signaling. (*A*) MDA-MB-231 cells expressing control or LRP6 shRNA were transfected with GFP along with vector control or shRNA-resistant LRP6 (LRP6-Res). The transfection efficiencies were similar under different conditions (85–90%). (*B*) Cells were treated with L or Wnt3a CM. Expression of LRP6-Res in MDA-MB-231 cells restored Wnt signaling, detected by GST–E-cadherin pull-down assay. (*C*) MDA-MB-231 cells expressing control or LRP6 shRNA were transduced with retrovirus expressing IRES-GFP vector control or CA β-catenin. The levels of LRP6, β-catenin, and CA β-catenin were determined by Western blot analysis.

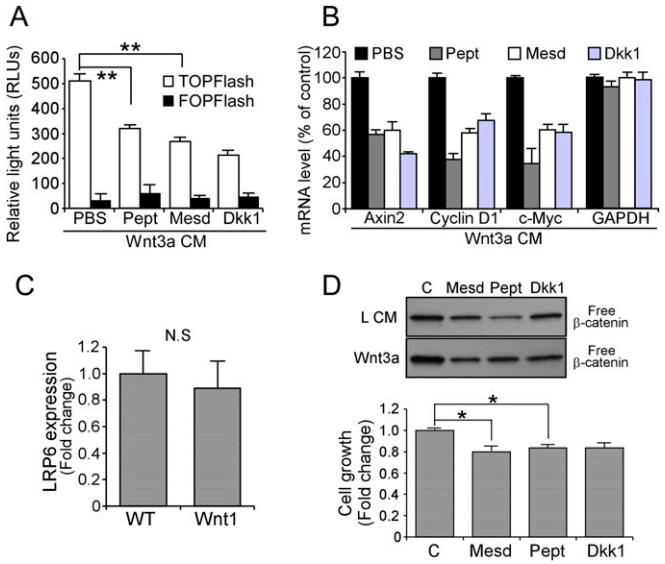

Fig. S6. Treatment with Mesd or Mesd peptide decreases Wnt signaling and cell growth in HCC1187 and T-47D breast cancer cells. (*A*) Mesd (5 μM), Mesd peptide (5 μM), and Dkk1 (50 nM) suppressed Wnt signaling activation in HCC1187 cells in the presence of Wnt3a ligands examined by TOPFlash reporter assay. (*B*) Wnt target gene expression (*c-Myc, Cyclin D1,* and *Axin2*) was down-regulated in HCC1187 cells treated with Mesd, Mesd peptide, and Dkk1. (*C*) LRP6 expression levels in mammary glands of MMTV-Wnt1 and age-matched wild-type mice examined by Western blot analysis using LRP6 specific antibody (Abcam). Results are shown as densitometric analysis of Western blots from triplicate samples. (*D*) Treatment with Mesd, Mesd peptide, or Dkk1 decreased Wnt signaling shown by GST–E-cadherin pull-down assay (*Upper*) and cell growth assessed by MTT assay (*Lower*) in T-47D breast cancer cells.

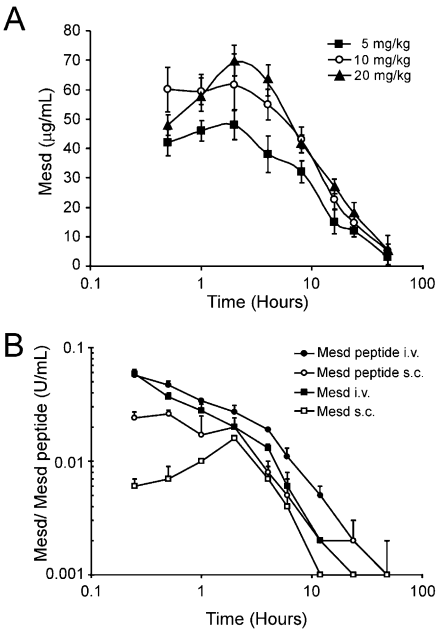

Fig. S7. Examination of pharmacokinetics of Mesd peptide and Mesd. (*A*) Amount of Mesd in serum following a single dose of protein administration. Mice were dosed at 5, 10, 20 mg/kg of Mesd via i.p. and the serum samples were collected from individual mice at the indicated time points. Levels of Mesd in serum were quantified by comparison with Mesd pure protein with known concentrations in Western blot analysis. Values are the average of triple determinations with SD indicated by error bars. After dosing, the protein was rapidly absorbed, with the highest concentration at ~2 h (*Tmax*). (*B*) To achieve a better sensitivity for detecting the pharmacokinetics of Mesd, each mouse was injected with ^{125}I-labeled Mesd or Mesd peptide via i.v. (tail vein) or s.c. routes. Samples were obtained by terminal bleed under anesthesia at each of the indicated time points (n = 3 animals per time point). Radioactivity was measured using a gamma counter and DPM converted to mass units per milliliter blood using the specific activity (DPM/mass) of each test substance. Data are plotted as mass units per milliliter test substance with standard deviation indicated by error bars. Distribution of Mesd and peptide into tissues (e.g., thyroid/parathyroid, liver, kidney, stomach, gastrointestinal tract) was rapid and widespread, with bioavailability averaging 60%.

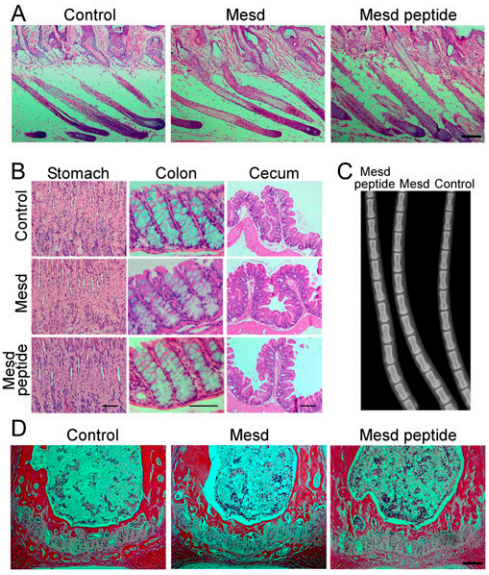

Fig. S8. Effect of Mesd and Mesd peptide administration on skin hair follicle and bone. (*A*) In mice treated with Mesd and Mesd peptide, skin hair follicles and sebaceous glands exhibit no apparent gross defects when examined by H&E staining (*B*) No significant adverse effect on stomach and large intestine with Mesd and Mesd peptide administration is apparent upon gross examination. (Scale bars, 50 μm.) (*C*) Representative x-ray image of tail vertebrae. No osteolytic bone lesion or outgrowth was observed upon Mesd and Mesd peptide administration. (*D*) H&E staining of tail vertebrae treated with PBS, Mesd, or Mesd peptide.

Table S1. Expression of Wnt-related genes in human breast cancer cell lines by real-time PCR–based SuperArray analysis

Cell line	*LRP6*	*LRP5*	*Dkk1*	*MYC*	*CCND1*	ER status
MDA-MB-157	0.35	1.15	9.85	4.92	0.29	−
MDA-MB-231	9.22	5.85	78.64	43.35	12.66	−
HCC1143	7.46	2.46	0.20	34.30	17.15	−
HCC1806	6.06	7.46	11.31	18.38	4.59	−
HCC38	6.06	2.83	2.46	13.00	0.41	−
HCC1187	25.99	6.06	0.27	36.76	8.57	−
SKBR3	0.10	6.96	2.46	42.22	0.71	−
HCC1937	4.57	6.00	16.53	12.31	2.83	−
MDA-MB-435s	1.23	0.57	4.29	4.29	0.44	−
MDA-MB-361	9.20	6.34	0.64	5.30	4.37	+
HCC1395	6.62	12.03	0.72	11.19	0.45	+
T-47D	1.71	6.28	0.63	5.93	1.84	+
CAMA1	1.74	9.85	0.13	19.70	2.83	+
MCF-7	3.03	4.92	22.63	21.11	6.06	+

Cell line	*Wnt2B*	*Wnt3a*	*Wnt4*	*Wnt7B*	*Wnt8A*	*Wnt16*
MDA-MB-157	25.48	702.35	45.25	0.38	0.52	0.81
MDA-MB-231	34.30	274.37	356.00	22.83	157.59	18.38
HCC1143	21.11	10.56	0.82	5.28	0.87	103.97
HCC1806	55.72	310.19	398.98	73.52	3.73	6.06
HCC38	222.86	14.93	16.02	0.44	1.87	3.48
HCC1187	6.96	311.01	776.05	17.15	12.13	8.57
SKBR3	1.74	17.15	289.31	42.22	9.85	4.29
HCC1937	47.16	464.94	325.88	21.05	8.01	0.68
MDA-MB-435s	2.83	9.19	1.32	0.12	3.03	6.96
MDA-MB-361	2.36	231.83	316.62	7.67	10.35	7.50
HCC1395	17.23	15.53	25.74	0.19	6.85	3.83
T-47D	1.49	38.39	297.97	6.78	7.85	1.15
CAMA1	19.70	7.46	1097.50	45.25	5.66	1.25
MCF-7	17.15	207.94	194.01	13.00	19.70	8.57

Numbers represent fold changes compared to the gene expression levels in nontransformed MCF-10A cells.