Supporting Information

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SI Materials and Methods

Transplantation Analysis. Northwestern University's Animal Care and Use Committee approved all procedures involving animals in this study. Cultured leiomyoma (LM) cells infected with adenovirus or freshly isolated LM side population (LMSP) or LM mature population (LMMP) cells with primary myometrial (MM) cells were used for xenotransplantation. Cells were suspended into rat-tail collagen (type I) solution (BD Biosciences) at 10^6 cells/10 μL (for cultured LM cells with adenoviral infection) or 10^5 cells/10 μL (for freshly isolated LMSP or LMMP cells cultured with primary MM cells). We have used this method successfully to study the hormonal response of human endometrial and LM tissue in the past (1–3). With this technique, the low-density collagen gel consists mostly of water, and thus the pellet volume (10 μL) does not reflect the starting volume of the tumor. When cell pellets are incubated at 37 °C overnight as floating cultures, they become smaller than 1 mm in diameter because of the contraction of the collagen by the LM and/or MM cells. Therefore, the estimated starting volume of the cell graft is smaller than 0.6 mm^3. The grafting procedure was performed as described previously (1). Cell pellets were grafted onto opposing kidneys of adult female NOD-SCID (*IL2Rγ*^{null}) mouse hosts (Jackson Laboratory). The cell pellets are high in water content and become smaller under the pressure of the subrenal capsule. The estimated starting volume of tissue grafts under the renal capsule was ∼1 mm^3. According to our previous report, estrogen and progesterone promote the growth of cells derived from uterine LM when transplanted under the kidney capsule (1). For this reason, all hosts were ovariectomized and supplemented with or without s.c. implantation of 80 mg progesterone (P) Sigma-Aldrich plus 80 μg estrogen (E) (Sigma-Aldrich). These doses were chosen because previous studies demonstrated that they were able to sustain systemic E_2 and P_4 levels within cycling women (4). The effects of ovariectomy and hormone treatments were confirmed by the gross appearance and histology of the host female reproductive tracts. The presence of hormone pellets also was confirmed at when the mice were killed.

Transfections and β-Catenin/T-Cell Factor Reporter Assay. MM and LM cells were transfected with a TCF Optimal luciferase Promoter plasmid (TOPflash) containing four consensus T-cell factor (TCF)-binding sites upstream of a minimal c-fos promoter or FOPflash containing four mutant TCF-binding sites (kindly provided by H. Clevers, Utrecht, The Netherlands), using Lipofectamine 2000 (Invitrogen). A thymidine kinase-Renilla plasmid (50 ng) also was included to normalize luciferase values to the efficiency of transfection. Cells were treated 24 h after transfection. Cells were solubilized 2 d after transfection using the Dual-Luciferase Assay Kit (Promega), and luciferase activity was quantified with a Synergy-HT plate reader (Bio-Tek Instruments, Inc.). Each reporter plasmid/condition was transfected into cells plated in triplicate wells, and data are expressed as the mean ± SD.

Cadherin-Free β-Catenin–Binding Assays. To evaluate activation of canonical wingless-type (WNT) signaling, intracellular levels of cadherin-free β-catenin were determined by pull-down assays using GST/β-catenin–interacting protein (ICAT), as previously described (5). Briefly, at the end of the experiments, cells were harvested, solubilized in a nonionic detergent buffer [1% Nonidet P-40 (Nonidet P-40), 50 mM Tris (pH 7.5), 150 mM NaCl, and 2 mM EDTA including protease inhibitors], and centrifuged at 14,000 × *g*. Cellular β-catenin was affinity-precipitated using GST-ICAT immobilized to glutathione-coupled Sepharose (Sigma), washed with buffer A [10 mM Tris (pH 8.0), 140 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, and 10 μg/mL leupeptin and aprotinin], and subjected to SDS/PAGE and immunoblot analysis using β-catenin antibody (BD Biosciences).

Immunofluorescence and Histological Analysis. Indirect immunofluorescence staining was performed as described previously (6, 7). Background fluorescence was determined by applying the secondary conjugated antibody alone and by replacement of the primary antibody with nonimmune serum. Slides were successively stained with various antibodies as listed in Table S1, followed by incubation with secondary antibodies. Images were collected using a confocal laser scanning system (LSM 510, Karl Zeiss) in combination with an inverted microscope (Axiovert 200, Karl Zeiss).

Hoechst 33342 Staining and FACS Analysis. The dissociated cells were resuspended at a concentration of 2×10^6 cells/mL in FACS solution (calcium- and magnesium-free HBSS containing 2% FBS). Hoechst 33342 (Sigma-Aldrich) was added at a final concentration of 5 μg/mL, and the sample was incubated at 37 °C for 90 min. A parallel aliquot was stained with Hoechst 33342 dye in the presence of 50 μM reserpine (Sigma-Aldrich). After incubation, the cells were centrifuged at 1,500 rpm for 7 min, resuspended in 2 mL cold FACS solution, and further incubated with 1 μg/mL propidium iodide (PI) (Sigma-Aldrich) to label nonviable cells. The cells were kept on ice at all times after staining with the Hoechst 33342 dye. The Hoechst dye- and PI-treated cells were subjected to flow cytometric analysis to separate LMSP cells from LMMP cells. The cells were subjected to FACS sorting and analysis by FACSAria (BD Biosciences), MoFlo (Cytomation), and LSR Fortessa (BD Biosciences). LM cells were sorted by FACSAria and MoFlo and analyzed using the FlowJo software (Tree Star). After collecting 1×10^5 events, the SP population was defined as previously reported (3, 6, 8–10). Forward scatter, side scatter, and PI gating excluded residual erythrocytes, debris, doublets, and dead cells. The purity of cell populations was verified directly after sorting as >98%. The viability of sorted cells exceeded 90% as assessed by trypan blue exclusion.

Ki-67 and TO-PRO-3 Staining. After mixed coculturing, PKH-26$^+$ cells were harvested and fixed in cold 70% ethanol. Cells then were stained with antibodies against Ki-67 (BD Biosciences) and with TO-PRO-3 iodide (Life Technologies). All data were collected on an LSR Fortessa and analyzed with FlowJo software.

Lactate Dehydrogenase Cell-Death Assay. A cell-death assay was performed on mixed cocultured cells as previously described (11). Lactate dehydrogenase activity was measured using a Cytotoxicity Detection Kit (Roche). OD_{490} was read using a Synergy-HT plate reader and software (Bio-Tek Instruments, Inc.).

WNT Quantitative RT-PCR Array and Quantitative Real-Time RT-PCR. Total RNA was extracted using the RNeasy Mini Kit, (Qiagen) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of RNA using the RT^2 First Strand cDNA synthesis kit (SA Biosciences). The RT^2 Profiler PCR arrays specific for the WNT signaling pathway (PAHS-043) was purchased from SA Biosciences and used according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed as

described previously (12, 13). *GAPDH* transcripts were measured as an internal control. Primer pairs used for each PCR are listed in the Table S2. Amplification was performed using the ABI 7900HT (SA Biosciences) for 40 cycles, with each cycle consisting of a 15-s denaturation at 95.0 °C followed by 1 min of annealing at 60.0 °C.

Statistical Analysis. Tumor volume was measured using the program ImageJ (National Institutes of Health, http://rsbweb.nih.gov.ezproxy.galter.northwestern.edu/ij/index.html). P values were calculated using the unpaired Student t test. One-way ANOVA followed by Turkey's test and nonparametric t test were used for other statistical analysis. Values of $P < 0.05$ are considered significant.

1. Ishikawa H, et al. (2010) Progesterone is essential for maintenance and growth of uterine leiomyoma. *Endocrinology* 151(6):2433–2442.
2. Kurita T, et al. (2005) The activation function-1 domain of estrogen receptor alpha in uterine stromal cells is required for mouse but not human uterine epithelial response to estrogen. *Differentiation* 73(6):313–322.
3. Ono M, et al. (2012) Role of stem cells in human uterine leiomyoma growth. *PLoS ONE* 7(5):e36935.
4. McDonnel AC, Van Kirk EA, Isaak DD, Murdoch WJ (2005) Effects of progesterone on ovarian tumorigenesis in xenografted mice. *Cancer Lett* 221(1):49–53.
5. Gottardi CJ, Gumbiner BM (2004) Role for ICAT in beta-catenin-dependent nuclear signaling and cadherin functions. *Am J Physiol Cell Physiol* 286(4):C747–C756.
6. Masuda H, et al. (2010) Stem cell-like properties of the endometrial side population: Implication in endometrial regeneration. *PLoS ONE* 5(4):e10387.
7. Kurita T (2010) Developmental origin of vaginal epithelium. *Differentiation* 80(2-3): 99–105.
8. Mas A, et al. (2012) Identification and characterization of the human leiomyoma side population as putative tumor-initiating cells. *Fertil Steril* 98(3):741–751.e746.
9. Gargett CE, Chan RW, Schwab KE (2008) Hormone and growth factor signaling in endometrial renewal: Role of stem/progenitor cells. *Mol Cell Endocrinol* 288(1-2): 22–29.
10. Chang HL, et al. (2010) Uterine leiomyomas exhibit fewer stem/progenitor cell characteristics when compared with corresponding normal myometrium. *Reprod Sci* 17(2):158–167.
11. Flozak AS, et al. (2010) Beta-catenin/T-cell factor signaling is activated during lung injury and promotes the survival and migration of alveolar epithelial cells. *J Biol Chem* 285(5):3157–3167.
12. Xue Q, et al. (2007) Transcriptional activation of steroidogenic factor-1 by hypomethylation of the 5′ CpG island in endometriosis. *J Clin Endocrinol Metab* 92(8): 3261–3267.
13. Ishikawa H, et al. (2008) CCAAT/enhancer binding protein beta regulates aromatase expression via multiple and novel cis-regulatory sequences in uterine leiomyoma. *J Clin Endocrinol Metab* 93(3):981–991.

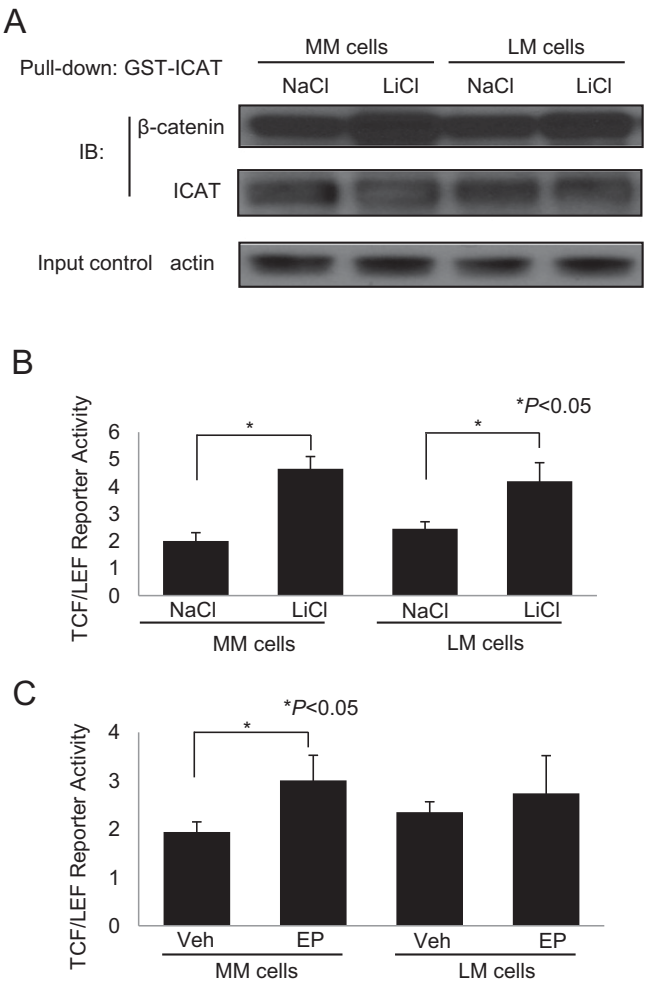


Fig. S1. β-Catenin transcriptional activity in MM and LM cells. (*A*) Whole-cell lysates were subjected to GST/β-catenin–interacting protein (ICAT) pull-down assays. Active β-catenin binding to ICAT was probed with a β-catenin antibody. Representative immunoblots (IB) from three independent samples are shown. Lysates from MM and LM cells treated with or without LiCl were affinity-precipitated with GST-ICAT to isolate cytosolic β-catenin. Actin-loading controls were taken from inputs. (*B*) LiCl induced WNT activity in both MM and LM cells. Cells were transiently cotransfected with a Renilla luciferase reporter construct (pRL-CMV) and either the firefly luciferase TOPflash TCF reporter plasmid or the FOPflash TCF mutant reporter plasmid before treatment with LiCl or NaCl for 24 h. Firefly luciferase activities first were normalized to the corresponding Renilla luciferase activity to normalize for transfection efficiency. The adjusted TOPflash activity then was normalized to the corresponding adjusted FOPflash activity to account for background reading of the TOPflash construct. The results are representative of six separate experiments. (*C*) E+P treatment induced WNT activity in MM cells. $n = 6$. $^*P < 0.05$.

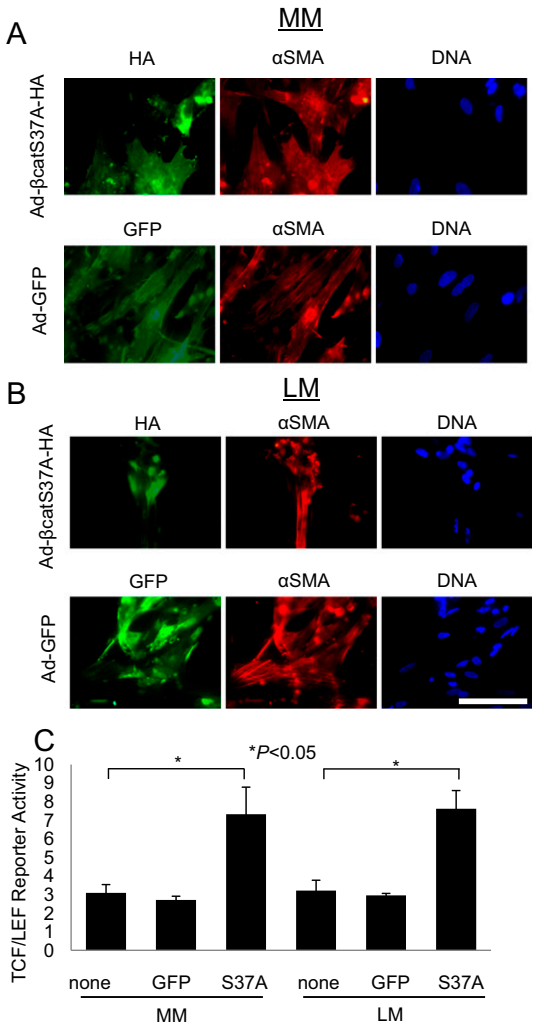


Fig. S2. Nondegradable β-catenin can activate the TCF/LEF reporter activity but cannot alter WNT secretion patterns in MM and LM cells. (*A* and *B*) Immunofluorescence of MM and LM cells infected with Ad-GFP and Ad-S37A–β-catenin–HA for 48 h. Immunostaining was performed to confirm the presence of HA, GFP, and α-smooth muscle actin (α-SMA); cell nuclei were stained with Hoechst. (Scale bar, 100 μm.) (*C*) Activation of β-catenin/TCF signaling in MM and LM cells by adenoviruses expressing S37A–β-catenin shows increased signaling and stabilization of β-catenin. $*P < 0.05$.

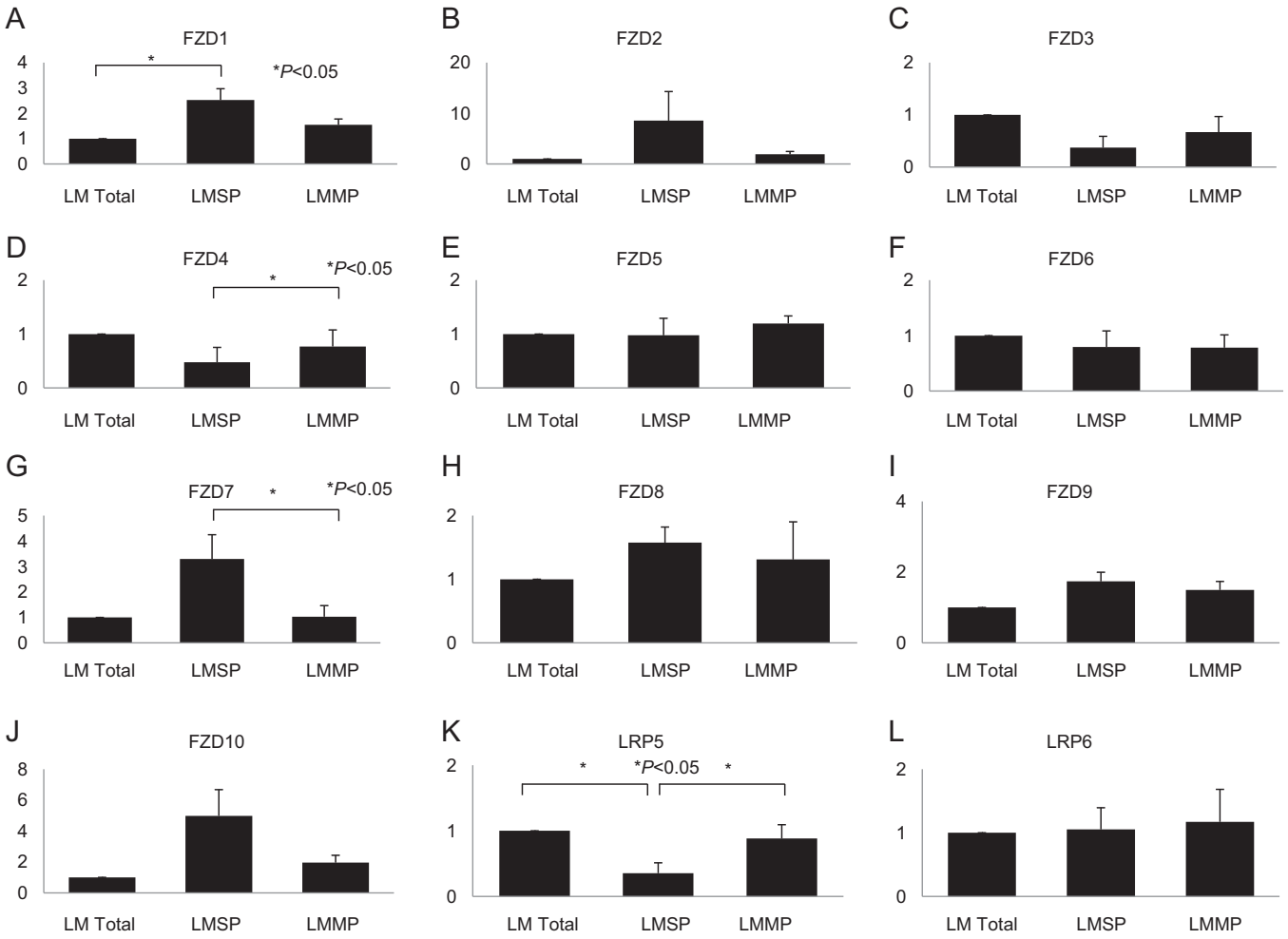
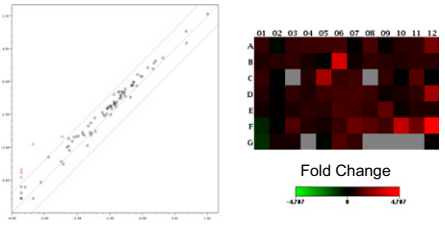


Fig. S3. Expression of Frizzled (FZD) and low-density lipoprotein-related receptor protein 5/6 (LRP5/6) genes in LMSP and LMMP cells. RNA was extracted from LMSP, LMMP, and total LM cells and then was subjected to reverse transcription to the first-strand cDNA. PCR amplification of the cDNA was conducted using specific primers for human *FZD1* through *FZD10* (*A–J*) or LRP5 versus LRP6 (*K–L*).



mRNA	E+P FOLD INDUCTION
WNT1	2.09
WNT10A	8.61
WNT11	3.29
WNT16	17.6
WNT2	0.54
WNT2B	1.38
WNT3	1.4
WNT3A	1.43
WNT4	0.83
WNT5A	2.42
WNT5B	1.86
WNT6	1.43
WNT7A	1.43
WNT7B	1.43
WNT8A	1.43
WNT9A	1.12

Fig. S4. E+P treatment alters the expression of WNT genes in MM cells. MM cells were treated with E+P or vehicle. RNA was isolated using the RNeasy Mini Kit (Qiagen), and a cDNA library was prepared using the RT^2 First Strand Kit (SA Biosciences). The WNT quantitative RT-PCR array (PAHS-043; SA Biosciences) was performed according to the manufacturer's specifications and used internal controls. Red designates genes that are up-regulated more than twofold in cells relative to controls; black designates genes that show a fold change between −2 and +2.

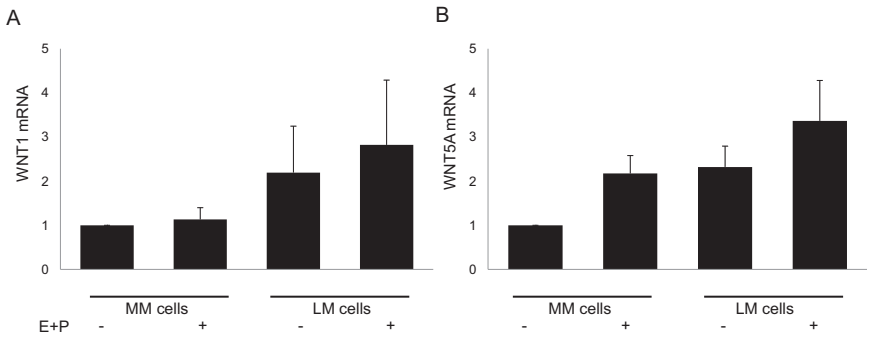


Fig. S5. E+P treatment does not alter the expression of *WNT1* and *WNT5A* genes in MM cells. (*A* and *B*) MM and LM cells were treated with E+P or vehicle before isolation of RNA and evaluation of WNT expression by real-time quantitative PCR as described in *SI Materials and Methods*. Results represent the mean of a minimum of four independent experiments. Error bars indicate SD. $*P < 0.05$.

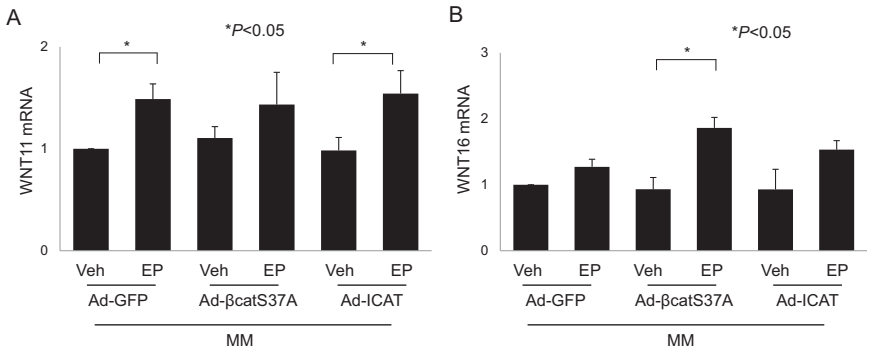


Fig. S6. Nondegradable β-catenin can activate the TCF/LEF reporter activity but cannot alter WNT secretion patterns in MM cells. (*A* and *B*) MM cells infected with adenovirus expressing S37A–β-catenin or ICAT for 48 h did not alter the pattern of *WNT11* and *WNT16* gene expression pattern after E+P treatment. Error bars indicate SD. $*P < 0.05$.

Table S1. Antibodies used in this study

Antgigen	Clone	Isotype	Supplier
β-Catenin	14	Mouse IgG1	BD Biosciences
ICAT	Polyclonal	Rabbit IgG	Santa Cruz Biotechnology
β-Actin	AC-15	Mouse IgG1	Sigma-Aldrich
HA	16B12	Mouse IgG1	Covance
α-SMA	1A4	Cy3-conjugated mouse IgG2a	Sigma-Aldrich
GFP	MAB3580	Mouse IgG1	Millipore
Ki-67	B56	FITC-conjugated mouse IgG1	BD Biosciences

Table S2. Primer sets used in this study

Gene	Primer sets	Accession No.
WNT1	TTCTCCGGGTCCTCCTAAGT TTTTGCCCTGTAACCTCCTG	NM_005430
WNT5A	TAAGCCCAGGAGTTGCTTTG GCAGAGAGGCTGTGCTCCTA	NM_1256105
WNT10A	ACACAGTGTGCCTAACATTGC TGGATGGCGATCTGGATGC	NM_25216
WNT11	CTGACCTCAAGACCCGATACC CGAGTTCCGAGTCCTTCACAG	NM_004626
WNT16	GCCCAAGGAAACTGGATGTG GCTCCCGCACTCCTGAATG	NM_57168
GAPDH	GAAGGTGAAGGTCGGAGTC GAAGATGGTGATGGGATTTC	NM_002046
WNT16A (gel)	CAGAAAGATGGAAAGGCACC ATCATGCAGTTCCATCTCTC	NM_16087
WNT16B (gel)	TGCTCGTGCTGTTCCCCTAC ATCATGCAGTTCCATCTCTC	NM_57168
GAPDH (gel)	TCACCATCTTCCAGGAGCG CTGCTTCACCACCTTCTTGA	NM_002046
AXIN2	ACAACAGCATTGTCTCCAAGCAGC GCGCCTGGTCAAACATGATGGAAT	NM_004655
FZD1	ACTGACCAAATGCCAATCCA CCTACCTCAACTACCACTTCCT	NM_003505
FZD2	CCCACCCGTCTTTATCACTT TTTCCCTGCAGTCTATTCACTC	NM_001466
FZD3	GTAGTCTCTCCTCCATTCCTCT GAAAGCATCCACAGCAAAGTG	NM_145866
FZD4	CTTCAGGACGGGTTCACA TCATCCAGTACGGCTGCT	NM_012193
FZD5	ACCGTCCAAAGATAAACTGCT GGACTGTCTGCTCTTCTCG	NM_003468
FZD6	CAGGAACAGTCTCATCACAGT ACCTTGTCGTAAACTTTGTGAGA	NM_001164615
FZD7	GCAGTTCTTTTCCCTACCGT CTACCACAGACTTAGCCACAG	NM_003507
FZD8	GAATACCCAAACCGTGCAATC TTCTCCCTCGACTCTTCCTAC	NM_031866
FZD9	CGCCAGAAGTCCATGTTGA GTCTTCTCCATCCTCTACACG	NM_003508
FZD10	TCTTCCAGTAATCCATGTTGAGG AGAAGCTCATGGTGCGTATC	NM_007197
LRP5	GCTGTAGATGTCGATGCTGAG AGAACATCAAGCGAGCCAA	NM_002335
LRP6	ACGCCAATAACAGAACCAACT GATTGCAGTGACAAGTCAGATG	NM_002336