FZD5 regulates cellular senescence in human mesenchymal stem/stromal cells

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Supplemental Information
• Supplemental Figures S1-6
• Supplemental Tables S1-2
• Supplemental Materials and Methods
• Supplemental References
Supplemental Fig. S1. Screening REC-specific genes.

Search for REC-specific genes using microarray analysis. The population of LNGFR⁺THY-1⁺ BM-MNCs was sorted into 96 well culture dishes. Single LNGFR⁺THY-1⁺-derived clones were divided into distinct REC, MEC and SEC subgroups [1]. (A) Morphology, adipocyte differentiation, osteoblasts and chondrocytes stained with Giemsa, Oil Red-O, ALP and Alcian blue, respectively. Senescence was assessed using SA-b-gal and F-actin (Phalloidin, green), α-tubulin (red), and Hoechst DNA (blue) staining. Intravenous infusions of hMSCs and localization of REC or MEC and SEC with Venus-ffLuc engrafted in mice. Scale bars, 1 mm for morphology and 100 μm for others. (B) Line plots of genes with differentially regulated expression in REC, MEC, and SEC (number of probes, 41,000). Gene expression is shown in red (> 2.0-fold higher in REC), blue (< 2.0-fold lower in REC), and yellow (intermediate in REC). (C) Genes (n = 57) were clustered as follows. Expression of genes in group 1 was higher in REC than in MEC and SEC (fold change, > 2-fold). Expression of genes was moderate (group 2, 1- to 2-fold) and others (group 3 and 4). We confirmed gene expression using quantitative PCR (Group 1: FZD5, KLRC4, BG199525, and METTL7A, Group 2: HMGN2, PABPN1, AK025353, COX4I1, LOC652489, A24_P315644, FZD2, C11orf51, CCDC23, CNP, GABARAP, HNRPR, RNF181, LRRC29, MRPS15, NFYB, TARDBP, TINP1, and TRADD). (D) Top 21 REC-related pathways derived from gene ontology analysis using Ingenuity Pathway Analysis (IPA). Identified pathways are shown on x-axis. Left y-axis, -log of P. Threshold (yellow line) indicates minimum significance (-log P; Fisher exact tests, set here at 1.3). Right y-axis displays ratios of input genes mapped to considered pathways divided by total number of genes from IPA database (orange dots connected by a line). Red squares, Wnt-related signals.
Supplemental Fig. S2. Expression of FZD5 in mouse MSCs.

Mouse bone marrow cells were sorted from the tibias and femurs of 8- to 10-week-old C57BL/6 mice. Levels of FZD5 expression in PDGFR-a^{-}Sca-1^{+} (purified mouse MSCs [2]), PDGFR-a^{-}Sca-1^{-}, PDGFR-a^{-}Sca-1^{+}, and PDGFR-a^{-}Sca-1^{-} fractions were determined by quantitative PCR (n = 3). *P < 0.05, n.d., not detected.
Supplemental Fig. S3. Canonical WNT pathway involvement in REC.

(A–C) Relative AXIN2 (A), LRP5 (B), and LRP6 (C) mRNA expression in REC, MEC, and SEC. Expression of AXIN2, LRP5, and LRP6 mRNA is normalized against that of GAPDH mRNA. Mean expression in REC was adjusted to 100. Three clones of each cell type are shown. (D) Western blots of active (dephosphorylated) and total β-catenin in REC, MEC, and SEC. Internal control was α-tubulin. (E) TOP/FOP flash assay of REC, MEC, and SEC (n = 3). Three clones of each cell type are shown. (F) TOP/FOP flash assays of cultured REC cells with various added recombinant proteins. n.s., not significant. *P < 0.05.
Supplemental Fig. S4. Expression of WNT in REC and WNT5A in BM-MNCs.

(A) Quantitative PCR of several WNTs expressed in REC, MEC, and SEC. Ratios of WNT mRNA to GAPDH mRNA were calculated from three clones in each condition (n = 3). (B) Q-PCR analysis of WNT5A expressed in each fraction immediately after sorting from BM-MNCs. Expression of the LNGFR+THY-1+ fraction was set to 100 (n = 3). (C) Western blotting analysis of WNT5A expression in RECs (20%FBS or 1%FBS containing culture medium). The internal control was GAPDH. *P < 0.05, **P < 0.01, ***P < 0.001. n.s., not significant. n.d., not detected.
Supplemental Fig. S5. Analysis of RNA sequences in shFZD5-REC and shCTRL.

(A) Senescence and cell cycle-related genes (RB1, RB2, CBX3, and CDK2). (B) Non-canonical Wnt signal-related genes (AP1, PLCB1, SDC1, PRKACA, and PRKCA).
Supplemental Fig. S6. Western blots of non-canonical WNT related pathways in clonal MSCs.

(A–F) Western blots of non-canonical WNT related pathways: JNK (A), p38 MAPK (B), PKC (C), CaMKII (D), ERK (E) and c-Jun (F). Average values calculated for each band: p-JNK, REC:1.00/SEC:1.49, p-p38MAPK, REC:1.00/MEC:1.23/SEC:1.35, p-PKC, REC:1.00/MEC:1.61/SEC:2.14; p-CaMKII, REC:1.00/MEC:1.17/SEC:1.54, p-ERK, REC:1.00/SEC:2.63; p-c-Jun, REC:1.00/SEC:0.00. (G, H) Activation of RAC1 and CDC42 by Wnt5A analyzed in REC using pull-down assays.
Supplemental Table S1. Primers and shRNA sequences.

| Gene          | Forward primer                      | Reverse primer                      |
|---------------|-------------------------------------|-------------------------------------|
| GAPDH         | CGAAGATCCCTCAAACTCA                | CATGAGTCCCTCTACGAGCA               |
| F2DC          | TCTTGTATGAGACCTGTG                 | CTGCTGACCTGCTGCTCTCTCTCTCTCTCTCTCT |
| ROR2          | CGTACCGGAGGCTGCTG                  | ATGTGACGCTGCTGCTGCTGCTGCTGCTGCTGCTG |
| PTEN          | TCTGCTGACGCTGCTGCTG                | CGTCTGACGCTGCTGCTGCTGCTGCTGCTGCTG |
| PRKC          | GACAGCTCGAGGCTGCTG                  | TTGGTACGCTGCTGCTGCTGCTGCTGCTGCTG |
| PPARG         | GACCACCACTCCACTCTCTGTA             | GGAGCACTGATCTGTCTGCTGCTGCTGCTGCTG |
| C3BP          | GAACATGTCTGTGCTGCTG                 | CAGCTGACGCTGCTGCTGCTGCTGCTGCTGCTG |
| PHI           | GAGAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
## Supplemental Table S2. Primary antibodies and suppliers

| Antibody Name | Supplier | Catalog Number | Host/Isotype | Working Dilution |
|---------------|----------|----------------|--------------|-----------------|
| GAPDH, 14C10 | Cell Signalling Technology | 2118 | Rabbit IgG | WB 1:2000 |
| FZD5, 6F5B9  | Medical and Biological Laboratories | W362-3 | Mouse IgG2a | WB 1:1000-500, ICC 1:250 |
| β-catenin    | BD Transduction Laboratories | 610154 | Mouse IgG1 | WB 1:1000 |
| active β-catenin, 8E7 | Millipore | 05-665 | Mouse IgG1 | WB 1:1000 |
| α-Tubulin, DM1A | Sigma | T9026 | Mouse IgG1 | WB, ICC 1:2000 |
| β-Actin, AC-15 | Sigma | A1978 | Mouse IgG1 | WB 1:2000 |
| ROR2, 6F12A2 | Medical and Biological Laboratories | W360-3 | Mouse IgG2b | WB 1:500, ICC 1:250-500 |
| ROR2        | Cell Signalling Technology | 4105 | Rabbit IgG | WB 1:500 |
| F-actin, NH3 | Abcam | Ab6205 | Mouse IgM | ICC 1:250 |
| vH2AX       | Millipore | 05-636 | Mouse IgG1 | ICC 1:1000 |
| 53BP1       | Novus Biologicals | N1B100-304 | Rabbit IgG | ICC 1:1000 |
| HA-Tag, C29F4 | Cell Signalling Technology | 3724 | Rabbit IgG | WB 1:1000, ICC 1:1600 |
| anti-GST tag mAb | Medical and Biological Laboratories | PM4013 | Rabbit IgG | WB 1:1000 |
| WNT5A       | Abcam | ab68720 | Mouse IgG1 | WB 1:1000 |
| JNK         | Cell Signalling Technology | 9252 | Rabbit IgG | WB 1:1000 |
| p-JNK       | Abcam | ab4821 | Rabbit IgG | WB 1:2000 |
| p38 MAPK    | Cell Signalling Technology | 9212 | Rabbit IgG | WB 1:1000 |
| p-p38 MAPK  | Cell Signalling Technology | 9211 | Rabbit IgG | WB 1:500 |
| PCK         | Novus Biologicals | NB100-92211 | Rabbit IgG | WB 1:1000 |
| p-PCK, 190D10 | Cell Signalling Technology | 2060 | Rabbit IgG | WB 1:1000 |
| CaMKII, D11A10 | Cell Signalling Technology | 4436 | Rabbit IgG | WB 1:1000 |
| p-CaMKII, Thr286 | Cell Signalling Technology | 3361 | Rabbit IgG | WB 1:1000 |
| ERK1/2, 137F5 | Cell Signalling Technology | 4695 | Rabbit IgG | WB 1:1000 |
| p-ERK1/2, D13,14,14E | Cell Signalling Technology | 4370 | Rabbit IgG | WB 1:2000 |
| c-JUN, 60A8 | Cell Signalling Technology | 9165 | Rabbit IgG | WB 1:1000 |
| p-c-JUN, 54B3 | Cell Signalling Technology | 2361 | Rabbit IgG | WB 1:1000 |
| CDC42       | Cytoskeleton | ARC03-A | Mouse IgG1 | WB 1:1000 |
| ROR2-APC, 7C9D6 | Cell Signalling Technology | 2462S | Rabbit IgG | WB 1:1000 |
| LN5FR-PE    | Medical and Biological Laboratories | W361-3 | Mouse IgG2a | - |
| THY-1-APC   | Miltenyi Biotec | 130-098-111 | Mouse IgG1 | - |
| THY-1-FITC  | BD Pharmingen | 555595 | Mouse IgG1 | - |
| CD45-PE/Cy7 | BD Pharmingen | 557748 | Mouse IgG1 | - |
| GPA-PE/Cy7  | BD Pharmingen | 563666 | Mouse IgG1 | - |
| mouse IgG-APC | BD Pharmingen | 550854 | Mouse IgG1 | - |
| CD45-PE     | BD Pharmingen | 553081 | Rat IgG | - |
| Ter-119-PE  | BD Pharmingen | 553673 | Rat IgG | - |
| CD31-PE     | BD Pharmingen | 550274 | Rat IgG | - |
| CD29-PE     | BD Pharmingen | 562801 | Rat IgG | - |
| CD49c-PE    | BD Pharmingen | 553319 | Rat IgG | - |
| Gr-1-PE     | BD Pharmingen | 553128 | Rat IgG | - |
| CD90-PE     | BD Pharmingen | 553005 | Rat IgG | - |
| B220-PE     | BD Pharmingen | 561878 | Rat IgG | - |
| Sca-1-FITC  | BD Pharmingen | 557405 | Rat IgG | - |
| PIGFRa-APC  | BD Pharmingen | 562777 | Rat IgG | - |

*WB: western blot, ICC: immunocytochemistry*
SUPPLEMENTAL MATERIALS AND METHODS

Cell preparation and cell sorting with flow cytometry
We used Poietics™ human BM-MNCs (Lonza Group Ltd., Basel, Switzerland). Human MSCs were sorted from human BM-MNCs (Lonza), as we previously described [1]. Human BM-MNCs (2M-125C) was incubated at 37°C. At the moment of melting, Hank’s Balanced Salt Solution (HBSS; Nacalai Tesque, Kyoto, Japan) containing 2% fetal bovine serum (FBS; HyClone Laboratories Inc., South Logan, UT, USA) was added to the vial at 37°C, then the cells were transferred to tubes and pelleted by centrifugation at 1,200 rpm (630 g) at room temperature for 5 minutes. The supernatant was removed and the cells were incubated with HBSS containing antibodies to LNGFR-PE (Miltenyi Biotec, Biotec, Bergisch Gladbach, Germany) and THY-1-APC (BD Pharmingen, San Diego, CA, USA) for 30 minutes on ice. The cells were pelleted by centrifugation, the supernatant was removed, then the cells were suspended in HBSS containing propidium iodide (PI, Sigma-Aldrich) and filtered through a cell strainer (BD Falcon, Franklin Lakes, MA, USA). The cells were separated into subpopulations using triple-laser MoFlo (Beckman Coulter, Brea, CA, USA), FACS Vantage SE, or FACS Aria III (Becton Dickinson and Co., Franklin Lakes, MA, USA) cell sorters. Data were analyzed using FlowJo software (BD).

Cell culture and differentiation assays
Human MSCs were cultured in DMEM (Nacalai Tesque) containing 20% FBS and 10 mM HEPES (all from Nacalai Tesque), 10 ng/mL basic fibroblast growth factor (bFGF, recombinant human FGF-basic) (Peprotech, Inc., Rocky Hill, NJ, USA), and 1% penicillin-streptomycin (Nacalai Tesque) [1]. When WNTs are analyzed, the cells were cultured in DMEM containing 1% FBS, 10 mM HEPES, and 1% penicillin-streptomycin. Human MSCs were cultured with osteogenic induction medium (Lonza), for two weeks and differentiated into osteoblasts. Likewise, adipocytes were induced and maintained with adipogenic induction and maintenance medium (Lonza), respectively. Human MSCs were incubated in chondrogenic induction medium (Lonza) containing 10 ng/mL of transforming growth factor-β3 (TGF-β3; Lonza) and 500 ng/mL of bone morphogenetic protein-6 (BMP-6; R&D Systems Inc., Minneapolis, MN, USA) for three weeks for chondrogenic differentiation. Adipocytes were stained with Oil red O (Muto Pure
Chemicals Ltd., Tokyo, Japan). Osteoblasts were stained using an alkaline phosphatase staining kit (Nichirei Corp., Tokyo, Japan). Chondrocytes were stained with alcian blue solution (Diagnostic Biosystems, Pleasanton, CA, USA).

**RNA purification, cDNA synthesis and quantitative PCR**

We purified total RNA using RNeasy Mini or RNeasy Micro Kits (Qiagen GmbH, Hilden, Germany) and synthesized first-strand cDNA with an AffinityScript multiple-temperature cDNA synthesis kit (Agilent Technologies Inc., Santa Clara, CA, USA). We synthesized cDNA for quantitative PCR using ReverTra Ace qPCR RT master mix (Toyobo Co., Ltd., Osaka, Japan), and fast SYBR Green or Power SYBR Green PCR master mix (Life Technologies Corporation, Carlsbad, CA, USA) and HT7900 fast or ViiA7 real-time PCR systems (both from Applied Biosystems, Waltham, MA, USA). Table S1 shows the primer sequences.

**Microarray analysis**

hMSCs (RECs, MECs, and SECs) without abnormal copy numbers were cultured, then total RNA was extracted. Total RNA (300 ng) was labeled with Cy3, hybridized to human whole-genome chips (4x44K, AMADID, 14850; Agilent Technologies), then gene expression was determined using a microarray scanner system and the genes were screened using GeneSpring GX10 software (Agilent Technologies). We normalized the signal intensity of each chip at the 75th percentile according to the signals of all 41,000 spots on each chip. We then selected candidate genes as follows: 1) created from advanced analysis, significance; 2) entity list, significantly high in at least one sample; 3) interpretation, type; 4) experiment, GE per gene norm; 5) cut off, p = 0.05; 6) post-hoc test, Tukey HSD; 7) selected test, one-way ANOVA; 8) p computation, asymptotic; 9) multiple-test correction, Benjamini-Hochberg. The candidate genes were split into four clusters according to K-means (n = 57). We confirmed that their expression was > 2-fold (Group 1) and 1- to 2-fold (Group 2) higher in REC than in MEC/SEC using quantitative PCR. The results are described in our previous report (Gene Expression Omnibus (GEO) number GSE86369).

**RNA sequencing**

We sequenced RNA using TruSeq RNA Sample Prep Kits (Illumina Inc., San Diego, CA,
USA) as described by the manufacturer. The generated library was sequenced using a HiSeq 2500 platform (Illumina). Base-calling and chastity filtering were accomplished using Real-Time Analysis Software version 1.18.61 and raw reads were mapped to reference genome hg19 using Sailfish (v0.7.6) with default settings. Gene expression profiles of indicated genes were evaluated and visualized using Exatlas (https://lgsun.irp.nia.nih.gov/exatlas/).

**TOP/FOP flash assay**

Human MSCs (RECs, MECs, and SECs; each $1 \times 10^5$/well) were seeded into 24-well plates. On the following day, the medium was changed to OPTI-MEM (Gibco Laboratories, Gaithersburg, MD, USA). In total, 0.98 µg/well of TOPV-tk-Luc or FOP-tk-Luc and 0.02 µg/well EF-Rluc were used for transfection. The cells were co-transfected with stabilized β-catenin using 0.49 µg/well TOPV-tk-Luc or FOP-tk-Luc, 0.49 µg/well stabilized β-catenin, and 0.02 µg/well EF-Rluc plasmids and ViaFect transfection reagent (Promega Corp., Madison, WI, USA) as described by the manufacturer. The medium was replaced with medium optimized for each condition 24 hours after transfection. Luciferase activity was assayed 48–72 hours later using the Dual-luciferase reporter assay and GloMax-multi detection system (Promega) that includes cells transfected with the Super TOP Flash reporter gene carrying the wild-type TCF binding region (CCTTTGATC). Quantifiable changes in luciferase activity (TOP activity) in samples reflect TCF/β-catenin transcriptional activities that are the furthest downstream from the Wnt signal. Those that selectively increased only TOP activity (not affecting FOP) were considered to have TCF/β-catenin transcriptional inhibition (Wnt signal inhibition) activity [3,4].

**G-tail telomere hybridization protection assay (Gt-telomere HPA)**

The Gt-telomere HPA has the advantage of accurate and sensitive short G-tails of ≤ 20 nucleotides [5]. We extracted DNA from shCTRL-REC and shFZD5-REC, and the amount of telomere probes bound to the DNA was measured as luminescence intensity using an EnVision® (PerkinElmer Life and Analytical Sciences Inc., Waltham, MA, USA) plate reader. The results were calculated as luminescence values (relative light units, RLU) of telomere length and telomere G-tail length per µg of DNA.
**Plasmid construction**

We designed an shRNA for FZD5 (shFZD5) using Block-iT RNAi designer (Invitrogen, Carlsbad, CA, USA) and recombined shFZD5 into pENTR4-H1. We conducted LR recombination as using LR Clonase II (Invitrogen), and shFZD5 was recombined into CS-RfA-EG or CS-RfA-EF-mRFP to construct CS-shFZD5-EG and CS-shFZD5-EF-mRFP. Control vectors (CS-shCTRL-EG and CS-shCTRL-EF-mRFP) were similarly constructed with pENTR4-H1-shCTRL and either CS-RfA-EG or CS-RfA-EF-mRFP.

The cDNA of FZD5 was cloned from cDNA of hMSC, and a 2xHA tag was added to its 3' tail. The cDNA was recombined into PB513-RfA-EGFP using LR Clonase II as described above to generate PB513-FZD5-2xHA. We constructed five silent mutations in PB513-FZD5-2xHA using PCR with specific primers. This mutated vector (PB513-FZD5mut-2xHA) did not have knockdown activity with shFZD5.

**Knockdown and overexpression**

We knocked down FZD5 using the lentiviral vectors CS-shFZD5-EG and CS-shFZD5-EF-mRFP. Human MSC (2 × 10^5/well) were seeded on six-well plates, then ~24 hours later, appropriate amounts of CS-shFZD5-EG or CS-shFZD5-mRFP lentivirus were added and the cells were incubated for 12 hours. The medium containing lentivirus was removed, and cells were passaged if necessary. Four days after viral infection, EGFP-positive or mRFP-positive hMSC were sorted by flow cytometry. Ten days after viral infection, FZD5 was overexpressed using the PiggyBac transposon vector system (System Biosciences LLC, Palo Alto, CA, USA). PB513-FZD5-2xHA or PB513-FZD5mut-2xHA was transfected with the PB200 vector (System Biosciences LLC). The molar ratio of PB513-FZD5-2xHA: PB200 or PB513-FZD5mut-2xHA to PB200 was 5:1. These vectors were transfected into hMSC using a Human Mesenchymal Stem Cell Nucleofector™ Kit (Lonza) or the ViaFect Transfection Reagent (Promega).

**Analysis of proliferation capacity**

Human MSC (1 × 10^5/flask) transfected with shCTRL (CS-shFZD5-EG) or shFZD5 (CS-shFZD5-EF-mRFP) were seeded in T75 flasks then trypsinized, harvested and counted 7 days later. The hMSC (1 × 10^5/flask) were seeded again, and repeatedly counted and seeded until the number of hMSC fell to < 2 × 10^5 indicating that cell growth had stopped. Human MSC transfected with wt-FZD5 (PB513-FZD5-2xHA) or mut-FZD5 (PB513-
FZD5mut-2xHA) were seeded in T25 flasks at a density of $1 \times 10^4$ cells/flask. Changes in the sample group are indicated relative to that in control cells numbered as 1.

**Immunocytochemistry**

Cells were seeded onto eight-well chamber slides (Iwaki & Co., Ltd., Tokyo, Japan) or glass coverslips (Matsunami Glass Ind., Ltd., Osaka, Japan) coated with poly-L-ornithine (Sigma-Aldrich) and fibronectin (Sigma-Aldrich). The cells were incubated with PBS containing 4% paraformaldehyde for 10 minutes at room temperature, washed three times with PBS, then incubated with PBS containing 0.3% Triton X-100 then washed again three times with PBS. The cells were incubated with PBS containing 5% FBS for 30–60 minutes, followed by the primary antibody overnight. The samples were washed three times with PBS, then incubated with secondary antibody at room temperature for one hour and mounted with PermaFluor (Thermo Fisher Scientific Inc., Waltham, MA, USA). F-actin was stained with Alexa fluor 555 Phalloidin (Molecular Probes Inc., Eugene, OR, USA). Cell areas were quantified using ImageJ, and 300–500 cells were counted and classified per sample. Anti-FZD5 (#6F5B9) and anti-ROR2 (#6F12A2) monoclonal antibodies were prepared at a commercial laboratory (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan). Table S2 shows the antibody information. The proximity ligation assay (PLA) visualizes interactions when a pair of oligonucleotide probes that are in close proximity to each other. We assayed such interactions using the Duolink In Situ kit as described by the manufacturer (Olink Bioscience, Uppsala, Sweden). Interactions between non-canonical Wnt pathway-related factors (FZD5/ROR2) in REC cultured in 1% serum were determined using PLA in situ. Dots were counted as signals generated by interactions when target proteins are within 40 nm of each other. An average of > 200 cells was scored per assay.

**Lentivirus preparation**

We transiently transfected 293T cells with the lentivirus constructs pCMV-VSV-G-RSV-Rev and pCAGHIVgp48 using Gene Juice (Novagen) as described [6]. High-titer, concentrated stocks were prepared by ultracentrifugation and suspended in Dulbecco’s PBS (2.68 mM KCl, 1.47 mM KH$_2$PO$_4$, 136.89 mM NaCl and 8.1 mM Na$_2$HPO$_4$).

**Western blotting and pull-down assays**
Cells in lysis buffer (20 mM Tris-HCl pH 8.0, 10% Glycerol, 137 mM NaCl, 1% NP-40, 1% cOmplete mini (Roche Holdings AG, Basel, Switzerland) and 1% PhosStop mini (Roche) were scraped into tubes, then centrifuged at 20,000 × g for 10 minutes at 4°C. Supernatants were incubated with Laemmli buffer (120 mM Tris-HCl (pH7.6), 4% SDS, 20% glycerol) at 95°C for 5 minutes, then proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. Nonspecific protein binding was blocked by incubating the membranes with Tris-buffered saline containing 0.2% Tween20 (Bio-Rad) and 2% nonfat dry milk (Cell Signaling Technology) for one hour at room temperature. Primary antibody was added, and the membranes were incubated for 48 hours for ROR2, and overnight for other proteins at 4°C. The membrane was washed three times with Tris-buffered saline containing 0.2% Tween20, then incubated with secondary antibody conjugated with horseradish peroxidase (Jackson Laboratory, Bar Harbor, ME, USA) for 60–90 minutes. the membrane was washed three times with Tris-buffered saline containing 0.2% Tween20. The secondary antibody was detected by western blotting using ECL Prime (GE Healthcare, Chicago, IL, USA). The activation of RAC1 and CDC42 was assayed using RAC Activation Assay Biochem Kits (Cytoskeleton, Denver, CO, USA). The GTP loading of RAC and CDC42 was determined using the RAC/CDC42 binding domain of p21-activated kinase 1 (PAK)-PBD-GST fusion protein beads, as described by the manufacturer. The amount of PAK PBD/GTP-RAC or GTP-CDC42 that was pulled down was determined by western blotting using RAC1- or CDC42-specific antibodies. Before the experiment, REC were starved in 1% FBS for 24 h, and treated with 200 ng/mL recombinant mouse/human Wnt5a (R&D Systems) for a predetermined period.

**Supplemental References**

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