FZD5 regulates cellular senescence in human mesenchymal stem/stromal cells

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Supplemental Information

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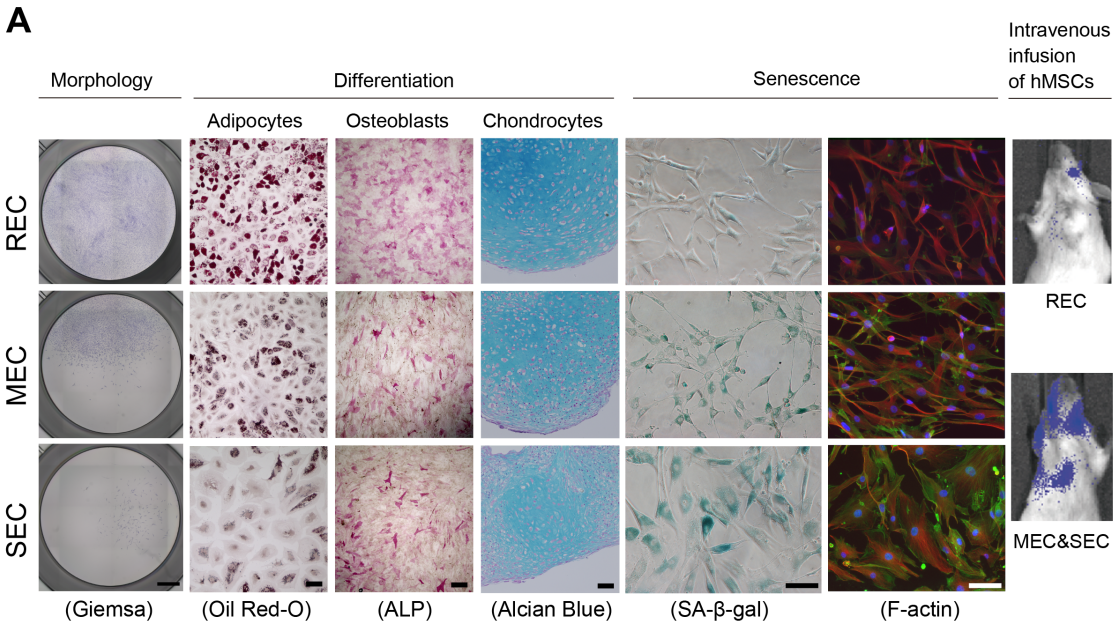
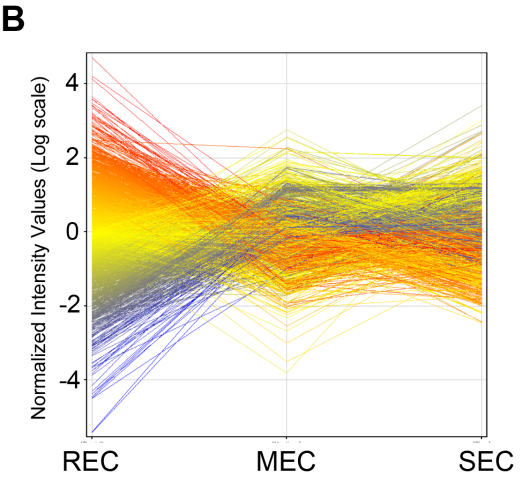
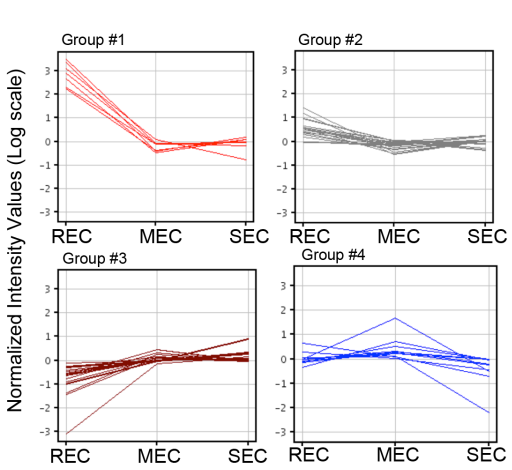
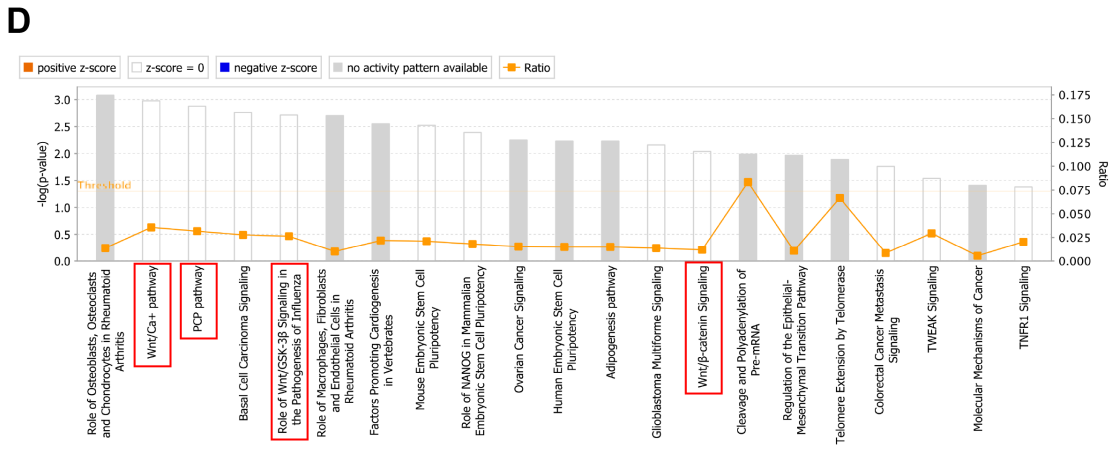

A
Morphology
Differentiation
Adipocytes
Osteoblasts
Chondrocytes
Senescence
Intravenous infusion of hMSCs
REC
MEC
SEC
REC
MEC&SEC
(Giemsa)
(Oil Red-O)
(ALP)
(Alcian Blue)
(SA-β-gal)
(F-actin)

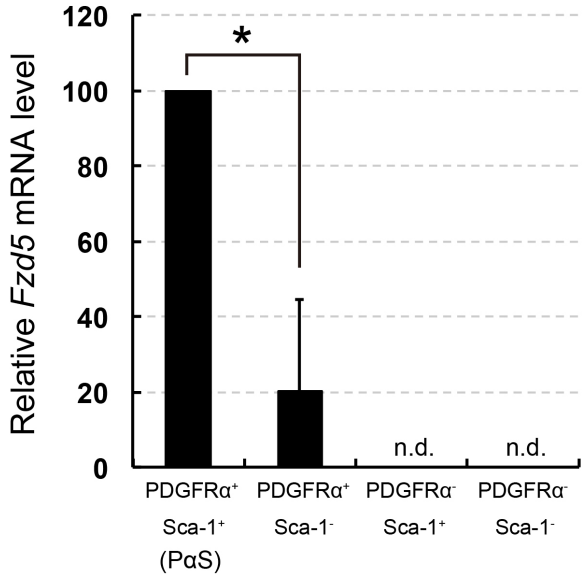

B
Normalized Intensity Values (Log scale)
REC
MEC
SEC


C
Group #1
Group #2
Group #3
Group #4
Normalized Intensity Values (Log scale)
REC
MEC
SEC


D
positive z-score
z-score = 0
negative z-score
no activity pattern available
Ratio
-log(p-value)
Threshold
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis
Wnt/Ca+ pathway
PCP pathway
Basal Cell Carcinoma Signaling
Role of Wnt/GSK-3β Signaling in the Pathogenesis of Influenza
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis
Factors Promoting Cardiogenesis in Vertebrates
Mouse Embryonic Stem Cell Pluripotency
Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency
Ovarian Cancer Signaling
Human Embryonic Stem Cell Pluripotency
Adipogenesis pathway
Glioblastoma Multiforme Signaling
Wnt/β-catenin Signaling
Cleavage and Polyadenylation of Pre-mRNA
Regulation of the Epithelial-Mesenchymal Transition Pathway
Telomere Extension by Telomerase
Colorectal Cancer Metastasis Signaling
TWEAK Signaling
Molecular Mechanisms of Cancer
TNFR1 Signaling

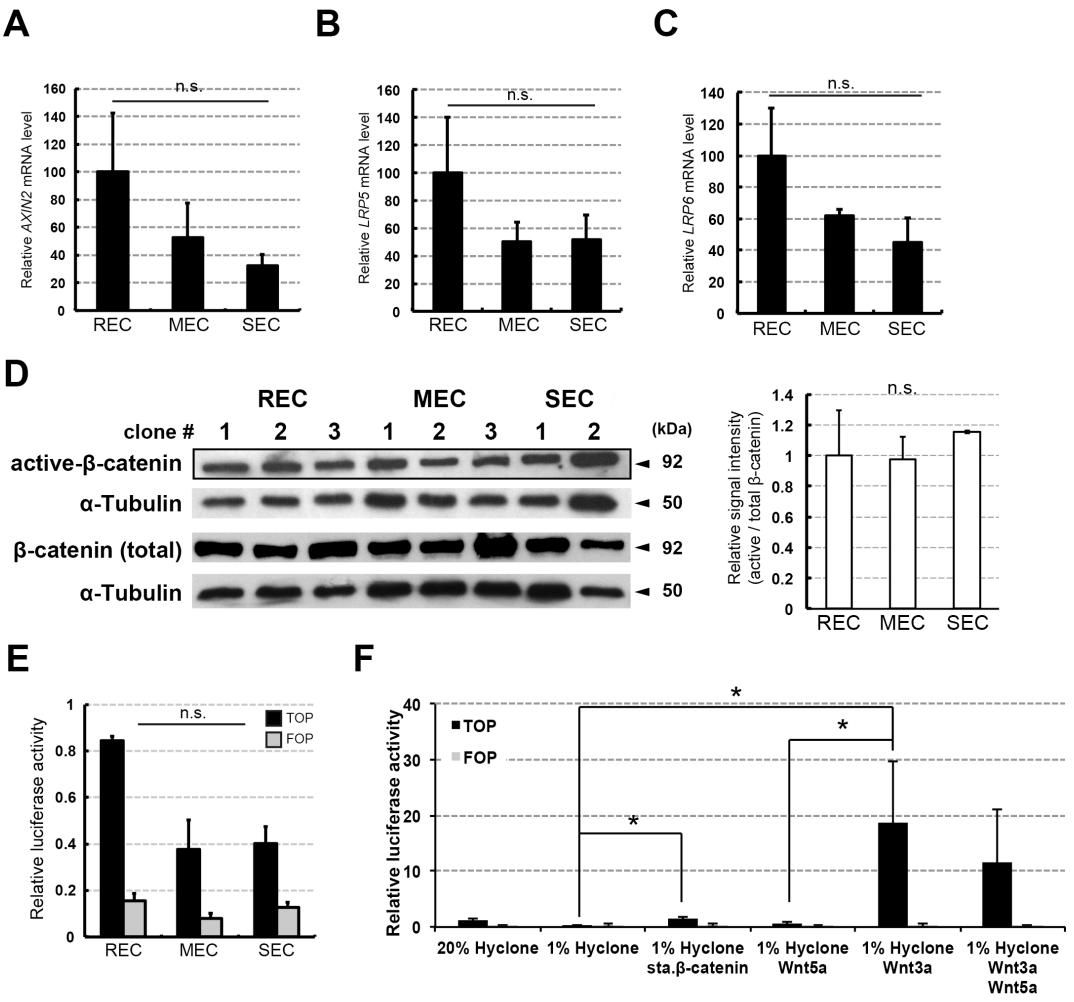
Supplemental Fig. S1. Screening REC-specific genes.

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.



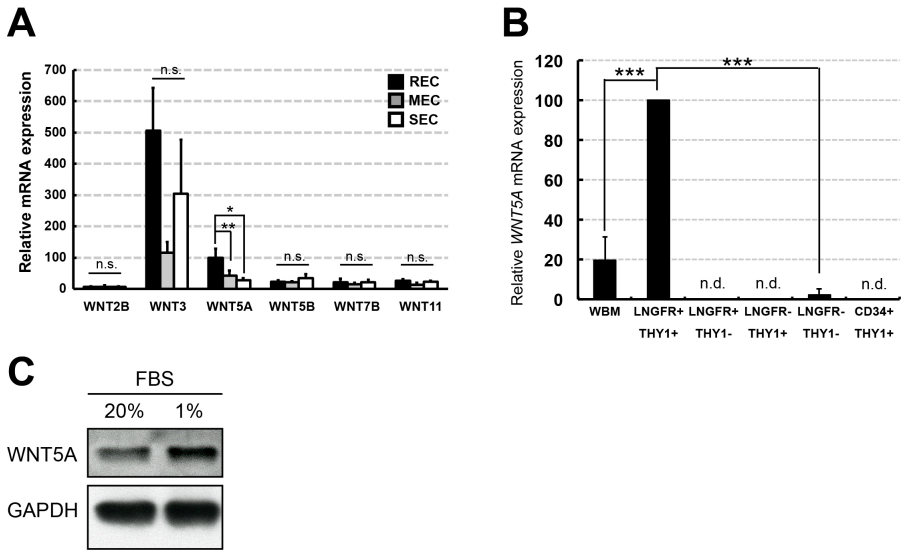
Supplemental Fig. S2. Expression of FZD5 in mouse MSCs.

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.



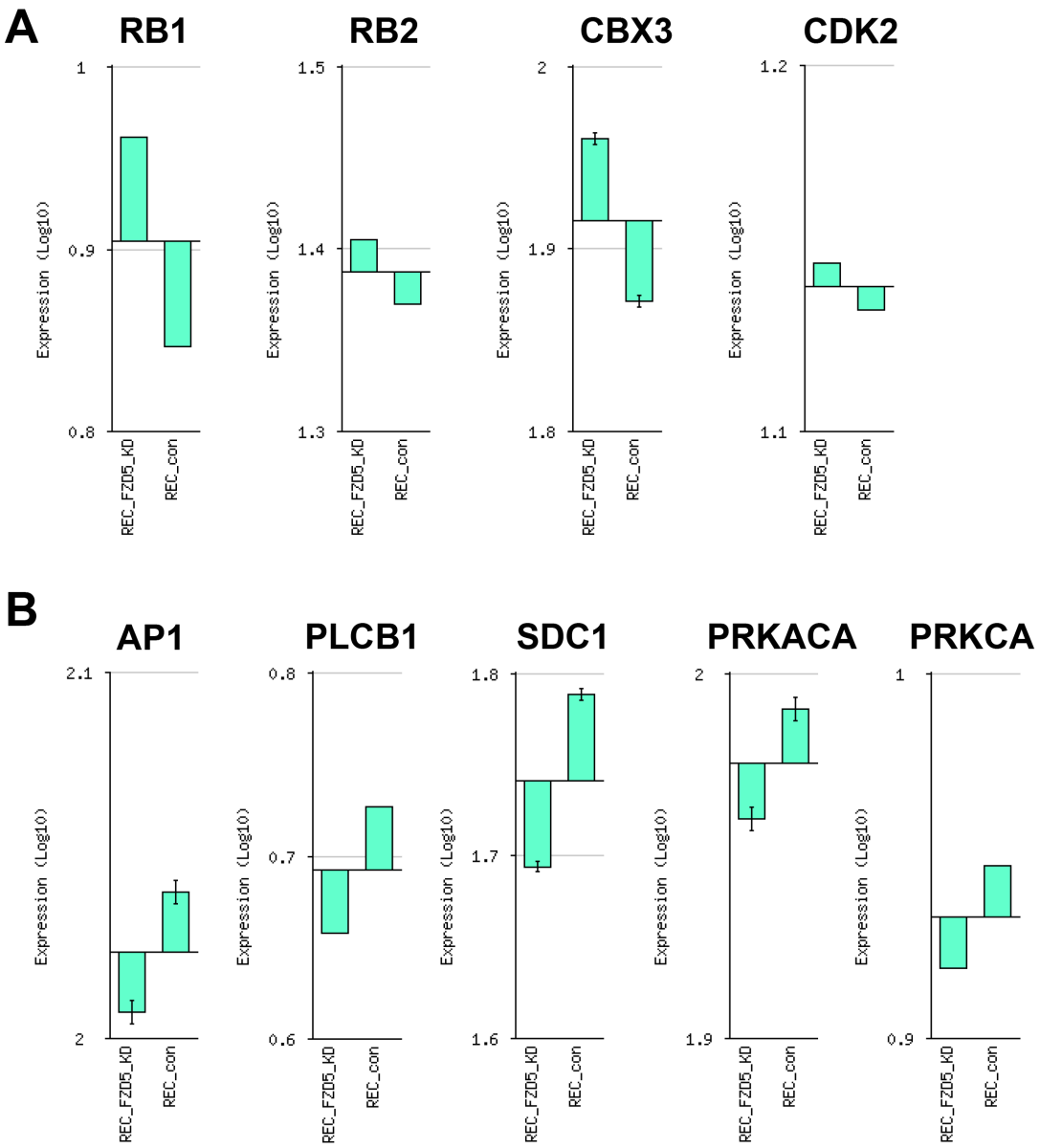
Supplemental Fig. S3. Canonical WNT pathway involvement in REC.

(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. *$P < 0.05$.

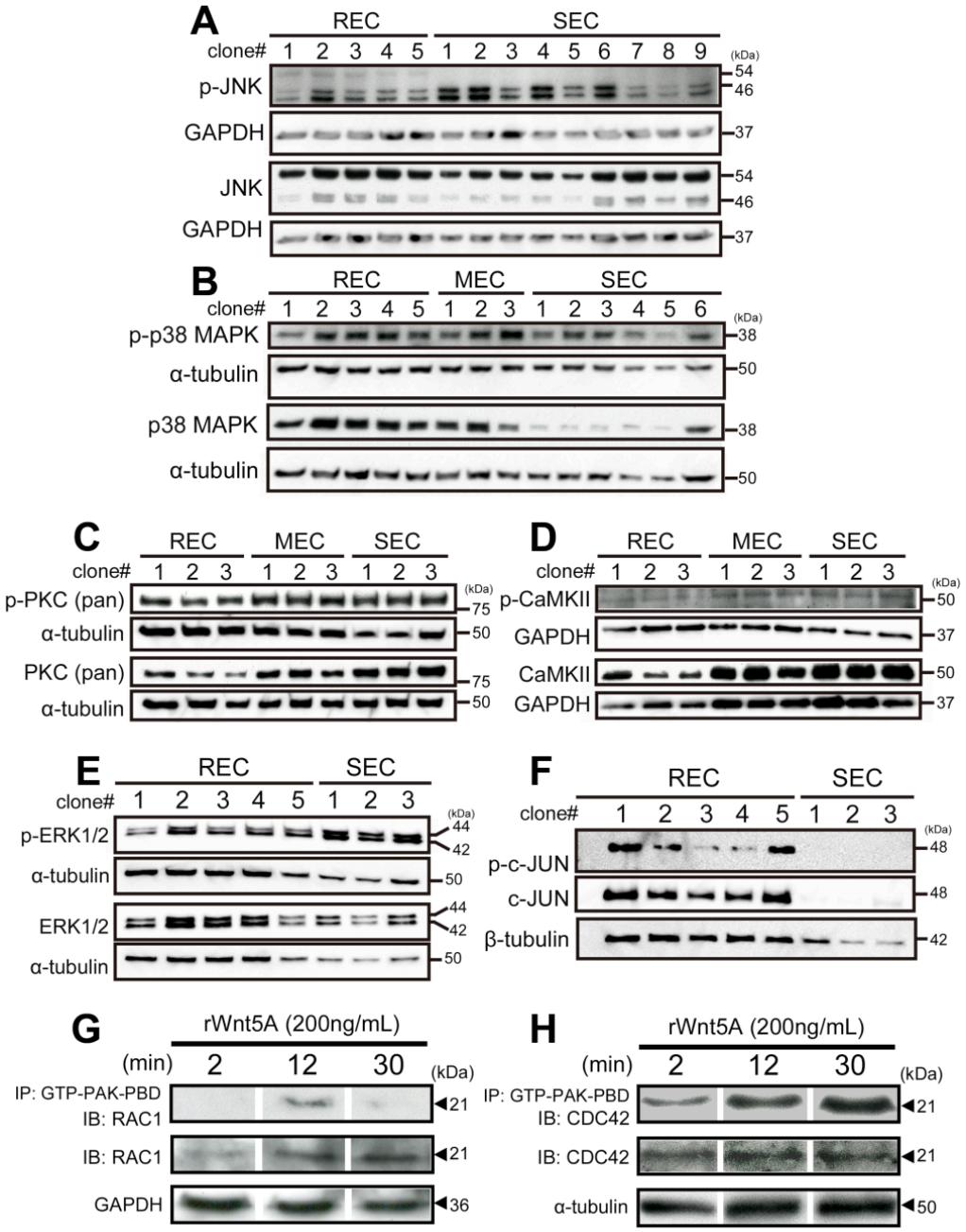


Supplemental Fig. S4. Expression of WNT in REC and WNT5A in BM-MNCs.

(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.

(For Q-PCR)

Gene	Forward primer	Reverse primer
GAPDH	CGAGATCCCTCCAAAATCAA	CATGAGTCCTTCCACGATACCAA
FZD5	TCTTGTTTCCAAAGTCCAATCA	GCTTTTGAGAGTCTTGACTCAGGT
ROR2	CTCATGATCGAGTGCTGGAA	CGTTGCTCACATTGCTCACT
P14ARF	TACTGAGGAGCCAGCGTCTA	TGCACGGGTCGGGTGAGAGT
P16INK4A	AGCATGGAGCCTTCGGCTGA	CCATCATCATGACCTGGATCG
P21	GAGACTCTCAGGGTCGAAAA	TTAGGGCTTCCTCTTGGAGA
PROGERIN	GCGTCAGGAGCCCTGAGC	GACGCAGGAAGCCTCCAC
LMNA	GGTGGTGACGATCTGGGCT	CCAGTGGAGTTGATGAGAGC
PPARG	GACCACTCCCACTCCTTTGA	CGACATTCAATTGCCATGAG
CEBPA	GAAAGCTAGGTCGTGGGTCA	TCATAACTCCGGTCCCTCTG
CEBPB	CTCTCTGCTTCTCCCTCTGC	CAACAAGCCCGTAGGAACAT
RUNX2	TCAACGATCTGAGATTTGTGGG	GGGGAGGATTTGTGAAGACGG
BGLAP	GACTGTGACGAGTTGGCTGA	CTGGAGAGGAGCAGAACTGG
ACAN	TATGTGAGGAGGGCTGGAAC	CCAGTTCTCAAATTGCATGG
COL10A1	GCTAAGGGTGAAAGGGGTTC	CTCCAGGATCACCTTTTGGA
AXIN2	CTGGCTCCAGAAGATCACAAAG	CATCCTCCCAGATCTCCTCAAA
LRP5	CTCCGACGAGCTCATGTGT	CGGAGCTCATCATGGACTTT
LRP6	TGACATGACAGGTCGAGAGG	CCAAGCCACAGGGATACAGT
HMGN2	GGAGGAGCAGGTTTAGCAGA	CACCATGCCCAAGAGAAAG
HNRPA2B1	GCCACCAATAAAGAGCTTACG	CGCGGAGATCTCTCTCATCT
C11ORF86	GAGAGCTTTGTCGCCATCTT	AGCCAAAGTCCAGAGAGCTG
KLRC4	TCCACTCCTCAGGACAATGG	CTTGTATTGGAGTACTGGAGCAGA
PABPN1	GGGAATACCATGATGTCGCT	ACCAACTACAACAGCTCCCG
BG199525	GGACCCAACTTGGAGAATCA	TTTATTGGCCCTTCTTGCAC
BQ017638	GGGGGTGGACCAAATAACTG	CCCACTCCCTCTGGACAATA
AK025353	AGTCAGAGGGACCCAGGATT	CACAAAACCACAATGGCAAG
HRASLS5	GCACTGTGCTAGGCATTTCA	TCTGATGGCACTCAAGCATC
COX4I1	ACGCCGATCCATATAAGCTG	TTTAGCCTAGTTGGCAAGCG
METTL7A	GATCCAGGACTTGTTGCCAG	AGGTGTGCAGAGTGCTGAGA
LOC643744	AAGATCCGCGAGGTTGTCT	GCAGGGTTATTTCCCTCCTT
LOC652489	GCAGTTTAAGATTAAGAGGCATACA	TTCTCCATTTCCAACTGTGC
A_24_P633825	GCCATGTAGAAACTTCAAAAGAAA	CCGCACCACTTCATCACTG
A_24_P803809	TCTTCCTTGCTAATGCACCA	TGACCTATAGTGACAGATGACATCAC
THC2631465	TGCATAAGATTCCTGCCAGTT	TCAACCAACTTCTCTTCATTTCC
A_24_P315644	AGAGAAGTACCGGGGTCGTG	TCTTCTCTTCTCTGCAGCTGAT
FZD2	CTCCGTGCTCTACACAGTGC	ACGATGAGCGTCATGAGGTA
C11ORF51	TATGCAGGACATGGACGAGA	CTGGTCCTGATCCTGTTCGT
CCDC23	CAGAGATCTATGCCCTCAACAG	ACTGGCAACACCCTCTCAAA
CNP	AAGAGGCAATGAGTGTGTTGG	CCATGTTACTTGCCCTGCTC
GABARAP	TGTCTACGGTCTGTGAAGCTG	GCCACAAACATTAAGAAGTGC
HNRPR	TGAAATTCGCCCTGGTAAAC	CCACCAAACCCTCTGTGACT
RNF181	TAGATTGGGACCACCACCTG	AGGCATCTCAATGGCAGTCT
LRRC29	CTCGTGAGTTGGGCTTGGTA	CCAGAGCTGTACATCCTGCAT
MRPS15	GAAGATTGTTGCAAACCCAGA	TGGTCAATGCTCATTAGCAGATAG
NFYB	TCCACTTGATCCAAGCAGTG	ACCTGGCCTCAAATTCCTTT
TARDBP	AAATGGTACTCGGGGACCTC	ACCCGTATCTATGCAGTACCA
TINP1	CAACAATCCTGAAAATGATGGA	TGCAGACATGTATGGCTGTTT
TRADD	GCTTTGGAGATCAGCCTCAC	GTATCTGCAGCACCCAGGAT
WNT1	CGGCGTTTATCTTCGCTATC	GCCTCGTTGTTGTGAAGGTT
WNT2	CAAGAACGCTGACTGGACAA	TGACTGCAGAACACCAGGAG
WNT2B	TGCCAAGGAGAAGAGGCTTA	CTGCACAGCCCCATCATAG
WNT3	GAGTTCTTTCCCCCAGCTCT	GCCTCCCTTTTGCTAGCTTT
WNT3A	CATATCCCTGGTTGCCTCAT	AACTCCGTTGGACAGTGGAT
WNT4	CTAGCCCCGACTTCTGTGAG	GCACTTGACGAAGCAGCAC
WNT5A	GCACATGCTTTTATCCGTGA	TGTGGAGCACTGTCCAGATT
WNT5B	CGGTGCAGAGACCCGAGATG	GTCTCTCGGCTGCCTATCTG
WNT6	GTCACGCAGGCCTGTTCTAT	CGTCCATAAAGAGCCTCGAC
WNT7A	GCTGTGACCTCATGTGCTGT	TCCTCCCAGCAATCTGACTT
WNT7B	CAAGGGAACCTGAGCAACTG	GGCCTCATTGTTATGCAGGT
WNT8A	TGCAAGTTCCAGTTTGCTTG	ATCCTTTCCCCAAATTCCAC
WNT8B	CCATGAACCTGCACAACAAC	TGAGTGCTGCGTGGTACTTC
WNT9A	GGGTGTGAAGGTGATCAAGG	GCAGAAGCTAGGCGAGTCAT
WNT9B	GAGGACTCACCCAGCTTCTG	TAGGCCTAGTGCTTGCAGGT
WNT10A	AAGCTGCACCGCTTACAACT	ATTCTCGCGTGGATGTCTCT
WNT10B	AATGCGAATCCACAACAACA	GGGTCTCGCTCACAGAAGTC
WNT11	CAGGATCCCAAGCCAATAAA	TATCGGGTCTTGAGGTCAGC
WNT16	GCTCCTGTGCTGTGAAAACA	ACCCTCTGATGTACGGTTGC
β-Actin	AGACCTTCAACACCCCAGCCATGT	GGCCAGCCAGGTCCAGACGCAG
Fzd5	AGGCATCCCGATTTTCTTTT	TGAGCGAGGGCAGAGTATTT

(For cloning)

Name	Sequence
5'-BamHI-Kozak-FZD5cds	CGGGATCCGCCACCATGGCTCGGCCTGACCCATC
3'-XhoI-TAG-2xHA-FZD5cds	CGGCTCGAGCTAAGCGTAATCTGGAACATCGTATGGGTAAGCGTAATCTGGAACATCGTATGGGTACACGTGCGACAGGGACACC
FZD5cds-mut	GCTGAAGTATTTCATGTGCCTGG ATCAAGACCCAGTACTCGGGC

(For shRNA)

shRNA Name	Target	Sequence
CTRL	none	ACTACCGTTGTTATAGGTGttcaagagaCACCTATAACAACGGTAGT
FZD5	FZD5	GGGTGCTCATGCTCAAGTACTgagaAGTACTTGAGCATGAGCACCC

Supplemental Table S2. Primary antibodies and suppliers

antibody name	Supplier	Catalog number	Host/isotype	Working dillution
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	Ab205	Mouse IgM	ICC 1:250
γH2AX	Millipore	05-636	Mouse IgG1	ICC 1:1000
53BP1	Novus Biologicals	NB100-304	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	PM013	Rabbit IgG	WB 1:1000
WNT5A	Abcam	ab86720	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, D13.14.4E	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
RAC1	Cytoskeleton	ARC03-A	Mouse IgG1	WB 1:1000
CDC42	Cell Signalling Technology	2462S	Rabbit IgG	WB 1:1000
ROR2-APC, 7C9D6	Medical and Biological Laboratories	W361-3	Mouse IgG2a	-
LNGFR-PE	Miltenyi Biotec	130-098-111	Mouse IgG1	-
THY-1-APC	BD Pharmingen	559869	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	-
CD49e-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	-
PDGFRα-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 37ºC. 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 37ºC, 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 1×10^5/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 $< 2 \times 10^5$ 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 1×10^4 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_2PO_4, 136.89 mM NaCl and 8.1 mM Na_2HPO_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 4ºC. Supernatants were incubated with Laemmli buffer (120 mM Tris-HCl (pH7.6), 4% SDS, 20% glycerol) at 95ºC 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 4ºC. 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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