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Supplemental Information

Long-Term Expansion, Enhanced Chondrogenic Potential, and Suppression of Endochondral Ossification of Adult Human MSCs via WNT Signaling Modulation

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Supplemental figures and legends

Figure S1

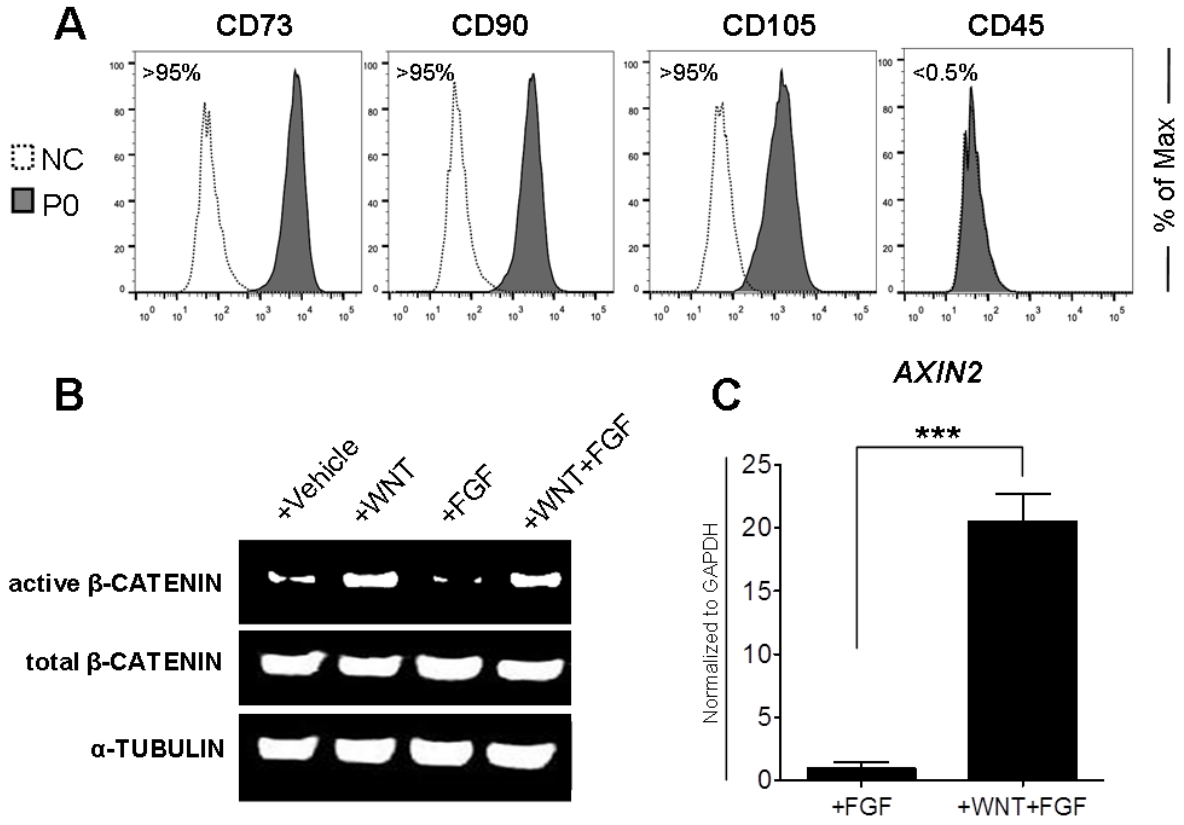

Figure S1, related to Figure 1. WNT3A induces WNT signalling activation of human MSCs. (A) MSCs were characterized for their surface marker expression by flow cytometry. Numbers indicate the percentage of positive cells for the indicated markers. NC = negative control, P0 = after selection by plastic adherence. (B) After expansion for one passage in the indicated media, active (non-phosphorylated) and total (phosphorylated) β-CATENIN accumulation in the MSCs was detected by western blot. (C) Transcript analysis of WNT target gene *AXIN2* on MSCs expanded in the indicated conditions. Values represents means ± s.e.m. of 3 MSC donors (n=3) in biological triplicate. ***P<0.001.

Figure S2

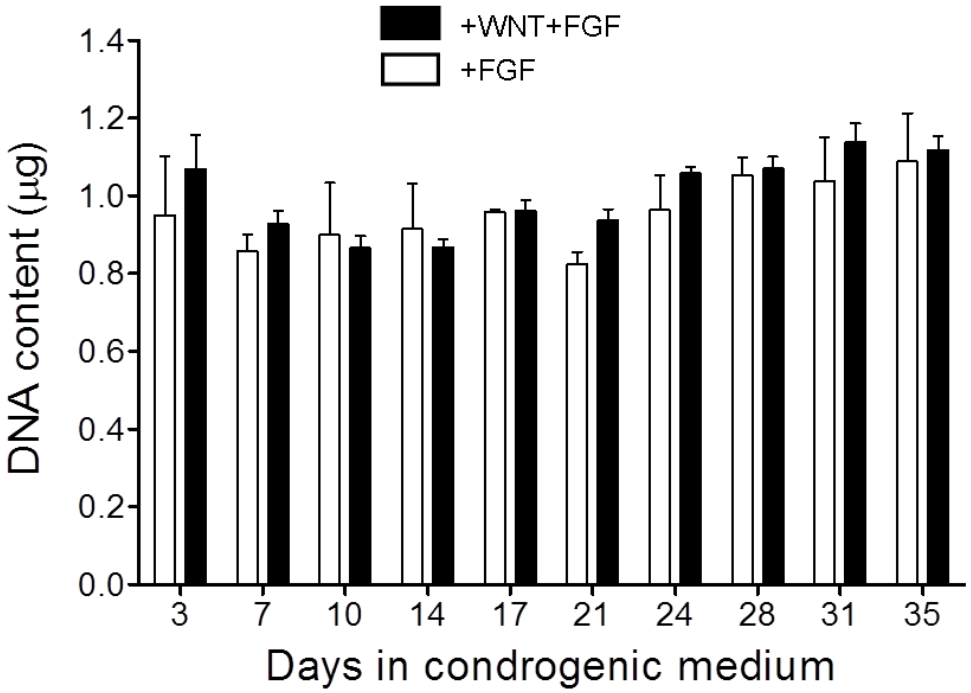

Figure S2, related to Figure 2. Pellets formed from MSCs expanded in the indicated conditions contain comparable amounts of DNA over time. Values represent means ± s.d. of 3 to 4 pellets from the same MSC donor.

Figure S3

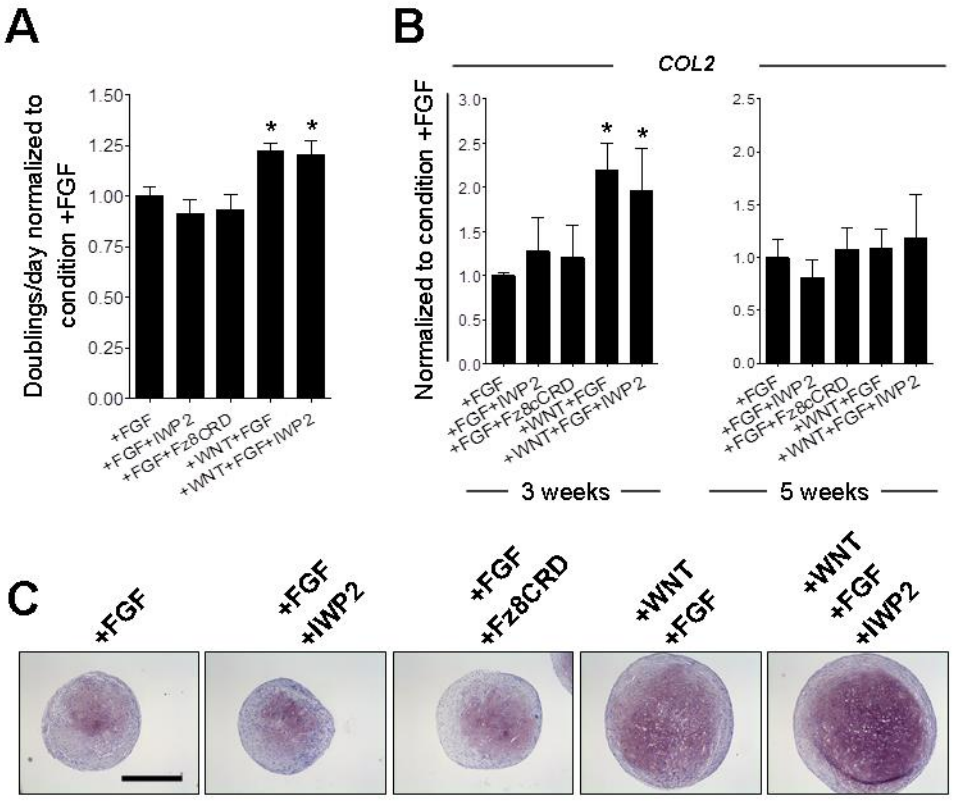

Figure S3, related to figure 3. Endogenous WNT does not influence expansion and chondrogenic potential of MSCs. (A) Proliferation rate of MSCs during expansion in the indicated media (n=4 donors). (B) Relative gene expression levels of *COL2* in pellet cultures from cells expanded in the indicated conditions, after 3 or 5 weeks of chondrogenic induction (n=3 donors with 3 pellets per donor). (C) Thionine staining (glycosaminoglycan) of representative sections from cartilage pellets formed with cells expanded in the indicated conditions, after 5 weeks of chondrogenic induction (n=3 donors with 2 to 3 pellets per donor). Values represent means ± s.e.m. *$P<0.05$ compared to the conditions with FGF ± IWP2 or Fz8CRD. Scale bar, 1 mm. IWP2 = Wnt secretion inhibitor (blocker of acyltransferase enzyme porcupine), Fz8CRD = soluble WNT receptor.

Figure S4

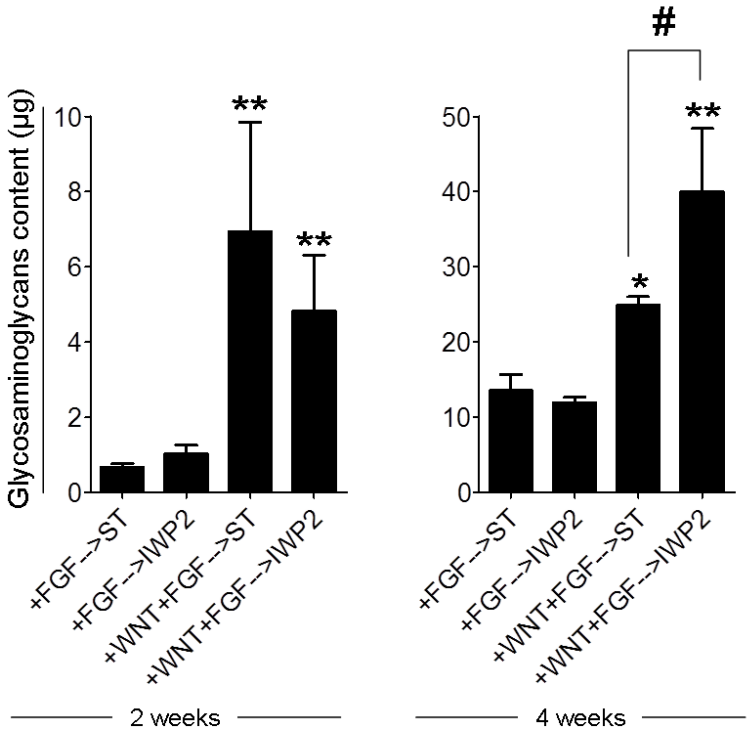

Figure S4, related to Figure 4. Treatment with IWP2 enhances and accelerates glycosaminoglycan production of pellet formed by WF-MSCs. Glycosaminoglycan content in pellet cultures after 2 or 4 weeks in standard (→ST) or IWP2-supplemented (→IWP2) chondrogenic medium. Values represent means ± s.e.m of 3 MSC donors with 2 to 3 pellets per donor. *#P<0.05. *P<0.05* and ***P<0.01* compared to both +FGF→ST and +FGF→IWP2.

Figure S5

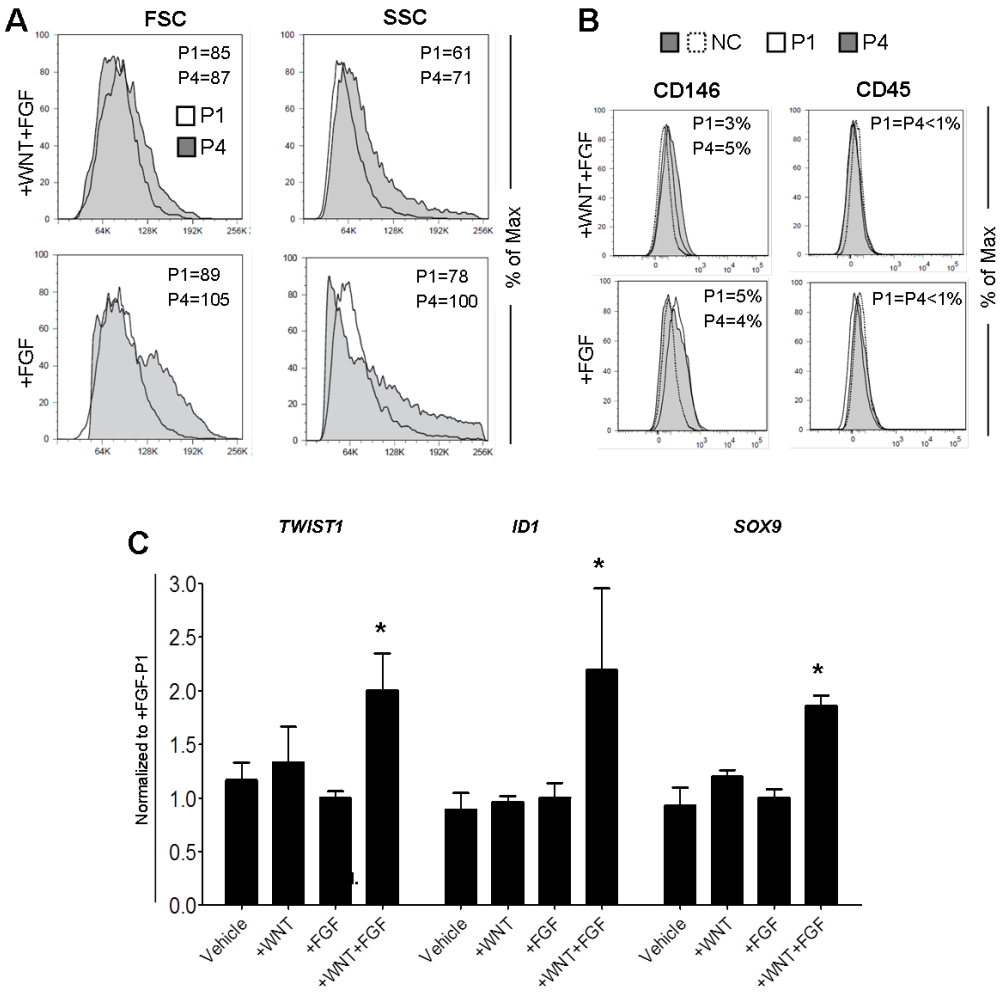

Figure S5, related to Figure 5. MSCs expanded with WNT3A and FGF2 display a unique phenotype. (A) Size distribution analysis (FCS=forward scatter) and cell granularity analysis of (SSC=side scatter) of MSCs expanded as indicated. Values represent the mean obtained from 2 independent donors. NC = negative control, P1 = passage 1, P4 = passage 4. (B) Flow cytometry analysis of markers not affected by the different expansion conditions. Values represent the mean obtained from 3 independent donors. (C) Transcript analysis on P1-expanded MSCs in the indicated conditions. Values represent means ± s.e.m. of 3 MSCs donors, 2 pellets per donor. *$P<0.05$ compared to the other conditions.

Supplemental Experimental Procedures

Western blot. After P1-expansion, and 3 h after last medium renew, MSC of two donors were harvested using M-PER Protein extraction reagent (Thermo Scientific, Rockford, IL, USA) with 1% protease inhibitor (Roche, Mannheim, Germany). Equal amounts of protein lysate were subjected to 10% SDS-PAGE gels and transferred on PVDF membranes. Membranes were blocked (0.1% tris/tween buffer containing 5% dry milk powder) and then incubated overnight at 4°C with primary antibody (**Table S2**). Afterwards, anti-rabbit secondary antibody was added for 1 h at room temperature. Antibodies were purchased from Cell Signaling Technology (Leiden, the Netherlands). The blots were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) following manufacturer's instructions.

Osteogenic differentiation. Cells were seeded at 3,000 cells cm^{-2} and cultured for 18 days with DMEM-high glucose (Gibco) + 10% FCS, 50 μg ml^{-1} gentamicin, 1.5 μg ml^{-1} fungizone, β-glycerophosphate 10 mM, dexamethasone 0.1 μM and L-ascorbic acid 2 phosphate 0.5 mM (all from Sigma-Aldrich). Cultures were fixed in 10% formalin, hydrated with water and treated with 5% silver nitrate solution (Sigma-Aldrich) for 10 minutes. Excess staining was removed with 5% sodium-thiosulphate (Sigma-Aldrich) followed by counterstaining with azophloxine (Sigma-Aldrich).

Adipogenic differentiation. Cells were seeded at 20,000 cells cm^{-2} and cultured for 3 weeks in DMEM-high glucose (Gibco) + 10% FCS, supplemented with 50 μg ml^{-1} gentamicin, 1.5 μg ml^{-1} fungizone, dexamethasone 1 μM, indo-methacin 0.2 mM, insulin 0.01 mg ml^{-1}, and 3-isobutyl-l-methyl-xanthine 0.5 mM (all from Sigma-Aldrich). Cultures were fixed in 10%

formalin, treated with 0.3% Oil red O solution (Sigma-Aldrich) for 15 min, and then washed with tap water.

Supplemental tables

Table S1 - SYBR/Taqman primer and probe sequences

Gene name	Description	Primers Forward (Fw) and Reverse (Rv)	Probe
COL2	Chondrogenic marker	Fw: GGCAATAGCAGGTTCACGTACA Rv: CGATAACAGTCTTGCCCCACTT	CCGGTATGTTTCGTGCAGCCATCCT
COL2B	Chondrogenic marker	Fw: GCTGTCCTTCGGTGTCA Rv: GTTCTCCTTTCTGTCCCTTTGGT	CTGGTTGCCGGACATC
SOX9	Chondrogenic marker	Fw: TCCACGAAGGGCCGC Rv: CAACGCCGAGCTCAGCA	TGGGCAAGCTCTGGAGACTTCTGAACG
MMP13	Hypertrophic marker	Fw: AAGGAGCATGGCGACTTCT Rv: TGGCCCAGGAGGAAAAGC	CCCTCTGGCCTGCGGCTCA
COL10	Hypertrophic marker	Fw: CAAGGCACCATCTCCAGGAA Rv: AAAGGGTATTTGTGGCAGCATATT	TCCAGCACGCAGAATCCATCTGA
ALP	Hypertrophic/ osteogenic marker	Fw: GACCCTTCACCCCCACAAT Rv: GCTCGTACTGCATGTCCCCT	TGGACTACCTATTGGGTCTCTTCGAGCCA
FABP4	Adipogenic marker	Fw: TGTCTCCAGTGAAAACTTTGATGATTA Rv: CCATGCCAGCCACTTTCC	N.A.
AXIN2	β-CATENIN target gene	Purchased by AB applied Biosistem (Axin2 FAM, Hs00610344_m1)	Purchased by AB applied Biosistem (Axin2 FAM, Hs00610344_m1)
TWIST1	Uncommitted marker	Purchased by AB applied Biosistem (Twist1 FAM, Hs01675818_s1)	Purchased by AB applied Biosistem (Twist1 FAM, Hs01675818_s1)
ID1	*TWIST1* target gene	Purchased by AB applied Biosistem (ID1 FAM, Hs03676575_s1)	Purchased by AB applied Biosistem (ID1 FAM, Hs03676575_s1)
MYC	Proliferative marker	Fw: GGCTCCTGGCAAAAGGTC Rv: CTGCGTAGTTGTGCTGATGT	N.A.
MYCL1	Proliferative marker	Fw: CTGCGGGGAGGATTTCTACC Rv: CATGCAGTCACGGCGTATGAT	N.A.
NMYC	Proliferative marker	Fw: ACCCGGACGAAGATGACTTCT Rv: CAGCTCGTTCTCAAGCAGCAT	N.A.
GAPDH	Refer gene	Fw: ATGGGGAAGGTGAAGGTCG Rv: TAAAAGCAGCCCTGGTGACC	CGCCCAATACGACCAAATCCGTTGAC

N.A. = not applicable. This is valid for the SYBR green-based primers

Table S2 - Western Blot antibodies

Antibody name	Type	Cat#
Active-β-CATENIN	Primary	8814
Total-β-CATENIN	Primary	9587
α-TUBULIN	Primary	2125
Anti -rabbit/HRP-linked	Secondary	7074

All antibodies used for western Blot were purchased from Cell Signalling