Supporting Information

Quarto et al. 10.1073/pnas.1113442109

SI Methods

Cell Lines and Culture Conditions. Marfan syndrome human embryonic stem cells, designated MFS5 (referred as to MFS cells in this manuscript), were derived from a donated IVF blastocyst determined by preimplantation genetic diagnosis as having a mutation (c.1747delC) in the 5' region of the *FBN1* gene. Wild type human embryonic stem cells (referred as to WT cells) were derived from a blastocyst donated for research under informed consent. MFS and WT embryonic stem cells were derived as follows: The zona pellucida was removed by briefly incubating the blastocyst in an acid tyrodes solution. Six days later, the blastocyst was plated on mitotically inactivated mouse embryonic fibroblast feeders (MEFs) and fed daily with 50% W8 50% hESC conditioned media. At day 12, putative trophectoderm cells were scraped away from the initial outgrowth. Three weeks after plating the blastocyst, an outgrowth with hESC morphology was manual dissected onto MEFs.

To obtain iPS cells, MFS fibroblasts harboring a *FBN1* splice-site mutation (c.3839-1 g>t) that causes skipping of exon 31 (FB1121) (1), or harboring a *FBN1* frame-shift mutation (c.1642del3ins20bp) (2), and control human fibroblasts were transfected with the pMX-based retroviral vectors encoding human *SOX2*, *OCT4*, *KLF4*, and c-*MYC* (Addgene Inc., Cambridge, MA), as previously described (3, 4); two rounds of viral transduction of 10^5 fibroblast cells were performed. Patient-specific iPS and control iPS cells are referred as to MFSiPS and WTiPS respectively. Five days posttransduction, the cells were resuspended with trypsin, counted, and seeded onto 10-cm dishes preplated with mitomycin C inactivated MEF feeders; 10^5 transduced cells were seeded per biological replicate. Colonies with hESC-like morphology were manually picked and transferred to 12- or 6-well plates preplated with mitomycin C inactivated MEF feeders after 3 wk. Differentiation assays were performed as previously described (5). RNA was collected from each clone and analyzed by quantitative RT-PCR for the mRNA of the exogenous reprogramming factors *SOX2*, *OCT4*, *KLF4*, and c-*MYC*. All cells were maintained on inactivated mouse embryonic fibroblasts (MEFs) in medium consisting of Knockout DMEM/F12 supplemented with 20% Knockout Serum Replacer, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 0.1 mM β-mercaptoethanol, and 10 ng/ml rhFGF-2 (Invitrogen). Medium was changed every 3 d. Cells were routinely passaged using 1 mg/ml type IV collagenase (Invitrogen). The results are presented as means ± SD of three independent experiments.

Teratoma Formation and Karyotype. Assessing the in vivo ability of MFS and MFSiPS cells to differentiate into cells from all three germ layers was accomplished by engraftment of cells in immuno-deficient mice. For each graft, approximately 10^6 cells were manually harvested, washed, and resuspended in a 1.5-ml tube containing 300 μl medium and then injected subcutaneously into female SCID mice (Charles River Laboratories International, Inc.). Any visible tumors 4–8 wk posttransplantation were dissected and fixed overnight with 4% paraformaldehyde/PBS solution. The tissues were then paraffin embedded, sectioned, stained with hematoxylin and eosin or safranin O, and examined for the presence of tissue representatives of all three germ layers. Spectral karyotyping (SKY) was performed as previously described (4).

Alkaline Phosphatase Staining and Immunocytochemistry. AP staining was done using the Vector Red Alkaline Phosphatase Sub-strate Kit I (Vector Laboratories), according to the manufacturer's protocol. Standard immunostaining techniques were applied for surface, cytoplasmic, and nuclear protein localization as previously described (4). Primary antibodies and dilutions were as follows: OCT4 (1:200) was obtained from Santa Cruz Biotechnology; SSEA4 (1:200), TRA1-60 (1:200), and TRA1-81 (1:200) were obtained from Millipore; NANOG (1:300) was obtained from ReproCell, Inc.

Fluorescence-Activated Cell Sorting (FACS). Cells were dissociated by incubation with collagenase IV and then with accutase. Single cells were stained using PE-conjugated anti-human CD73 (Becton Dickinson) and analyzed and sorted with FACS Aria II and FACSDiva software (Becton Dickinson). Cells positive for CD73 were plated in αMEM+20%FBS (Invitrogen) on Matrigel GFR coated plates and supplemented with ROCK inhibitor (Sigma;Y0503) to promote cell survival. After first passage, cells were passed with accutase onto 0.25% gelatin-coated plates and maintained in αMEM+10%FBS. Three independent FACS products of CD73$^+$ MFS and CD73$^+$ WT cells were analyzed. The three batches are referred as to #1, #2, and #3 in *Results*. For MFSiPS and WTiPS cells, three independent FACS products of CD73$^+$ cells derived from two different clones were analyzed, both with similar results.

Osteogenic Differentiation, Alkaline Phosphatase Activity and Mineralization Assay. Osteogenic differentiation was induced by culturing cells in α-MEM supplemented with 10% FBS, 100 IU/ml penicillin, 100 IU/ml streptomycin, 10 mM β-glycerophosphate, and 100 μg/ml ascorbic acid (Sigma-Aldrich). Where required, SB-431542, TGF-β1, and noggin (R&D Systems) were added, respectively, at a concentration of 10μM, 10 ng/ml, and 150 ng/ml. Medium was changed every 3 d. Alkaline phosphates enzymatic activity and Alizarin red staining were performed as described previously (6). All values were normalized against protein concentration obtained from triplicate wells.

RNA Isolation, Reverse-Transcriptase Polymerase Chain Reaction and Quantitative PCR Analysis. RNA isolation, reverse transcription (RT), and quantitative real time PCR (qPCR) were described previously (7, 8). Briefly, qPCR was performed using the ABI Prism 7900 Sequence Detection System, TaqMan Gene Expression Master Mix, and TaqMan Gene Expression Assays (Applied Biosystems). Primers were designed using Applied Biosystems Primer Express software. Primers sequences are listed in Table S1. Cycling conditions were initial denaturation at 95°C for 3 min, followed by 30 cycles consisting of a 15-s denaturation interval at 95°C and a 30-s interval for annealing and extension at 60°C. The relative mRNA level in each sample was normalized to its *GAPDH* content. Values are given as relative to *GAPDH* expression. The results are presented as means ± SD of three independent experiments.

Immunoblotting Analysis. Immunoblotting analysis was performed using the following primary rabbit antibodies: anti-p-SMAD2, anti-SMAD2, anti-pSMAD5, anti-SMAD5 (1:1000; Cell Signaling Technology), anti-TGFβ-1, and anti-β-ACTIN (1:400; ab9758; 1:5000; ab8227, Abcam). Eighty micrograms of cell lysate protein isolated from subconfluent cells cultured for 24 hr in αMEM+1%FBS was resolved by 12% Tris-HCl sodium dodecyl sulfate (SDS)-polyacrylamide gel. Proteins were transferred to an Immobilon-P membrane (Millipore). Membranes were probed with specific antibody. A horseradish peroxidase-conju-

gated secondary anti-rabbit was used (1:2000; Cell Signaling Technology). Immunoblotted proteins were visualized by enhanced chemiluminescence (Amersham Biosciences). To assess for the total amount of endogenous SMAD2 and SMAD5 and to control for equal loading and transfer of the samples, the membranes were reprobed either with anti-SMAD2 or -SMAD5 antibodies and anti-β-ACTIN antibody. Densitometry analysis of electrophoretic bands was performed using the ImageJ software program, (National Institutes of Health). The density of pSMAD2 and pSMAD5 bands were normalized to the loading controls (β-ACTIN) and presented as percentage increase. The results are the mean ± SD of three independent experiments.

Preparation of Cell-Conditioned Media. Cell-conditioned media (CM) were obtained from WT and MFS cells. Briefly, 8×10^5 cells were plated in 100-mm dishes. Upon subconfluence, the cells were washed three times with sterile PBS prior to addition of serum free medium. After culture in serum free medium for 48 hours, the cell-conditioned media were collected and concentrated 50-fold using Centricon filters (Centricon-3, 3000 NMWL, Millipore Corporation). Collection and concentration of the media were carried out at 4°C. The volumes of the conditioned media were normalized by cell numbers so that an equal volume of WT and MFS medium was produced by an equal number of WT or MFS cells. Protein concentration was determined by Bicinchoninic Acid (BCA) protein assay (Pierce). For the stimulation experiments, an equal amount of proteins from each medium was applied to subconfluent WT and MFS cells starved for 24 h in serum free medium. After 18 h, stimulation cells were analyzed for SMAD2 phosphorylation as described above. All experiments were performed three times using freshly harvested media.

TGF-β1 Enzyme-Linked Immunosorbent Assay. Serum free medium samples (conditioned for 48 h) were analyzed for active TGF-β1 concentrations by enzyme-linked immunosorbent assay (ELISA) (Quantakine #DB100B, R&D Systems) according to the manufacturer's instructions. The assay was repeated twice. Analysis of active TGF-β1 during the osteogenic differentiation assay was performed on media supplemented with 2% FCS and collected every 3 d.

Immunofluorescent Staining. Cells grown for 2 d in αMEM+1% FBS were fixed with Methanol for 5 min at −20 °C then with Acetone for 2 min at −20 °C. After washing five times with cold PBS, cells were incubated with 2% normal goat serum/PBS for 30 min at room temperature to block nonspecific binding of antibodies. Subsequently, the cells were incubated with rabbit primary anti-pSMAD2 antibody (1:50; Cell Signaling Technology) overnight at 4 °C followed by a fluorescein-conjugated Alexa Fluor 568 anti-rabbit secondary antibody (1:400; Molecular Probes, Invitrogen) for 1 hr at room temperature. Nuclear counterstaining was performed using Vectashield H-1200 mounting medium with DAPI (Vector Laboratories), and a Zeiss Axioplan microscope equipped with an Axiocam HRc digital camera was used for imaging.

Inhibition of Endogenous Phosphorylated SMAD2 with Neutralizing Anti-TGFβs Antibodies. Subconfluent MFS cells were maintained for two days in 1% FCS-growth medium in absence or presence of 1.2 μg/ml neutralizing mouse monoclonal anti-human TGF-β1, -2 and -3 antibodies (clone 1D11 #MAB1835, R&D Systems). Samples were processed for immunoblotting analysis as described above.

Chondrogenic Differentiation. For in vitro chondrogenic differentiation of high-density micromass cultures, 200,000 cells per 10-μl spot were plated in the center of well (20-multiwell plates). The cells were allowed to attach for 1.5 h at 37 °C and 5% CO_2. The plates were then flooded with DMEM supplemented with 1% FBS, 6.25 mg/ml human recombinant insulin, 6.25 mg/ml human transferrin, 6.25 ng/ml selenious acid, 5.35 mg/ml linoleic acid (1% ITS premix, BD Biosciences), 50 nM sodium L-ascorbate, 100 nM dexamethasone (Sigma-Aldrich), and ±2 ng/mlTGFβ-1 (hrTGFβ-1, R&D Systems #240-B). Culture media were changed every 2–3 d for 21 d. Where requested, 10 μM SB-431542 was added to the medium.

Alcian Blue Staining and Glycosaminoglycan Production. The content of sulfated glycosaminoglycans (GAGs) was investigated by Alcian blue staining. For Alcian blue staining micromass cultures were washed with ice-cold PBS and fixed for 20 min in 100% ethanol, then stained for 4 h at room temperature with 0.1% Alcian blue/80% ethanol-20% acidic acid. The micromasses were rinsed several times with 100% ethanol to eliminate nonspecific staining. Photographs were obtained with a LeicaMZ16 stereomicroscope. The synthesis of GAGs was investigated as follows: 10 micromasses per each time point were harvested (in triplicate). Each replicate was digested separately at 65 °C using a 0.1% papain solution (pH 6). The sGAG quantification was performed on each replicate using an sGAG assay kit according to the manufacturer's protocol (Kamiya Biomedical). The deoxyribonucleic acid (DNA) quantities of total micromasses were simultaneously measured using a Quant-iT kit (Molecular Probes). All sGAG quantities were normalized according to the corresponding DNA content. Experiments were repeated three times. Significance (*$P \le 0.05$) was assessed using a Student t test.

Statistical Analysis. The results are presented as the mean ± SD of three independent experiments. Statistical differences between the means were examined by Student's t test. *$P < 0.05$ was considered statistically significant.

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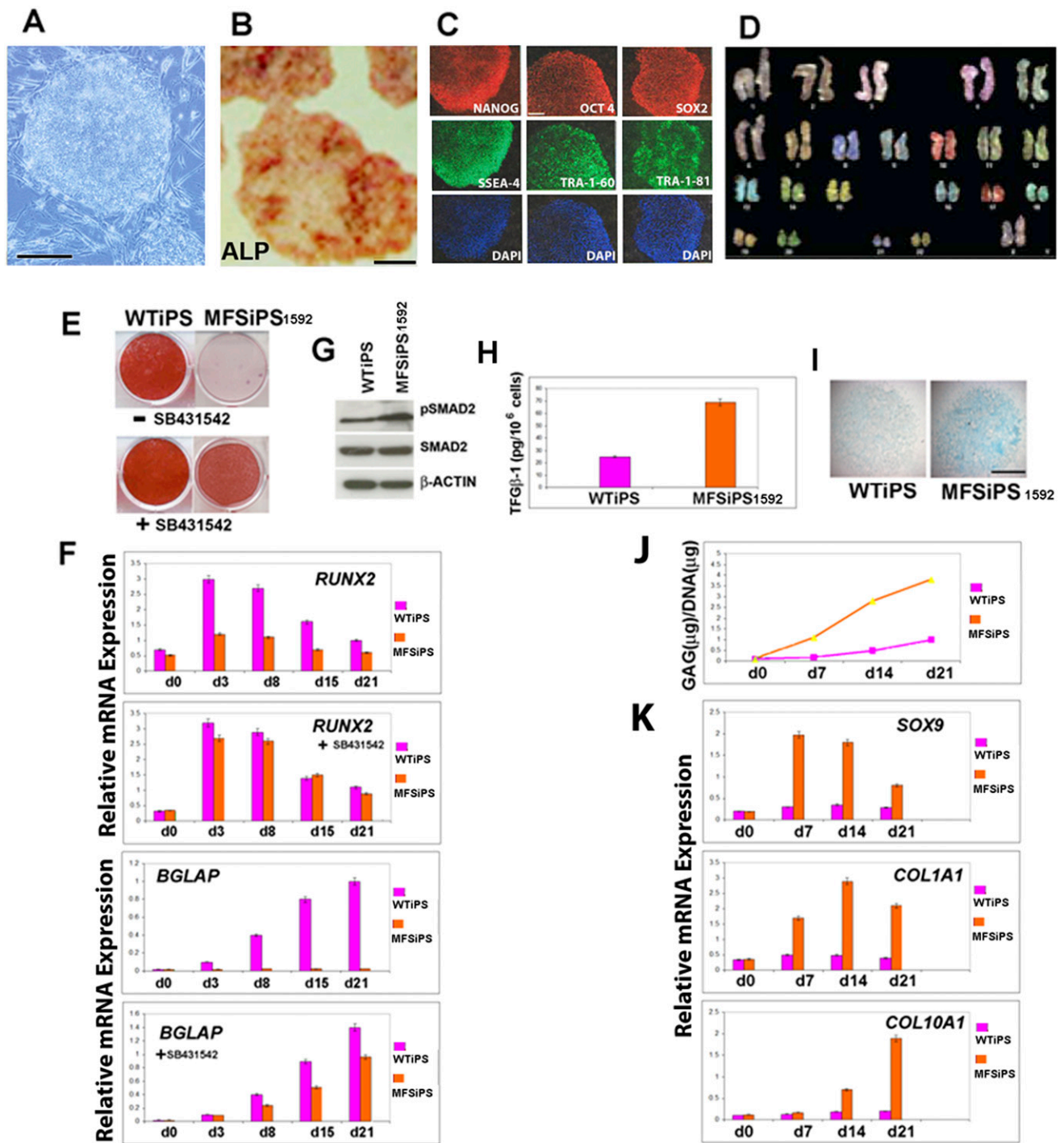
Fig. S1. Analysis of an independent MFSiPS 1592 cell line derived from MFS-patient fibroblasts harboring a *FIBRILLIN-1* (*FBN1*) frame-shift mutation (1642del3ins20bp) resulting in a premature stop codon in exon 14. (*A*) Morphology of MFSiPS1592 cells. (*B*) Alkaline phosphatase (ALPL) staining. (Scale bar, 100 μm.) (*C*) Immunofluorescence staining for embryonic stem cell markers in MFS-iPS1592. Nuclear counterstaining was performed with DAPI. (Scale bar, 250 μm.) (*D*) Spectral karyotyping analysis of MFSiPS1592 cells. (*E*) Alizarin red staining performed at day 21 of osteogenic differentiation assay shows impairment of osteogenesis in MFSiPS1592 cells compared with WTiPS cells. Treatment with 10 μM SB 431542 rescues the osteogenic differentiation of MFSiPS1592 cells. (*F*) qPCR analysis of osteogenic markers. (*G*) Immunoblotting analysis of pSMAD2 performed on subconfluent cells cultured for 24 h in αMEM +1%FBS indicates enhanced activation of TGF-β1 signaling in MFSiPS1592 cells. The membrane was stripped and subsequentially incubated with SMAD2 and β-actin antibody to assess for the total amount of endogenous SMAD2 and to control for equal loading and transfer of the samples. β-Actin was used as loading control. (*H*) ELISA performed on serum-free medium samples (conditioned for 48 h) reveals higher levels of active TGF-β1 in MFSiPS1592 cells. (*I*) Chondrogenic assay performed in absence of exogenous TGF-β1 demonstrates that MFSiPS1592 cells undergo robustly to chondrogenic differentiation compared with WTiPS cells. (Scale bar, 250 μm.) (*J*) GAG synthesis and (*K*) qPCR confirming the chondrogenic differentiation of MFSiPS1592 in absence of exogenous TGF-β1.
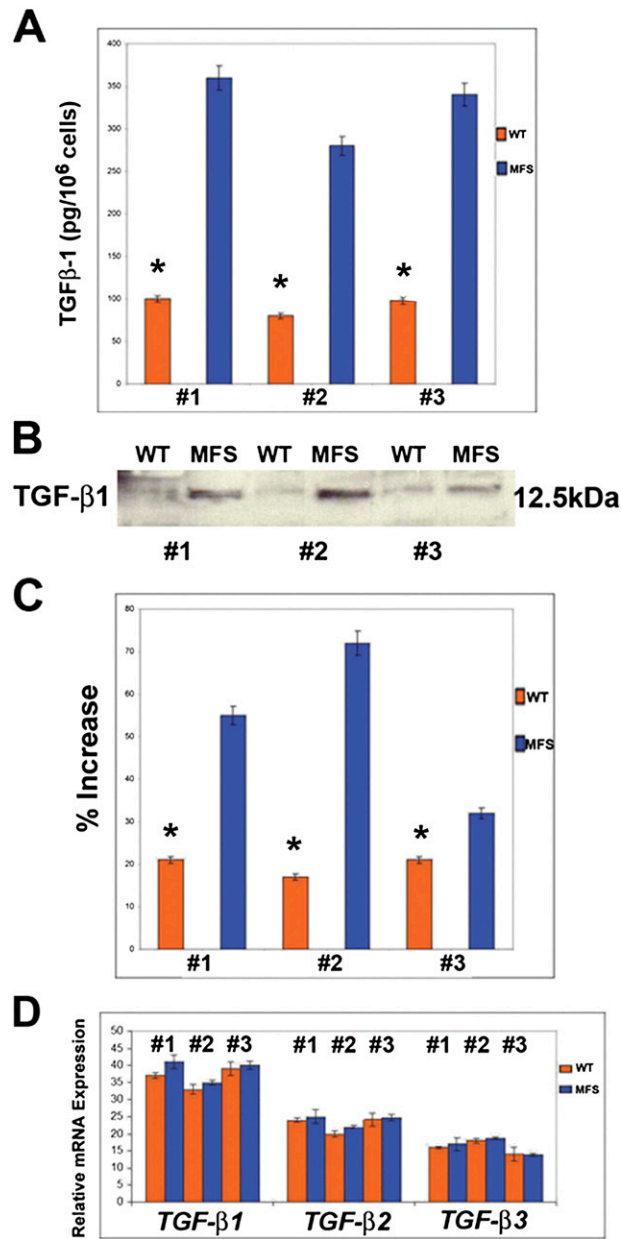
Fig. S2. Determination of active TGF-β1 and TGF-β transcripts in MFS and WT cells. (*A*) ELISA detects higher levels of active TGF-β1 in MFS than WT medium. *$P < 0.05$. (*B*) Immunoblotting analysis performed using antibody against TGF-β1 reveals larger amount of TGF-β1 monomer form in MFS medium. (*C*) Histogram showing densitometry analysis of immunoblot in *B*. *$P < 0.05$. (*D*) qPCR of TGF-β1, -2, and -3 genes does not reveal significant differences in the expression level between WT and MFS cells.
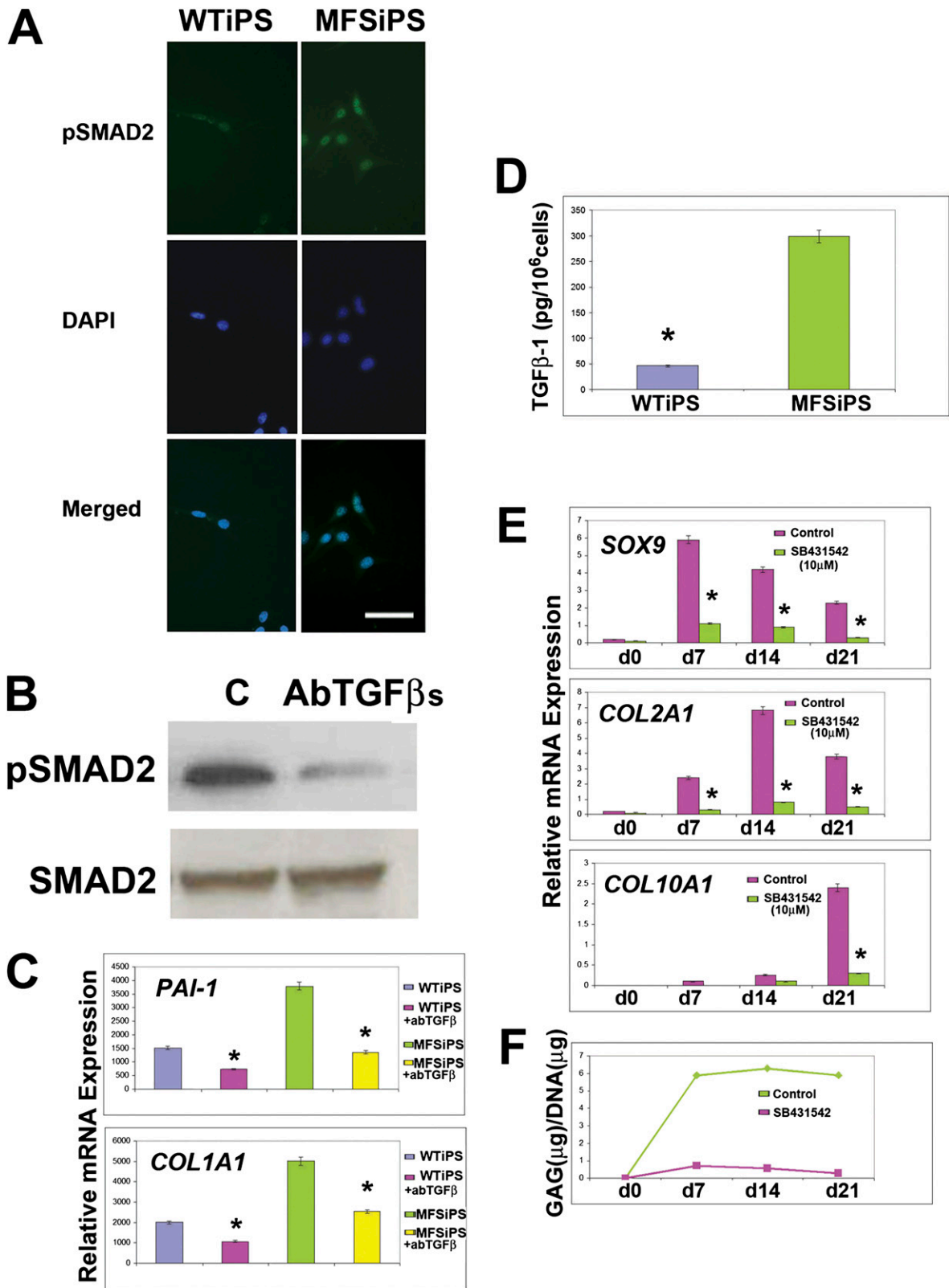
Fig. S3. Enhanced activation of TGF-β signaling and effect of its inhibition on MFS-induced pluripotent-stem cells (MFSiPS) chondrogenic differentiation. (*A*) Immunofluorescence performed with anti-pSMAD2 antibody reveals intense staining in MFSiPS cells. (Scale bar, 50μm.) (*B*) Immunoblotting analysis showing inhibition of SMAD2 phosphorylation in MFSiPS cells treated with pan–TGF-β-neutralizing antibody (1.2 μg/mL). (*C*) Treatment with neutralizing anti–TGF-β antibody also decreases up-regulation of *PAI-1* and *COL1A1* genes, as assessed by qPCR analysis. (*D*) ELISA detects higher levels of active TGF-β1 in media from MFSiPS cells than WTiPS. (*E*) qPCR analysis showing inhibition of chondrogenic markers in MFSiPS cells when treated with 10 μM SB431542. (*F*) Treatment with 10 μM SB431542 also inhibits GAG synthesis in MFSiPS cells.

Fig. S4. Enhanced activation of TGF-β signaling during osteogenic differentiation assay. (*A*) ELISA detects higher levels of active TGF-β1 in MFS than WT media throughout osteogenic differentiation. (*B*) Immunoblotting analysis performed on cell lysates (80 μg total protein) showed sustained phosphorylation of SMAD2 in MFS cells during the osteogenic assay. To assess for the total amount of endogenous SMAD2 and to control for equal loading and transfer of the samples the membrane was reprobed with anti-SMAD2 and anti–β-actin antibody. (*C*) ELISA detects higher levels of active TGF-β1 also in MFSiPS media compared with WTiPS. *$P < 0.05$. (*D*) Immunoblotting analysis reveals sustained phosphorylation of SMAD2 also in MFSiPS cells.

Table S1. qPCR primers

Gene	Forward	Reverse	Accession no.
COL1A1	CTCGAGGTGGACACCACCCT	CAGCTGGATGGCCACATCGG	NM_000088
RUNX2	CCAACCCACGAATGCACTATC	TAGTGAGTGGTGGCGGACATAC	NM_00102463.02
BGLAP	AAACCCAGCGGTGCAGAGT	GGCTCCCAGCCATTGATACA	NM_199173.3
TGFβ1	GACTACTACGCCAAGGAGGTCA	GAGCTCTGATGTGTTGAAGAACATATA	NM_011577.1
PAI 1	GGCTGACTTCACGAGTCTTTC	TTCGAGACTTTCTGCAGCT	M16006
TGFβ2	GCTAATGTTGTTGCCCTCCT	GCAGCAATTATCCTGCACATT	NM_009367.1
TGFβ3	GCACTTGCAAAGGGCTC	TTGGCATAGTATTCCGA	MN_003239
ALPL	ATGGGATGGGTGTCTCCACA	CCACGAAGGGGAACTTGTC	NM_001632
GAPDH	CCACGAAGGGGAACTTGT	GGGGTCATTGATGGCAACAATA	NM_002046
SOX9	CCCCAACAGATCGCCTACAG	GAGTTCTGGTCGGTGTAGTC	BC 056420
COL2 A1	GGCAATAGCAGGTTCACGTACA	GATAACAGTCTTGCCCCACTTACC	NM_001844
COL10A 1	ACGCTGAACGATACCAAATG	TGCTATACCTTTACTCTTTATGGTGTA	NM_000493