Supporting information

A reciprocal role of the Smad4-Taz axis in osteogenesis and adipogenesis of mesenchymal stem cells

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Supporting information includes

1. **Supporting information; Materials and Methods**
2. **Supporting information figures S1-S9, including legends**
3. **Supporting information Table S1-S5**

Supporting information; Materials and Methods

Transfection and reporter assay

Plasmids were transiently transfected into HEK293 cells using PEI (Polyethyleneimine). siRNAs (Qiagen, Hilden, Germany) sequences specific for endogenous SMAD4 (Supporting information Table S3) were reverse transfected into hASCs by Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA). Luciferase activity was performed with a dual-luciferase reporter assay system (Promega, Medison, WI).

Subcellular fractionation, immunoblot and immunoprecipitation

For subcellular fractionation, the Subcellular Proteome Extraction Kit (Thermo scientific, Waltham, MA) was used. For immunoblot analysis, cells were lysed in lysis buffer (1 % Triton X-100, 20 mM Hepes at pH 7.4, 150 mM NaCl, 12.5 mM b-glycerol phosphate, 1.5 mM $MgCl_2$, 10 mM NaF, 2 mM DTT, 1 mM NaOV, 2 mM EGTA, 1mM PMSF, protein inhibitor cocktail). Protein extracts were separated by SDS-PAGE, transferred to a PVDF membrane filter, and subjected to immunoblot analysis. For immunoprecipitation, lysates were incubated with protein G agarose beads (Gendepot) at 4 °C for 12 h. Immunocomplexes were washed twice with lysis buffer, and separated from the beads by adding 2X sample buffer and boiling. Immunoblot analysis was performed using the indicated antibodies.

RNA extraction and quantitative real-time RT-PCR

Total RNAs were isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA). The Superscript kit (Invitrogen, Carlsbad, CA) was used for reverse transcription. Primer sequences of the Smad4, Taz, ALP, OC, OSX, Runx2, aP2, LPL, C/EBPα, PPARγ, and GAPDH genes are described in Supporting information Table S4. For quantitative RT-PCR, an iCycler real-

time PCR machine and iQ SYBR Green Supermix (Bio-Rad) were used to measure the expression of genes under the following conditions: 40 cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s. All reactions were independently repeated at least three times to ensure reproducibility.

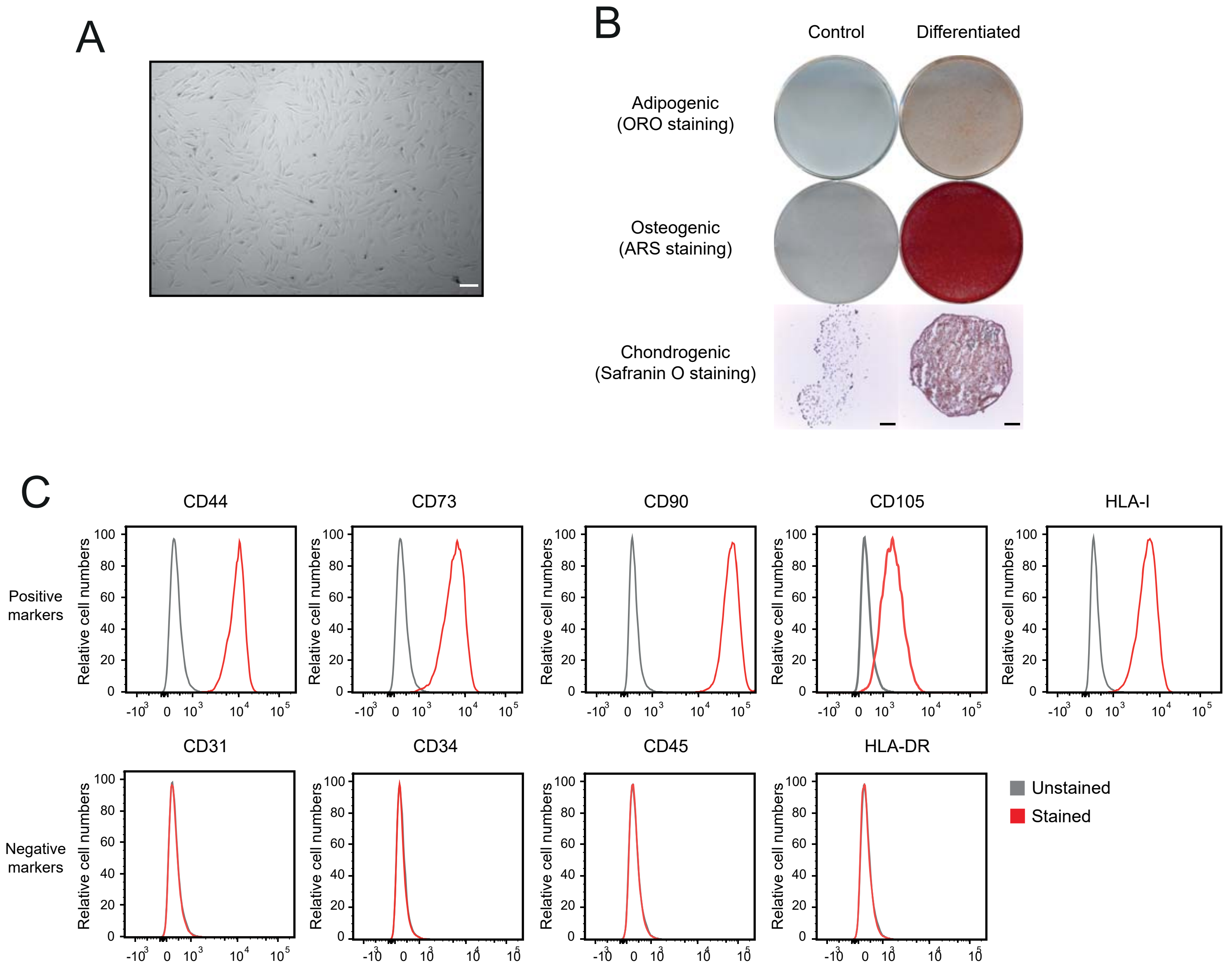
Immunofluorescence and FACS analysis

For immunofluorescence assays, cells were fixed in cold methanol at -20 °C for 7 min, followed by blocking (5 % BSA in PBS) at room temperature for 30 min and incubation with anti-Flag (F3165, Sigma) and anti-Taz (#4883, cell signaling) primary antibodies at 4 °C for 15 h. After washing with PBS, cells were stained with secondary antibodies at room temperature for 2 h. Alexa Fluor-488-conjugated goat anti-mouse IgG (A11001, Invitrogen), Alexa Fluor-594-conjugated goat anti-Rabbit IgG (A11012, Invitrogen) were used. Next, cells on coverslips were stained with DAPI (Sigma) and mounted on glass slides. Cells were examined with a laser-scanning confocal microscope (Carl-Zeiss). For the FACS assay, shGFP- and shSmad4-expressing C3H10T1/2 cells were stained against PerCP Cy5.5-CD11b, APC-CD44, FITC-CD45, APC-CD105, PE-Sca-1 and FITC-MHCII for 30 min at 4 ºC. Stained cells were washed in cold FACS buffer (3 % FBS in PBS) and analyzed on a FACS Canto II.

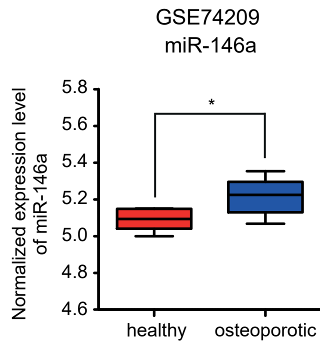
Chromatin immunoprecipitation (ChIP)

ChIP assays were performed with minor modifications based on the EZ-ChIP (Cat No.17-371) kit (Upstate Biotechnology, Charlottesville, VA). Differentiated C3H10T1/2 cells were crosslinked for 10 min with 1 % formaldehyde at 37 ˚C and quenched for 5 min with 125 mM glycine at 37 ˚C. Fixed cells were scrapped and sonicated on wet ice in lysis buffer (1 % SDS, 10 mM EDTA, 50 mM Tris at pH 8.1 and protease inhibitor cocktail). Sheared DNA-protein complexes were incubated for 15 h at 4 °C with rabbit anti-Taz antibody (#4883, CST) and

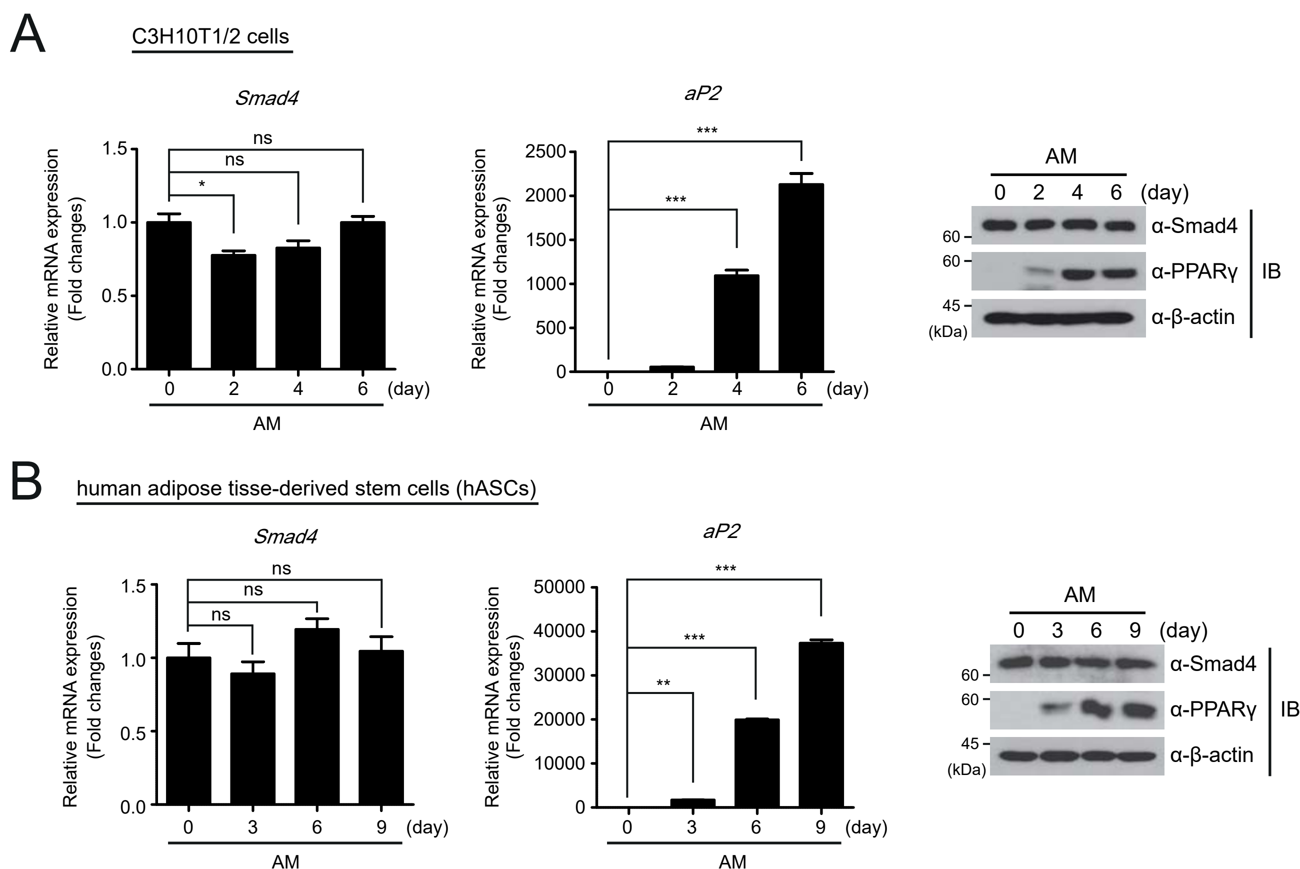
incubated with Protein A/G agarose beads (sc-2003, Santa Cruz) for 1 h at 4 °C. The immunoprecipitated material was washed with low salt wash buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl at pH 8.1, 150 mM NaCl), high salt wash buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl at pH 8.1, 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1 % NP-40, 1 % deoxycholic acid, 1 mM EDTA, 10 mM Tris at pH 8.1) and TE buffer (10 mM Tris-HCl at pH 8.0, 1 mM EDTA). After washing, DNA-protein complexes were eluted with 1 % SDS, 100 mM $NaHCO_3$ and reverse-crosslinked with 200 mM NaCl at 65 °C for 12 h. RNA and proteins were removed by treating with RNase A (iNtRON) and Proteinase K (TaKaRa), and DNA was purified by a purification kit (iNtRON). Purified DNA was amplified and analyzed by PCR or qRT-PCR. Primer sequences for the ChIP assay in this study are described in Supporting information Table S5.



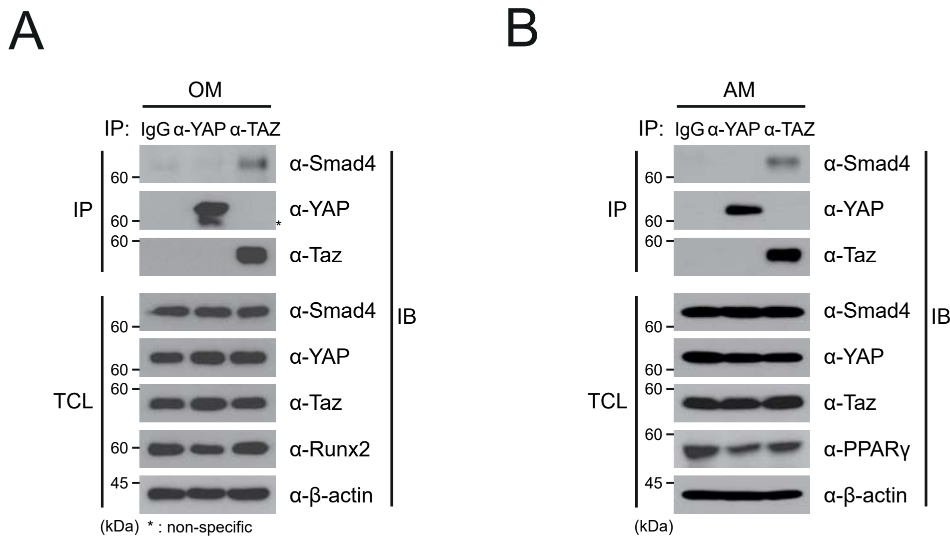
Supporting information figure S1. Characterization of human adipose tissue-derived stem cells (hASCs). (**A**): Fibroblast-like morphology of plastic adherent of hASCs were observed by microscope. Scale bars, 100μm. (**B**): Multilineage potential of hASCs was verified by cell type specific stainings using Oil Red O (ORO), Alizarin Red S (ARS), and Safranin O. Scale bars, 100μm. (**C**): Expression of several MSC markers were analyzed by flow cytometry. The images in flow cytometry are representative of three independent experiments.



Supporting information figure S2. Expression of miR-146a targeting Smad4 mRNA is increased in osteoporotic bones.
Box-plot analysis of miR-146a expression in healthy and osteoporotic femoral neck trabecular bones from 12 postmenopausal women from a GEO dataset (GSE74209, healthy people n=6, osteoporotic patients n=6). The data were statistically analyzed by unpaired, two-tailed Student's *t*-test (*$P < 0.05$ compared to healthy people). The boxes represent the interquartile range, centre is the median, and the minimum and maximum values are represented by the whiskers.

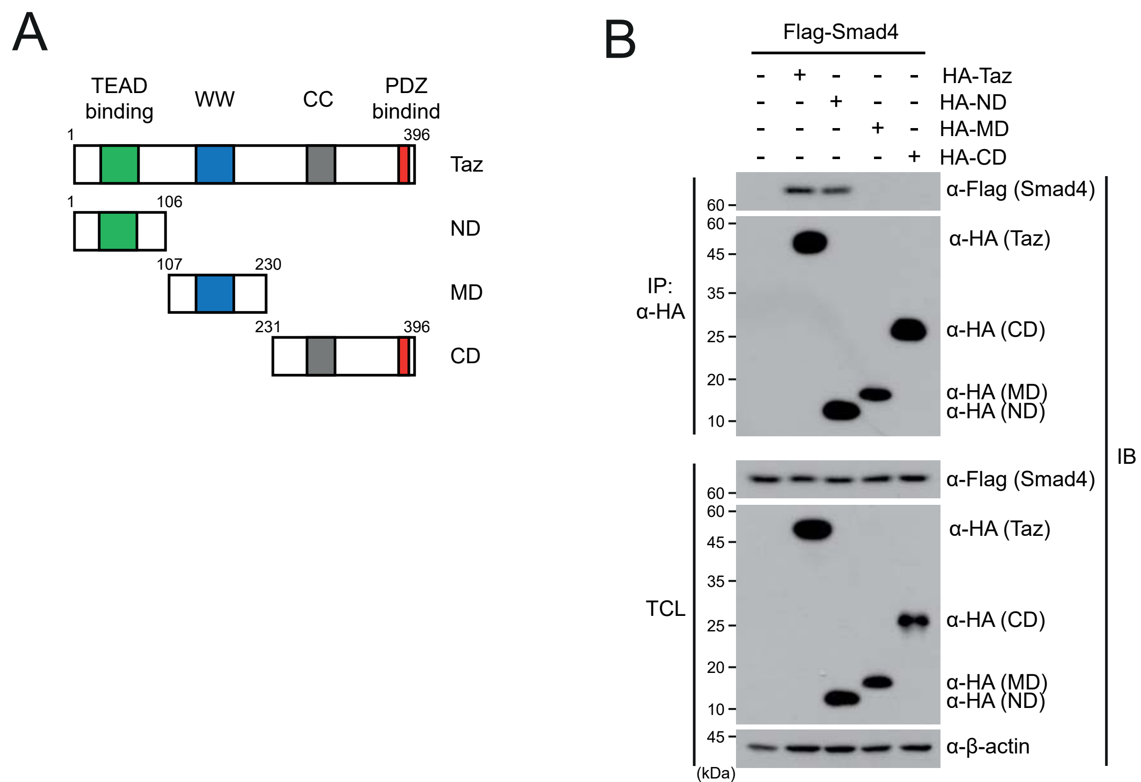


Supporting information figure S3. Expressions of Smad4, aP2, and PAPRγ during adipogenic differentiation. (**A, B**): Expression of Smad4 and aP2 mRNAs detected by qRT-PCR at the indicated time points during adipogenic differentiation of C3H10T1/2 cells (**A**) and human adipose tissue-derived stem cells (hASCs) (**B**). Expression of the aP2 gene was used as a positive control for adipogenesis. The data were statistically analyzed by one-way ANOVA followed by Tukey's multiple comparison test (**P* < 0.05, ***P* < 0.01 and ****P* < 0.001 compared to apidpogenic differentiation at day 0, *n*=3). Bars represent the mean ± SD. ns; not significant. Expression of the Smad4 and PPARγ proteins were analyzed by immunoblotting at the indicated time points during adipogenic differentiation of C3H10T1/2 cells and hASCs. Expression of β-actin was used as a loading control. AM; adipogenic differentiation medium. Expression of the PPARγ protein was used as a positive control for adipogenesis. The immunoblot images are representative of three independent experiments.

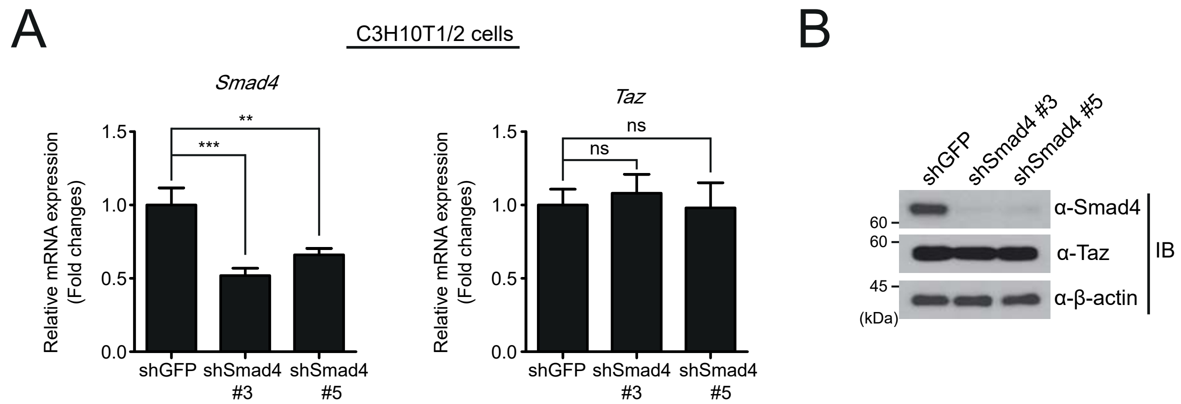


Supporting information figure S4. Smad4 only interacts with Taz at osteogenic and adipogenic differentiation, but not with YAP.

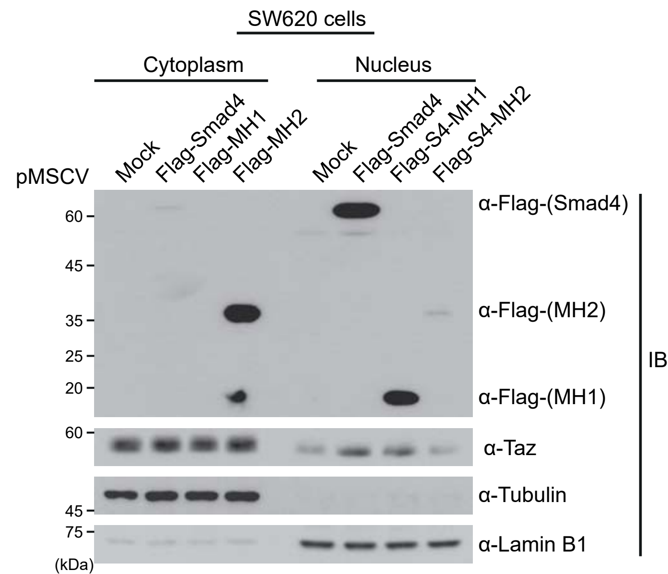
(**A**, **B**): Immunoprecipitation assay during osteogenic (**A**) and adipogenic (**B**) differentiation of C3H10T1/2 cells. Immunoprecipitation (IP) and immunoblot (IB) analysis were performed with the indicated antibodies against endogenous proteins. The immnoblot images are representative of three independent experiments. OM; osteogenic differentiation medium, AM; adipogenic differentiation medium, TCL; total cell lysates.



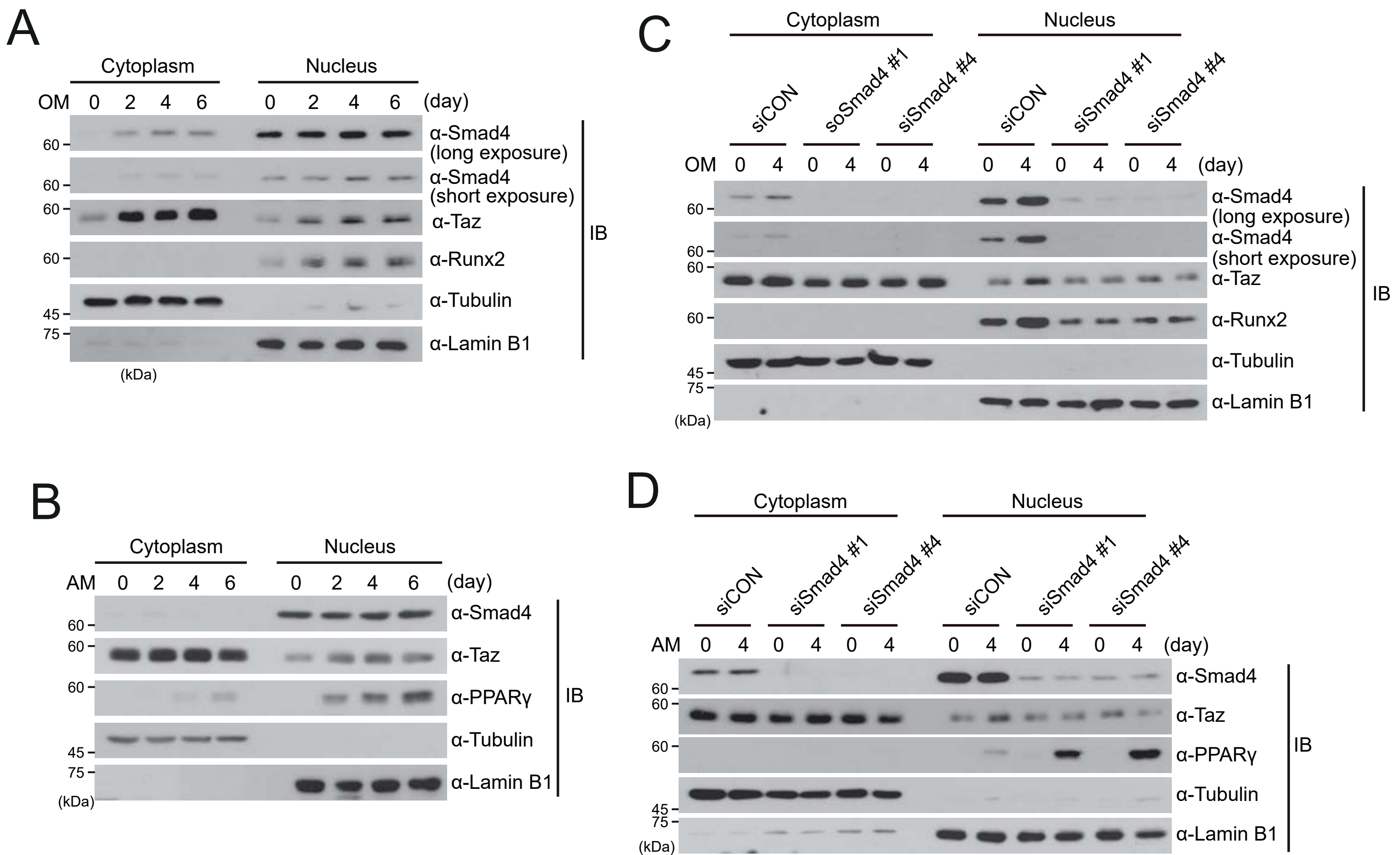
Supporting information figure S5. N-terminal domain of Taz interacts with Smad4.
(**A**): Schematic diagram of truncated Taz mutants. (**B**): Plasmids encoding Taz truncated mutants were respectively co-transfected with Flag-Smad4 plasmid into HEK293 cells. Cell lysates were immunoprecipitated (IP) with anti-HA antibody and subsequently immunoblotted with the indicated antibodies. TCL; total cell lysates. The immunoblot images are representative of three independent experiments.



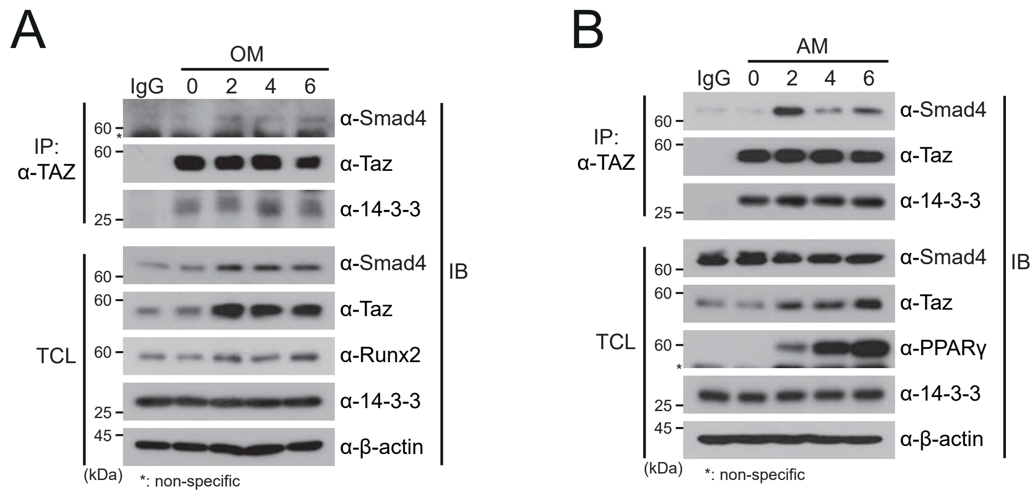
Supporting information figure S6. Smad4 does not affect expression of Taz mRNA and protein.
(**A**): Total RNA was isolated from Smad4-knockdown and shGFP-expressing C3H10T1/2 cells. Expression of the Smad4 and Taz mRNAs were analyzed by qRT-PCR. The data were statistically analyzed by one-way ANOVA followed by Tukey's multiple comparison test (** $P < 0.01$ and *** $P < 0.001$ compared to shGFP, n=3). Bars represent the mean ± SD. ns; not significant. (**B**): Immunoblot analysis of endogenous Smad4 and Taz proteins in Smad4-knockdown and shGFP-expressing cells. Expression of β-actin was used as a loading control. The immunoblot images are representative of three independent experiments.



Supporting information figure S7. Ectopic expression of Smad4 and the Smad4 MH1 domain enhance Taz retention in the nucleus.
Stable SW620 cells expressing full-length Smad4, or the MH1 and MH2 domain by the infection of recombinant retroviruses were fractionated into cytoplasmic and nuclear extracts. Both extracts were immunoblotted with the indicated antibodies. Expression of α-tubulin and α-lamin B1 were used as cytoplasmic and nuclear markers and loading controls. The immunoblot images are representative of three independent experiments.



Supporting information figure S8. Smad4 facilitates the nuclear localization of Taz during osteogenesis and adipogenesis of C3H10T1/2 cells and hASCs.
(**A**): C3H10T1/2 cells were incubated in osteogenic differentiation medium. Cell lysates at the indicated time points after osteogenic differentiation were fractionated into cytoplasm and nuclei. Taz localization was analyzed by immunoblots. (**B**): C3H10T1/2 cells were differentiated into adipocytes. Cell lysates at the indicated time points after adipogenic differentiation were fractionated into cytoplasmic and nuclear extracts. Both extracts were immunoblotted with the indicated antibodies. (**C**, **D**): Smad4-knockdown and siCON-expressing hASCs were differentiated into osteoblasts (**C**) and adipocytes (**D**). Differentiated Smad4-knockdown and control cells were fractionated into cytoplasmic and nuclear extracts on day 4. Taz localization was analyzed by immunoblottinng with the indicated antibodies. OM; osteogenic differentiation medium. AM; adipogenic differentiation medium. Expression of α-tubulin and α-lamin B1 in this fiugre were used as cytoplasmic and nuclear markers and loading controls. The immunoblot images are representative of three independent experiments.



Supporting information figure S9. Smad4 does not affect Taz interaction with the 14-3-3 protein.
(**A**, **B**): C3H10T1/2 cells were differentiated into osteoblasts (**A**) and adipocytes (**B**) for the indicated times, respectively. Cell lysates were immunoprecipitated with anti-Taz antibody against endogenous Taz protein and subsequently immunoblotted with the indicated antibodies. TCL; total cell lysates. Expression of β-actin was used as a loading control. The immunoblot images are representative of three independent experiments. OM; osteogenic differentiation medium, AM; adipogenic differentiation medium.

Supporting information Table S1. Primer sequences used to construct plasmids in this study

Construct	**Direction**	**Sequence (5' - 3')**
Flag-Smad4, HA-Smad4	Forward	ATGGACAATATGTCTATAACAAAT
	Reverse	TCAGTCTAAAGGCTGTGGGT
pMSCV-Flag-Smad4	Forward	GCCACCATGGATTACAAGGATGACGACGATAAGATG GACAATATGTCTATAACAAAT
	Reverse	TCAGTCTAAAGGCTGTGGG
pMSCV-Flag-S4-MH1	Forward	GCCACCATGGATTACAAGGATGACGACGATAAGATG GACAATATGTCTATAACAAAT
	Reverse	CTATGAGACAACCCGCTCATAGT
pMSCV-Flag-S4-MH2	Forward	GCCACCATGGATTACAAGGATGACGACGATAAGCCT GAGTACTGGTGCTCCA
	Reverse	TCAGTCTAAAGGCTGTGGG
HA-S4-MH1	Forward	ATGGACAATATGTCTATAACAAAT
	Reverse	CTATGAGACAACCCGCTCATAGT
HA-S4-Linker	Forward	CCTGGAATTGATCTCTCAGG
	Reverse	TCAAGGAGCAGGATGATTGGAAAT
HA-S4-MH2	Forward	CCTGAGTACTGGTGCTCCA
	Reverse	TCAGTCTAAAGGCTGTGGGT
HA-S4-ΔMH1	Forward	CCTGGAATTGATCTCTCAGG
	Reverse	TCAGTCTAAAGGCTGTGGGT
HA-S4-ΔMH2	Forward	ATGGACAATATGTCTATAACAAAT
	Reverse	TCAAGGAGCAGGATGATTGGAAAT
pMSCV-Flag-hSmad4	Forward	GCCACCATGGATTACAAGGATGACGACGATAAGATG GACAATATGTCTATTACGAAT
	Reverse	TCAGTCTAAAGGTTGTGGGTC
pMSCV-Flag-S4-NLSm	Forward	CCATTTGTAGTTATAGCTGTTATTAAAGAATCCAATT CATCTGCTGCCTCTGCCAGCGCCGCTACCAAACTCT CAATTGCTCTTTTTGCAAAGGTTTCACTTTCCCC
	Reverse	GGGGAAAGTGAAACCTTTGCAAAAAGAGCAATTGA GAGTTTGGTAGCGGCGCTGGCAGAGGCAGCAGATG AATTGGATTCTTTAATAACAGCTATAACTACAAATGG
Flag-TAZ, HA-TAZ	Forward	ATGAATCCGTCCTCGGTGCC
	Reverse	TTACAGCCAGGTTAGAAAGGG

Table S1. – continued

HA-ND	Forward	ATGAATCCGTCCTCGGTGCCCC
	Reverse	TTAAGGGCCTCCAGCGGCTCCCG
HA-MD	Forward	ATGGCACAGCAGCATGCACATCTCCG
	Reverse	TTACTGCTGCTGCTGAGTGGTCAG
HA-CD	Forward	ATGCAGCAGAAACTGCGGCTTCAGAGG
	Reverse	TTACAGCCAGGTTAGAAAGGGCTCGC
HA-Runx2, pcDNA-Runx2	Forward	ATGGCGTCAAACAGCCTCTT
	Reverse	TCAATATGGCCGCCAAACAG
Flag-PPARγ, HA-PPARγ, pcDNA-PPARγ	Forward	ATGGGTGAAACTCTGGGAGA
	Reverse	CTAATACAAGTCCTTGTAGATC
pGL3-mOC-Luc	Forward	CCAAGACCATGGCCCAGGG
	Reverse	GACTTGTCTGTTCTGCACCC

Supporting information Table S2. Primary antibodies used in this study.

Antibody name	Company	Cat No.	Species	Assay
HA	Covance	16B12	Mouse	IB, IP
Tubulin	Millipore	05-829	Mouse	IB
Lamin B1	Abcam	ab16048	Rabbit	IB
Taz	BD bioscience	560235	mouse	IB
Smad4	Santa Cruz Biotechnology	sc-7966	Mouse	IB
Smad1/5/8	Santa Cruz Biotechnology	sc-6031	Rabbit	IB
PPARγ	Santa Cruz Biotechnology	sc-7273	Mouse	IB
Runx2	Santa Cruz Biotechnology	sc-390715	Mouse	IB
14-3-3	Santa Cruz Biotechnology	sc-133233	Mouse	IB
GFP	Santa Cruz Biotechnology	sc-9996	Mouse	IB
Smad2	Cell signaling	#3103	Mouse	IB
Smad3	Cell signaling	#9523	Rabbit	IB
Smad4	Cell signaling	#9515	Rabbit	IB
PPARγ	Cell signaling	#2443	Rabbit	IB
Runx2	Cell signaling	#12556	Rabbit	IB
Taz	Cell signaling	#4883	Rabbit	IB, IP, IF, ChIP
Flag	Sigma	F3165	Mouse	IB, IP, IF
Flag	Sigma	F7425	Rabbit	IB
β-actin	Sigma	A5316	Mouse	IB
Sca-1	BD bioscience	562059	Mouse	FACS
CD44	BD bioscience	561882	Mouse	FACS
CD45	BD bioscience	561088	Mouse	FACS
CD31	BD bioscience	555445	Human	FACS
CD34	BD bioscience	555821	Human	FACS
CD44	BD bioscience	550989	Human	FACS
CD45	BD bioscience	347463	Human	FACS
CD73	BD bioscience	550257	Human	FACS
CD90	BD bioscience	555595	Human	FACS
CD105	BD bioscience	561443	Human	FACS
HLA-DR	BD bioscience	347363	Human	FACS
HLA-I	BD bioscience	555552	Human	FACS
CD105	Thermo Scientific	17-1051	Mouse	FACS
CD11b	Thermo Scientific	45-0112	Mouse	FACS
MHCII	Thermo Scientific	11-5322	Mouse	FACS

IB: immunoblot, IP: immunoprecipitation, IF: immunofluorescence

Supporting information Table S3. The target sequences of shRNAs and siRNAs used in this study.

Lentiviral shRNA	Species	Target Sequences
shSmad4 #3	Mouse	GCCAGCTACTTACCATCATAA
shSmad4 #5	Mouse	ATCCAACACCCGCCAAGTAAT
siRNA	**Species**	**Target Sequences**
siSmad4 #1	Human	AAGCAGCGTCACTCTACCTAA
siSmad4 #4	Human	CCCTGTTAAACAGTAGTTGTA

Supporting information Table S4. Primer sequences for real-time RT-PCRs used in this study.

Construct	Species	Direction	Sequence (5' - 3')
Smad4	Mouse	Forward	GTCTAACGCCACCAGCAC
		Reverse	GGAGGCTGGAATGCAAGC
TAZ	Mouse	Forward	GTGAAGGCTTCTCGGTTGAG
		Reverse	ACGTGTTGCTTGCCTTGTCT
ALP	Mouse	Forward	CCAGCAGGTTTCTCTCTTGG
		Reverse	CTGGGAGTCTCATCCTGAGC
OC	Mouse	Forward	CTGACCTCACAGATGCCAAGC
		Reverse	TGGTCTGATAGCTCGTCACAAG
OSX	Mouse	Forward	CCCCTTGTCGTCATGGTTACAG
		Reverse	AGAGAAAGCCTTTGCCCACCTA
Runx2	Mouse	Forward	CACGGTGACTCCCGTTACTT
		Reverse	ATACGTGTGTGACCCAGTGCAA
aP2	Mouse	Forward	GATGCCTTTGTGGGAACCTG
		Reverse	TCCTGTCGTCTGCGGTGATT
LPL	Mouse	Forward	ATTGAAACACCTGGCCTTTG
		Reverse	TGAGCCATGTCTTCAACTGC
C/EBPα	Mouse	Forward	GCCGAGATAAAGCCAAACA
		Reverse	CCTTGACCAAGGAGCTCTCA
PPARγ	Mouse	Forward	CTTATTTATGATAGGTGTGATCTTAACTGC
		Reverse	GTGATATGTTTGAACTTGATTTTATCTTCT

Supporting information Table S5. Primer sequences for ChIP-PCRs used in this study.

Position relative to TSS	Direction	Sequence (5' - 3')
mouse *OC* promoter	Forward	CTGAACTGGGCAAATGAGGACA
	Reverse	AGGGGATGCTGCCAGGACTAAT
mouse *aP2* promoter	Forward	GCTTGATTGTTACAAGGCAAGGAAGG
	Reverse	CCAGCAGGAATCAGGTAGCTGGAG