Supplementary Table. Representative genes regulated by the SOX trio

Gene name	Gene symbol	Accession no.	Fold change
Genes up-regulated more than 5-fold by the SOX trio			
S100 calcium binding protein, beta	*S100B*	NM_006272	28.0
S100 calcium binding protein A1	*S100A1*	NM_006271	15.1
serine (or cysteine) proteinase inhibitor, clade A, member 3	*SERPINA3*	NM_001085	14.1
prodynorphin	*PDYN*	NM_024411	10.9
scrapie responsive protein 1	*SCRG1*	NM_007281	10.6
matrilin 3	*MATN3*	NM_002381	9.2
dermatan sulfate proteoglycan 3	*DSPG3*	NM_004950	7.8
chromobox homolog 3 (HP1 gamma homolog, Drosophila)	*CBX3*	NM_007276	7.1
empty spiracles homolog 1 (Drosophila)	*EMX1*	NM_004097	7.0
chitinase 3-like 1 (cartilage glycoprotein-39)	*CHI3L1*	NM_001276	6.5
nuclear DNA-binding protein	*C1D*	NM_006333	6.4
etoposide-induced mRNA	*EI24*	NM_004879	6.3
nth endonuclease III-like 1 (E. coli)	*NTHL1*	NM_002528	6.2
mitofusin 2	*MFN2*	NM_014874	5.8
collagen, type IX, alpha 1	*COL9A1*	NM_001851	5.6
matrilin 1, cartilage matrix protein	*MATN1*	NM_002379	5.2
casein kinase 2, beta polypeptide	*CSNK2B*	NM_001320	5.0
Other extracellular matrix genes up-regulated by the SOX trio			
cartilage linking protein 1	*HAPLN1*	NM_001884	3.1
aggrecan 1	*AGC1*	NM_001135	2.2
collagen, type IX, alpha 3	*COL9A3*	NM_001853	2.1
collagen, type II, alpha 1	*COL2A1*	NM_001844	2.0
collagen, type XI, alpha 1	*COL11A1*	NM_001854	2.0
Genes down-regulated more than 5-fold by the SOX trio			
spindlin family, member 2	*SPIN2*	NM_019003	0.07
translocation associated membrane protein 1	*TRAM1*	NM_014294	0.14
ATPase, H+ transporting, lysosomal 9kDa, V0 subumit e1	*ATP6V0E1*	NM_003945	0.16
ATP6V1B2 ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B2	*ATP6V1B2*	NM_001693	0.17
ADP-ribosylation factor 3	*ARF3*	NM_001659	0.19

Genes up- or down-regulated more than 5-fold in human mesenchymal stem cells with adenoviral introduction of SOX trio in comparison with the LacZ-introduced control and other up-regulated extracellular matrix genes were shown. All results of the microarray analysis are provided at ArrayExpress (accession number: E-MEXP-960).

Supplementary Fig S1

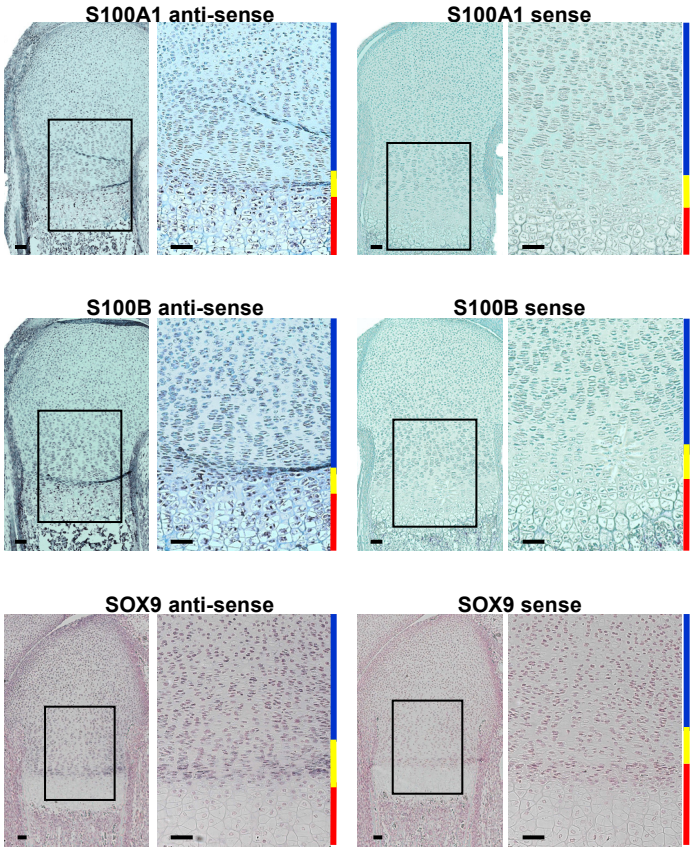

Fig S1. Co-expressions of S100A1, S100B, and SOX9 in differentiated chondrocytes of the fetal mouse growth plate by in situ hybridization using the specific anti-sense probes and the respective sense probes as the negative controls. Inset boxes in the left figures indicate the regions of the respective right figures. Blue, yellow and red bars indicate layers of proliferative, prehypertrophic and hypertrophic zones, respectively. Scale bars, 50 μm.

Supplementary Fig S2

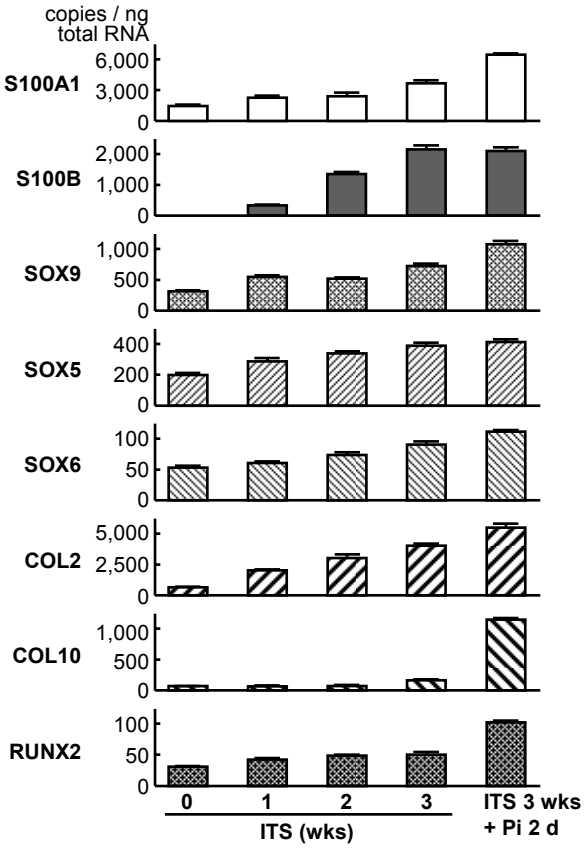

Fig S2. Time course of mRNA levels of S100A1, S100B, SOX9, 5, 6, COL2, COL10 and RUNX2 in cultured ATDC5 cells during chondrogenic and hypertrophic differentiation. The cells were cultured in the differentiation medium containing ITS for the indicated periods or ITS and Pi, and the levels were determined by real-time RT-PCR analysis. Data are expressed as means (bars) ± SDs (error bars) for 3 wells / group.

Supplementary Fig S3

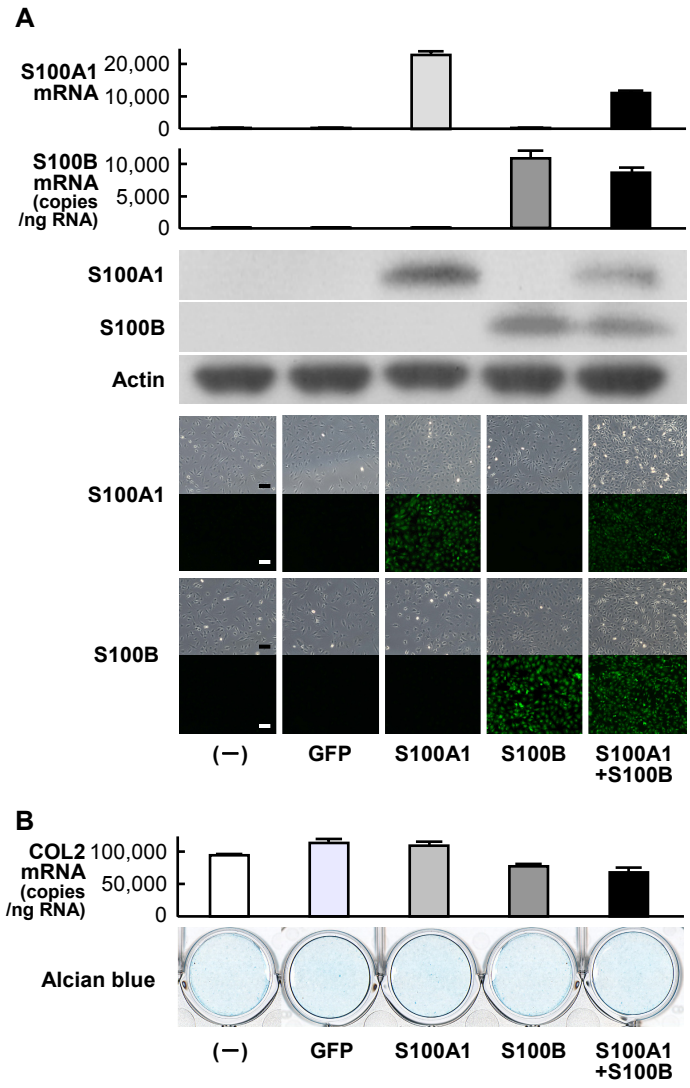

Fig S3. (**A**) Specific expressions of S100A1 and S100B determined by real-time RT-PCR, Western blot, and immunocytochemical analyses in stable lines of ATDC5 cells with retroviral transfection of GFP, S100A1, S100B or their combination, and in non-transfected parental cells (−) after 3 d of culture with the maintenance medium. Immunocytochemistry is shown by immunofluorescence under non-fluorescence (upper panels) and fluorescence (lower panels) microscope. Scale bars, 50 μm. (**B**) The early differentiation markers COL2 mRNA level and Alcian blue staining in the five kinds of cells above after 1 week of culture with ITS. The graphs are expressed as means (bars) ± SDs (error bars) for 3 wells / group.

Supplementary Fig S4

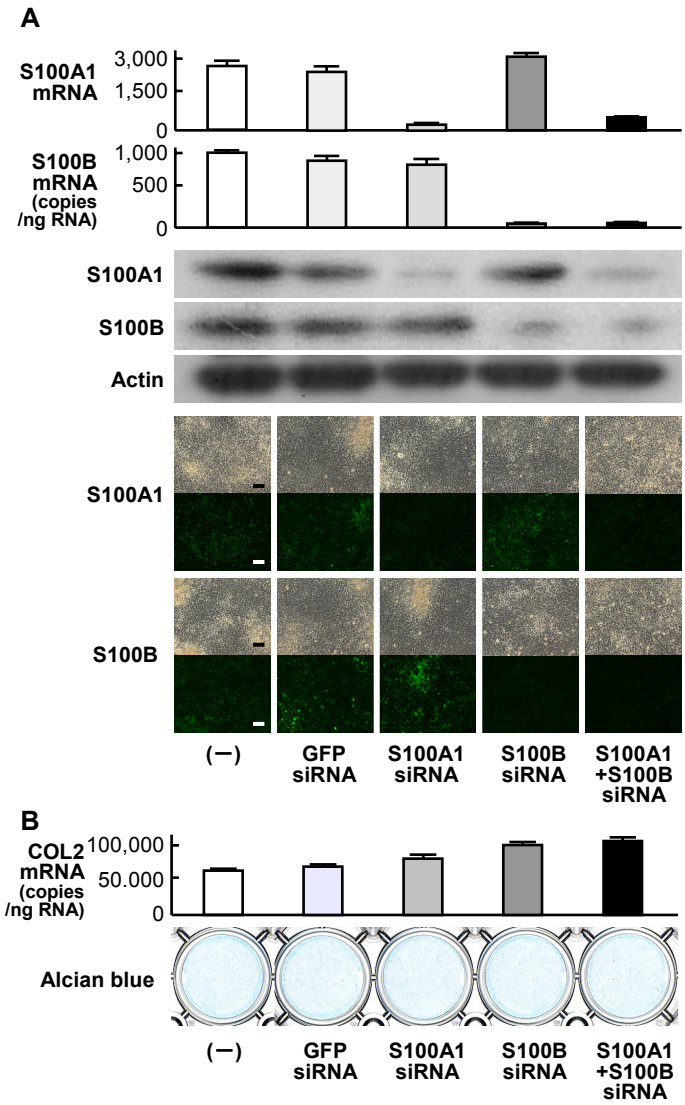

Fig S4. (**A**) Specific expressions of S100A1 and S100B determined by real-time RT-PCR, Western blot, and immunofluorescence analyses in stable lines of ATDC5 cells retrovirally transfected with siRNA of GFP, S100A1, S100B, or their combination, and in non-transfected parental cells (−) after 2 weeks of culture with ITS. Scale bars, 50 μm. (**B**) The early differentiation markers COL2 mRNA level and Alcian blue staining in the five kinds of cells above after 1 week of culture with ITS. The graphs are expressed as means (bars) ± SDs (error bars) for 3 wells / group.

Supplementary Fig S5

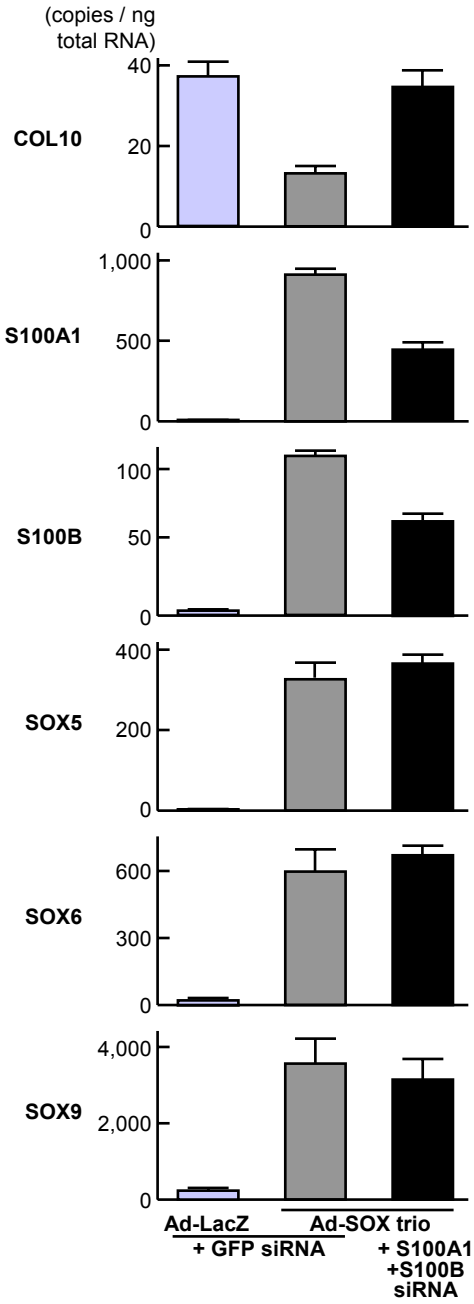

Fig S5. COL10, S100A1, S100B, SOX5, SOX6, and SOX9 mRNA levels in cultured hMSC co-transfected with LacZ or the SOX trio by adenovirus, and siRNA of GFP or S100A1+S100B by retrovirus. After the transfections, the cells were cultured in the differentiation medium containing dexamethasone, β -glycerophosphate and ascorbic acid-2-phosphate for 2 weeks. mRNA levels were determined by real-time RT-PCR, and data are expressed as means (bars) $\pm$ SDs (error bars) for 3 wells / group.

Supplementary Fig S6

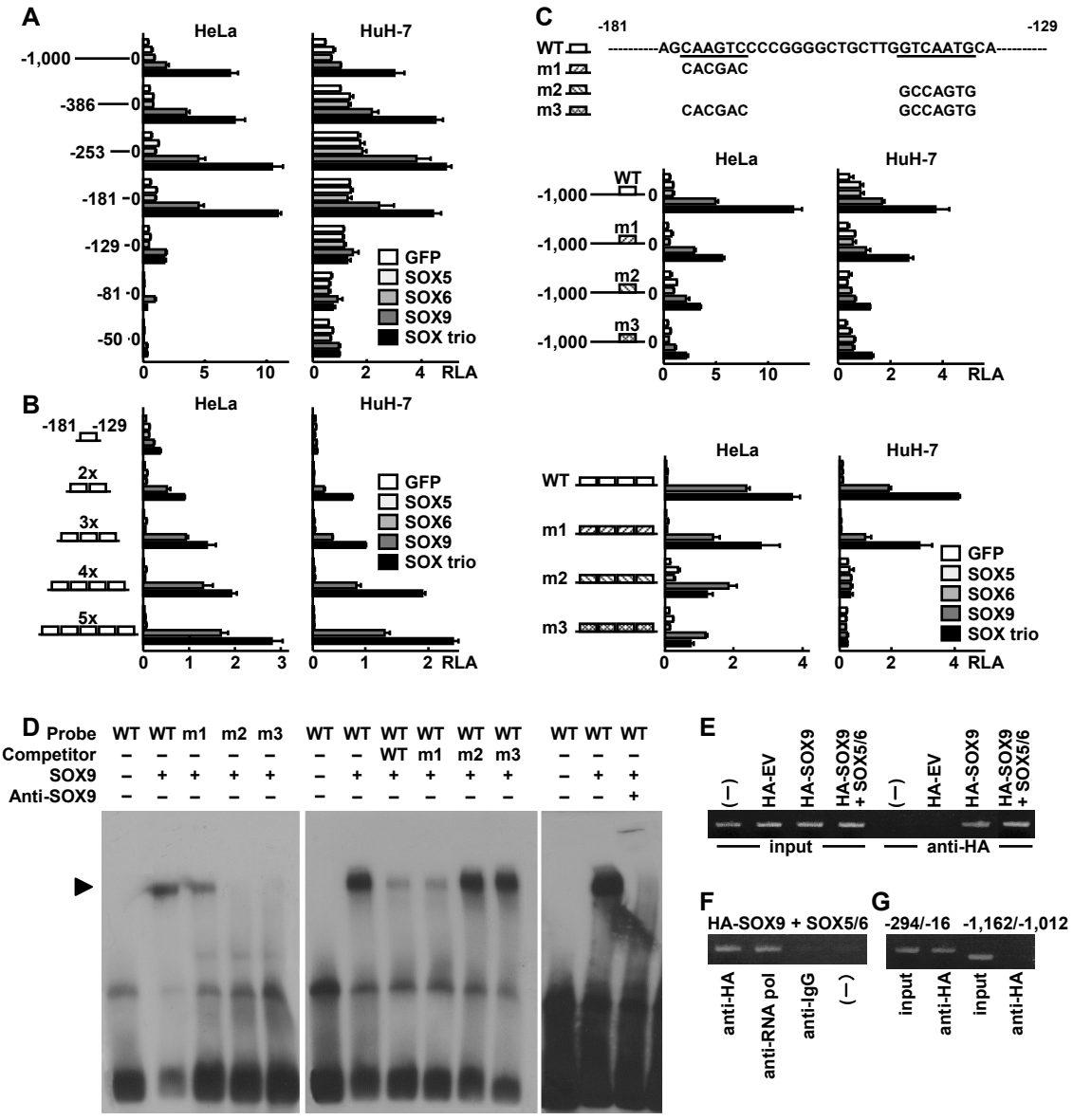

Fig S6. The SOX trio transcriptionally induces S100B through activation of a SOX9 enhancer element in the 5'-end flanking region by luciferase-reporter (**A**-**C**), EMSA (**D**), and ChIP (**E**-**G**) analyses. Similarly to S100A1 in Fig 4, deletion (**A**), tandem-repeat (**B**), and site-directed mutagenesis (**C**) analyses of the S100B regulatory region were performed by the luciferase-reporter assay in HeLa and HuH-7 cells transfected with the SOX trio or the GFP control. (**D**) EMSA for specific binding of the SOX9 protein with the WT and the mutated oligonucleotide probes of the identified responsive element (-181/-129) (left), cold competition with 50-fold excess of unlabeled WT and mutated probes (middle), and the supershift by an anti-SOX9 antibody (right). (**E**) ChIP assays for specific binding of SOX9 to the responsive element (-181/-129). Lysates of ATDC5 cells transfected with HA-tagged empty vector (HA-EV) or HA-tagged SOX9 in the presence or absence of SOX5 and 6 (HA-SOX9 + SOX5/6 or HA-SOX9, respectively) were amplified by a primer set spanning the responsive element (-294/-16) before (input) and after (anti-HA) the immunoprecipitation with an anti-HA antibody. The specificity of binding was confirmed by changing the antibodies for immunoprecipitation (**F**) and the primer sets for amplification (**G**), as described in Fig 4. The graphs are expressed as means (bars) ± SDs (error bars) for 3 wells / group.

SUPPLEMENTARY METHODS

Construction of expression vectors. Full-length human S100A1 and S100B cDNA were amplified by PCR and cloned into pShuttle vectors (Clontech) and pMx vectors (Kitamura, 1998). Adenovirus vectors expressing S100A1 and S100B were constructed with the AdenoX Expression system (Clontech), according to the manufacturer's instructions. Adenoviruses were packaged and amplified in HEK293 cells and purified with an AdenoX virus purification kit (Clontech). The viral titers were estimated with an AdenoX rapid titer assay kit (Clontech). Production of retrovirus vectors was performed as described (Morita et al., 2000). 2 x 10^6 Plat-E cells were plated in 60-mm dishes 1 d before transduction. On the following day, cells were transfected with 2 μg pMx vector using Fugene (Roche). After 24 h, the medium was replaced with fresh medium, which was collected and used as the retrovirus supernatant 48 h after the transfection. Retrovirus expressing GFP was also constructed for the control. Puromycine resistance gene was inserted into pMx vector of GFP and that of S100A1, and blasticidin resistance gene was inserted into that of S100B for selection of stable cells. RNAi sequence was designed for the mouse S100A1 gene (nucleotides 423-443), the mouse S100B gene (nucleotides 216-236), the human S100A1 gene (nucleotide 263-281), the human S100B gene (nucleotide 220-240) and GFP (GCTACGTCCAGGAGCGCACCA). siRNA expression vectors for these genes were constructed with piGENEmU6 vector (for mouse) or piGENEhU6 vector (for human) (iGENE Therapeutics) as described(Miyagishi and Taira, 2004). For retrovirus expressing siRNA, U6 promoter and inserts in piGENE vectors were cloned into pMx vectors. Puromycine resistance gene was inserted into pMx vector of GFP-siRNA and that of S100A1-siRNA, and blasticidin resistance gene was inserted into that of S100B-siRNA for selection of stable inhibition cells. The expression vectors and adenoviruses of SOX5, SOX6,

SOX9, LacZ and GFP were created as previously described (Ikeda et al., 2004). For chromatin immunoprecipitation assay, cDNA of SOX9 was inserted into pCMV-HA vector (Clontech).

Cell cultures. For generation of the stable cell lines, 3 x 10^5 ATDC5 cells were plated and cultured in 60-mm dishes for 1 d, and the retrovirus supernatant was added to the cells with polybrene (8 μg / mL final concentration). After 2 d, the cells were passaged into 100-mm dishes and cultured with the medium containing 3 μg / mL puromycin and/or 10 μg / mL blasticidin until confluency. Selective transduction of S100A1 and/or S100B was confirmed by real-time RT-PCR, Western blotting and immunocytochemistry. For the stable inhibition of S100, retroviruses expressing siRNA of S100A1 and/or S100B were used. The selective inhibition was confirmed after 2 weeks culture with ITS supplement.

For the pellet culture, ATDC5 cells were cultured in 60-mm dishes until confluency, and adenoviruses expressing SOX or S100 genes were transduced at 100 multiplicities of infection (MOI). Two days after transduction, cells were trypsinized and gently centrifuged to form pellets, which were cultured in the maintenance medium. Cells were collected at 10 d after pellet formation for real-time RT-PCR and Western blot analysis.

For hMSC differentiation experiment, 1 x 10^5 hMSC were plated and cultured in 60-mm dishes for 1 d, and the retrovirus supernatant expressing S100 siRNA was added to the cells together with polybrene (8 μg / mL final concentration). After 2 d, the medium was replaced with fresh medium containing 1 μg / mL puromycin and/or 10 μg / mL blasticidin. After 5 d, adenovirus expressing SOX genes were transduced at 100 MOI. Two days after adenovirus transduction, the medium was replaced with α-MEM/10% FBS supplemented with dexamethasone (100 nM), β-glycerophosphate (10 mM) and ascorbic acid-2-phosphate (0.05 mM). After 2 weeks, the cells were collected for real-time RT-PCR.

Microarray analysis. hMSC were infected with the adenoviruses carrying the SOX trio or the LacZ-control at 100 MOI, and total RNA was isolated from hMSC after 2 weeks of culture with an RNeasy mini kit (Qiagen), according to the manufacturer's instructions. Total RNA samples were treated with DNase I. ^{33}P-labeled cDNA probes were prepared using the Atlas Pure Total RNA Labeling System from total RNA samples. Labeling of probes, hybridization, exposure to screen and analysis were carried out under contract by Clontech using the Atlas Plastic Human 12K Microarray (Clontech, containing long oligonucleotides of 11,904 genes) and AtlasImage software (Clontech). Precise experimental conditions are described in the user manual of Atlas Plastic Microarrays (available at the Clontech homepage). The ratio of signal was calculated as adjusted intensity in the experimental (the SOX trio) array / adjusted intensity in the control (LacZ) array. Adjusted intensity equals the raw intensity minus the background value, multiplied by the normalization coefficient. GAPDH was used as a normalization standard, and the normalization coefficient was 1.16. Genes up- or down-regulated more than 2-fold were picked up.

Real-time RT-PCR analysis. Total RNAs from cells were isolated with an RNeasy mini kit (Qiagen), according to the manufacturer's instructions. All total RNA samples were treated with DNase I. Total RNAs (50 ng to 1 μg) were reverse-transcribed with MultiScribe reverse transcriptase (ABI) and random hexamers in a 50 μL reaction volume, according to the manufacturer's instructions, and 1 μL of each reverse transcriptase reaction was used as a template for the second-step SYBR Green real-time PCR. The full-length or partial-length cDNA of target genes, including PCR amplicon sequences, were amplified by PCR, cloned into pCR-TOPO Zero II or pCR-TOPO II vectors (Invitrogen), and used as standard templates after linearization. QuantiTect SYBR Green PCR Master Mix (Qiagen) was used for the second-step

SYBR Green real-time PCR according to the manufacturer's instructions. SYBR Green PCR amplification and real-time fluorescence detection were performed with an ABI 7700 Sequence Detection system. All reactions were run in quadruplicate. Copy numbers of target gene mRNA in each total RNA were calculated by reference to standard curves and were adjusted to the human or mouse standard total RNA (ABI) with the human GAPDH or rodent Gapdh as an internal control. Each primer position in the coding sequences of target genes is described below. The human set was as follows: for S100A1 (NM_006271), 140-354; for S100B (NM_006272), 125-331; for COL10 (NM_000493), 1641–1843; for GAPDH (NM_002046), 108-333. The mouse set was as follows: for S100A1 (NM_011309), 238-433; for S100B (NM_009115), 138-300; for SOX9 (NM_011448), 1675-1862; for SOX5 (NM_011444), 1887-2122; for SOX6 (NM_011445), 2287-2444; for RUNX2 (NM_009820), 1407-1556; for COL2 (NM_031163), 3713–3951; for COL10 (NM_009925), 910-1120; for GAPDH (NM_ 001001303); 506-682.

Western blot analysis. Western blot analysis was performed with cell extracts from ATDC5 cells. Whole cell lysates were separated by 4–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride filters. The filters were incubated with an antibody against S100A1 (1:500; Upstate), S100B (1:500; Sigma), and actin (1:1000; Sigma). Antigen–antibody complexes were detected with HRP–conjugated secondary antibodies (Promega) and visualized with the use of an ECL-Plus system (Amersham).

In situ hybridization. Tissues from C57BL6 mouse embryos (E17.5) were fixed in 4% paraformaldehyde/PBS overnight at 4°C, embedded in paraffin, and cut into 5-μm sections. In situ hybridization for S100A1, S100B and SOX9 was performed as described previously (De Block and Debrouwer, 1993) using sense and anti-sense DIG-labeled RNA probes for mouse S100A1 (nucleotides 43-658), S100B (nucleotides 61-397) and SOX9 (nucleotides 2848-3130).

The signals were detected with ALP–conjugated anti-DIG antibodies (Roche). The sections were then counterstained with methylgreen (S100A1 and S100B) or fast red (SOX9).

Luciferase assay. The human S100A1 and S100B promoter regions from -1,000 bp relative to the transcriptional start site were obtained by PCR using human genomic DNA as a template and were cloned into the pGL3-Basic vector (Promega). Deletion and mutation constructs were created by PCR technique. Tandem-repeat constructs were created by ligating the double strand oligonucleotides of A1 box or B box into pGL3-Basic vector. HeLa and HuH-7 cells were used for the luciferase assay because of the low level expressions of endogenous SOX proteins and the excellent transfection efficiency. Transfection of the cells was performed in triplicate in 24-well plates using Fugene (Roche) with a total amount of 300 ng of plasmid DNA, 200 ng of pGL3 reporter vector, 100 ng of effector vector, and 8 ng of pRL-TK vector (Promega) for internal control. Cells were harvested 48 h after the transfection. The results were shown as relative luciferase activities (RLA), the ratio of the firefly activities to the Renilla activities (internal control to minimize experimental variability caused, for instance, by differences in cell viability and transfection efficiency. Our preparatory experiments have confirmed that the RLA showed similar results to those of the absolute firefly activity values.

EMSA. Binding reactions were incubated for 30 min at room temperature. For competition analyses, 50-fold excess of unlabeled competitor probe was included in the binding reaction. For the supershift experiments, 1 μL of anti-SOX9 antibody (Santa Cruz Biotechnology) was added after 30 min of the binding reaction, and the reaction was incubated for an additional 30 min at room temperature.

ChIP assay. HeLa cells were transfected with HA-tagged empty vector (HA-EV) or HA-tagged SOX9 in the presence or absence of SOX5 and 6 (HA-SOX9 + SOX5/6 or HA-SOX9,

respectively) using Fugene. In vivo crosslinking was performed 48 h after the transfection. To shear genomic DNA, the lysates were then sonicated on ice for 10 sec X 5 times. For immunoprecipitaion, antibodies against HA (Abcam), RNA polymerase II (Upstate, for positive control), and the non-immune rabbit IgG (Promega, for negative control) were used. Two kinds of primer sets, one spanning and the other not spanning the identified enhancer element; -233/-29 and -1,300/-1,143 for S100A1, and -294/-16 and -1,162/-1,012 for S100B; were used.

References

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