Supplementary Materials and Methods

Construction of expression vectors

Expression vectors were prepared for GFP and RUNX1 in pEGFP (Clontech) and siRNA expression vectors for mouse *Sox5* (GCGCTGGAGTCTCAGAGTCAA), *Sox6* (GTTATGTTCCCTATATTCC), *Sox9* (GGCTACGACTGGACGCTGGTG), *Runx1* (TGACCACC CTGGCGAGCTA), and *GFP* (GCTGGAGTACAACTACAACAG) in piGENEmU6 vector (iGENE Therapeutics). All siRNA vectors were confirmed to knock down the targets using immunoblotting. Adenovirus vectors were prepared as previously described.[1] Cells were transduced with adenoviral vectors at MOI (a multiplicity of infection) 100.

Cell cultures

We maintained C3H10T1/2, COS-7, HuH-7 and ATDC5 cells (Riken BRC, Tsukuba, Japan) in a monolayer culture as previously described.[2 3] Human articular chondrocytes were purchased from Lonza Walkersville Inc (Wakersville, MD, USA). Primary mouse chondrocytes were isolated cultured as previously described.[3 4] Toluidine blue, alcian blue, and alizarin red stainings were used to assess cartilaginous matrix synthesis and mineralization. A WST-8 Assay Kit was used to determine proliferation (Dojindo, Mashiki, Japan).

Screening of small compound libraries

Natural small compounds were purchased from InterBioScreen, Inc. (Chernogolovka, Russia); synthetic small compounds were provided by Takeda Chemical Industries (Osaka, Japan). We previously reported the monitoring system of chondrogenic differentiation using Col2GFP-ATDC5 cells. Briefly, we established ATDC5 cells that were stably expressed with 4 tandem repeats of enhancer region of Sox9 to the COL2A1 (+2,126/+2,174) cloned upstream of the COL2A1 basal promoter (-1,031/+37) in combined with the EGFP gene (called

Col2GFP–ATDC5 cells). We confirmed that Col2GFP–ATDC5 cells showed time-dependent increases in the specific green fluorescence during the cultures, without being destroyed or fixed, and the fluorescence was enhanced by stimulation with insulin, a known chondrogenic stimulator of ATDC5 cells. This system allowed us to monitor the complex process of chondrogenic differentiation of living cells in real time without analyzing differentiation markers or staining the cells.[5] Using Col2GFP–ATDC5 cells, we screened natural and synthetic compound libraries to identify the preeminent chondrogenic compound among them. In the first screening, Col2GFP-ATDC5 cells were treated with each compound at 20 mg/ml for 6 days and GFP fluorescence observed every 3 days. To analyse the initial hit compounds, Col2GFP-ATDC5 cells were treated with each compound at 2 mg/ml for 7 days, and cartilaginous matrix synthesis assessed by toluidine blue staining. To analyse the second hit compounds, Col2GFP-ATDC5 cells were treated with each compound at 2 mg/ml for 7 days, and the mRNA expression of *Col2a1* examined by real-time RT-PCR analysis. Finally, to analyse the third hit compounds, Col2GFP-ATDC5 cells were treated with each compound at 10^{-6} M, and fluorescence assessed within 2 days after commencing the treatment.

Real-time RT-PCR analysis

Total RNA was extracted from cells using an RNeasy Mini kit (Qiagen, Valencia, CA, USA). One microgram of RNA was reverse-transcribed with QuantiTect Reverse Transcription (Qiagen) to produce single-stranded cDNA. Real-time RT-PCR was performed with an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using FastStart Universal SYBR Green Master (Roche, Tokyo, Japan) with rodent actin or human GAPDH as the internal control. Primer sequence information is available upon request.

Biochemical measurement of glycosaminoglycan

Whole cell lysates were collected from C3H10T1/2 cells or primary mouse chondrocytes, using an M-PER kit (Pierce Chemical, Rockford, IL, USA) and glycosaminoglycan (GAG) contents were evaluated by the Alcian blue-binding assay (Wieslab, Lund, Sweden).

Metatarsal organ culture

The second, third and fourth metatarsals were dissected from mouse embryonic hindlimbs under sterile conditions and cultured in 1 mL of MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 50 μg/ml ascorbic acid, 1 mM β-glycerophosphate, and 0.25% fetal bovine serum (Gibco BRL, Rockville, MD, USA) in the presence or absence of TD-198946.

Metatarsal Staining

Metatarsal rudiments were cultured for 7 days and then harvested and fixed in 4% paraformaldehyde buffered with PBS. *In situ* hybridization was performed for Col10a1 using DIG-labelled RNA probes. von Kossa staining was performed as described previously.[6 7]

Microarray analysis

Total RNA was isolated from C3H10T1/2 cells treated with TD-198946 (10^{-6} M) or dimethyl sulfoxide (DMSO) as a vehicle for 48 h. A microarray analysis was then performed using the Gene Chip Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA, USA), and analysed with the GeneChip Operating Software 1.2 (Affymetrix). Gene ontology analysis was undertaken with GOTERM_BP_ALL using the DAVID v6.7 (The Database for Annotation, Visualization and Integrated Discovery).

Luciferase reporter assay

A human *COL2A1* promoter region was constructed (from −1,031 to +37 bp relative to the transcriptional start site) via PCR using human genomic DNA as a template and cloned into pGL3-Basic vector (Promega). Deletion and mutation constructs were also created by PCR,

and the 12xGCCG-luc, 9xCAGA-luc, Top flash, and constitutively active TCF (TCF-CA) expressing plasmids were prepared as previously described.[3] C3H10T1/2 cells were treated with vehicle, TD-198946 (10^{-6} M), BMP2 (100 ng/ml, R&D Systems, Minneapolis, MN, USA) or TGF-β1 (1 ng/ml, R&D Systems), or transfected them with TCF-CA expressing plasmid six hours after transfection. We performed luciferase assays with the PicaGene Dual SeaPansy Luminescence Kit (Toyo Ink, Tokyo, Japan) using HuH-7 cells and showed the data as the ratio of the firefly activities to the Renilla activities.

EMSA

Nuclear extracts were prepared from COS-7 cells that were transfected with plasmids expressing GFP-tagged Runx1 (Runx1-GFP) and EMSA was performed using a DIG gel shift kit (Roche). The region of the oligonucleotide probe ranged from −304 to −275 bp relative to the transcriptional start site. For competition analysis, a 50-fold excess of unlabelled competitor probe containing the binding reaction was used. For the supershift experiment, 2 μg of an antibody to Runx1(C-19X; Santa Cruz Biotechnology, Santa Cruz, USA) was added.

ChIP assay

HeLa cells were transfected with GFP or Runx1-GFP using a Shearing ChIP kit and a One day ChIP kit (Diagenode). Immunoprecipitation was performed using antibodies to Runx1 (H-65X; Santa Cruz Biotechnology), GFP (JL-8; Clontech), and normal IgG (Promega). Primer sets, one spanning and the other not spanning the identified binding region of Runx1, ranged from −400 to −200, and from −1,685 to −1,467, respectively.

Animals and materials

All experiments were approved by the Animal Care and Use Committee of the University of Tokyo. WT C57BL/6N male mice were obtained from the Charles River Laboratories

(Wilmington, MA, USA). Human samples were obtained from OA patients undergoing total knee arthroplasty after obtaining written informed consent as approved by the Ethics Committee of the University of Tokyo.

References

1. **Chikuda H,** Kugimiya F, Hoshi K*, et al.* Cyclic GMP-dependent protein kinase II is a molecular switch from proliferation to hypertrophic differentiation of chondrocytes. *Genes Dev* 2004;**18**:2418-29.
2. **Buttery LD,** Bourne S, Xynos JD*, et al.* Differentiation of osteoblasts and in vitro bone formation from murine embryonic stem cells. *Tissue Eng* 2001;**7**:89-99.
3. **Yano F,** Kugimiya F, Ohba S*, et al.* The canonical Wnt signaling pathway promotes chondrocyte differentiation in a Sox9-dependent manner. *Biochem Biophys Res Commun* 2005;**333**:1300-8.
4. **Gosset M,** Berenbaum F, Thirion S*, et al.* Primary culture and phenotyping of murine chondrocytes. *Nat Protoc* 2008;**3**:1253-60.
5. **Kan A,** Ikeda T, Saito T, et al. Screening of chondrogenic factors with a real-time fluorescence-monitoring cell line ATDC5-C2ER: identification of sorting nexin 19 as a novel factor. *Arthritis Rheum* 2009;**60**:3314-23.
6. **Lee K,** Deeds JD, Chiba S*, et al.* Parathyroid hormone induces sequential c-fos expression in bone cells in vivo: a model for intercellular communication in bone. *Miner Electrolyte Metab* 1995;**21**:120-2.
7. **Ohba S,** Kawaguchi H, Kugimiya F*, et al.* Patched1 haploinsufficiency increases adult bone mass and modulates Gli3 repressor activity. *Dev Cell* 2008;**14**:689-99.