Functional Gene Screening System Identified TRPV4 as a Regulator of Chondrogenic Differentiation*  

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Sox9 is a transcription factor that is essential for chondrocyte differentiation and chondrocyte-specific gene expression. However, the precise mechanism of Sox9 activation during chondrogenesis is not fully understood. To investigate this mechanism, we performed functional gene screening to identify genes that activate SOX9-dependent transcription, using full-length cDNA libraries generated from a murine chondrogenic cell line, ATDC5. Screening revealed that TRPV4 (transient receptor potential vanilloid 4), a cation channel molecule, significantly elevates SOX9-dependent reporter activity. Microarray and quantitative real time PCR analyses demonstrated that during chondrogenesis in ATDC5 and C3H10T1/2 (a murine mesenchymal stem cell line), the expression pattern of TRPV4 was similar to the expression patterns of chondrogenic marker genes, such as type II collagen and aggrecan. Activation of TRPV4 by a pharmacological activator induced SOX9-dependent reporter activity, and this effect was abolished by the addition of the TRPV antagonist ruthenium red or by using a small interfering RNA for TRPV4. The SOX9-dependent reporter activity due to TRPV4 activation was abrogated by both EGTA and a calmodulin inhibitor, suggesting that the Ca²⁺/calmodulin signal is essential in this process. Furthermore, activation of TRPV4 in concert with insulin activity in ATDC5 cells or in concert with bone morphogenetic protein-2 in C3H10T1/2 cells promoted synthesis of sulfated glycosaminoglycan, but activation of TRPV4 had no effect alone. We showed that activation of TRPV4 increased the steady-state levels of SOX9 mRNA and protein and SOX6 mRNA. Taken together, our results suggest that TRPV4 regulates the SOX9 pathway and contributes to the process of chondrogenesis.

Chondrogenesis is an important biological event for endochondral bone development, skeletogenesis, and tissue patterning (1, 2). The first step in chondrogenesis is the aggregation of mesenchymal cells into prechondrogenic condensations. These condensations start to express cartilage-specific genes and further differentiate into mature chondrocytes. In the growth plate, chondrocytes proliferate and further differentiate into hypertrophic chondrocytes. The control of chondrogenic differentiation and hypertrophy plays a pivotal role in the process. Dysregulation of either step leads to severe skeletal dysplasia in both mice and humans (3).

The transcription factor Sox9 (SRY (sex-related Y)-type high mobility group box), which contains a SRY-related high mobility group box, has an essential role in the chondrocyte differentiation pathway (4, 5). Sox9 regulates the transcription of cartilage-specific extracellular matrix molecules, such as collagen type II (6), IX (7), and XI (8) and aggrecan (9). Heterozygous mutations in the Sox9 gene cause campomelic dysplasia characterized by severe chondrodysplasia (10). Sox9 heterozygous mutant mice and mice lacking SOX9 function show impaired endochondral bone formation (4, 5). Sox9 is also involved in the expression of Sox5 and Sox6, both of which form the transcriptional complex with Sox9 and control the expression of type II collagen and aggrecan (4, 11). These findings indicate that Sox9 plays essential roles in chondrogenesis. Although several molecules involved in chondrocyte differentiation have been identified, the mechanism of chondrogenesis is not fully understood. Identification of the mechanisms that control expression and activity of Sox9 would provide important insights into the regulation of chondrogenesis.

We recently established a powerful functional cDNA screening system to identify molecules involved in NF-κB2 and MAPK signaling pathways, and we successfully identified many potential activators of these signals (12). That study and others provided a genome-wide screening method based on large scale cDNA transfection and showed that, as a functional genomics method, large scale transfection linked to functional screening is an effective approach for searching for genes related to specific functions (12–16).

In the present study, we used a similar approach for identifying additional components and modulators that are involved

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in the regulation of Sox9 and chondrocyte differentiation. We constructed full-length cDNA libraries derived from ATDC5 cells using the oligo-capping method (17, 18) and screened the libraries by performing a luciferase reporter assay using the SOX9-dependent type II collagen gene promoter. We isolated several positive cDNA clones that clearly stimulated the reporter gene and identified TRPV4 (transient receptor potential vanilloid 4) acting as a SOX9 regulator during chondrogenesis. Thus, our findings reveal novel functional roles for TRPV4 in chondrocyte differentiation.

**Experimental Procedures**

**Cell Culture and Analysis of Chondrocytic Differentiation—**ATDC5, a murine chondrogenic cell line, was obtained from the RIKEN cell bank (Tsukuba, Japan) and cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (Invitrogen) containing 5% FBS, 10 μg/ml human transferrin (Sigma), and 3 × 10^{-8} M sodium selenite (Wako) (19). A murine mesenchymal stem cell line, C3H10T1/2, was obtained from the RIKEN cell bank (Tsukuba, Japan) and cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium containing 10% FBS. To induce chondrogenic differentiation, bovine insulin (Sigma) was added to the medium at a concentration of 10 μg/ml human transferrin (Sigma), and 3 × 10^{-8} M sodium selenite (Wako) (19). A murine mesenchymal stem cell line, C3H10T1/2, was obtained from the RIKEN cell bank (Tsukuba, Japan) and cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS. To induce chondrogenic differentiation, bovine insulin (Sigma) was added to the medium at a concentration of 10 μg/ml ATDC5 cells, and an appropriate concentration of human recombinant bone morphogenetic protein-2 (BMP-2) (R & D Systems) was added to C3H10T1/2 cells. The medium was replaced once every 2 days. To evaluate chondrocyte differentiation, cells were fixed with 95% methanol and stained with 0.1% Alcian blue 8GS (Sigma) in 0.1N HCl overnight. Alcian blue-stained cultures were extracted with 6 M guanidine HCl for 2 h at room temperature. Optical density of the extracted dye was measured at 620 nm.

**Construction of Full-length cDNA Library and Arrayed cDNA Pool—**ATDC5 cells were cultured with or without 10 μg/ml bovine insulin for 4 days. RNA samples were prepared from both samples and used for the construction of full-length cDNA libraries in the pME18S-FL3 mammalian expression vector (GenBank™ accession number AB009864). The procedure for constructing the full-length cDNA library using the oligo-capping method is described elsewhere (17, 18). We randomly isolated 40,000 cDNA clones from the cDNA library of ATDC5 cells stimulated with insulin and 80,000 cDNA clones from the library without insulin. In total, 120,000 cDNA clones were isolated and used to construct an arrayed cDNA pool in 96-well microtiter plates. Plasmid DNAs were purified using QIAwells of 96 Ultra Plasmid Kits (Qiagen) according to the manufacturer’s instructions.

**Screening of the Full-length cDNA Library—**SOX9-dependent transcription was measured by using a reporter construct, tentatively named 4Col2E-Luc, which contained four tandem 48-bp chondrocyte-specific enhancer segments of type II collagen α1 (Col2a1) in the pGL3 Basic vector (Promega), as previously reported (6, 20). Reporter assays were performed using transient transfection. In each well of a 96-well microtiter plate, ATDC5 cells were inoculated at a density of 7.5 × 10^{3} cells/well and cultured overnight prior to transfection. Cells were transfected with 50 ng of each cDNA clone and 100 ng of reporter plasmid using 0.3 μl of FuGENE 6 (Roche Applied Science). Immediately after transfection, ATDC5 cells were stimulated with 5 ng/ml insulin-like growth factor-1 (Roche Applied Science). At 48 h after transfection, cells were harvested, and luciferase activity was measured using PicaGeneLT2.0 (Toyo B-Net). cDNAs that produced more than 2-fold induction of luciferase activity relative to the parental plasmid (pME18S-FL3), the mock control, were defined as positive clones.

**Preparation of Tissue Samples—**Primary murine chondrocytes were prepared from the rib cages of 4-week-old DDY mice (Nihon SLC) by collagenase digestion (0.2% collagenase (Wako) in phosphate-buffered saline) after adherent connective tissue and muscle was thoroughly removed by trypsin and collagenase pretreatment. Then cells were subjected to RNA extraction. Articular cartilage tissues were surgically prepared from femoral condyles and the tibial plateaus of 12-week-old ICR mice (Clea Japan). Embryonic hind limb buds were surgically prepared from embryonic day 12 embryos of DDY mice (Clea Japan). Embryonic hind limb buds were surgically prepared from embryonic day 12 embryos of DDY mice. Both tissues were then subjected to RNA extraction.

**Quantitative and Conventional Reverse Transcription-PCR Analysis—**Total RNA was extracted from cells using an RNase kit (Qiagen) with DNase I (Qiagen) treatment, and 0.5 μg of total RNA was used to synthesize cDNA using SuperScript III reverse transcriptase (Invitrogen). The cDNAs were then used for quantitative RT-PCR, the products of which were analyzed using an ABI PRISM 7000 sequence detection system. Expression values were normalized to ribosomal protein RPL19. The following oligonucleotides were used. The dual fluorophore-labeled probe for mouse TRPV4 was 5’-FAM-TCAGGCACTTGAGAGGCACGC-TAMRA-3’, and the PCR primers were 5’-TCTTACCCTCACGGCTACT-3’ and 5’-TCCACTGTTGGTCCGGTAAAG-3’; for mouse COL2A1, the dual fluorophore-labeled primer was 5’-FAM-CTTGAGGCTTCCACGACCCGACATCACCCTA-TAMRA-3’, and the PCR primers were 5’-TCCAGATGACTTTTCTCCGTCTA-3’ and 5’-AGGTAGCGATGTCTGTTTACA-3’; for mouse aggrecan, the dual fluorophore-labeled primer was 5’-FAM-CGGTGAAGAAAGGAGCACCGTGGGCC-TAMRA-3’, and the PCR primers were 5’-GCATGAGAGAGGCGAATGGA-3’ and 5’-CTGGGCCATGCTGACAAAGC-3’; for mouse osteocalcin, the dual fluorophore-labeled primer was 5’-FAM-CTTGCTGCTCAGAATCTACCACTACCTGATCTACCACTTCCTCC-TAMRA-3’, and the PCR primers were 5’-TCTTACTAGGGATTGAGATGAG-3’ and 5’-TTTTAGCGATGAGCTTCTGAA-3’; for mouse SOX6, the dual fluorophore-labeled primer was 5’-FAM-CTTGCTGCTCAGAATCTACACCTCCTGACCCCTCCCTTCTCC-TAMRA-3’, and the PCR primers were 5’-CCATGTTGGCCACGACATG-3’ and 5’-TTTACACATGGAGATGCTTGGAA-3’; for mouse RPL19, the dual fluorophore-labeled primer was 5’-FAM-CTTCCCGGGCTGTGGTGAC-3’, and the PCR primers were 5’-ATCCGAAGCTCTGGTACTGCT-3’ and 5’-TGGATGCTGCTGCTGACAAAGC-3’; for mouse alkaline phosphatase, the dual fluorophore-labeled primer was 5’-FAM-CGCTGGGAACGGCTGCTGGCC-TAMRA-3’, and the PCR primers were 5’-CATGTCCTCAAGGAGAACGATG-3’ and 5’-GGCTGCACCCCTCCCTTCTCC-TAMRA-3’, and the PCR primers were 5’-ATGGCTGTCGTCGAGAGTGGTCTGTTTT-3’ and 5’-GGCTGCACCCCTCCCTTCTCC-TAMRA-3’, and the PCR primers were 5’-CATGTCCTCAAGGAGAACGATG-3’ and 5’-GGCTGCACCCCTCCCTTCTCC-TAMRA-3’, and the PCR primers were 5’-CATGTCCTCAAGGAGAACGATG-3’ and 5’-GGCTGCACCCCTCCCTTCTCC-TAMRA-3’, and the PCR primers were 5’-CATGTCCTCAAGGAGAACGATG-3’ and 5’-GGCTGCACCCCTCCCTTCTCC-TAMRA-3’, and the PCR primers were 5’-CATGTCCTCAAGGAGAACGATG-3’ and 5’-GGCTGCACCCCTCCCTTCTCC-TAMRA-3’, and the PCR primers were 5’-CATGTCCTCAAGGAGAACGATG-3’ and 5’-GGCTGCACCCCTCCCTTCTCC-TAMRA-3’, and the PCR primers were 5’-CATGTCCTCAAGGAGAACGATG-3’ and 5’-GGCTGCACCCCTCCCTTCTCC-TAMRA-3’, and the PCR primers were 5’-CATGTCCTCAAGGAGAACGATG-3’ and 5’-GGCTGCACCCCTCCCTTCTCC-TAMRA-3’, and the PCR primers were 5’-CATGTCCTCAAGGAGAACGATG-3’ and 5’-GGCTGCACCCCTCCCTTCTCC-TAMRA-3’, and the PCR primers were 5’-CATGTCCTCAAGGAGAACGATG-3’ and 5’-GGCTGCACCCCTCCCTTCTCC-TAMRA-3’, and the PCR primers were 5’-CATGTCCTCAAGGAGAACGATG-3’ and 5’-GGCTGCACCCCTCCCTTCTCC-TAMRA-3’, and the PCR primers were 5’-CATGTCCTCAAGGAGAACGATG-3’ and 5’-GGCTGCACCCCTCCCTTCTCC-TAMRA-3’, and the PCR primers were 5’-CATGTCCTCAAGGAGAACGATG-3’ and 5’-GGCTGCACCCCTCCCTTCTCC-TAMRA-3’, and the PCR primers were 5’-CATGTCCTCAAGGAGAACGATG-3’ and 5’-GGCTGCACCCCTCCCTTCTCC-TAMRA-3’, and the PCR primers were 5’-CATGTCCTCAAGGAGAACGATG-3’ and 5’-GGCTGCACCCCTCCCTTCTCC-TAMRA-3’, and the PCR primers were 5’-CATGTCCTCAAGGAGAACGATG-3’.
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GAGTCTGTTCAGCTACTTTT-3’. Reaction conditions were 60 °C for 2 min and then 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. For analysis of TRPV4 gene expression in cartilaginous tissues, conventional RT-PCR was employed. For amplification of the TRPV4 gene, the PCR primers used were 5’-GGTACCAAGTCATGTTGTTCAAGTGG-3’ and 3’-CCCAAGTTGTTGTTCCAGTGAG-3’. For the β-actin gene, the PCR primers were 5’-CTAGACTTTCGAGCAGGAGATG-3’ and 3’-GACTCATCGTACTCCTGGTTG-3’. Reaction conditions were 94 °C for 3 min and then 35 cycles for TRPV4 and 30 cycles for β-actin of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The PCR products were resolved on 1.2% agarose gels and visualized by ethidium bromide staining.

Measurement of Intracellular Ca2+—Cellular Ca2+ was estimated using the ratiometric fluorescence Ca2+ indicator Fura-2. ATDC5 cells were incubated at 37 °C for 30 min in assay buffer (20 mM HEPES [pH 7.4], 115 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 1.8 mM CaCl2, 13.8 mM glucose, and 0.1% bovine serum albumin), containing 5 μM Fura-2 AM (Dojindo) and 0.2% Pluronic F-127 (Molecular Probes). The cells were then washed and resuspended in assay buffer. Cellular Ca2+ was measured by ratio imaging of Fura-2 fluorescence (emission at 510 nm with excitation at 340 and 380 nm) using the Functional Drug Screening System 3000 (Hamamatsu Photonics).

Reporter Gene Assay—ATDC5 cells were inoculated at a density of 7.5 × 104 cells/well in 96-well microtiter plates and cultured overnight prior to transfection. Cells were transfected with 100 ng of the 4Col2E-Luc reporter plasmid and 10 ng of phRL-TK (Promega) using 0.5 μl of FuGENE 6 (Roche Applied Science). Six hours after transfection, the medium was replaced by Dulbecco’s modified Eagle’s medium/F-12 containing 0.5% or 2% FBS, and cells were cultured overnight. On the following day, cells were treated with various concentrations of EGTA or the calmodulin inhibitor W-7 (Calbiochem) for 0.5 to 1 h and then treated with an appropriate concentration of a pharmacological activator of TRPV4, 4α-phorbol 12,13-didecanoate (4α-PDD; Calbiochem) overnight. Reporter activity originating from 4Col2E-Luc and the internal control, phRL-TK, was measured using the dual luciferase reporter assay system according to the manufacturer’s instructions (Promega).

Preparation of the Adenovirus Vector and RNA Interference—A short hairpin RNA (shRNA) expression vector was constructed as described previously (21). The shRNA expression cassette was then transferred into the Swal site of the pAxew cosmord vector (TaKaRa Bio Inc.). A control adenovirus was constructed using an shRNA expression cassette without the RNA interference sequence. Propagation and generation of recombinant adenoviruses were performed according to the manufacturer’s instructions (TaKaRa Bio Inc.). The RNA interference target sequence for mouse TRPV4 mRNA was 5’-CTGGCAAGAGGTAAATCCTACCGATA-3’. For the RNA interference experiments, ATDC5 cells were infected with the adenovirus construct at a multiplicity of infection (MOI) of 300. The transfection experiment was carried out 3 days after adenovirus infection, and luciferase activity was measured the following day.

Western Blot Analysis—Cells were lysed with Tris-SDS sample buffer, and cell lysates were electrophoretically separated on a 4–20% SDS-polyacrylamide gel (Daichi Pure Chemical) and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked with Immuno Block (Dainippon Sumitomo Pharmaceutical) for 1 h at room temperature and incubated with anti-SOX9 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-α-tubulin primary antibody for 1 h at room temperature. The membrane was then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody for 1 h at room temperature. Immunoreactive bands were visualized by ECL (Amersham Biosciences).

RESULTS

Genome-wide Identification of Full-length cDNA Involved in Chondrogenesis—In order to identify genes that are involved in chondrocyte differentiation, we screened full-length cDNA libraries using luciferase reporter assays. In these assays, we used a murine chondrogenic cell line, ATDC5, which mimics chondrogenic differentiation by insulin stimulation, as a host cell and 4Col2E-Luc as a SOX9-dependent Col2a1 reporter construct (Fig. 1). We prepared RNAs from ATDC5 cells treated with and without 10 μg/ml bovine insulin and constructed full-length cDNA libraries on the mammalian expression vector, pME18S-FL3. We randomly isolated 40,000 cDNA clones from the cDNA library of ATDC5 cells treated with insulin and 80,000 cDNA clones from the library of cells without insulin. In total, 120,000 cDNA clones were isolated and individually arrayed in 96-well microtiter plate format. Plasmid DNA from each cDNA clone was prepared and co-transfected into ATDC5 cells together with a reporter plasmid, 4Col2E-luc (6, 20). The activity of each cDNA clone for the SOX9-dependent Col2a1 promoter was measured by luciferase reporter assay. Each clone with an activity more than 2-fold above that of the control was defined as a positive clone.
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**TABLE 1**

Complete list of genes that activated the SOX9-dependent Col2a1 promoter

| Gene ID      | Gene name                                      | Induction | Clone number |
|--------------|------------------------------------------------|-----------|--------------|
| 18747        | Protein kinase A                               | 50        | 5            |
| 63873        | TrpV4                                          | 42        | 1            |
| 16653        | Ki-ras                                         | 17        | 1            |
| 18176        | N-ras                                          | 16        | 2            |
| 73835        | Interferon-induced transmembrane protein 5     | 14        | 1            |
| 20462        | Splicing factor, arginine/serine-rich 10       | 14        | 10           |
| 20779        | Src                                            | 12        | 1            |
| 209446       | Transcription factor E3                        | 12        | 2            |
| 21415        | TCF-3                                          | 10        | 5            |
| 14158        | fer (fms/fps-related) protein kinase, testis-specific 2 | 8        | 1            |
| 15162        | Hck                                            | 7.2       | 1            |
| 22629        | 14-3-3                                        | 6.8       | 4            |
| 192662       | Rho GDI a                                      | 6         | 14           |
| 330171       | Potassium channel tetramerization domain-containing 10 | 5.8 | 1            |
| 93834        | Peli2                                          | 5.3       | 2            |
| 18596        | PDGFR β                                        | 5.1       | 1            |
| 12005        | Axin1                                          | 5         | 1            |
| 26397        | MKK3                                           | 4.7       | 1            |
| 20678        | Sox5                                           | 4.4       | 2            |
| 232334       | Vestigial-like 4                               | 4.3       | 1            |
| 226591       | TOR signaling pathway regulator-like           | 4.2       | 1            |
| 224619       | TRAF7                                          | 4.1       | 1            |
| 16601        | Kruppel-like factor 9                          | 4         | 1            |
| 20679        | Sox6                                           | 4         | 1            |
| 12045        | B-cell leukemia/lymphoma 2-related protein A1B | 3.9       | 2            |
| 12609        | CCAAT/enhancer binding protein, δ              | 3.8       | 1            |
| 26406        | MEK3                                           | 3.8       | 1            |
| 12321        | Calumenin                                      | 3.7       | 1            |
| 17210        | Myeloid cell leukemia sequence 1               | 3.6       | 1            |
| 17872        | Myd116                                         | 3.3       | 2            |
| 20204        | Paired related homeobox 2                     | 3.2       | 1            |
| 110157       | Raf-1                                          | 3.2       | 1            |
| 73181        | NFATc4                                         | 3         | 1            |
| 67201        | RIKEN cDNA 2700085E05 gene                     | 2.9       | 1            |
| 14182        | FGER1                                          | 2.8       | 2            |
| 80912        | Pumilio 1                                      | 2.8       | 1            |
| 74168        | Zinc finger, DHHC domain-containing 16         | 2.7       | 1            |
| 192176       | Filamin, α                                     | 2.5       | 1            |
| 65247        | Ankyrin repeat and SOCS                       | 2.5       | 1            |
| 32161        | box-containing protein 1                       | 2.5       | 1            |
| 54601        | AFX/FOXO4                                      | 2.4       | 1            |
| 71966        | NF-κB inhibitor-interacting Ras-like protein 2 | 2.3       | 1            |
| 13682        | Eukaryotic translation initiation factor 4A2    | 2.2       | 1            |
| 234594       | CCR4-NOT transcription complex, subunit 1      | 2.2       | 1            |
| 229589       | Prune homolog                                  | 2.2       | 1            |
| 12387        | β-Catenin                                      | 2.1       | 1            |
| 20853        | Staufen (RNA-binding protein) homolog 1        | 2.1       | 1            |

criptional co-activators for Sox9 and are essential for chondrogenic differentiation (22). Although many of the genes shown in Table 1 have not been previously reported as being related to chondrogenesis, our result suggests that some genes identified here might be involved in the course of chondrocyte differentiation.

**Gene Expression Analysis of the Identified Genes during Chondrogenic Differentiation**—We next examined the expression profiles of genes listed in Table 1 during chondrogenic differentiation of ATDC5 cells stimulated with insulin. For these experiments, we employed cDNA microarray analysis and found that the mRNA levels of TRPV4, interferon-induced transmembrane protein 5, SOX9, and MYD116 were increased during the differentiation of ATDC5 cells (data not shown).

In this report, we investigated the TRPV4 cation channel molecule in detail, since it had a strong effect on SOX9-dependent reporter activity (42-fold increase) (Table 1) and its relevance to chondrogenesis remains poorly understood.

To confirm the increase in TRPV4 mRNA observed using microarray analysis, we performed quantitative RT-PCR and compared the expression pattern of TRPV4 with that of other chondrogenic marker genes. The expression levels of well-known marker genes, such as COL2A1 and aggrecan, were elevated on day 12 after insulin stimulation (Fig. 2A) (19, 23). The elevation in the levels of the two mRNAs reached about 45-fold for COL2A1 and 110-fold for aggrecan on day 21. TRPV4 mRNA was expressed at significantly high levels on day 12 and peaked on day 14 (Fig. 2A). The maximum induction level of TRPV4 was about 7-fold on day 14. We further examined the expression pattern of the TRPV4 gene using another cell line, C3H10T1/2, a murine mesenchymal stem cell line that is known to differentiate into chondrocytes when stimulated with BMP-2. Expression of the COL2A1 gene was elevated by BMP-2 stimulation and reached about 30-fold on day 7 (Fig. 2B). Although expression of the aggrecan gene was not detected on day 0, it was evident on day 3 after BMP-2 stimulation and increased to about 24-fold on day 7 when compared with day 3 (Fig. 2B). We also observed that TRPV4 gene expression had increased about 30-fold by day 3. TRPV4 and COL2A1 mRNAs had increased significantly on day 1, and an increase in aggrecan mRNA was evident on day 3 after BMP-2 stimulation, suggesting that chondrocyte differentiation in C3H10T1/2 cells proceeds faster than in ATDC5 cells. These results indicate that TRPV4 mRNA is elevated during chondrocyte differentiation.

**Gene Expression of TRPV4 in Cartilage Tissues**—The expression of TRPV4 in several murine tissues has been previously reported; however, its expression in cartilage tissues is still uncertain (24). To determine the importance of TRPV4 in chondrogenesis in vivo, we examined the expression of TRPV4 in murine cartilage tissues using RT-PCR. As shown in Fig. 2C, amplified DNA fragments of predictable size were detected in cartilage tissue of the hind limb in embryonic day 12 embryos and in cartilage tissues of knee joints and primary chondrocytes prepared from the rib cage in adults (Fig. 2C). From these observations, we concluded that the TRPV4 gene is expressed in cartilage tissues as well as in chondrogenic cell lines. These results prompted us to investigate the function of TRPV4 in chondrogenesis.

**Activation of TRPV4 Promotes SOX9-dependent Transcription**—TRPV4 was identified by its ability to elevate SOX9-responsive reporter activity in an ectopic expression experiment. We next examined whether the activation of endogenous TRPV4 resulted in the elevation of SOX9-dependent luciferase activity. For this purpose, we used a pharmacological activator of TRPV4, 4α-PDD, which is a non-protein kinase C-activating phorbol ester derivative, in the following experiments (25). We examined the efficacy of 4α-PDD on 4Col2E-Luc reporter gene.
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A. ATDC5

![Graph A] (Relative mRNA level vs Days)

B. C3H10T1/2

![Graph B] (Relative mRNA level vs Days)

C. TRPV4 and β-actin

![Image C]

FIGURE 2. TRPV4 gene expression during chondrogenesis. A, confluent ATDC5 cells were stimulated with 10 μg/ml insulin or cultured without insulin for the indicated numbers of days. Expression of each gene was measured by quantitative RT-PCR as described under “Experimental Procedures.” The closed circles represent cell samples stimulated with insulin, and open circles represent those without insulin. B, confluent C3H10T1/2 cells were stimulated with 1000 ng/ml BMP-2 or cultured without BMP-2 for the indicated numbers of days. Expression of each gene was measured as described above. The closed circles represent cell samples stimulated with BMP-2, and open circles represent those without BMP-2. After normalization to RPL19 mRNA, values were expressed relative to those measured in control unstimulated cells (day 0), except for the aggrecan gene in C3H10T1/2 cells. Expression of aggrecan mRNA in C3H10T1/2 cells was expressed as a ratio to that in BMP-2-stimulated cells on day 3, because no mRNA was detected in the day 0 sample. These data are representative of two separate experiments. C, RNAs were prepared from murine cartilage tissues as described under “Experimental Procedures.” RT-PCR for the TRPV4 gene and an internal control, β-actin, was performed, and PCR products were resolved and visualized on agarose gels. DNA fragments of sizes corresponding to TRPV4 (465 bp) and β-actin (434 bp) are indicated by the arrowheads. Lane 1, negative control; lane 2, primary chondrocytes prepared from the rib cages of adult mice; lane 3, hind limb buds from embryonic day 12 embryos; lane 4, cartilage tissues prepared from knee joints of adult mice.

activity. As shown in Fig. 3A, 4α-PDD strongly increased 4Col2E-Luc reporter activity in a dose-dependent manner in ATDC5 cells, and this effect was abolished by the addition of 10 μM ruthenium red (RR), a TRPV antagonist (25). Similar results were also obtained when C3H10T1/2 cells were examined (Fig. 3B). To further confirm that the effect of 4α-PDD on 4Col2E-Luc reporter activity was mediated by TRPV4, we generated an adenovirus expressing an shRNA against the TRPV4 gene (Ad-shVR4) and used it to attempt to inhibit TRPV4 expression. When ATDC5 cells were infected with Ad-shVR4 at an MOI of 300, expression of endogenous TRPV4 mRNA was reduced to about 35% of that in cells infected with the control adenovirus (Fig. 3C). When cells were stimulated with various concentrations of 4α-PDD, Ad-shVR4-infected cells completely failed to respond to 4α-PDD, whereas control virus-infected cells retained their response (Fig. 3D). Together, these results suggested that endogenous TRPV4 promotes SOX9-dependent reporter activity in ATDC5 and C3H10T1/2 cells.

Ca2+ Influx Is an Essential Event for SOX9-dependent Transcription in Response to TRPV4 Activation—Since TRPV4 is considered to be a cation channel, we examined the intracellular Ca2+ level in ATDC5 cells upon TRPV4 activation. As shown in Fig. 4A, stimulation with 10 μM 4α-PDD increased intracellular Ca2+ level in ATDC5 cells, and this effect was blocked by the addition of 10 μM RR. We next examined whether the increased Ca2+ level affects SOX9-dependent reporter activity. ATDC5 cells were cultured in medium containing various concentrations of Ca2+ and stimulated with 120 nM 4α-PDD, and then SOX9-dependent luciferase activity was measured (Fig. 4B, top). When ATDC5 cells were maintained in Ca2+-free medium, stimulation with 120 nM 4α-PDD had no effect on SOX9-dependent reporter activity. However, luciferase activity was induced about 3-fold when cells were stimulated with 120 nM 4α-PDD in the presence of 62.5 μM CaCl2, and the response reached 15-fold in the presence of 1,000 μM CaCl2. Without stimulation by 4α-PDD, no elevation in SOX9-dependent reporter activity was observed regardless of Ca2+ concentration. We also examined the effect of Ca2+ on SOX9-dependent reporter activity in the presence of a calcium chelator, EGTA. ATDC5 cells were cultured in medium containing various concentrations of EGTA and stimulated with 120 nM 4α-PDD, and then SOX9-dependent luciferase activity was measured (Fig. 4C, top). When ATDC5 cells were cultured in medium containing 0.5 mM EGTA, SOX9-dependent reporter activity was about half of the maximum response, and in medium containing 1 mM EGTA, reporter activity was reduced to basal level (Fig. 4C, top). In both experiments, cell toxicity caused by a low concentration of Ca2+ was monitored using Renilla reporter activity as an internal control, and no toxic events were observed (Fig. 4, B and C, bottom). Next, we examined whether calmodulin, a calcium effector, could be a downstream target molecule of TRPV4. ATDC5 cells were treated with various concentrations of the calmodulin inhibitor W-7 and stimulated with 360 nM 4α-PDD, and then
SOX9-dependent reporter activity was measured. W-7 caused dose-dependent inhibition of reporter activity (Fig. 4D, top). W-7 did not cause toxicity at concentrations up to 10 μM (Fig. 4D, bottom). Taken together, our data indicate that the Ca<sup>2+</sup>/calmodulin pathway might mediate the TRPV4 activation signal.

**Functional Analysis of TRPV4 in Chondrogenic Differentiation**—We next examined the effect of 4α-PDD on chondrogenic differentiation of ATDC5 cells (Fig. 5). After stimulation with 10 μg/ml insulin for 10 days, cells clearly stained with Alcian blue, indicating an accumulation of sulfated glycosaminoglycan (GAG), as previously described (Fig. 5, A and B) (23). Co-stimulation with 4α-PDD at concentrations of 40–120 nM produced a significant increase in GAG accumulation as compared with insulin alone, whereas 4α-PDD alone had no effect (Fig. 5, A and B). We obtained a similar result when C3H10T1/2 cells were examined using BMP-2 (Fig. 5, C and D).

To confirm these observations, we measured the amounts of mRNAs of two chondrogenic marker genes, COL2A1 and aggrecan, by quantitative RT-PCR. When ATDC5 cells were stimulated with insulin for 10 days, COL2A1 and aggrecan mRNAs were elevated about 14- and 7-fold as compared with cells without insulin, respectively (Fig. 5, E and F). Co-stimulation with 120 nM 4α-PDD resulted in an increase in the expression of both genes about 2-fold as compared with insulin alone. We observed no effects on gene expression when cells were stimulated with 4α-PDD alone.

To confirm that the effect of 4α-PDD on chondrogenic differentiation was mediated by TRPV4, we also used Ad-shVR4 (Fig. 6). When C3H10T1/2 cells were infected with Ad-shVR4 at a MOI of 200, expression of endogenous TRPV4 mRNA was reduced to about 55% of that in cells infected with the control adenovirus (Fig. 6A). C3H10T1/2 cells were infected with Ad-shVR4 or control adenovirus prior to stimulation with various concentrations of 4α-PDD and 1,000 ng/ml BMP-2 for 5 days, and GAG was measured (Fig. 6B). In C3H10T1/2 cells infected with control adenovirus, stimulation with BMP-2 alone increased GAG accumulation about 4-fold as compared with unstimulated cells. Co-stimulation with 120 nM 4α-PDD further elevated the GAG content about 2.5-fold as compared with BMP-2 alone. However, infection with Ad-shVR4 inhibited the elevation of GAG content caused by the co-stimulatory effect of 4α-PDD. Ad-shVR4 had no effect on the GAG accumulation induced by BMP-2 alone. These results demonstrate that activation of TRPV4 promotes chondrogenesis of ATDC5 and C3H10T1/2 cells in association with insulin and BMP-2, respectively, but that activation of TRPV4 alone is not sufficient.

**TRPV4 Activator Increases the Amount of Sox9**—To determine how TRPV4 contributes to chondrogenesis, we next examined the relation between TRPV4 and SOX9. First, to determine whether stimulation of TRPV4 by 4α-PDD affects protein levels of SOX9, we performed Western blot analysis using anti-SOX9 antibody. At ADTC5 cells, 24 h of treatment with 4α-PDD increased the amount of SOX9, and this effect was blocked in the presence of 10 μM RR (Fig. 7A). Insulin alone also induced the expression of SOX9, and a further increase was observed when cells were co-stimulated with 120 nM 4α-PDD. Similarly, when C3H10T1/2 cells were stimulated with 4α-PDD, an increase in SOX9 protein was observed, which was abolished by the addition of 10 μM RR (Fig. 7B). We next performed quantitative RT-PCR to deter-
that activation of TRPV4 increases the level of functional SOX9 molecules.

**DISCUSSION**

Chondrogenesis has recently been studied from various perspectives, and Sox9 is well established as an essential transcription factor that controls chondrocyte fate and differentiation. However, the molecular basis of the initiation of chondrogenesis remains poorly understood. To better understand this process, we attempted to identify genes that activate the Sox9 pathway.

We screened full-length cDNA libraries generated from ATDC5 cells using the SOX9-dependent reporter construct 4Col2E-Luc and identified 46 genes that can activate SOX9-dependent reporter activity. Some genes listed in Table 1 are known or thought to be involved in chondrogenesis. For example, protein kinase A, which had the strongest effect on SOX9-dependent transcription in our assay (Table 1), has been found to induce phosphorylation of SOX9 on serine residues Ser64 and Ser181 and to enhance the activity of SOX9 with respect to the Col2a1 chondrocyte-specific enhancer (26). Our screening also identified SOX5 and SOX6, both of which are well known to be required in chondrogenesis. Sox5−/− and Sox6−/− double knock-out mice have chondrodysplastic phenotypes and die at birth (22).

In vitro studies have shown that Sox5 and Sox6 cooperate with Sox9 to activate the Col2a1 enhancer in chondrogenic cells (11). Furthermore, in many studies, cross-talk between Sox9-dependent transcription and various intracellular signaling cascades has been identified, including the Rho-ROCK pathway (27), MAPK pathway (28, 29), and Wnt pathway (30–32). In this regard, identification of RhoGDIα, a negative regulator of the Rho-ROCK pathway, is reasonable, since pharmacological inhibition of ROCK signaling results in an increase in SOX9 mRNA and GAG production in murine mesenchymal limb bud cells (27). We also identified a number of genes involved in the MEK1-MAPK and p38 MAPK pathways, including Ki-ras, N-ras, Raf-1, MKK3, MEKK3, PDGFRβ, and FGFR1. It has previously been demonstrated that constitutive
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activation of the MEK1-MAPK pathway in chondrocytes in transgenic mice inhibits hypertrophic chondrocyte differentiation and causes a dwarf phenotype without a decrease in cell proliferation (28). The p38 MAPK pathway has also been shown to affect chondrocyte differentiation (29). The canonical Wnt signaling pathway has also been implicated in the Sox9 pathway (30–32). As shown in Table 1, we identified downstream molecules in the canonical Wnt pathway, including TCF-3, AXIN1, and β-catenin. These findings indicate that our screening method worked properly, and we thus conclude that the genes identified here might somehow be involved in the process of chondrogenic differentiation. In this regard, it would be valuable to analyze the function of the RIKEN cDNA 2700085E05 gene, which we have identified in our screening (Table 1), since it encodes the hypothetical protein LOC67201, and a possible relationship between the RIKEN cDNA 2700085E05 gene and chondrogenesis has not been hitherto found.

In the present study, we examined the TRPV4 cation channel molecule in detail because of its strong effect on SOX9-dependent transcription (Table 1), increased mRNA expression during chondrocyte differentiation, and its expression pattern being similar to those of marker genes for chondrogenesis, including COL2A1 and aggrecan in the ATDC5 chondrogenic cell line and the C3H10T1/2 mesenchymal stem cell line (Fig. 2, A and B). Detailed expression analysis of the TRPV4 gene in mice confirmed that TRPV4 mRNA is expressed in cartilage tissues of the knee joint in adults and in the hind limb buds of embryonic day 12 embryos (Fig. 2C). TRPV4 mRNA was also detected in murine primary chondrocytes prepared from adult rib cage (Fig. 2C). These results suggest that TRPV4 might have an important role in chondrogenesis in vivo.

We showed that activation of endogenous TRPV4 increased SOX9-dependent luciferase activity in both ATDC5 and C3H10T1/2 cells (Fig. 3, A and B) and increased Ca2+ influx in ATDC5 cells (Fig. 4A). We also demonstrated that the increased Ca2+ influx followed by TRPV4 activation is an essential event in enhancing SOX9-dependent promoter activity (Fig. 4, B and C). In our study, a calmodulin inhibitor, W-7, inhibited the induction of SOX9-dependent reporter activity by TRPV4 activation, suggesting that Ca2+/calmodulin signaling might mediate the TRPV4 pathway.

FIGURE 5. Analysis of chondrogenic differentiation in the presence of the TRPV4 activator 4α-PDD. A and B, ATDC5 cells were cultured with various concentrations of 4α-PDD in the presence (10 μg/ml) or absence of insulin for 10 days. The cells were stained with Alcian blue (A), and GAG was quantified (B). Representative data from three independent experiments are shown. C and D, C3H10T1/2 cells were cultured with various concentrations of 4α-PDD in the presence (1000 ng/ml) or absence of BMP-2 for 4 days. The cells were stained with Alcian blue (C), and GAG was quantified (D). Representative data from three independent experiments are shown. E and F, ATDC5 cells were cultured with various concentrations of 4α-PDD in the presence (10 μg/ml) or absence of insulin for 10 days. Levels of COL2A1 (E) and aggrecan (F) mRNA were determined by quantitative RT-PCR. The values were normalized to RPL19 mRNA and are expressed as ratios relative to values in unstimulated samples. Solid bars represent cells with insulin, and open bars represent cells without insulin. These data are representative of three separate experiments (E and F).

FIGURE 6. Inhibition of chondrogenic differentiation by Ad-shVR4. A, C3H10T1/2 cells were infected with Ad-shVR4 or control adenovirus at an MOI of 200. Three days after infection, total RNA was extracted, and the amount of TRPV4 mRNA was measured by quantitative RT-PCR. The values were normalized to the internal control, RPL19 mRNA. B, C3H10T1/2 cells were infected with Ad-shVR4 or control adenovirus at an MOI of 200. Cells were stimulated with various concentrations of 4α-PDD and 1000 ng/ml BMP-2 for 5 days. Cells were stained with Alcian blue. GAG accumulation was quantified, and relative values were indicated. Representative data from three independent experiments are shown.
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TRPV4 was originally identified as a channel molecule activated by hypotonic cell swelling (24, 33, 34). Later studies showed that it could also be activated by temperature (35, 36), acidic pH (37), and a synthetic activator, such as 4α-PDD (25). However, the physiological stimulus for TRPV4 during chondrogenesis remains unknown. TRPV4 is considered to be a mechanosensor for shear stress (38). Compressive force increases the mRNA levels of SOX9, type II collagen, and aggrecan, resulting in the promotion of chondrogenesis in murine embryonic limb bud mesenchymal cells (39). Furthermore, elevation of aggrecan mRNA by compressive forces is mediated by a transient increase in intracellular Ca\textsuperscript{2+} and Ca\textsuperscript{2+}/calmodulin level in bovine articular chondrocytes (40). Given these observations, it is conceivable that TRPV4 might sense mechanical stress in the articular cartilage and be involved in the maintenance of cartilage homeostasis.

In the present study, we identified TRPV4 via its effect on SOX9-dependent transcription. Our data suggest an important role for TRPV4 in early chondrogenesis. It would also be worthwhile to examine the role of TRPV4 during hypertrophic differentiation in late chondrogenesis, since SOX9 functions as a negative regulator in that process. Via investigation of the mechanism of chondrogenesis and pathogenesis of cartilage diseases, study of TRPV4 may provide new insights for future study.

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