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Transcriptional induction of SOX9 by NF-κB family member RelA in chondrogenic cells

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Summary

Objective: Although SOX9 is a key molecule for chondrogenic differentiation, little is known about the upstream signal. The present study attempted to identify transcription factors to induce SOX9 expression and examined the mechanism.

Methods: Sequences of about 1 kb of 5′-end flanking regions were compared between human and mouse SOX9 genes. *In vivo* localization was examined by immunohistochemistry in the limb cartilage of fetal mice. Promoter activities of the SOX9, SOX6, and type II collagen (COL2A1) genes were determined in human non-chondrogenic HeLa cells and mouse chondrogenic ATDC5 cells transfected with a luciferase-reporter gene containing the promoter fragments. Protein–DNA binding was examined by electrophoretic mobility shift and chromatin immunoprecipitation assays. The chondrogenic differentiation was assessed by endogenous SOX9, SOX6, and COL2A1 mRNA levels, and by Alcian blue staining and alkaline phosphatase activity.

Results: Among transcription factors whose binding motifs were identified in the highly-conserved regions between human and mouse SOX9 promoters, a nuclear factor kappa B (NF-κB) member RelA strongly activated the promoter activity. RelA and SOX9 were co-localized in the limb cartilage. Deletion, mutagenesis, and tandem-repeat analyses identified the core region responsive to RelA at the NF-κB binding motif to be around −250 bp of the human SOX9 promoter, and this was confirmed to show specific binding to RelA. RelA induced the chondrogenic differentiation parameters in HeLa and ATDC5 cells.

Conclusion: We have identified RelA as a transcriptional factor for SOX9 induction and chondrogenic differentiation *via* binding to an NF-κB binding motif in the SOX9 promoter.
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Key words: SOX9, RelA, Chondrocyte, Transcription.

Introduction

Skeletal development is initiated by the recruitment of undifferentiated mesenchymal cells into condensations, which differentiate into pre-chondrocytes and then chondrocytes that produce cartilage-specific extracellular matrix proteins like type II collagen (COL2A1)[1]. Sex-determining region Y-type high mobility group box 9 (SOX9) is expressed in the mesenchymal cells, pre-chondrocytes and chondrocytes[2,3], and functions as a master transcriptional activator of COL2A1 and other chondrocyte-specific matrix proteins, in cooperation with the co-factors SOX6 and L-SOX5[4–9]. Expressions of the SOX6 and L-SOX5 are also controlled by SOX9[4,10]. Studies in mice have shown that SOX9 is essential for multiple steps in the chondrogenic differentiation pathway: conditional ablation of the SOX9 gene in the limb buds before mesenchymal condensation resulted in a complete absence of chondrocytes, whereas the conditional ablation after mesenchymal condensation resulted in a severe generalized chondrodysplasia[10–12]. In humans as well, heterozygous mutations of the SOX9 gene cause a severe chondrodysplasia, known as campomelic dysplasia[11,13]. Furthermore, we previously reported that SOX9 in combination with SOX6 and L-SOX5 (the SOX trio) stimulated chondrogenesis even from non-chondrogenic cells of mouse and human origins, implicating a possible clinical application of this signal to cartilage regeneration[14]. Despite the substantial information about the expression profiles and the target genes of SOX9, little is known about the upstream signaling or the functional regulation of the SOX9 promoter. To identify transcription factors that induce SOX9 expression, the present study initially compared the genomic sequences of proximal promoter regions between human and mouse SOX9 genes, and identified several highly-conserved regions containing putative transcription

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factor-binding motifs. Among the candidate transcription factors, our further analyses found that nuclear factor kappa B (NF-κB) family member RelA (NF-κB p65) most strongly activated the human SOX9 promoter activity.

The NF-κB family of transcription factors plays a crucial role in a broad range of biological processes, including immune responses, inflammation, proliferation, differentiation and apoptosis[15–17]. The family includes RelA, RelB, Rel, p105/p50 and p100/p52, each of which contains a Rel homology domain that mediates DNA binding and dimerization. Numerous studies have established that IκB proteins are phosphorylated and degraded by a large protein complex IκB kinase (IKK) in response to several signals, thereby allowing free NF-κB complexes to translocate from the cytoplasm into the nucleus, leading to target gene transactivation[18,19]. The NF-κB family genes are expressed in the chick limb cartilage, and the blockage of the NF-κB activity caused the arrest of the limb outgrowth[20]. The IKKα-deficient mice also exhibited suppression of limb outgrowth[21,22]. Since these lines of evidence implicate the interaction between NF-κB and SOX9 signals during skeletal development, the present study investigated the mechanism underlying the transcriptional regulation of the SOX9 promoter by RelA.

Materials and methods

COMPARISON OF THE PROXIMAL PROMOTER SEQUENCES OF THE HUMAN, MOUSE AND CHICK SOX9 GENES

We compared the sequences of the 5′-end flanking regions relative to the transcription start site among 4 kb human, 4 kb mouse and 300 bp chick SOX9 gene, using BLASTN search[23]. The detected sequences were aligned by the Vector-NTI software (Invitrogen), and the transcription factor-binding motifs were predicted using the TFSEARCH web site (Computational Biology Research Center, AIST, Japan).

CELL CULTURES

The human epithelial cell line HeLa (RIKEN Cell Bank, Tsukuba, Japan) was cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Mouse chondrogenic ATDC5 cells (RIKEN Cell Bank) were grown and maintained in DMEM/F12 (1:1) with 5% FBS. To induce chondrogenic differentiation, ATDC5 cells were cultured in the presence of insulin-transferring-sodium selenite media supplement (ITS) (Sigma) for 3 weeks and replaced by αMEM/5% FBS with 4 mM inorganic phosphate (Pi) for 2 d[24].

CONSTRUCTION OF EXPRESSION VECTORS

Full-length human cDNA sequences of the transcription factors were polymerase chain reaction (PCR)-amplified and cloned into pCMV-HA vector (Clontech, Palo Alto, CA, USA). The Gene Bank access numbers are as follows: NFAT1 NM_012340.3, NFAT2 NM_006162.3, NFAT3 NM_004554.4, NFAT4 NM_004555.2, NFAT5 NM_138714, Fos NM_005252.2, Jun NM_002228.3, Fra-1 NM_005438.3, RelA NM_021975.2, RelB NM_006509.2, Rel NM_002908.2, p105 NM_003998.2, p100 NM_001077493.1, ATF1 NM_005171.3, ATF2 NM_001880.2, ATF4 NM_001675.2, ATF6 NM_007348.2, ATF7 NM_001130059.1, CREB NM_004379.2, C/EBPα NM_004364.2, C/EBPβ NM_005194.2, C/EBPδ NM_005195.3, C/EBPε NM_001805.2, GATA-1 NM_ 002049.3, GATA-2 NM_032638.3, GATA-3 NM_001002295.1, GATA-4 NM_002052.3, GATA-5 NM_080473.4, GATA-6 NM_005257.3. The primer sequences are available upon request.

LUCIFERASE ASSAY

The human SOX9 promoter region from −927 to +84 bp relative to the transcriptional start site (TSS) was obtained by PCR using human genomic DNA as a template and were cloned into the EcoRI and HindIII sites of the modified pGL3 vector containing additional cloning sites between the XhoI and HindIII sites of the original plasmid, the pGL3-basic vector (Promega, Madison, WI, USA). Deletion and mutation constructs were created by PCR technique. Tandem-repeat constructs were created by ligating the double strand oligonucleotides into EcoRI site of the modified pGL3 vector. Transfection of HeLa and ATDC5 cells was performed in quadruplicate in 48-well plates using FuGENE 6 transfection reagent (Roche, Mannheim, Germany): FuGENE 6 with a total amount of 150 ng of plasmid DNA, 100 ng of pGL3 reporter vector, 50 ng of effector vector, and 4 ng of pRL-TK vector (Promega) for internal control per well. Cells were harvested 48 h after the transfection. The luciferase assay was performed with a dual-luciferase-reporter assay system (Promega) using a GloMax 96 Microplate Luminometer (Promega). The results were shown as the ratio of the firefly activities to the renilla activities. For the SOX6 promoter assay, a luciferase-reporter construct containing the human SOX6 promoter and exon 1 region (−517 to IVS1 + 23) was generated and transfected in HeLa and ATDC5 cells as reported previously[25]. For the COL2A1 promoter assay, a luciferase-reporter construct containing the four repeats of the 49 bp SOX9 enhancer and the basal promoter (from −183 to +23) in the human COL2A1 gene was generated and transfected in the cells[26].

IMMUNOHISTOCHEMISTRY

Tissues from C57BL6 mouse embryos (E17.5) were fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) overnight at 4°C, embedded in paraffin and cut into 5 μm sections. Sections were incubated overnight at 4°C with primary antibodies to RelA (C-20) (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and SOX9 (1:500; Santa Cruz Biotechnology), as well as the non-immune serum as the control. The localizations were detected with horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, WI, USA).

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

RelA protein was prepared by *in vitro* translation using the TNT T7 Coupled Reticulocyte Lysate System (Promega) and pCITE4 vector (Novagen, Milwaukee, WI, USA) into which RelA complementary DNA was cloned. The translation product was verified by Western blotting. Nuclear extracts were prepared from undifferentiated and differentiated ATDC5 cells before and after the culture with ITS for 3 weeks and Pi for 2 d, respectively. EMSA was carried out using a DIG gel shift kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Binding reactions were incubated for 30 min at room temperature. For competition analyses, 100-fold excess of unlabeled competitor probe was included in the binding reaction. For the supershift experiments, 1 μL of the antibody to RelA (C-20) above was added after 30 min of the binding reaction, and the reaction was incubated for an additional 30 min at room temperature. Samples were loaded onto Novex 6% TBE gels (Invitrogen), and electrophoresed at 100 V for 60 min.

CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

The ChIP assay was performed with a OneDay ChIP kit (Diagenode, Liege, Belgium) according to the manufacturer's instructions. *In vivo* cross-linking was performed 2 d after the transfection of HeLa cells with EV, RelA using FuGENE 6, and then the lysates were sonicated to shear genomic DNA. For immunoprecipitation, an antibody to RelA and the control normal rabbit immunoglobulin G (IgG) were used. Two primer sets, one spanning (−478/−239 bp) and the other not spanning (−3781/−3625 bp) the identified NF-κB motif, were employed. PCR was performed using Ex Taq (Takara Bio, Otsu, Japan) in the presence of 10% dimethyl sulfoxide.

GENE TRANSFER

For transient gene transfer, 2×10^5 HeLa cells were cultured in 6-well plates to subconfluency, and transfected with 1 μg of expression vector of RelA or the control empty vector (EV) using FuGENE 6. After 48 h, total mRNA of harvested cells was extracted and analyzed by real-time RT-PCR as described below.

Production of retroviral vectors was performed as described previously[27]. For retroviral gene transfer, 2×10^6 Plat-E cells were plated in 6-well plates, transfected with 2 μg pMx vector of RelA or the control green fluorescent protein (GFP) using FuGENE 6, and the conditioned medium was collected after 48 h. On the day before retroviral transfection, 3×10^5 of ATDC5 cells were plated onto a 60-mm culture dish. For the transfection, 4 mL of the conditioned medium containing the retrovirus was added to the cells with 32 μg of polybrene. Selection of the retrovirus-introduced cells was started 48 h after transfection in the medium containing 10 μg/mL of blasticidin.

REAL-TIME RT-PCR

Total RNA from cells was isolated with an RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and an aliquot (1 μg) was reverse-transcribed with QuantiTect Reverse Transcription (Qiagen) to make single-stranded cDNA. Real-time RT-PCR was performed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using QuantiTect SYBR Green PCR Master Mix (Qiagen)

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