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TASR-1 regulates alternative splicing of collagen genes in chondrogenic cells

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Abstract

During the differentiation of chondroprogenitors into mature chondrocytes, the alternative splicing of collagen genes switches from longer isoforms to shorter ones. To investigate the underlying mechanisms, we infected mouse ATDC5 chondroprogenitor cells with retrovirus for stable expression of two closely related SR splicing factors. RT-PCR analysis revealed that TASR-1, but not TASR-2, influenced alternative splicing of type II and type XI collagens in ATDC5 cells. The effect of TASR-1 on splicing could be reversed with the addition of insulin. Results from our microarray analysis of ATDC5 cells showed that TASR-1 and TASR-2 differentially affect genes involved in the differentiation of chondrocytes. Of special interest is the finding that TASR-1 could down-regulate expression of type X collagen, a hallmark of hypertrophic chondrocytes. Immunohistostaining demonstrated that TASR-1 protein is more abundantly expressed than TASR-2 in mouse articular chondrocytes, raising the possibility that TASR-1 might be involved in phenotype maintenance of articular chondrocytes.

Keywords: SR protein; Splicing factor; Collagen genes; Chondrocyte differentiation; Chondrogenesis; Articular chondrocyte

Chondrogenesis is initiated during embryonic development when mesenchymal cells first condense then differentiate into chondrogenic cells [1]. The relatively few proliferating chondrocytes present at the epiphyseal extremity of long bone anlagen develop into articular chondrocytes. These cells give rise to articular cartilage, produce abundant extracellular matrix, and maintain normal joint function throughout life [1]. Proliferating chondrocytes in the center of the long bone anlagen become organized into growth plates and eventually differentiate into hypertrophic chondrocytes. These hypertrophic cells mineralize the surrounding matrix before undergoing apoptotic cell death. The cartilage matrix left behind then provides a scaffold for growth of osteoblasts and osteoclasts along with blood vessels [1]. Ultimately, this process of endochondral ossification replaces all remaining cartilage except at the articular surface of the joints.

Developmentally regulated alternative splicing of type II collagen from COL2A in chondroprogenitor cells to COL2B in mature chondrocytes is a hallmark of chondrocyte differentiation [2]. Structurally these two COL2 splicing products differ only by one exon: COL2A includes exon 2 while COL2B lacks this exon [3]. The region encoded by exon 2 is homologous to a conserved cysteine-rich globular domain which is characteristic of many fibrillar collagens. Type II

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collagen forms a triple helix consisting of three identical alpha-1 helical strands and is the predominant form of collagen in the cartilaginous extracellular matrix.

While signal transduction pathways involved in the transcriptional control of chondrogenesis have been studied extensively [1], much less is known about how pre-mRNA splicing is regulated during the differentiation of chondrocytes. Analogous to gene transcription, RNA splicing is controlled by both cis-acting elements and trans-acting factors. Recently, a mouse COL2 minigene construct was generated and exogenous COL2 transcripts from this minigene were shown to mimic endogenous splicing switch from COL2A to COL2B during insulin-induced differentiation of ATDC5 chondroprogenitor cells [4]. The availability of such COL2 minigenes has made it possible to further analyze the cis-acting RNA elements required for COL2 alternative splicing in chondrogenic cells [5,6].

In an attempt to understand how trans-acting factors regulate RNA splicing and differentiation of chondrogenic cells, we have investigated the effects of different serine–arginine (SR) proteins on the alternative splicing of pre-mRNAs generated from endogenous collagen gene. SR proteins are important for splice site selection and appear to be functionally interchangeable when tested by an *in vitro* splicing assay [7]. Here we report that when stably expressed via retroviral transduction in ATDC5 cells, the translocation liposarcoma protein (TLS)-Associated SR protein-1 (TASR-1, also called SRp38-2) can have an effect on the alternative splicing of collagen genes. In addition, TASR-1 is found to be expressed in articular chondrocytes *in vivo* and may be able to influence expression of genes known to be important to chondrogenesis.

Materials and methods

Cell culture. ATDC5 cells, a chondrogenic cell line derived from mouse embryonal carcinoma [8], were cultured in a 1:1 mixture of DME and Ham's F-12 (Cambrex Bio Science Inc., Walkersville, MD) supplemented with 5% FBS (Invitrogen Co., Carlsbad, CA), 10 μg/ml human transferrin (Sigma–Aldrich Co., St. Louis, MO), 3×10^{-8} M sodium selenite (Sigma–Aldrich Co.) at 37 °C under 5% CO_2. To induce chondrogenic differentiation, 10 μg/ml of bovine insulin (Sigma–Aldrich Co.) was added to confluent cells and the culture media were changed every other day.

Extraction of RNA and RT-PCR. Total RNAs were extracted with RNeasy Mini Kit (QIAGEN Inc., Valencia, CA), RT-PCR was performed using SuperScript one-step RT-PCR with Platinum *Taq* (Invitrogen Co.). The primers for type II collagen (COL2) mRNA were 5′-cag gcc tcg cgg tga gcc atg at-3′ and 5′-gtt ctc cat ctc tgc cac g-3′. The primers for type XI collagen (COL11A2) were 5′-cag act cag aag cct cac ag-3′ and 5′-tcc ctc tac aaa cat acc ag-3′. The primers for GAPDH were 5′-gtg gat att gtt gcc atc att-3′ and 5′-tga tgg caa caa tat cca ctt-3′. RT-PCR conditions were described previously [4].

Plasmid constructs. cDNAs for mouse TASR-1 and TASR-2 were cloned into the *Xba*I–*Bam*HI sites of the pCG vector [9] which tags the N-terminal ends of TASR proteins with a T7 epitope. For LXSN retroviral constructs expressing T7-tagged TASR-1 and TASR-2, T7-tagged TASR cDNAs were amplified by PCR and cloned into the *Eco*RI–*Bam*HI sites of pLXSN retroviral vector (Clontech Laboratories, Inc., Mountain View, CA).

Retroviral transduction. BOSC23 retrovirus packing cells were obtained from the American Type Culture Collection (Manassas, VA), and maintained in DME supplemented with 10% FBS and 0.025 mg/ml mycophenolic acid (Sigma–Aldrich Co.) at 37 °C under 5% CO_2. The BOSC23 cells were transfected with pLXSN-T7-TASR constructs with Lipofectamine 2000 Transfection Reagent (Invitrogen Co.). After 48 h, retrovirus in the supernatant was collected and used to infect ATDC5 cells. Infected cells were selected in media containing 0.5 mg/ml G418 (Sigma–Aldrich Co.), and G418 resistant clones were pooled in this study to avoid clonal variations.

Western blot analysis. To confirm retroviral expression of T7-tagged SR proteins, 30×10^6 ATDC5 cells were collected and lysed with 5 ml NP-40 cell lysis buffer (10 mM Tris, pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl, 0.5% NP-40). The resultant nuclear pellet was resuspended in 0.2 ml Buffer X (50 mM Tris, pH 7.4, 270 mM NaCl, 0.5% Triton X-100) supplemented with protease and phosphatase inhibitors (Sigma–Aldrich Co.). After separation on a 12.5% SDS–PAGE and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA), the proteins were blotted with the mouse monoclonal horse radish peroxidase-conjugated anti-T7 antibody (EMD Biosciences, Inc., San Diego, CA), and protein bands were visualized by the ECL Plus Western Blotting Detection Reagents (Amersham Biosciences Corp., Piscataway, NJ).

Antibody production and purification. Rabbit polyclonal antibodies were raised using keyhole limpet haemocyanin peptide conjugates. The peptide antigen CNTQYSSAYYTSRKI was specific for the C-terminal end of TASR-1, and the peptide antigen CSRSRSWTSPKSSGH was specific for the C-terminal end of TASR-2. For affinity purification of the antibodies, peptide antigen was conjugated to NHS-activated sepharose beads (Pierce Biotechnology Inc., Rockford, IL) according to instructions given by the manufacturer. The purified antibodies were concentrated to 0.5 mg protein/ml and their specificities were confirmed with T7-TASR proteins by Western blotting.

Immunohistochemistry. The decalcified paraffin-embedded longitudinal sections (5 μm) of mouse (3 month old, BALBc) knee joints were blocked with 5% normal goat serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h following deparaffinization and hydration, and incubated with 0.5 μg/ml of rabbit polyclonal anti-TASR-1 or TASR-2 antibody at 4 °C overnight. As a negative control, a serial section was also incubated with 1.0 μg/ml of preimmune IgG. The sections were incubated with a 1:200 dilution of Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) for 1 h at RT, then covered with VECTASHIELD Mounting Medium (Vector Laboratories, Inc., Burlingame, CA) and examined under a fluorescence microscope (BX-51, Olympus Corp., Tokyo, Japan) with 20×/0.70 objectives at RT and captured with a digital CCD camera (CoolSNAP ES, Photometrics, Tucson, AZ) and image analysis software (MetaMorph version 6.2, Universal Imaging Corp., Downingtown, PA).

DNA microarray analysis. Total RNAs from retrovirus transduced ATDC5 cells harboring an empty vector or expressing T7-tagged TASR-1 and TASR-2 were isolated, from duplicate experiments, for DNA array analysis at the University of Washington Center for Expression Array. Target labeling and hybridization of Affymetrix GeneChips (mouse genome 430 array, version 2.0) were carried out with minor modifications from procedures recommended by the manufacturer. The chips were scanned using the GeneChip Scanner, and the CHP files were generated using Affymetrix GCOS 1.1 software. The expression settings for scaling were set for all probe sets with a target value of 250, and normalization was also set for all probe sets. Default values were used for all other parameters. Gene expression in cells with empty LXSN vector was used as the baseline control for comparison analysis. Analysis of the array data was carried out as previously described [10].

Results

Retroviral expression of TASR-1 protein influences alternative splicing of type II collagen gene in ATDC5 cells

Since chondrocyte differentiation is marked by a splicing switch from COL2A to COL2B, we hypothesized that the