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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

INTRODUCTION

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

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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 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 transacting 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 μ g/ml human transferrin (Sigma-Aldrich Co., St. Louis, MO), 3 $\times 10^{-8}$ M sodium selenite (Sigma-Aldrich Co.) at 37°C under 5% CO₂. To induce chondrogenic differentiation, 10 μ g/ml of bovine insulin (Sigma-Aldrich Co.) was added to confluent cells and the culture media was 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 *XbaI-BamHI* 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 *EcoRI-BamHI* 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% CO₂. The BOSC23 cells were transfected with pLXSN-T7-TASR constructs with Lipofectamine 2000 Transfection Reagent (Invitrogen Co.). After 48hs, 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 × 10⁶ ATDC5 cells were collected and lysed with 5 ml NP-40 cell lysis buffer (10mM Tris, pH7.4, 3mM MgCl₂, 10mM NaCl, 0.5% NP-40). The resultant nuclear pellet was resuspended in 0.2 ml Buffer X (50mM Tris, pH 7.4, 270mM 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 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 1h 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 1h 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 alternative splicing of chondrocyte-specific genes is controlled by splicing factors such as those with multiple SR repeats. It is known that SR proteins display unique roles in cell growth [11] and development [12]. The translocation liposarcoma protein (TLS)-associated SR proteins TASR-1 and TASR-2, originally cloned by our group [13,14], are alternative splicing products of the same gene [15] and may play important roles in oncogenesis [16], control of cell cycle [17], cellular response to heat-shock [18], and inhibition of neurogenesis [19].

A schematic representation of TASR-1 and TASR-2 is shown in Fig. 1A. To differentiate endogenous TASR proteins from exogenous ones, we added a T7-epitope tag at the N-terminal end of these proteins. The T7 tag did not appear to influence splice site selection of TASR proteins in an *in vivo* splicing assay [13,14]. For stable expression, cDNAs encoding T7-tagged TASR-1 and -2 proteins were subcloned into the pLXSN vector for retroviral infection. Following retroviral transduction of ATDC5 cells, G418 selection identified lines that stably express these two proteins. To rule out variations due to differences among individual clones, ATDC5 clones were pooled after G418 selection. Western blotting with the mouse monoclonal anti-T7 antibody showed that T7-tagged TASR-1 and TASR-2 exist mainly as doublets around 25, and 39 kDa (Fig. 1B) respectively, possibly as the results of protein phosphorylation [18].

The structural differences between COL2A and COL2B splicing isoforms were shown in Fig 2A. To investigate whether stable expression of TASR proteins had any effect on splice site selection of endogenous COL2 pre-mRNA, total RNA was isolated from the pooled, confluent ATDC5 clones at different time points with or without insulin treatment. RT-PCR analysis revealed that the COL2A to COL2B splicing switch occurred even in the absence of insulin (Fig. 2B, lanes 1&6, top panels), but insulin appears to accelerate such a switch (Fig. 2B, top panels, lanes 7&12). Stable expression of TASR-1 prevented the splicing switch from COL2A to COL2B when the cells were cultured without insulin (Fig. 2B, middle panels, lanes 1&6). However, the inhibitory effect of TASR-1 on COL2 splicing was abrogated by the addition of insulin to the culture medium (Fig. 2B, middle panels, lanes 7&12). In comparison, stable expression of TASR-2 in ATDC5 cells had minimal effects on the splicing of endogenous COL2 pre-mRNA (Fig. 2B, bottom panels, lanes 1&12).

Retroviral expression of TASR-1 affects alternative splicing of COL11A2 transcripts

To examine whether the splicing effect of TASR-1 is unique to COL2 transcripts, the alternative splicing of COL11A2 exon 5â10 was also examined in ATDC5 cells (Fig. 3A). Like pre-mRNA for type II collagen, COL11A2 transcripts reportedly also undergo alternative splicing switch from longer isoforms to shorter ones during chondrocyte differentiation [20]. In ATDC5 cells either harboring the empty LXS vector or expressing TASR-2, a switch in splicing also took place for COL11A2 transcripts after prolonged culture without insulin, but the addition of insulin accelerated such a switch (Fig. 3B, top and bottom panels). Retroviral TASR-1 expression prevented such a change in splicing of COL11A2 transcripts in the absence of insulin (Fig. 3B, middle panel, lanes 1â6), but such an effect on COL11A2 splicing was again overcome by insulin (Fig. 3B, lanes 7â12). We speculate that the default function of TASR-1 is to prevent splicing switch from taking place in these cells, and insulin signaling may promote splicing switch (at least in part) through functional inactivation of the TASR-1 protein.

TASR-1 and TASR-2 alter the expression of two sets of genes in ATDC5 cells

TASR-1 and TASR-2, alternative splicing products of the same *TASR* gene, appear to have different effects on splicing switch of collagen genes such as COL2 and COL11A2. To further investigate their differences, we carried out DNA microarray experiments with RNAs from ATDC5 cells expressing retroviral TASR-1 and TASR-2. With the Affymetrix Mouse GeneChip that covers 45,000 probes corresponding to 39,000 transcripts, global gene expression was compared between these two proteins with cells harboring the empty LXS viral vector as the baseline control. After comparison analysis with the empty vector control, our microarray data revealed that 219 genes in TASR-1 cells and 566 genes in TASR-2 cells were up- or down-regulated at least twofold in un-induced cells. After ATDC5 cells were induced with insulin for 21 days, 585 genes in TASR-1 cells and 302 genes in TASR-2 cells were found to be up- or down-regulated at least twofold in comparison with the control cells.

To better understand how TASR-1 and TASR-2 differentially affect genes involved in chondrogenesis, a partial list of chondrocyte-related genes was compiled to illustrate their regulation by TASR-1 and TASR-2 in ATDC5 cells (Table 1). Notably, our microarray data showed that type X collagen (COL10) was down regulated more than 11 fold by TASR-1 in insulin-induced ATDC5 cells, an observation further confirmed by RT-PCR analysis (data not shown). TASR-1 decreased Sox6 and Sp7 expression, with no effect on Runx1 and Runx2 in insulin induced cells. TASR-1 also down-regulated Fgfr3 and parathyroid hormone receptor 1, whereas TASR-2 up-regulated Fgfr3 and cyclin-dependent kinase inhibitor 1C.

TASR-1 is more abundantly expressed than TASR-2 in mouse articular chondrocytes

Expression of COL10 is a hallmark of hypertrophic chondrocytes during endochondral ossification in growth plates. On the other hand, articular chondrocytes do not normally undergo hypertrophic differentiation or express COL10 [21,22]. As TASR-1 protein can down-regulate COL10 expression in insulin-induced ATDC5 cells, we speculated that TASR-1 may contribute to the phenotypic maintenance of articular chondrocytes *in vivo*, and such a role for TASR-1 would require its expression in articular cartilage. To examine TASR proteins *in vivo*, we generated rabbit polyclonal antibodies against the unique C-terminal ends of TASR-1 and TASR-2. The specificities of these antibodies were confirmed by recognizing specifically against T7-tagged TASR-1 and TASR-2 in western blotting (data not shown).

Using these same antibodies or a pre-immune IgG, immunohistochemical staining was performed with serial sections of normal mouse tibia. Our results showed that TASR-1 protein was expressed in articular chondrocytes (Fig. 4, panel A), with little or no detectable expression of TASR-2 (Fig. 4, panel B). Staining with the pre-immune IgG was used as a negative control

in these experiments (Fig. 4, panel C), and the H&E staining was performed to show articular chondrocytes (Fig. 4, panel D).

DISCUSSION

Despite advances in our understanding of skeletal morphogenesis, important aspects of endochondral ossification and articular chondrocytes remain unclear. These critical issues include the control of chondrocyte hypertrophy and maintenance of the phenotype of articular chondrocytes [22,23]. In this manuscript we have presented findings that the SR family of splicing factors, especially TASR-1, can influence alternative splicing of collagen genes and may contribute to the decision of chondrocytes to remain proliferating or entering into the hypertrophic phase of terminal differentiation.

Insulin-treatment of ATDC5 cells represents a well-known *in vitro* model of growth factor-induced chondrogenic differentiation. ATDC5 cells, originally isolated from a mouse embryo, can mimic chondrogenesis with hallmark events such as cellular condensation to form cartilage nodules, splicing switch from COL2A to COL2B, and the ability to undergo hypertrophic differentiation. In this study we have examined the effects of TASR proteins on the alternative splicing of COL2 and COL11A2 transcripts in ATDC5 cells. Interestingly, the alternative splicing patterns of other matrix protein-encoding genes (collagen type IX, fibronectin, and tenascin C) are very similar in that exon(s) encoding globular domains are excluded from mRNAs in mature chondrocytes. This raises the intriguing possibility that a common mechanism may be involved in the alternative splicing of all matrix genes.

During skeletal development, growth plate chondrocytes undergo many rounds of proliferation, then become hypertrophic and eventually undergo apoptosis at puberty. In contrast, articular chondrocytes normally do not undergo hypertrophic differentiation, thus helping to maintain the articular surface throughout life. Even though they are of similar developmental origin, chondrocytes in the growth plate and articular cartilage have different lineage-specific fates. There are probably multiple factors that determine the ultimate lineage. It was reported that the chicken *ets* transcription factor (*ch*-ERG), an Ets-related DNA-binding protein, is expressed prominently in prehypertrophic chondrocytes in the growth plate. On the other hand, C-1-1, a splice variant of the *ch*-ERG, is preferentially expressed in articular cartilage, and C-1-1 over-expression in chondrocytes leads to failure of hypertrophic differentiation [24]. It is notable that TASR-1 but not TASR-2 inhibits expression of type X collagen (a marker for hypertrophic chondrocytes). In addition, TASR-1 is more abundantly expressed than TASR-2 in articular chondrocytes. These data suggest that TASR-1 protein may play a role in determining whether mature chondrocytes enter into the hypertrophic phase.

How does TASR-1 act differently from TASR-2 in differentiating chondrocytes? Though they are both splicing factors derived from the same gene, TASR-1 and TASR-2 differ from each other in their arginine-serine domains and favor different splice sites [14]. We speculate that TASR-1 target genes are critical to chondrocyte differentiation, whereas TASR-2 target genes have minimal effect on chondrocytes and instead play important roles in the differentiation of other cell types such as neurons [19]. Down-regulation of chondrogenic genes by TASR-1 in ATDC5 cells is likely mediated through splicing inhibition as the primary mechanism. Since transcription and splicing are tightly coupled processes inside live cells [25], it is also possible that splicing inhibition by TASR-1 can cooperate with transcriptional repression to shut down genes critical for mature chondrocytes to enter into hypertrophic differentiation.

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Fig 1. Retroviral expression of T7-tagged TASR proteins in ATDC5 cells

(A) The RNP consensus sequences shared by TASR-1 and TASR-2 are shown in gray boxes. RS domains are in hatched boxes. (B) Nuclear extracts from ATDC5 cells harboring the empty LXSN retroviral vector (lane 1), T7-TASR-1 (lane 2), or T7-TASR-2 were separated on a 12.5% SDS-polyacrylamide gel. The proteins were blotted with a mouse monoclonal anti-T7 antibody, and their positions are indicated to the right.

Fig 2. Effects of TASR proteins on the alternative splicing of endogenous COL2 transcripts
 (A). Schematic of mouse COL2 transcripts. (B) RNAs were obtained from the ATDC5 cells harboring the empty retroviral vector (top panels), T7-TASR-1 (middle panels) or T7-TASR-2 (bottom panels). RT-PCR analysis of COL2 transcripts were performed using samples from cells cultured without insulin (lanes 1-6) or with insulin (lanes 7-12). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to demonstrate that similar amounts of RNAs were present in these samples.

Fig 3. Effects of TASR proteins on the alternative splicing of endogenous COL11A2 transcripts (A). Schematic of mouse COL11A2 transcripts. (B) RNAs were obtained from ATDC5 cells harboring the empty retroviral vector (top panel), T7-TASR-1 (middle panel) or T7-TASR-2 (bottom panel). RT-PCR analysis of COL11A2 transcripts were performed using samples from cells cultured without insulin (lanes 1â6) or with insulin (lanes 7â12).

Fig 4. Immunohistostaining of mouse articular cartilage with anti-TASR antibodies

Sections of normal mouse knee joint were incubated with the rabbit anti- TASR-1 (A), anti-TASR-2 (B), pre-immune IgG (C) or stained with hematoxylin&eosin (D). After several washes with PBS, the sections were incubated with 1:200 dilution of Cy3-conjugated Goat Anti-Rabbit IgG, and protein expression was examined under a fluorescence microscope. Joint surface is indicated by arrows. Bars, 20 μ m.

Table 1
Partial List of Genes Reported to be Relevant to Chondrocytes

ProbeÂID	Gene	Symbol	Fold Change vs LXSN Control			
			DayÂ0	DayÂ21	TASR1	TASR2
			TASR1	TASR2	TASR1	TASR2
<i>Extracellular matrix proteins</i>						
1418477_at	Matrilin 1, cartilage matrix protein 1	Matn1	- *	-	â2.6	-
1450567_a_at	Procollagen, type II, alpha 1	Col2a1	-	5.4	-	-
1422253_at	Procollagen, type X, alpha 1	Col10a1	-	-	â11.3	-
1418599_at	Procollagen, type XI, alpha 1	Col11a1	-	-	â2.1	-
1423578_at	Procollagen, type XI, alpha 2	Col11a2	-	-	â3.7	-
1477484_at	IntegrinÂbindingÂsialoprotein	Ibsp	-	-	â10.2	-
1449827_at	AggrecanÂ1	Agc1	-	-	â4.6	-
1426294_at	Hyaluronan and proteoglycan link protein 1	Halpn1/ Crtl1	-	-	â2.3	-
1417389_at	GlypicanÂ1	Gpc1	â2	â2	-	-
1423516_a_at	NidogenÂ2	Nid2	â2	-	â2.3	-
1449880_s_at	BoneÂgamma-carboxyglutamateÂprotein	Bglap	-	-	â3.5	-
1419608_a_at	Melanoma inhibitory activity 1	Mia1/ Cdrap	-	-	â2	-
1419527_at	Cartilage oligomeric matrix protein	Comp	-	-	â3.2	-
<i>Transcription and regulatory factors</i>						
1434918_a_at	SRY-box containing gene 6	Sox6	-	-	â2.3	-
1424950_at	SRY-box containing gene 9	Sox9	-	2.3	-	-
1418425_at	Trans-acting transcription factor 7 (Osx)	Sp7/Osx	-	-	â2.1	-
<i>Growth factors, receptors, kinases and phosphatases</i>						
1421841_at	Fibroblast growth facto receptor 3	Fgfr3	-	-	â2.1	2.5
1450922_a_at	Transforming growth factor, beta 2	Tgfb2	-	2	-	-
1417092_at	Parathyroid hormone receptor 1	Pthr1	-	â2.3	â2.5	-
1417649_at	Cyclin-dependent kinase inhibitor 1C (p57)	Cdkn1c	-	2.8	-	2.3
1423611_at	Alkaline phosphatase 2, liver	Akp2	â2.1	â2.3	â2.5	-

* - indicates fold changes < 2.