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Biochemical and Biophysical Research Communications 341 (2006) 167–174
BBRC
www.elsevier.com/locate/ybbrc

Screening for genes preferentially expressed in the early phase of chondrogenesis

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Received 20 December 2005
Available online 6 January 2006

Abstract

This study reports a new system that is very effective in identifying genes closely related to the early phase of chondrogenic differentiation. While studying chondrogenesis in a progenitor cell line, ATDC5, we found that the amount of culture media overlying an ATDC5 monolayer affected the extent to which differentiation occurred. Therefore, to gain insight into the molecular mechanisms of chondrogenic differentiation, differential gene expression between differentiating and non-differentiating ATDC5 cultures was examined by suppression subtractive hybridization analysis. In this study, we focused on transcription factors that were identified in differentiating cultures, and found that activating transcription factor 5, ATF5, exhibited a conspicuous activation pattern using two methods to induce chondrogenesis of ATDC5 cells. Furthermore, ATF5 was found to be elevated in the developing limb bud by in situ hybridization in a pattern that was highly restricted to the cartilaginous anlagen, suggesting a positive association with ATF5 expression and chondrogenesis.

Keywords: Chondrogenesis; ATDC5 cells; Inclined culture; Activating transcription factor 5; Glucocorticoid-induced leucine zipper; Pleiotrophin

Knowledge of genes that are activated in chondrogenesis is of fundamental importance to understanding cartilage development. Recent studies have shown that the expression of type II collagen and aggrecan, both considered hallmarks of cartilage differentiation [1], is coregulated by Sox9, a transcription factor with a high mobility group box harboring sequence-specific DNA binding activity [2]. The Sox9 gene is expressed predominantly in mesenchymal condensations which occur early in cartilage development [3] and, when mutated, causes campomelic dysplasia [4]. These observations suggested that Sox9 was essential for both the onset of chondrogenesis and the activation of the type II collagen and aggrecan genes. However, type II collagen is transiently expressed in the wide variety of embryonic tissues such as heart, skin, kidney, aorta, notochord, epithelium, and some basement membrane [5]. Aggrecan is also present in non-cartilaginous tissues such as brain, notochord, and heart [6,7]. These observations suggested that type II collagen and aggrecan expression was not necessarily synchronized with chondrogenesis and that their expression was regulated in a different way in different tissues. Therefore, in this report, we have re-examined, in detail, the processes of chondrogenic differentiation. For this purpose, we used a chondrogenic clonal cell line, ATDC5, that was established from mouse embryonal carcinoma cells [8]. This cell line enables us to survey the multistep chondrogenic differentiation process in culture [9].

Chondrogenesis of ATDC5 is induced by both insulin and BMP2. Insulin signaling induces cellular condensation before cartilage nodule formation. However, chondrogenesis of ATDC5 induced by BMP2 skips this condensation

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.12.169

stage [10]. In addition, we recently found that chondrogenic differentiation is enhanced by the amount of culture medium given to the cells. To confirm this, we cultured a flask of ATDC5 cells on a slight incline and observed that extent of chondrogenesis was directly related to the depth of medium. Therefore, we hypothesized that a comparison of gene expression in cultures with different amounts of medium would provide us with information on genes that are expressed in proportion to the extent of differentiation and hence, with insight into the molecular mechanisms of chondrogenic differentiation.

We performed a suppression subtractive hybridization analysis to identify differences in gene expression between the two culture conditions which differed in the degree of chondrocyte differentiation. We report here details that lead to the finding that glucocorticoid-induced leucine zipper, GILZ [11], and activating transcription factor 5, ATF5 [12], were preferentially expressed in differentiating cells along with the activation of several cartilage characteristic genes including pleiotrophin (PTN) [13].

Materials and methods

Cell culture. Mouse ATDC5 cell line, originally established by T. Atsumi, was maintained in DF5 medium, a 1:1 mixture of DMEM and Ham's F12 (Gibco, Rockville, MD, USA) supplemented with 5% fetal bovine serum (Gibco) and 0.03 mM Na_2SeO_3 (Sigma, St. Louis, MO, USA) as described previously [8]. The cells were cultured at an initial density of 1.6×10^4 cells/cm^2. For induction of chondrogenesis, the cells were cultured in DF5 medium until confluent, and then the culture medium was replaced with DF5 medium supplemented with 10 μg/ml bovine insulin (Sigma), DF5I, at either 0.47 ml/cm^2 (high medium) or 0.2 ml/cm^2 (low medium). For another condition of chondrogenic induction, cells were cultured in the DF5 medium (0.47 ml/cm^2) containing 50 ng/ml human recombinant bone morphogenetic protein 2 (BMP2) (a generous gift from Astellas Pharmaceutical, Tokyo, Japan). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air, and the medium was changed every other day. To verify chondrogenic differentiation of ATDC5 cells, the cultures were examined for the expression of cartilage characteristic genes by Northern blot analysis as described below or for intensity of Alcian blue staining at pH 1.0.

For the preparation of the inclined culture, the cells were inoculated into a tissue culture flask (Becton–Dickinson, Franklin Lakes, NJ, USA) as described above and cultured in DF5 medium until confluent. Then, the medium was replaced with DF5I, and the flask was tipped at slight angle and incubated at 37 °C in CO_2-incubator for 22 days.

For proliferation assays, 1.5×10^5 cells were plated in 35 mm dishes and cultured as described above. Cells were counted in a hemocytometer. The data shown represent means and SD from two independent experiments, each done in duplicate.

RNA preparation and Northern blot analysis. Total RNA from ATDC5 cells, embryonic (E14.5) mouse limb buds, and newborn (1-day-old) mouse epiphyseal cartilage was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocols.

Northern blot analysis was performed as described previously [14]. Briefly, 10 μg of total RNA was denatured and then resolved in a 1% agarose gel. After transferring the RNA samples onto a Hybond N membrane (Amersham Biosciences, Piscataway, NJ, USA), the blot was then cross-linked by UV irradiation.

The following cDNA fragments were synthesized by reverse transcriptase-polymerase chain reaction (RT-PCR) from ATDC5 cell total RNA and used as probes for hybridization; mouse type II collagen (Col II), type X collagen (Col X), Sox9, L-Sox5, pleiotrophin (PTN), glucocorticoid-induced leucine zipper (GILZ), activating transcription factor 5 (ATF5), and activating transcription factor 4 (ATF4). Briefly, purified total RNA (3 μg) from 12-day culture of chondrogenesis-induced ATDC5 cells was incubated with an oligo(dT) primer and SuperScript II RNase H$^-$ reverse transcriptase (Invitrogen). Aliquots of the resulting cDNA were used to amplify probes by PCR using specific primers as shown in Table 1. As a control probe, mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Ambion, Austin, TX, USA) was used.

Hybridization was performed as described previously [14]. Briefly, the blots were hybridized with the ^{32}P-labeled cDNA probe for 24 h at 42 °C in a solution containing 50% formamide, 5× SSPE (0.75 M NaCl, 50 mM sodium phosphate, pH 7.4, and 5 mM EDTA), 5× Denhardt's solution, 10% dextran sulfate, 0.1% SDS, and 20 μg/ml denaturated salmon sperm DNA. After hybridization, the membranes were washed three times at a high stringency of 65 °C in 0.1× SSPE containing 0.1% SDS and then exposed to X-ray film at −80 °C or to imaging plates (Fuji Film, Tokyo, Japan) at room temperature.

Table 1
Primers used to obtain cDNA probes for Northern blot analyses by RT-PCR

Probe[a]	GenBank number	Primer sequence[b] (5′ → 3′)	Size (bp)
Col II	BC052326	F: CCTGTCTGCTTCTTGTAAAACCCCCGAAC	400
		R: TACAGAGGTGTTTGACACAGAATAGCACC	
Col X	Z21610	F: AAGCTTACCCAGCAGTAGGTGCCCCCATC	556
		R: GGATCCTCACATACCCACTGTTACTGTTC	
Sox9	NM_011448	F: AGGAAGCTGGCAGACCAGTACCCGCATC	970
		R: GATGGTCAGCGTAGTCGTATTGCGAGCG	
L-Sox5	AJ010604	F: TAGCCATGGTGACAAGCAGACAGAAAGT	625
		R: CCTTGAACCTGGATCTGTTGCTGAAGCA	
PTN	BC002064	F: GATACCTGGAGTCTGCAGAAACCTCGCC	390
		R: CTGGTACTTGCACTCAGCTCCAAACTGC	
GILZ	NM_010286	F: TCGTGAAGAACCACCTGATGTACGCTGT	475
		R: CAGGCTCACTGGCTTGGTGTTACTAGGC	
ATF5	NM_030693	F: AGTCAGCTGCTCTCAGGTACCGCCAGAG	659
		R: TACCAACTACAATCACCCCTTCCTGTCC	
ATF4	NM_009716	F: CATGGCGTATTAGAGGCAGCAGTGCTGC	544
		R: TCATCCAACGTGGTCAAGAGCTCATCTG	

[a] Col II, type II collagen; Col X, type X collagen; L-Sox5, long form Sox5; PTN, pleiotrophin; GILZ, glucocorticoid-induced leucine zipper; ATF5, activating transcription factor 5; ATF4, activating transcription factor 4.
[b] Sequences are given as forward (F) and reverse (R) primers.