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Screening for genes preferentially expressed in the early phase of chondrogenesis

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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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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.

Construction and analysis of subtractive cDNA libraries. For the construction of a subtractive cDNA library, total RNA (250 ng) from ATDC5 cells cultured in different amounts of medium for 4 days was reverse-transcribed using a Super SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA). Transcribed cDNAs were then used for suppression subtractive hybridizations, which were performed using a PCR-Select cDNA Subtraction Kit according to the manufacturer's instructions (Clontech). Finally, the resulting subtracted cDNA was ligated into a T/A cloning vector (Invitrogen) to make a subtracted cDNA library. After construction of the library, bacterial colonies were randomly picked and cloned cDNA sequences were determined using the BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA) with either an M13 forward (5′-CGCCAGGGTT TTCCCAGTCACGAC-3′) or M13 reverse (5′-CACAGGAAACAGCT ATGACCATG-3′) primer. The resulting partial sequences were compared with sequences in GenBank using the NCBI BLAST program [15].

Microarray analyses. DNA microarray analyses were performed as described previously [16]. Briefly, total RNA samples were prepared from 2-day and 8-day cultures of ATDC5 cells in high medium, and 10 μg of each RNA sample was labeled with aminoallyl-dUTP using a CyScribe cDNA Post-Labeling Kit (Amersham Biosciences) according to the manufacturer's instructions. The purified cDNA was then labeled with Cy3 or Cy5 reactive dye (Amersham Biosciences), and the CyDye-labeled cDNA probes were hybridized to a mouse TF1560 array (Bio Matrix Research, Chiba, Japan) at 42 °C for 16 h. After being washed, the array was scanned using a ScanArray Express (Perkin-Elmer, Norwalk, CT, USA), and hybridization images were analyzed with QuantArray 3.0 software (Perkin-Elmer). The experiments were done in duplicate, and signal intensity data were analyzed by the program GeneSpring 6.0 (Silicon Genetics, Redwood City, CA, USA). The ratios of the signal intensities between the two cultures were calculated through Lowess normalization and expressed as fold difference.

Tissue preparation and in situ hybridization. E14.5 mouse embryonic limb buds were dissected and fixed in 10% formaldehyde in 0.1 M phosphate-buffered saline, pH 7.4. The tissues were then embedded in paraffin. Paraffin sections were prepared according to standard protocols and in situ hybridization was conducted essentially as described previously [17]. Briefly, sections were deparaffinized, treated with proteinase K, prehybridized, and then hybridized overnight at 50 °C. For the preparation of hybridization probes, the purified cDNAs used for Northern hybridization were cloned into pGEM-3Zf(−) (Promega, Madison, WI, USA), and then sense and antisense RNA probes were generated by in vitro transcription of the plasmid templates using a DIG RNA Labeling Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. After hybridization, signals were visualized with a DIG Nucleic Acid Detection Kit (Roche) and then some of the sections were counterstained with methyl green.

Results

Inclined culture of ATDC5 cells

Mouse ATDC5, an embryonal carcinoma-derived cell line, has been widely used as an in vitro model system for the study of chondrogenic differentiation [9]. In this system, type II collagen, whose expression is considered a hallmark of cartilage differentiation, is newly activated as shown in Fig. 1. Concomitantly, the expression of the Sox genes, Sox9, Sox6, and L-Sox5, thought to play an essential role in the activation of the type II collagen gene and also in chondrogenic differentiation [18], is observed throughout in both undifferentiated and differentiating ATDC5 cells (Fig. 1). The expression level of Sox9 is almost constant during chondrogenic differentiation. L-Sox5 is slightly

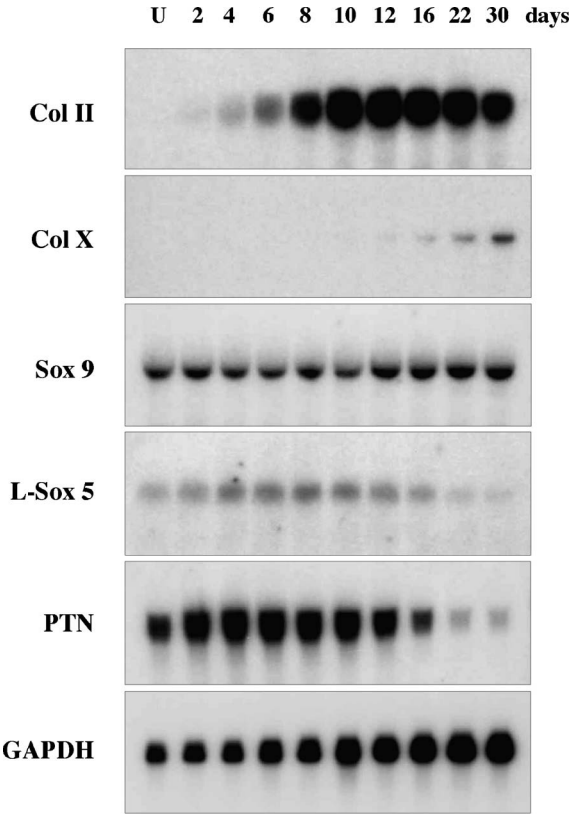

Fig. 1. Northern blot analyses. The gene expression patterns of type II collagen, Sox9, L-Sox5, and pleiotrophin (PTN) were analyzed during cartilage differentiation of ATDC5 cells. Total RNA was prepared from ATDC5 cells cultured in insulin-containing medium on days indicated at the top and from undifferentiated ATDC5 cells (U). The same amount of total RNA (10 μg) was used for each lane. Hybridization with a GAPDH probe is shown as a reference for the amount of RNA loaded in each lane.

up-regulated, but does not seem to be related to the activation of type II collagen gene. Sox6 was also detected by RT-PCR, but Northern blot analysis indicated that its expression level was very low in this system (data not shown). Therefore, these results suggested that expression of Sox genes alone was insufficient for the activation of the type II collagen gene and for the initiation of chondrogenesis in this system. Instead, other factors would be involved in the regulation of chondrogenic differentiation of ATDC5.

Although differentiation of ATDC5 cells is triggered by insulin, we have recently found that the differentiation is susceptible to the amount of medium overlying the cells as shown in Figs. 2A and B. This effect was due to the amount of medium and did not simply reflect the nutrient supply of medium. In other words, frequent replacement of culture medium with fresh medium had no effect on the differentiation. We initially investigated a possible relationship between the growth of ATDC5 cells and the amount of insulin-containing medium. As shown in Fig. 2C, initial growth rate is not dependent on the amount of medium. However, cells in low medium cease dividing after 4 days, but do not differentiate into chondrocytes. Day 4 cultures, then, provide us with ATDC5 cultures with predominantly

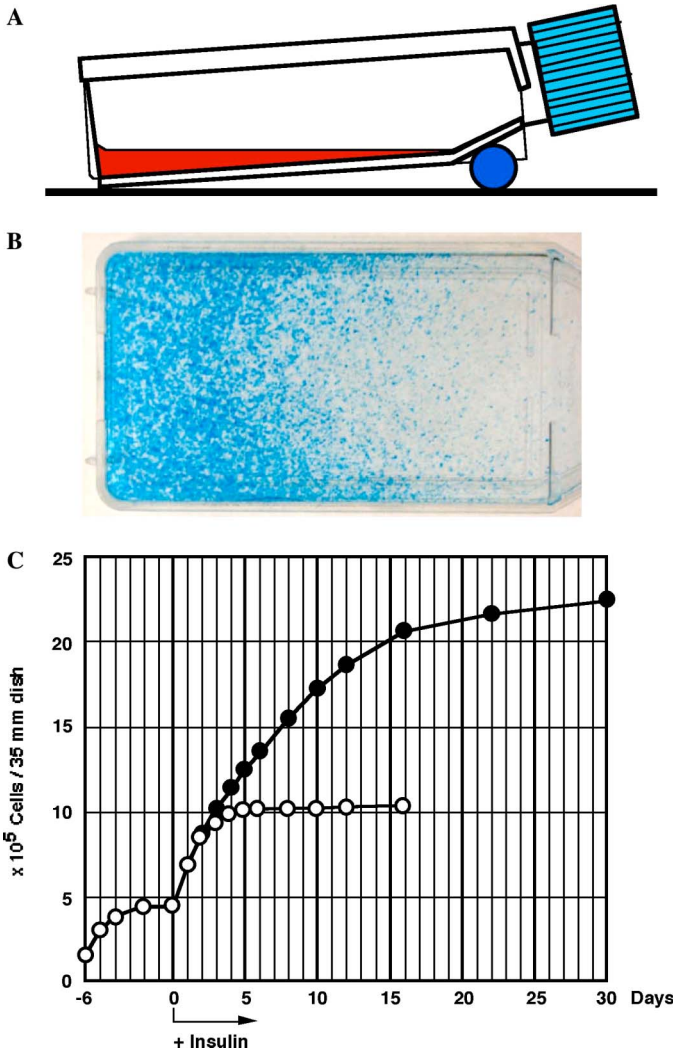

Fig. 2. Inclined culture of ATDC5 cells. (A) Confluent culture of ATDC5 cells 4 days after plating was tipped at slight angle and incubated in insulin-containing medium at 37 °C in CO_2-incubator. (B) After 22 days of culture, cells were fixed and stained with Alcian blue. (C) ATDC5 cells were cultured in 35 mm tissue culture dishes with 2 ml (open symbols) and 4.5 ml (filled symbols) of medium, and cell growth was monitored at the indicated time points.

differentiating cells (high medium) and cultures with entirely undifferentiated cells (low medium), providing us with cells differing primarily in the expression of genes related to chondrogenesis.

Screening of a subtracted cDNA library

A cDNA library enriched for genes characteristic of differentiating ATDC5 cells was constructed by suppression subtractive hybridization as described in Materials and methods. A total of 994 positive clones were randomly selected from the library and their cDNA inserts were partially sequenced and compared with sequences in GenBank using the BLAST algorithm [15]. These analyses revealed that 22% of cDNA inserts corresponded to mitochondrial and ribosomal RNA sequences. Of the remaining clones, 636 were found to correspond to known mRNA sequences and 139 clones represented hypothetical mRNA sequences or did not have any significant matches with entries in the database. Of the known sequences, 199 were redundant and the most frequently detected sequence (a total of 45 times) corresponded to pleiotrophin [13], which was transiently activated during chondrogenesis of ATDC5 (Fig. 1).

In order to understand the molecular mechanisms involved in the regulation of chondrogenic differentiation, we first focused on transcription factors identified by sequence homology searching as described above. A total of 31 different transcription-related factors were identified and are listed in Table 2. Northern blot analyses of all identified transcription factors revealed that glucocorticoid-induced leucine zipper, GILZ [11] and activating transcription factor 5, ATF5 [12] underwent unique temporal expression patterns during cartilage differentiation (Fig. 3). In contrast, the remaining factors were poorly detected on Northern blots or were either constitutively active or gradually suppressed.

Microarray analysis

In order to validate the results of Northern blot analyses that expression of most factors was poorly related to chondrogenic differentiation, we performed a microarray analysis using the mouse TF1560 array (Bio Matrix Research, Chiba, Japan) that contains 1560 genes encoding different transcription-related factors including 14 of 31 genes listed in Table 2. RNA was isolated from the cultures in high medium at day 2 and day 8, and then hybridized to the array. The observed signal intensities at day 8 were used to calculate fold changes relative to those at day 2 as recommended by manufacturer (Bio Matrix Research). Table 3 lists the 12 genes whose activation was most prominent; note that only five of these genes differed by more than 2-fold. Therefore, these results also indicated that the expression of most transcription-related factors was not significantly altered during cartilage differentiation of ATDC5.

Although some of the factors included in Table 2 and in the TF1560 array were known to be functioning in skeletal formation [19,20], we did not study them further and instead, focused on the two factors, GILZ and ATF5, that exhibited conspicuous activation patterns.

Activation of ATF5 by BMP2 but not of GILZ

Previous studies showed that BMP2 induced the chondrogenesis of ATDC5 cells [10]. Therefore, we examined the effects of BMP2 on the transcription of GILZ and ATF5 genes. As shown in Fig. 4, BMP2 induced the transient activation of ATF5 gene preceding type II collagen expression in ATDC5 cells. In contrast, the expression of GILZ was present at a low level or was poorly detected on Northern blots. These data suggested that

Table 2
Transcription factors identified by suppression subtractive hybridization

Gene name	Accession number	Hit number
Split hand/foot deleted gene 1	NM_009169	3
HMG box 1	NM_010439	2
Transcription factor 4	NM_013685	2
LIM domain, transcription factor	XM_194207	1
MSX2 homeobox gene	NM_013601	1
Zinc finger protein 142	XM_484882	1
Glucocorticoid-induced leucine zipper (GILZ)	NM_010286	1
Inhibitor of growth family, member 1	NM_011919	1
HMG nucleosomal binding domain 1	NM_008251	1
Tripartite motif protein 24	NM_145076	1
AE binding protein 1	NM_009636	1
Silencing mediator of retinoic acid and thyroid hormone	NM_011424	1
Gli3 gene	AF418601	1
TGF β1 induced transcript 1	NM_009365	1
Activating transcription factor 5 (ATF5)	NM_030693	1
A nuclear protein essential early in embryogenesis	NM_024428	1
Upstream transcription factor 2	NM_011680	1
Acidic nuclear phosphoprotein 32 family	NM_130889	1
General transcription factor II H	NM_008186	1
Putative transcriptional regulatory protein	NM_026453	1
Basic transcription factor 3	NM_145455	1
Proline-rich nuclear receptor coactivator 2	NM_026383	1
Zinc finger protein 95	NM_016683	1
CCR4-NOT transcription complex, subunit 10	NM_153585	1
Transcription factor PBX1a	NM_183355	1
A novel class of bHLH transcription factors	NM_025804	1
Zinc finger, C2H2 type containing protein	NM_172753	1
Paired related homeobox 1	AK048023	1
Nuclear factor NF-κB P65 subunit	NM_009045	1
CCR4-NOT transcription complex, subunit 7	NM_011135	1
Histone deacetylase 1	NM_008228	1

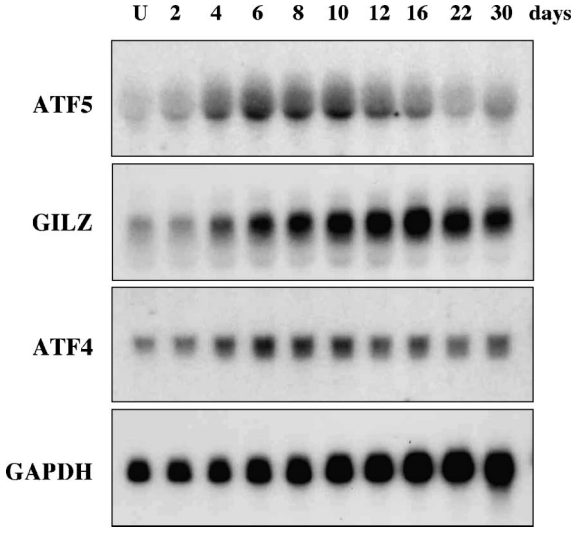

Fig. 3. Expression pattern of transcription factors during chondrogenic differentiation of ATDC5 cells. Gene expression of the transcription factors, ATF5 and GILZ, was analyzed during cartilage differentiation of ATDC5 cells by Northern blotting. Total RNA was prepared and used as described in Fig. 1. Hybridization with a GAPDH probe is shown as a reference for the amount of RNA loaded in each lane.

the expression of ATF5 was closely related to the initiation of chondrogenic differentiation while that of GILZ was not.

Table 3
Microarray analysis of differentiation-dependent gene expression for transcription-related factors

Gene name	Accession number	Fold difference
Insulin-like growth factor binding protein 4	NM_010517	5.0
Cyclin-dependent kinase inhibitor 1A	NM_007669	4.1
Transcription factor 7-like 2	NM_009333	2.6
Activating transcription factor 5 (ATF5)	NM_030693	2.3
DNA-damage inducible transcript 3	NM_007837	2.2
Distal-less homeobox 1	NM_010053	1.9
Kruppel-like factor 2	NM_008452	1.8
Lymphoid enhancer binding factor 1	NM_010703	1.7
Kruppel-like factor 4	NM_010637	1.7
bHLH domain containing, class B2	NM_011498	1.6
Early growth response 1	NM_007913	1.5
E74-like factor 5	NM_010125	1.5

Specific expression of ATF5 mRNA in mouse limb bud and newborn mouse epiphyseal cartilage

To examine whether ATF5 and GILZ are expressed in cartilage primordium, Northern blot analysis was performed using mRNA isolated from newborn mouse epiphyseal cartilages and embryonic mouse limb buds. Although the expression of ATF5 was evident in both the tissues, GILZ mRNA was at very low levels in limb buds

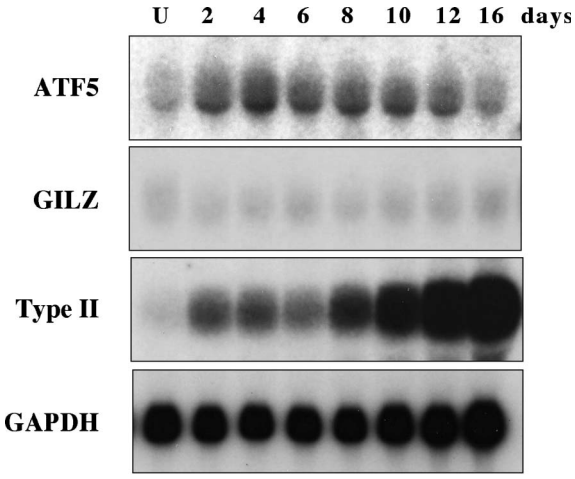

Fig. 4. Effects of BMP2 on the transcription of GILZ and ATF5 genes. ATDC5 cells were treated with BMP2 to induce chondrogenic differentiation, and gene expression of GILZ and ATF5 was analyzed by Northern blotting. Total RNA was prepared from ATDC5 cells cultured in BMP2-containing medium on days indicated at the top and from untreated ATDC5 cells (U). The same amount of total RNA (10 μg) was used for each lane. Hybridization with type II collagen and GAPDH probes is shown as evidence for the differentiation process and the amount of RNA loaded in each lane, respectively.

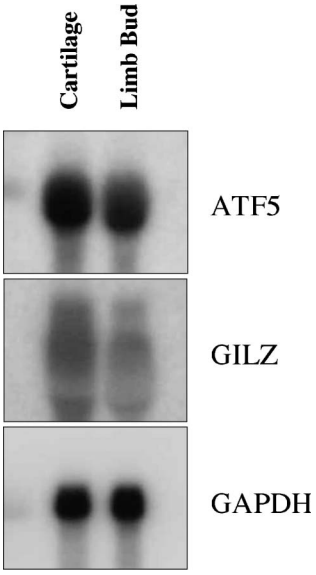

Fig. 5. Gene expression of ATF5 and GILZ in limb bud and cartilage. Total RNA was prepared from E14.5 embryonic mouse limb buds and newborn mouse epiphyseal cartilages, and subjected to Northern blot analyses using the cDNA probes indicated on the right. Hybridization with a GAPDH probe is shown as a reference for the amount of RNA loaded in each lane.

(Fig. 5). This was further supported by the in situ hybridization analysis of both transcripts in mouse limb buds. Specific expression of ATF5 transcripts was observed in cartilage primordium at E14.5 mouse embryonic limb buds (Fig. 6). However, GILZ mRNA was barely detectable in these tissues (data not shown).

At this stage, ATF5 is specifically expressed by chondrocytes in the reserve and proliferating zone of the growth plate. The staining pattern is almost the same as that of type II collagen (Figs. 6B and C). In contrast, the signal is almost negative to the zone of hypertrophic chondrocytes where type X collagen expression is localized (Fig. 6D).

Discussion

We have found several genes preferentially expressed in the early phase of differentiating ATDC5 cells by suppression subtractive hybridization. Most of genes that were detected with high frequency in a subtracted cDNA library are extracellular macromolecules such as osteoglycin [21], asporin [22], and osteonectin [23], and have been previously observed in cartilage. The most enriched gene is pleiotrophin that is found in abundance in fetal cartilage, but not in adult cartilage [13]. The detection of a large number of genes known to be associated with chondrogenesis strongly suggests that our subtractive hybridization has been effective in the selection of differentially expressed genes and, hence, that any novel genes appearing in this group are likely to be associated with the differentiation process.

In addition to extracellular molecules, our approach allowed the identification of many genes encoding transcription factors. Although most transcription factors were poorly detected on Northern blots or were not expressed synchronously with the chondrogenic differentiation of ATDC5 cells, many genes, such as split hand/foot deleted gene 1, Msx2, Gli 3, Pbx1, among others, have been reported to play significant roles in skeletal development [19,20]. Therefore, these data strongly suggested that our subtraction system was very effective in detecting those genes closely related to chondrogenesis. In this report, we have focused on ATF5 and GILZ because they are clearly activated in ATDC5 cells (Fig. 3) and their expression has not been previously reported in cartilage development. It is unlikely that we have identified all the important factors associated with chondrogenesis especially since 139 of the clones of differentially expressed genes contained sequences from either hypothetical or unidentified genes. Such clones might include new transcription factors that were critical for chondrogenesis and should be examined in the future.

Both ATF5 and GILZ are transiently up-regulated during insulin-induced chondrogenic differentiation of ATDC5 cells reaching maxima at different time points, day 8 and day 16, respectively. In contrast, BMP2 induced chondrogenic differentiation of ATDC5 cells accompanied by the activation of ATF5, but not of GILZ. These differences of the activation of ATF5 and GILZ in vitro might reflect their different gene regulation in vivo, such that ATF5 is readily detectable in embryonic limb buds while GILZ is not. Northern blot and RT-PCR (data not shown) analyses clearly indicated that GILZ is certainly expressed in cartilage tissue but not in the differentiation process,

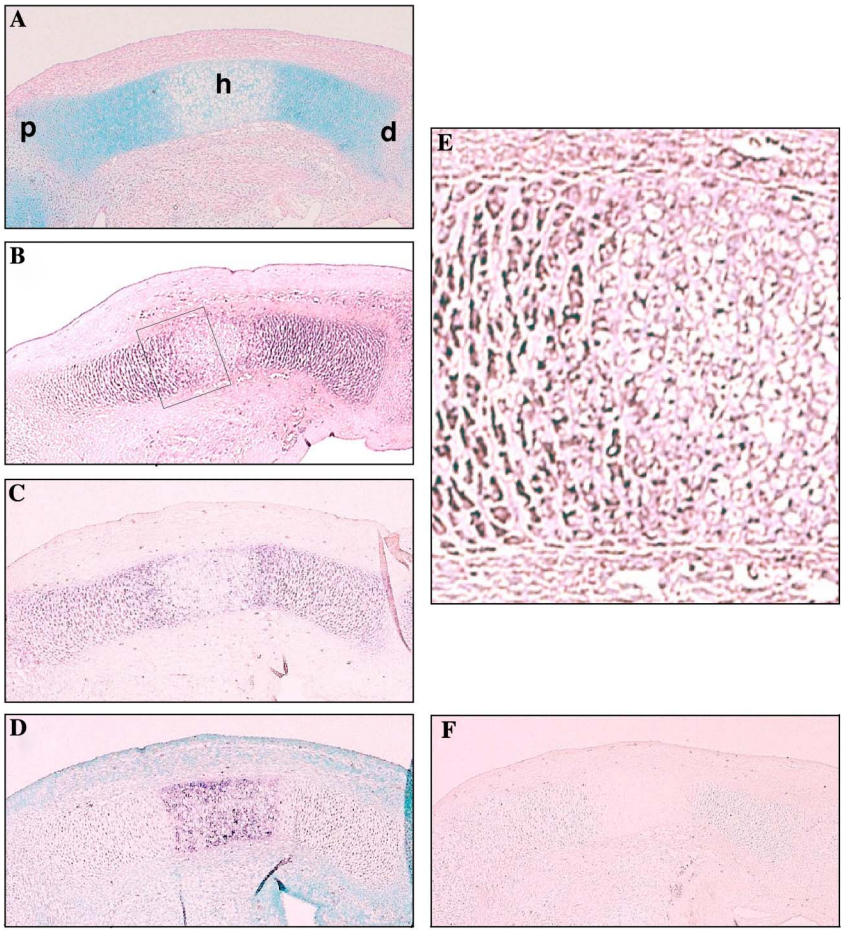

Fig. 6. Expression of ATF5 in mouse limb buds. In situ hybridization analyses were performed using longitudinal sections of the tibiotarsus at 14.5 days post-coitus mouse embryos. Cartilage primordium was clearly stained with Alcian blue (A). Proximal and distal regions are indicated by "p" and "d," respectively. Hypertrophic chondrocytes are labeled "h." The expression of ATF5 (B), type II collagen (C), and type X collagen (D) was detected with antisense RNA probes, and hybridization signals were observed in the cytoplasm of cartilage primodia (dark purple). A higher magnification of the boxed area in (B) is shown in (E). Staining with a sense probe of mouse ATF5 revealed no significant reaction (F). Methyl green was used as a counterstain (light green) (D).

indicating that further study will be necessary to clarify its significance in the tissue.

Recently, ATF family members have been found to be involved in the regulation of skeletal formation. ATF2 was shown to regulate proliferation of chodrocytes, and its deficiency causes chondrodysplasia [24]. Also, ATF4 regulates osteoblast differentiation, and its deficiency results in delayed bone formation [25,26]. Therefore, we analyzed the expression of other ATF family members in ATDC5 cells. Although ATF4 mRNA was transcribed in undifferentiated ATDC5 cells and its expression level was slightly increased after insulin induction (Fig. 3), ATF1, 2, and 3 were barely detectable on Northern blots (data not shown).

We showed here that regulation of chondrogenesis might be dependent on multiple transcription factors in addition to Sox genes. The system used here, ATDC5 cell differentiation under different amounts of culture media, has the advantage that it allows us to identify transcription factors involved in chondrogenic differentiation very efficiently leading us to feel certain that further characterization of the subtractive cDNA library will provide us more candidates for important genes involved in, if not regulating, chondrogenesis.

Acknowledgments

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