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Cell Density Dependent Regulation of AP-1 Activity is Important for Chondrogenic Differentiation of C3H10T1/2 Mesenchymal Cells

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Abstract The multipotential C3H10T1/2 mesenchymal cells undergo chondrogenic differentiation only when seeded as high-density micromass cultures, particularly upon treatment with bone morphogenetic protein-2 (BMP-2). The molecular mechanism(s) responsible for the cell density-dependent onset of cartilage-specific gene expression is presently unknown. Interestingly, a number of recent studies have indicated that activating protein-1 (AP-1), a well known downstream target of the mitogenic activated protein kinase (MAP kinase) signaling pathway, is a target of chondrogenic/osteogenic growth factors such as BMP-2, and plays a role in osteogenic gene regulation as well as in chondrogenic differentiation. The aim of this study is to examine the density-dependent alteration in the level and binding activity of AP-1 and its functional involvement in C3H10T1/2 mesenchymal chondrogenesis. To measure the activity of the AP-1 transcription factor, we generated a pool of stable C3H10T1/2 cell lines harboring a luciferase expression vector driven by a concatamer of an efficient AP-1 response element (AP1-10T1/2 cells). Luciferase activity of AP1-10T1/2 cultures was found to decrease sharply with increase in cell density, either as a function of culture time or initial cell seeding densities. In C3H10T1/2 micromass cultures undergoing chondrogenesis, AP-1 activity was further reduced and then maintained at a low, steady level for the entire 3–4 day culture period. AP-1 activity in micromass cultures was not significantly affected by BMP-2 treatment, but chondrogenesis was compromised upon competitive inhibition of AP-1 activity with a double-stranded AP-1 binding oligonucleotide. The level of AP-1 binding correlated with the activity of its response element but not with the levels of its leucine-zipper containing subunits, c-Jun and c-Fos. These findings suggest that a cell density-dependent, low but steady level of AP-1 binding and activity is required for promoting the chondrogenic potential of C3H10T1/2 cells. J. Cell. Biochem. 84: 237–248, 2002.

Key words: AP-1; chondrogenesis; C3H10T1/2 mesenchymal cells; micromass cultures; transcriptional regulation; MAP kinase; bone morphogenetic protein-2

Mesenchymal chondrogenesis, as observed in the developing embryonic limbs or in limb bud cells in vitro involves a requisite initial step of cellular condensation [Kosher, 1983; Solursh, 1983; DeLise et al., 2000]. This event appears to be essential for the initiation of chondrogenic cell signaling and the activation of cartilage-specific genes such as collagen type II and aggrecan [Oberlender and Tuan, 1994a,b; Seghatoleslami et al., 1995; Seghatoleslami and Kosher, 1996]. We have recently established a valid, convenient model for the study of chondrogenesis in vitro using the multipotential, murine embryonic mesenchymal cell line C3H10T1/2, which obviates the necessity of isolating primary cells and facilitates recombinant genetic manipulations. The pioneering work of Taylor and Jones [1979] showed that C3H10T1/2 cells, plated as monolayer cultures, underwent multiple differentiation events, under certain culture conditions, to give rise to cell types including myoblasts, adipocytes, and to a lesser extent, osteoblasts [Taylor and Jones, 1979]. Chondrocytes were also detected at a low frequency (0.5%). In our recent studies [Denker

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et al., 1995, 1999], we have shown that close juxtapositioning of the C3H10T1/2 cells, as in primary limb bud mesenchymal cells, is one of the key requirements for initiation of the process of chondrogenesis. Only when plated at high densities (e.g., 20×10^6 cells/ml), such as that commonly used in micromass cultures of limb mesenchymal cells [Ahrens et al., 1977; San Antonio and Tuan, 1986], were C3H10T1/2 cells, in the presence of growth factors (e.g., bone morphogenetic protein-2 (BMP-2) and transforming growth factor-β1 (TGFβ1)), able to initiate and maintain high expression of chondrogenesis-related genes, such as cartilage proteoglycan link protein and collagen type II [Denker et al., 1995, 1999]. The cell-density and cellular condensation dependence of embryonic, as well as C3H10T1/2 mesenchymal cells, therefore strongly implicates the critical involvement of cell–cell interaction and signaling in the initial stages of chondrogenic differentiation.

The signaling pathways that are activated or regulated at the cellular condensation phase of the mesenchymal chondrogenic differentiation are not well understood. Given the important and ubiquitous involvement of mitogen-activated protein kinases (MAP kinases) in the regulation of gene expression and cellular differentiation, we hypothesize that MAP kinase activity may play a key role in mediating and specifying the cell density dependent activation of mesenchymal commitment of differentiation along the chondrogenic lineage.

The nuclear effect of MAP kinases includes the alteration in levels, as well as activity, of different transcription factors [for review, see Hill and Treisman, 1995; Karin and Delhase, 1998]. Activating protein 1 (AP-1) is a transcription factor complex which has been characterized as one of the downstream targets for the MAP kinase signaling pathway. This complex DNA binding factor is composed of heterodimers of the Jun and Fos family of leucine-zipper proteins or homodimers of Jun proteins alone, with a preferential affinity to the phorbol 12-*O*-tetradecanoate-13-acetate (TPA)-responsive element. The components of AP-1 are affected by different MAP kinases. As a fast response to extracellular signals, the transcription of c-Fos is regulated by Elk1, a transcription factor from the family of ternary complex factors (TCF) whose activity is affected by the activated extracellular signal-regulated kinase (ERK). The transcription of c-Fos is also mediated by serum response factor (SRF) that is activated by exposure of cells to ultraviolet irradiation or to IL-1. These types of stimuli activate separate MAP kinases, JNK (Jun amino-terminal kinase) and p38, but not ERK. These MAP kinases, p38 and JNK, are also capable of stimulating Elk1, leading to c-Fos induction. As an immediate early response, in the absence of protein synthesis, the important component of the AP-1 complex, c-Jun, is activated by direct JNK (stress activated MAP kinase) phosphorylation of the serine 63 and 73 residues located at its N-terminal domain [for review, see Karin et al., 1997].

The components of AP-1 are shown to be expressed in most cells and to be essential for cellular proliferation, as well as playing a role in cellular differentiation. Transgenic mice with homozygous c-Fos null mutations are viable, but develop osteopetrosis and defects in the central nervous system [Johnson et al., 1992], whereas mice harboring c-Jun null mutations develop altered erythropoiesis, impaired hepatogenesis, and generalized edema [Hilberg et al., 1993]. Recent studies also show that overexpression of c-Fos results in inhibition of chondrogenesis in ATCD5 cells, a mouse carcinoma derived chondrogenic cell line [Thomas et al., 2000]. Moreover, regulation of the expression of collagen type I gene, an active gene in osteogenic as well as prechondrogenic cells, is also shown to be dependent on AP-1 enhancer binding and activity [Katai et al., 1992]. Interestingly, it has also been shown that cellular condensation in the pre-cartilage limb is characterized by an upregulation of expression as well as alternative splicing of fibronectin gene [Kulyk et al., 1989; Gehris et al., 1996, 1997]. Given that fibronectin–integrin interaction activates focal adhesion kinase (FAK), which in turn regulates MAP kinase signaling activity [for review, see Miyamoto et al., 1998; Giancotti and Ruoslahti, 1999; Schlaepfer et al., 1999], an important role for MAP kinase in pre-cartilage condensation perhaps in a tissue-specific manner, is further suggested.

In this investigation, our aim is to examine the binding and activity of the AP-1 transcription factor, components of which constitute the immediate early proto-oncogenes such as c-Jun and c-Fos, to test the hypothesis that initiation of cartilage specific AP-1 binding and activity requires a cell density-dependent alteration in the activity of the ubiquitous signaling pathway involving MAP kinase activities.

To analyze the functional role of AP-1, we have generated stable lines of C3H10T1/2 cells harboring a construct containing a concatamer of seven AP-1 consensus sequences followed by a TATA box fused to a luciferase marker gene (AP1-luc construct), as a reporter system for AP-1 activity. We report here that the level of AP-1 activity in C3H10T1/2 cells is dependent on cell density, and that this cell density-dependent change in AP-1 activity correlates with its level of DNA binding but not the levels of c-Jun and c-Fos, the major components of AP-1.

MATERIALS AND METHODS

Establishment of Stable AP-1 Cell Lines

C3H10T1/2 cells were transfected using FuGENE 6 transfection lipid (Boehringer Mannheim, Indianapolis, IN) as follows. Two 100 mm culture dishes plated with C3H10T1/2 cells, approximately 40% confluent, were transfected with 4 μg of plasmid DNA according to the manufacturer's protocol. The AP-1 plasmid vector (AP1-luc) contains a concatamer of seven AP-1 consensus binding sites followed by basic TATA transcriptional unit fused to luciferase marker gene (Fig. 1). The control plasmid vector (CT-luc) contains only the TATA box fused to luciferase marker gene (Fig. 1). Both AP1-luc and CT-luc constructs were obtained from Stratagene (LaJolla, CA). At a ratio of 10:1 (4 μg of reporter construct to 400 ng of the drug resistant selection construct), C3H10T1/2 cells were co-transfected as follows: 1) AP1-luc construct and a plasmid construct containing neomycin drug resistance gene, or 2) CT-luc and a plasmid construct containing puromycin resistance gene. Three days later, transfected cultures were split 1:10 and treated with G418 (500 μg/ml) or puromycin (2 μg/ml), respectively. For analysis of the incorporated promoter constructs, more than 20 positive clones of each cell type were pooled. Stable C3H10T1/2 cells harboring AP-1 construct were renamed as AP1-10T1/2 and the cells harboring the control construct as CT-10T1/2.

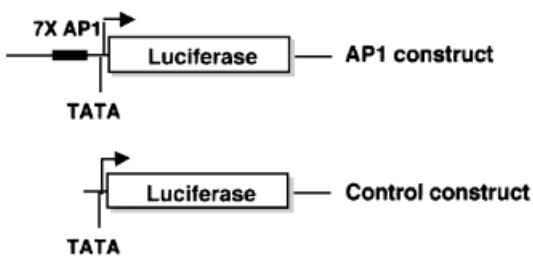

Fig. 1. Schematic of the AP1 and control reporter constructs. **A**: The AP-1 reporter construct (AP1-luc) contains seven tandem repeats of AP-1 consensus sequences upstream of the basal TATA promoter fused to a luciferase marker gene (obtained from Stratagene). **B**: The control vector is the same construct without the AP-1 binding sequences (Stratagene). Cells stably transfected with these constructs are denoted as AP1-10T1/2 and CT-10T1/2, respectively.

Analysis of AP-1 Activity

All cultures were maintained in Ham's F12 medium (Bio Whittaker, Walkersville, MD) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 50 U/ml penicillin G (Sigma, St. Louis, MO), and 50 μg/ml streptomycin sulfate (Sigma).

High density micromass cultures. Cells were spot-seeded as 10 or 40 μl drops containing 1×10^5 or 4×10^5 cells in 30 or 60 mm culture dishes, respectively. After cells had attached (1–2 h), cultures were fed with 2 ml (for 30 mm dish) or 5 ml (for 60 mm dish) of medium. The cultures were harvested at 4, 24, 48, and 72 h time points for luciferase assay.

Low density cultures. 1×10^5 or 4×10^5 cells were plated as monolayer cultures onto 30 or 60 mm dishes, respectively, and assayed for luciferase activity at 24 h time point.

Effect of plating density and culture time on AP-1 activity. To vary plating density, aliquots of 1×10^5, 5×10^5, 10×10^5, 20×10^5 cells, were seeded as monolayer culture into 24 well plates and harvested for luciferase activity 24 h later. To analyze the effect of time of culture, 1×10^5 cells were seeded as monolayer culture in 24 well dishes and assayed for AP-1 activity at 4, 24, 48, 72 h time points.

MEKK-1 activation of MAP kinases. In the following transient transfection experiments, to ensure equal transfection efficiencies in both the micromass and monolayer cultures, C3H10T1/2 cells were transfected in monolayer first and then pooled to establish the final monolayer or micromass cultures. Briefly, cells were plated on 100 mm dishes as 70% confluent cultures and transfected using FuGENE 6 with 50 ng of an expression plasmid vector containing a cDNA coding for the constitutively active form of MEKK1 (MEKK1) fused to CMV promoter (pFC-MEKK-1 encoding amino acids 360–672 of MEKK1; Stratagene). Transfection efficiency was optimized by adding pBluscript plasmid DNA to bring the total amount of DNA

to 4 μg. Controls consisted of transfection with 4 μg of Bluscript plasmid (pBS) alone. The next day, the transfected cells were trypsinized, replated as monolayer or micromass cultures (see above), and assayed at 24 h.

Luciferase assays. Cultures were scraped in 1×reporter lysis buffer (Promega), subjected to two freeze-thaw cycles, and the enzyme activity was assayed using the Luciferase Assay System kit from Promega. The light intensity generated was measured in 10 s intervals using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Protein content of each sample was measured using the Bradford protein assay system (BioRad, Hercules, CA).

Preparation of Nuclear Extracts

Five to ten micromass cultures of C3H10T1/2 cells were used, each initially plated at 10×10^5 cells/100 μl medium. A modified protocol of Andrews and Faller [1991] was used to prepare nuclear extracts. Briefly, cultures at different time points (4, 24, 48, 72 h) were washed twice in cold phosphate buffered saline (PBS), scraped into 1 ml of PBS, pelleted in microfuge tubes at low speed (3,000*g* for 15 s), and resuspended in 400 μl of Buffer A (10 mM HEPES-KOH, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5 mM DTT, 10 mM β-glycerophosphate), containing 50 μl/ml protease inhibitor cocktail for use with mammalian cell and tissue extracts (Sigma), 10 μl/ml phosphatase inhibitor Cocktail II (Sigma) and incubated on ice for 30 min. The cell suspensions were gently homogenized 30 times in a type B Dounce homogenizer. The nuclear fraction was collected by centrifugation at 600*g* for 10 min at 4°C, resuspended in 50 μl of Buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 10 mM β-glycerophosphate) containing the same protease and phosphatase inhibitors as in Solution A, and incubated on ice for 90 min, followed by centrifugation at high speed (12,000*g*) for 15 min. Aliquots of the supernatants containing the nuclear extract were frozen at −70°C until use. The BCA micro assay system (Pierce, Rockford, IL) was used to determine the protein content of the nuclear extracts.

Gel Shift Analysis

A double-stranded oligodeoxynucleotide (ODN) containing AP-1 consensus binding sequence (5′-CGCTTGATG**AGTCAG**CCGGAA-3′; Promega, Madison, WI) was used in binding reactions. End labeling of the ODN, binding, and the gel shift assays were done according to the protocol provided by the Gel Shift Assay System kit (Promega), using 2–4 μg protein aliquots of nuclear extracts.

Western Blot Analysis

A 4 μg aliquot of each nuclear extract was fractionated on a 10% SDS–polyacrylamide gel and electrophoretically transferred to nitrocellulose. Blots were blocked by incubation in 3% bovine serum albumin in Tris-buffered saline, pH 7.4, containing 0.05% Tween-20 and incubated with rabbit-derived polyclonal antibodies directed against the amino terminus of c-Fos (*SC-52*) and c-Jun (*SC-45*) factors (Santa Cruz; Santa Cruz, CA). Detection was done using species-specific horseradish peroxidase conjugated antibodies, followed by chemiluminescent detection using the Amersham ECL detection system (Piscataway, NJ). For some experiments, after immunodetection of c-Fos, the blot was stripped according to the protocol provided by the manufacturer and re-probed with c-Jun antibody as described above. Equal protein loading on the blots was verified with Colloidal Gold total protein stain (BioRad Laboratories, Hercules, CA).

Intracellular AP-1 Binding Competition

Double stranded ODN containing sequences responsive to AP-1 nuclear complex (AP-1 ODN), CTCTT**AGTCAG**GC, or AP-1 non-binding null sequences (null AP-1 ODN), CTCT-**GTACAA**GCG, were designed to correspond to the same sequences as the AP-1 binding site used in the AP1-luciferase construct purchased from Stratagene and were custom-synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). These double stranded sequences were used to compete for the endogenous AP-1 nuclear factor in BMP-2 treated micromass cultures of C3H10T1/2 cells. Briefly, in 100-mm dishes, separate monolayer cultures of C3H10T1/2 cells were transfected with 4 μg of the respective oligonucleotides, using FuGENE 6 transfection medium. A day later, cultures were trypsinized and micromass cultures established in medium supplemented with recombinant human BMP-2 (100 ng/ml kindly provided by Genetics Institute, Cambridge, MA). Micromass cultures were transfected again on the second day with an additional 4 μg of the ODN

as described above, and the effects on chondrogenic activity of the cultures were assayed as described below.

Metabolic Sulfate Labeling and Alcian Blue Staining

To assess the extent of chondrogenesis, cultures were metabolically labeled on day 4 for 24 h with [^{35}S]sulfate (1 μCi/ml; NEN, Boston, MA) to monitor sulfated proteoglycan synthesis. General protein synthesis was monitored by co-labeling with [^{3}H]leucine (5 μCi/ml; NEN). Incorporation of radioactivity was determined by dual isotope liquid scintillation counting and results are expressed as DPM ratio of ^{35}S:^{3}H. For Alcian blue staining, cultures were fixed in Kahle's fixative, washed in water and stained overnight with 1% Alcian Blue (Sigma) at pH 1.0 [Lev and Spicer, 1964].

Statistical Analysis

All data were analyzed for statistical significance using Student's *t*-test. Values of $P < 0.05$ are considered significantly different.

RESULTS

Chondrogenic Activity of C3H10T1/2 Cells Plated as High Density Micromass Cultures

When plated at high density as micromass cultures (1×10^5 cells in 10 μl), C3H10T1/2 cells spontaneously exhibited chondrogenic activity. For example, matrix staining with Alcian blue was seen (Fig. 2A), albeit at lower intensity than that seen upon treatment with BMP-2 [Denker et al., 1999]. Examination of mRNA expression by RT-PCR showed a gradual time-dependent increase in the expression of aggrecan mRNA in micromass cultures of C3H10T1/2 cells (Fig. 2B). The phenotype and specific gene expression profile in these cells are thus consistent with chondrogenesis in vitro.

Establishment of C3H10T1/2 Cell Lines Harboring an AP1-Luciferase Construct Responsive to the MAP Kinase Signaling Pathway

To analyze the molecular regulation of chondrogenic differentiation, experiments were designed to examine the involvement and the activity of the ubiquitous MAP kinase signaling pathway. The three well characterized MAP kinases target a number of cytoplasmic as well as nuclear factors [Treisman, 1996; Schaeffer and Weber, 1999]. In this study we have focused

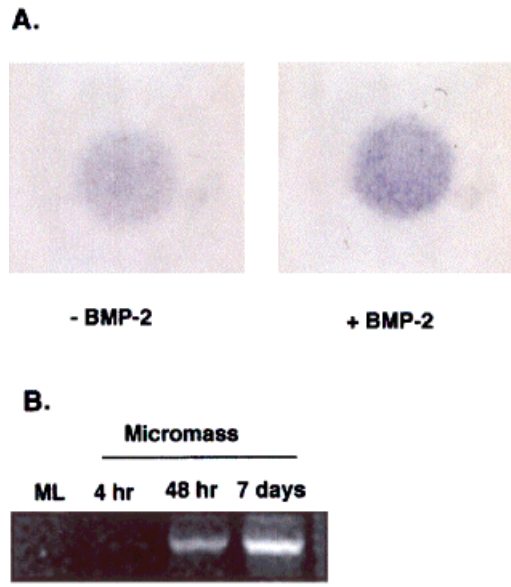

Fig. 2. Chondrogenic activity of micromass cultures of C3H10T1/2 cells. **A**: Alcian blue staining showed that high density micromass cultures (1×10^5 cells/10 μl) displayed chondrocyte phenotype after 4 days, particularly after BMP-2 treatment (100 ng/ml). **B**: Time-dependent chondrogenic differentiation of the untreated micromass cultures is further demonstrated by the expression of the cartilage matrix gene, aggrecan, assayed by RT-PCR. ML, monolayer. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.].

our analysis on the activity of AP-1 transcription factor, a well characterized downstream nuclear target of the MAP kinase pathway. For this purpose, a pool of C3H10T1/2 stable cell lines, AP1-10T1/2 cells, was created (see Materials and Methods), harboring a construct consisting of a simple promoter element containing a concatamer of seven AP-1 DNA binding sequences followed by a TATA box and fused to a luciferase marker gene (see Fig. 1). To test whether the incorporated construct is responsive to the MAPK signaling pathway, the AP1-10T1/2 cells were transiently transfected with a construct containing a constitutively active MEKK1 cDNA fused to CMV promoter. Exogenous expression of MEKK1 in AP1-10T1/2 cells resulted in high luciferase reporter activity, indicative of increased activity of the incorporated AP-1 promoter construct (Fig. 3). On the other hand CT-10T1/2 cells did not respond to the transient MEKK1 expression. Interestingly, this effect of MAP kinase signaling on the AP-1 activity appears to be dependent on the cell density of the cultures. Thus, when activated by MEKK1, monolayer

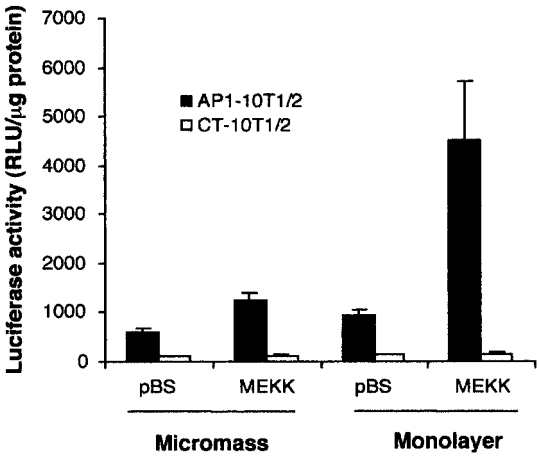

Fig. 3. AP-1 activity in AP1-10T1/2 cells is responsive to overexpression of MEKK1. AP1-10T1/2 or CT-10T1/2 cells were transiently transfected with a dominantly active form of MEKK1 kinase (pFC-MEKK1), an activator of multiple MAP kinases (see Materials and Methods), and plated in monolayer and micromass cultures. In control transfections Bluscript vector (pBS) instead of pFC-MEKK1 was used. Luciferase activities measured at 24 h and normalized to the protein content of each sample indicate that the AP1-luc construct in AP1-10T1/2 cells is responsive to the MAPK signaling pathway in both monolayer and micromass cultures. The results also indicate that the MEKK1 induction of AP-1 is sensitive to cell density such that higher activity of AP-1 is induced in low density monolayer cultures than in the high density micromass cultures. Background luciferase activity in CT-10T1/2 cells was not affected by the presence of exogenous MEKK1. Values are mean ± SD from triplicate experiments. $^*P < 0.05$, compared to pBS-transfected control.

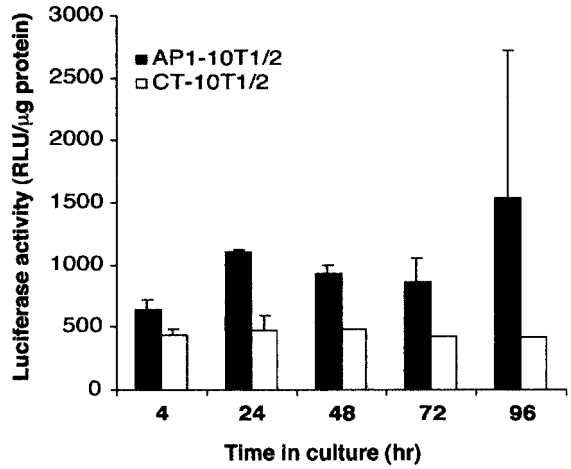

Fig. 4. AP-1 activity is maintained at a low steady level in chondrogenic micromass cultures of AP1-10T1/2 cells. AP1-10T1/2 cells were cultured at micromass densities and luciferase activities were measured at various times during the period of 3–4 days. AP-1 activity is maintained at a low (i.e., above background compared to CT-10T1/2 cells) but relatively steady level for the period of time tested. Note that this activity is substantially lower than the AP-1 activity in the densest cultures derived from monolayer plating (see in Figures 4A,B). Values are mean ± SD from duplicate experiments.

cultures of AP1-10T1/2 cells showed significantly higher luciferase activity than micromass cultures, indicating that cells cultured at lower densities were more responsive to MAP kinase signaling.

Decrease in AP-1 Activity in Chondrogenic Cultures of C3H10T1/2 Cells

To gain insight into the molecular pathways involved in the cell density-dependent chondrogenic differentiation of C3H10T1/2 cells, specifically with respect to the AP-1 activity, we examined the reporter activity in AP1-10T1/2 cells plated for various times in micromass cultures. AP-1 activity was found to be uniformly low throughout the entire culture period (up to day 4) (Fig. 4), in contrast to the high level seen in monolayer cultures (see Fig. 5). Given that the high cellular density is the key requirement for chondrogenesis, the steady, low AP-1 activity in these cultures could be important for the initiation and maintenance of the chondrogenic phenotype.

Low AP-1 Activity in C3H10T1/2 Cells is Dependent on Cell Density

To further investigate the cell density dependence of AP-1 activity in C3H10T1/2 cells, AP1-10T1/2 cells were seeded at lower density as monolayer cultures (1×10^5 cells/15 mm dish), allowed to proliferate to high density, and assayed for AP-1 reporter activities at various times. As shown in Figure 5A, the level of AP-1 activity was considerably higher in monolayer cultures than in micromass cultures (see Fig. 4), and dropped sharply as the proliferating cells increased in density and presumably established more contact (between days 1 and 2). At later time points, when the cultures reached higher densities, AP-1 activity continued decreasing, although at a considerably lower rate compared to the initial period of cell proliferation. To distinguish between high cell density and/or length of culture as the key contributing factor to the lowering of AP-1 activity, AP1-10T1/2 monolayer cultures were established at different densities and harvested for AP-1 reporter assay at the same time (24 h). As shown in Figure 5B, AP-1 activity was high only at low plating density (1×10^5 cells/ 30 mm dish), i.e., minimal cell contact, but was maintained at a low level at all higher cell plating

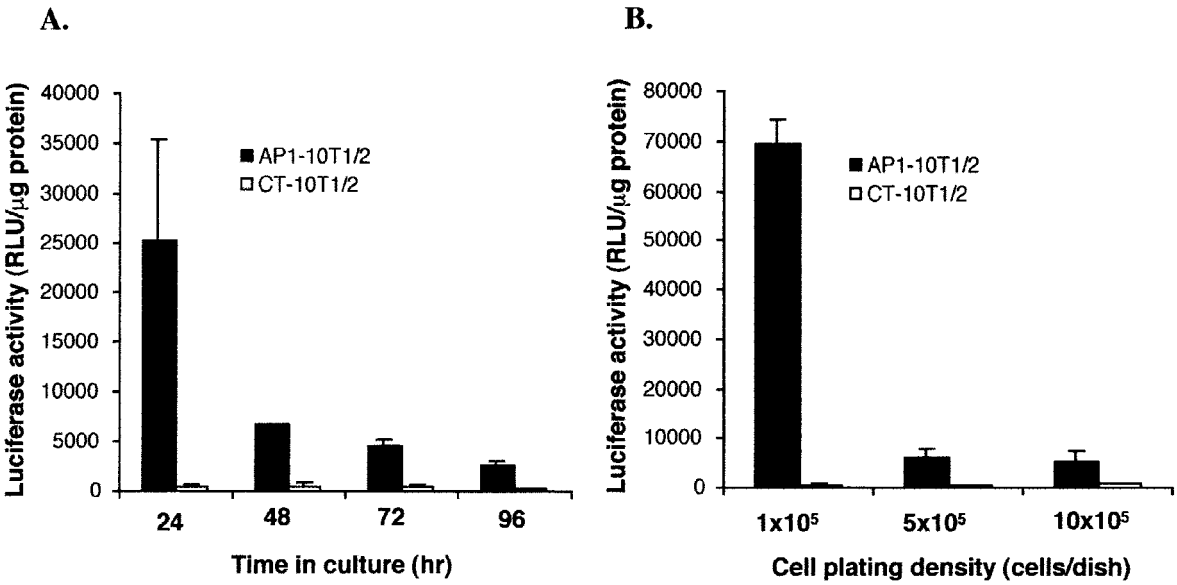

Fig. 5. AP-1 activity in AP1-10T1/2 cells is cell density but not time dependent. **A**: Low density cultures assayed as a function of culture time. Low density monolayer cultures were established at 1×10^5 cells/30 mm dish, and luciferase activities were measured at 4, 24, 48, 72 h time points. Luciferase activity dropped greatly within the first 24 h period during early proliferation of AP1-10T1/2 cells, and reached a low steady level at later time points during confluency. **B**: Activity of AP-1 in monolayer cultures of AP1-10T1/2 cells plated at different densities. Monolayer cultures of AP1-10T1/2 cells were plated at 1, 5, 10, and 20×10^5 cells/30 mm culture dish and assayed at 24 h. AP1-luc activity dropped as a function of increasing cell density, with the greatest decrease observed at 5×10^5 cell plating density. Luciferase activity in the control CT-10T1/2 cells was at background level and did not follow the same time and cell density profiles. Values are mean ± SD from duplicate experiments.

densities. Taken together, these data strongly suggest that high cellular density was the primary cause of diminished AP-1 activity in C3H10T1/2 cells.

DNA Binding Activity of AP-1 Correlates With its Transcriptional Activity in Chondrogenic Cultures of C3H10T1/2 Cells

To examine the molecular basis of the change in AP-1 reporter activity in C3H10T1/2 micromass cultures, the binding of AP-1 complex to its consensus binding sequence was assayed. Nuclear extracts isolated from cultures of C3H10T1/2 cells, corresponding to those analyzed for AP-1 reporter activity, were used in a DNA electrophoretic mobility shift assay using a double stranded oligonucleotide containing the consensus sequence for AP-1 binding (Fig. 6). The results showed a large decrease in AP-1 binding activity in the nuclear extracts of C3H10T1/2 cells cultured at micromass densities compared to those plated as low density monolayer cultures. This reduction in DNA binding thus correlated with the large drop in the activity of the AP-1 promoter in AP1-10T1/2 cells shown in Figures 4 and 5. In addition, the temporal profile of nuclear AP-1 DNA binding activity in C3H10T1/2 micromass cultures also correlated with that of the AP-1 promoter activity in AP1-10T1/2 cells (compare Figs. 4 and 6). These data therefore strongly suggest that the drop in the AP-1 promoter activity in high density cultures of C3H10T1/2 cells resulted in large part from the lack of AP-1 binding to its response element.

Drop in AP-1 Binding and Activity in Chondrogenic C3H10T1/2 Cultures is not Associated With Reduced Levels of c-Jun and c-Fos

Since the transcriptional and DNA binding activities of AP-1 are both dependent on its dimeric composition of c-Jun/c-Fos, i.e., heterodimeric AP-1 binds to DNA at a higher affinity than the homodimer [Karin et al., 1997], we examined whether the reduced binding of AP-1 in high density cultures of C3H10T1/2 cells observed here was due to a change in the levels of c-Fos or c-Jun. Western blot analysis of nuclear extracts used in the gel shift analysis described above revealed no significant time dependent difference in the levels of c-Jun that

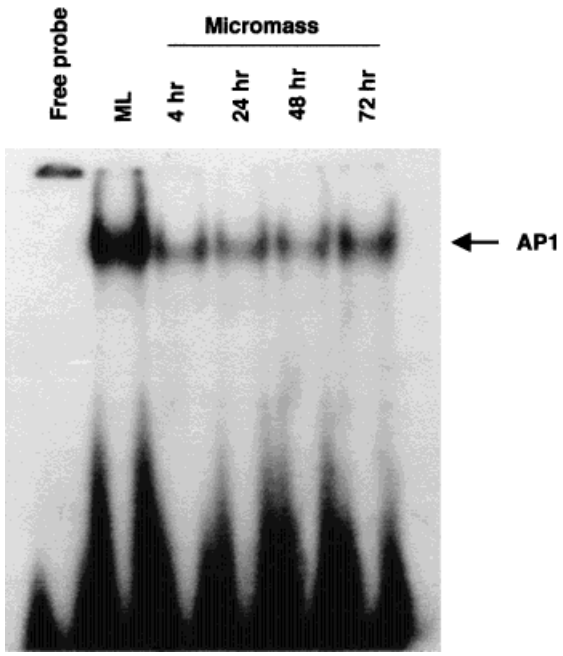

Fig. 6. Gel shift analysis of the effect of cell density and time of culture on nuclear AP-1 binding activity in C3H10T1/2 cells. Nuclear extracts were isolated from C3H10T1/2 cells cultured in monolayer for 24 h or as micromass for 4, 24, 48, and 72 h. Nuclear extracts isolated from monolayer cultures showed greater AP-1 binding capacity than those from micromass cultures at all time points. The low level of AP-1 binding activity persisted in the micromass cultures as a function of culture time, and paralleled the temporal profile of the activity of AP-1 promoter constructs shown in Figure 5. The pattern of binding shown in this figure was consistently reproduced when the same or different set of nuclear extracts was used. ML, monolayer.

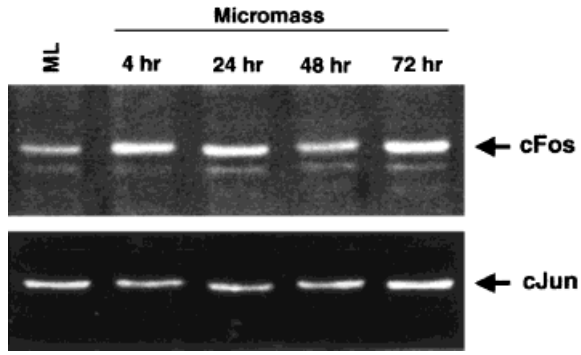

Fig. 7. Western analysis of c-Fos and c-Jun components of AP-1 in nuclear extracts of C3H10T1/2 cells. Equal amounts (4 μg) of the same nuclear extracts used in the gel shift analysis (Fig. 6) were analyzed for c-Fos and c-Jun by Western blotting. Detection of c-Fos was carried out first, after which the membrane was stripped and probed for c-Jun. **Upper panel**: c-Fos, 46 kDa; **lower panel**: c-Jun, 34 kDa. No detectable differences were seen in the amount or the electrophoretic mobility of the c-Jun band in the nuclear extracts isolated from monolayer (24 h culture; 80–90% confluent) or micromass cultures (4, 24, 48, and 72 h cultures). The levels of c-Fos, on the other hand, were upregulated in denser micromass cultures. In experiments in which monolayer cultures were seeded at lower densities (cellular contact minimized; 30–50% confluent) than shown in this figure, c-Fos levels were undetectable (data not shown). ML, monolayer.

could account for the large drop in AP-1 binding to DNA or its transcriptional activity observed in the high density micromass cultures (Fig. 7). Also there was no difference in c-Jun levels between low density monolayer and high density micromass cultures. Since the c-Jun/c-Fos heterodimer binds to DNA at a higher affinity, the pattern of expression of c-Fos in these cultures was also examined. The results show that the level of c-Fos protein was dependent on the cell plating density of the C3H10T1/2 cultures in that the denser cultures synthesize higher levels of this nuclear protein (Fig. 7; compare monolayer to micromass). Since a high level of c-Fos is in fact contrary to the reduction in AP-1 DNA binding or lower AP-1 transcriptional activity seen in high density C3H10T1/2 cultures (Figs. 4, 5, and 6), the regulation of AP-1 in these cultures is likely to be more complex than the consequence of a direct modulation of c-Fos or c-Jun expression.

Effect of BMP-2 on AP-1 Regulation and Stimulation of Chondrogenesis

The chondrogenic potential of C3H10T1/2 cells is greatly stimulated when micromass cultures of these cells are treated with BMP-2, as described previously [Denker et al., 1999] (Fig. 1). To examine whether the effect of BMP-2 is mediated via regulation of AP-1 activity, the AP1-10T1/2 cells were cultured at high density in the presence of BMP-2. We did not observe any significant change in the luciferase activity between the treated or untreated cells, with all cultures exhibiting similar, low, and steady levels of AP-1 activity (Fig. 8), suggesting that BMP-2 signaling was unlikely to influence directly the MAP kinases involved in AP-1 activity. On the other hand, this result suggests that chondrogenic differentiation requires the maintenance of AP-1 activity at low levels, even with BMP-2 stimulation.

Requirement of AP-1 Activity for Chondrogenesis

This requirement was tested by the introduction of double stranded oligonucleotides containing the AP-1 binding consensus sequence to BMP-2 treated micromass cultures in order to

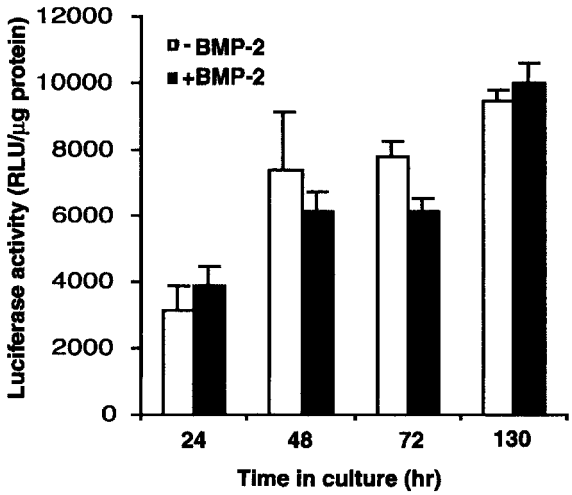

Fig. 8. Effect of BMP-2 on AP-1 activity in AP1-10T1/2 cells. Micromass cultures of AP1-10T1/2 cells treated with or without BMP-2 were harvested and their AP-1 activities assayed and compared at 4, 24, 48, 96 h time points. BMP-2 treatment did not significantly affect AP-1 activity. Values are mean ± SD for duplicate experiments.

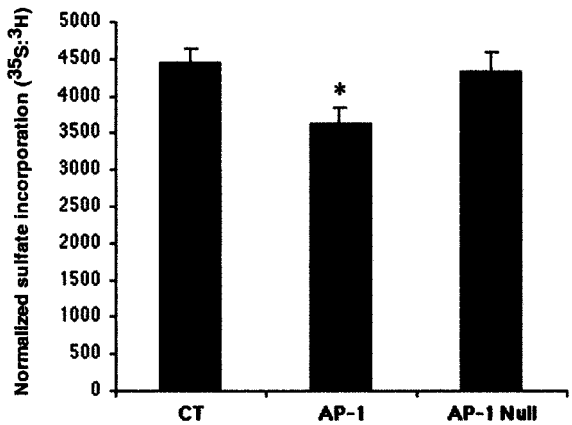

Fig. 9. Competition of AP-1 activity using a double stranded AP-1 binding oligonucleotide inhibited BMP-2 stimulation of chondrogenesis in micromass cultures. Micromass cultures of C3H10T1/2 cells were treated with double stranded AP-1 oligonucleotide as described in Materials and Methods. Chondrogenic activities of the controls (CT: no DNA; AP-1 Null: AP-1 non-binding oligodeoxynucleotide) and AP-1 competed cultures were measured on the basis of metabolic sulfate incorporation on culture day 4. A reduction of sulfate incorporation was seen upon AP-1 oligonucleotide treatment (18–20%). Values are mean ± SD for triplicate experiments; $*P < 0.01$.

compete for the activity of the AP-1 complexes. This competition resulted in the partial reduction of sulfate incorporation in C3H10T1/2 cells cultured for 4 days (Fig. 9). The intensity of Alcian blue staining in the AP-1 ODN treated cultures was also reduced (data not shown). Similarly transfected monolayer cultures of AP1-10T1/2 cells were also tested for specific AP-1 inhibitory effect of the AP-1 ODN. The effectiveness of the AP-1 ODN was indicated by the 10–20% reduction in AP-1 driven luciferase activity (data not shown), depending on the time of harvest, when compared to the CT (no DNA) or null AP-1 ODN transfected cultures. These results, therefore, suggest that low levels of AP-1 activity are required for optimal expression of the chondrogenic activity of C3H10T1/2 cells.

DISCUSSION

In this study we have set up a rapid and convenient system, using the mesenchymal C3H10T1/2 cell line, to analyze the effect of density-dependent cell–cell interaction, i.e., cellular condensation, during the early stage of chondrogenic differentiation, on the promoter regulation and DNA binding activities of the transcription factor, AP-1. The components of the AP-1, c-Jun and c-Fos, are well studied targets of the ubiquitous MAP kinase signaling pathway. A pool of stably transfected C3H10T1/2 cell line harboring an AP-1 response element-luciferase reporter construct was generated to measure the activity of AP-1 transcription factor. Initial analysis of this cell line confirmed the appropriate responsiveness of the AP1-luc construct to the MAP kinase signaling on the basis of the stimulatory effect of the expression of the constitutively active form of MEKK1, a nondiscriminatory MAP kinase activator [Lange-Carter et al., 1993; Yan et al., 1994; Guan et al., 1998; Yujiri et al., 1998]. Interestingly, this response was different depending on the initial plating density of the cells, i.e., the ratio of luciferase activity in MEKK1-activated versus non-activated AP1-10T1/2 cells was higher in monolayer compared to micromass cultures, suggesting different regulation of MAP kinase activity in the two types of culture. This difference may be the consequence of different components of the AP-1 dimer interactions with the AP1-luc construct. The lower level induction of the AP-1 activity by MEKK1 in micromass cultures also suggests a more stringent control of the MAP kinase signaling, perhaps necessary for promotion of chondrogenic activity in C3H10T1/2 cells.

To further analyze the relationship between cell density and MAP kinase regulation, we

have examined the effects of varying cell density, both as a function of initial plating density, as well as, time in culture. Compared to cultures plated at low density (1×10^5 cells/30 mm dish in Fig. 5B) and at early time in culture (24 h cultures in Fig. 5A), dense cultures (10×10^5 cells/30 mm dishes, Fig. 5B) and cultures that have reached higher density at later time in culture (96 h cultures in Fig. 5A) showed a 10- to 23-fold reduction in luciferase activity. Similarly, high density micromass cultures exhibited equally low AP-1 activity throughout the 4 day culture period (Fig. 4). Thus, regardless of whether AP1-10T1/2 cells reached high densities through increased time in culture or initial high density seeding, there is a similar sharp drop in AP-1 activity. This finding suggests that the drop in AP-1 activity is largely dependent on cell density and not on the time the cells spend in culture. Compared to the monolayer cultures that reached high cell density as a function of time, micromass cultures of AP1-10T1/2 cells showed an additional 2- to 4-fold reduction in the transcriptional activity of AP-1 (compare Fig. 5A,B with Fig. 4). This low AP-1 activity in micromass cultures is maintained at a steady level for the 72 h time course during which C3H10T1/2 cells begin to differentiate and progressively display chondrocyte-like phenotype. This time course is generally similar to that reported for growth factor-induced neuronal differentiation of PC12 cells [Marshall, 1995], and points to the importance of prolonged rather than short term effect of MAP kinase signaling in cellular differentiation, such as chondrogenic differentiation of C3H10T1/2 cells.

Consistent with the observed change in AP-1 transcriptional activity, the binding of the AP-1 complex to its response element is greatly reduced once the cells are plated as micromass cultures; during the 72 h course of micromass culture, the reduced binding of AP-1 to its consensus sequence remains relatively constant. It is noteworthy that reduction of AP-1 binding activity is a rapid response, observable by 4 h post-plating as micromass. Interestingly, this drop in AP-1 binding does not appear to be the result of reduced expression of c-Fos or c-Jun, components of the AP-1 complex. On the contrary, the expression of c-Fos in monolayer cultures, in which the binding and activity of AP-1 are the highest, is lower than in the micromass cultures; the expression of c-Jun remained similar in both monolayer and micromass cultures. This finding suggests that despite the availability of c-Jun and c-Fos in the micromass nuclear fractions, the formation of the stable form of AP-1 complex (c-Fos–c-Jun) and binding to its response element is not favored.

The fact that binding and activity of AP-1 is the highest in the monolayer cultures, which in fact have lower levels of c-Fos expression, suggests that members of the Fos/Jun family, other than c-Fos, might be involved in the binding and activity of this nuclear complex. The transcription of c-Fos gene is likely the target of regulation by the ERK group of MAP kinases [Hill and Treisman, 1995; Karin et al., 1997]. Interestingly, treatment of high density cultures of primary limb mesenchymal cells with EGF leads to the upregulation of ERK-1 and inhibition of chondrogenesis [Yoon et al., 2000]; whether EGF treatment in these cells leads to the upregulation of c-Fos is not known. In a recent study, direct overexpression of the c-Fos oncogene beyond its physiological levels is shown to inhibit the chondrogenic activity of ATCD5 chondroprogenitor cells [Thomas et al., 2000]. We observe here an increase in the endogenous level of c-Fos in C3H10T1/2 cells upon plating at micromass densities. This higher levels of c-Fos are maintained relatively constant over the 72 h period during which the cells undergo chondrogenic differentiation to form an Alcian blue stained matrix. However, despite the rise in c-Fos protein in the micromass cultures, the transcriptional and DNA binding activities of AP-1, compared to the monolayer cultures, are drastically reduced and then maintained at a steady low level for the 72 h period. Whether the excess unbound c-Jun and c-Fos participate in other nuclear regulatory functions, independent of DNA binding and formation of the AP-1 complex, is not known. Such an assumption is a formal possibility since, for example, the members of a superfamily of steroid receptors and c-Jun–c-Fos are shown to inhibit each other's binding and activity by direct protein–protein interaction [Schule et al., 1990; van der Burg et al., 1995; Karin, 1998; Zhou et al., 1999].

Results from this study also demonstrate that BMP-2 treatment, which stimulates chondrogenesis in C3H10T1/2 micromass cultures, does not significantly alter the AP-1 activity profile, i.e., maintenance of AP-1 activity at a low and steady level remains unchanged. However,

competing away and removing active endogenous AP-1 with the introduction of a double stranded oligonucleotide containing the AP-1 consensus sequence inhibits BMP-2 stimulated chondrogenesis (Note: The partial inhibition is likely due to the partial nature of the competition). These results suggest that while BMP-2 does not directly act on the MAP kinase involved in AP-1 activity, the final chondrogenic pathway is nevertheless dependent on a steady, low level of AP-1 mediated DNA binding or transcription, above or below which chondrogenic differentiation is compromised. Taken together, these data suggest that the DNA binding and function of the components of the AP1 nuclear factor complex play a pivotal role in the chondrogenic differentiation of C3H10T1/2 cells.

In summary, we have studied the activity of AP-1 nuclear factor in mesenchymal chondrogenesis using a simple promoter containing only AP-1 consensus sequence fused to a TATA box. Whether the activity and binding of AP-1 is further affected as a function of the complexity of native promoters of specific genes is not known; nevertheless, the use of such simple promoters provides a rapid and convenient assay for partial analysis of the signaling events involved in cell survival, proliferation, or differentiation.

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REFERENCES

Ahrens PB, Solursh M, Reiter RS. 1977. Stage-related capacity for limb chondrogenesis in cell culture. Dev Biol 60:69–82.

Andrews NC, Faller DV. 1991. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res 19:2499 p.

DeLise AM, Fischer L, Tuan RS. 2000. Cellular interactions and signaling in cartilage development. Osteoarthr Cartil 8:309–334.

Denker AE, Nicoll SB, Tuan RS. 1995. Formation of cartilage-like spheroids by micromass culture of murine C3H10T1/2 cells upon treatment with transforming growth factor-β1. Differentiation 59:25–34.

Denker AE, Haas AR, Nicoll SB, Tuan RS. 1999. Chondrogenic differentiation of murine C3H10T1/2 multipotential mesenchymal cells: I. Stimulation by bone morphogenetic protein-2 in high-density micromass cultures. Differentiation 64:67–76.

Gehris AL, Oberlender SA, Shepley KJ, Tuan RS, Bennett VD. 1996. Fibronectin mRNA alternative splicing is temporally and spatially regulated during chondrogenesis in vivo and in vitro. Dev Dyn 206:219–230.

Gehris AL, Stringa E, Spina J, Desmond ME, Tuan RS, Bennett VD. 1997. The region encoded by the alternatively spliced exon IIIA in mesenchymal fibronectin appears essential for chondrogenesis at the level of cellular condensation. Dev Biol 190:191–205.

Giancotti FG, Ruoslahti E. 1999. Integrin signaling. Science 285:1028–1032.

Guan Z, Buckman SY, Pentland AP, Templeton DJ, Morrison AR. 1998. Induction of cyclooxygenase-2 by the activated MEKK1→SEK1/MKK4→p38 mitogen activated protein kinase pathway. J Biol Chem 273: 12901–12908.

Hilberg K, Aguzzi A, Howlles N, Wagner EF. 1993. c-jun is essential for normal mouse development and hepatogenesis. Nature 365:179–181.

Hill CS, Treisman R. 1995. Transcriptional regulation by extracellular signals: mechanisms and specificity. Cell 80:199–211.

Johnson RS, Spiegelman BM, Papaionnou V. 1992. Pleiotropic effects of a null mutation in the c-fos protooncogene. Cell 71:577–586.

Karin M. 1998. New twists in gene regulation by glucocorticoid receptor: is DNA binding dispensable? Cell 93: 487–490.

Karin M, Delhase M. 1998. JNK or IKK, AP-1 or NF-kappaB, which are the targets for MEK kinase 1 action? Proc Natl Acad Sci USA 95:9067–9069.

Karin M, Zheng-gang Liu, Zandi E. 1997. AP1 function and regulation. Curr Opin Cell Biol 9:240–246.

Katai H, Stephenson JD, Simkevich CP, Thompson JP, Raghow R. 1992. An AP-1-like motif in the first intron of human Pro alpha 1(I) collagen gene is a critical determinant of its transcriptional activity. Mol Cell Biochem 118:119–129.

Kosher RA. 1983. The chondroblast and chondrocytes. In: Hall BK, editor. "Cartilage". Vol. 1: Structure, function, and biochemistry. New York: Academic Press. p 59–85.

Kulyk WM, Upholt WB, Kosher RA. 1989. Fibronectin gene expression during limb cartilage differentiation. Development 106:449–455.

Lange-Carter CA, Pleiman CM, Gardner AM, Blumer KJ, Johnson GL. 1993. A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. Science 260:315–319.

Lev R, Spicer S. 1964. Specific staining of sulfate groups with Alcian blue at low pH. J Histochem Cytochem 12: 309 p.

Marshall CJ. 1995. Specificity of receptor tyrosine kinase signaling; transient versus sustained extracellular signal-regulated kinase activation. Cell 80:179–185.

Miyamoto S, Katz BZ, Lafrenie RM, Yamada KM. 1998. Fibronectin and integrins in cell adhesion, signaling, and morphogenesis. Ann NY Acad Sci 857:119–129.

Oberlender SA, Tuan RS. 1994a. Spatiotemporal profile of N-cadherin expression in the developing limb mesenchyme. Cell Adhes Commun 2:521–537.

Oberlender SA, Tuan RS. 1994b. Expression and functional involvement of N-cadherin in embryonic limb chondrogenesis. Development 120:177–187.

San Antonio JD, Tuan RS. 1986. Chondrogenesis of limb bud mesenchyme in vitro: stimulation by cations. Dev Biol 115:313–324.

Schaeffer HJ, Weber MJ. 1999. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. Mol Cell Biol 19:2435–2444.

Schlaepfer DD, Hauck CR, Sieg DJ. 1999. Signaling through focal adhesion kinase. Prog Biophys Mol Biol 71:435–478.

Schule R, Rangarajan P, Kliewer S, Ransone LJ, Bolado J, Yang N, Verma IM, Evans RM. 1990. Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. Cell 62:1217–1226.

Seghatoleslami MR, Kosher RA. 1996. Inhibition of in vitro limb cartilage differentiation by syndecan-3 antibodies. Dev Dyn 207:114–119.

Seghatoleslami MR, Lichtler AC, Upholt WB, Kosher RA, Clark SH, Mack K, Rowe DW. 1995. Differential regulation of COL2A1 expression in developing and mature chondrocytes. Matrix Biol 14:753–764.

Solursh M. 1983. Cell–cell interaction in chondrogenesis. In: Hall BK, editor. "Cartilage". Vol. 2. Development, differentiation, and growth. New York: Academic Press. p 121–141.

Taylor SM, Jones PA. 1979. Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell 17:771–779.

Thomas DP, Saunters A, Gentry A, Grigoriadis AE. 2000. Inhibition of chondrocyte differentiation in vitro by constitutive and inducible overexpression of the c-fos proto-oncogene. J Cell Sci 113:439–450.

Treisman R. 1996. Regulation of transcription by MAP kinase cascades. Curr Opin Cell Biol 8:205–215.

Van der Burg B, Slager-Davidov R, van der Leede BM, de Laat SW, van der Saag PT. 1995. Differential regulation of AP1 activity by retinoic acid in hormone-dependent and -independent breast cancer cells. Mol Cell Endocrinol 112:143–152.

Yan M, Dal T, Deak JC, Kyriakis JM, Zon LI, Woodgett JR, Templeton DJ. 1994. Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. Nature 372:798–800.

Yoon YM, Oh CD, Kim DY, Lee YS, Park JW, Huh TL, Kang SS, Chun JS. 2000. Epidermal growth factor negatively regulates chondrogenesis of mesenchymal cells by modulating the protein kinase C-alpha, Erk-1, and p38 MAPK signaling pathways. J Biol Chem 275:12353–12359.

Yujiri T, Sather S, Fanger GR, Johnson GL. 1998. Role of MEKK1 in cell survival and activation of JNK and ERK pathways defined by targeted gene disruption. Science 282:1911–1914.

Zhou XF, Shen XQ, Shemshedini L. 1999. Ligand-activated retinoic acid receptor inhibits AP-1 transactivation by disrupting c-Jun/c-Fos dimerization. Mol Endocrinol 13: 276–285.